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# Molecular Studies of 5-Azacytidine-Induced Early-Flowering Lines of Flax

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# MOLECULAR STUDIES OF 5-AZACYTIDINE-INDUCED EARLY-FLOWERING LINES OF FLAX

by

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THESIS

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## ABSTRACT

Several early-flowering flax (*Linum usitatissimum* L.) lines were derived from treatment of germinating seeds with 5-azacytidine in 1990. These lines are also shorter, have fewer leaves, and their DNA is hypomethylated, relative to their corresponding controls. The work presented in this thesis used early-flowering and control lines of the Royal (R) flax genotype, and the Large (L) flax genotroph. Firstly, levels of cytosine methylation were measured over a 24-hour period in the early-flowering line RE2 and its control (RC), using an HPLC method. Secondly, to determine the response of the flax lines to short-day conditions, control and early-flowering lines from both L and R were grown in either 8-hour-day or ambient, long-day light conditions, and were compared in a number of aspects of development. Thirdly, primers for five putative flax flowering genes (*SOC1*, *COL*, *ADG1*, *GAI*, and *AP1*) were designed and a semi-quantitative PCR method was used to establish developmental expression profiles in leaves and shoot tips of RC and RE2 in order to detect differences in expression that may have resulted from the original demethylation treatment. Methylation was found to remain constant in RC and RE2 over the 24-hour period in all three tissues examined. In the short-day experiment the early-flowering lines differed from controls in a number of parameters, but the most notable were that the treatment delayed flowering and increased the number of leaves produced in all lines, but had less of an effect on the early-flowering lines. Expression patterns for the five genes examined indicated that they are all expressed in both leaves and shoot tips, and for most genes there was no indication that their expression had been altered by the demethylation treatment. However, *AP1* expression was higher in leaves of RE2 than those of RC, and was found to reach

higher levels in the buds of RE2 than those of RC. These expression differences may be the result of demethylation of a gene upstream of *AP1* that was affected by the demethylation treatment. The results of these experiments further demonstrate the differences between the early-flowering lines and their controls, and will help elucidate the genetic basis for these differences.

## ACKNOWLEDGEMENTS

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| | |
|---|---|
| *ADG1* | *ADP-GLUCOSE PHOSPHORYLASE 1* gene |
| ANOVA | Analysis of Variance |
| *AP1* | *APETALA1* gene |
| azaC | 5-azacytidine |
| *COL* | *CONSTANS-LIKE* gene |
| EST | Expressed Sequence Tag |
| *GAI* | *GIBBERELLIN INSENSITIVE* gene |
| HPLC | High-pressure liquid chromatography |
| L | Large fiber-type genotroph of flax |
| LC | Control line of Large |
| LE1 | An early-flowering line derived from L |
| LE2 | A second early-flowering line, independently derived from L |
| *LFY* | *LEAFY* gene |
| 5mC | Methylated cytosine |
| PCR | Polymerase chain reaction |
| R | Royal oilseed cultivar of flax |
| RC | Control line of Royal |
| RE1 | An early-flowering line derived from R |
| RE2 | A second early-flowering line, independently derived from R |
| RT-PCR | Reverse-transcriptase polymerase chain reaction |
| *SOC1* | *SUPPRESSOR OF OVEREXPRESSION OF CO 1* gene |
| TBE | Tris-borate EDTA |

# 1. INTRODUCTION

## 1.1 DNA Methylation

### *1.1.1 Cytosine Methylation*

The methylation of DNA, an example of an epigenetic change (a stable DNA modification that does not alter the sequence of base pairs), occurs when a methyl group is covalently linked to a nucleotide. Cytosine methylation, which is of most interest in the field of genetics, occurs when a methyl group is covalently added to the $5^{th}$ carbon of a cytosine pyrimidine ring forming 5-methylcytosine, a nucleic acid residue first discovered by Johnson and Coghill (1925). Methylation of cytosine residues has been found in both plants and animals but is found more frequently in plants where up to 50 % of the cytosine residues can be methylated (cited in Suzuki and Bird, 2008). Cytosine methylation typically takes place at symmetrical CG dinucleotides, but in plants it can also occur at CNG trinucleotides or in nonsymmetrical sequences such as CAT. Symmetrical sequences are of importance because they allow cytosine methylation to be transmitted to new daughter DNA strands through cell division (Bird, 1978) via maintenance methylation. This mechanism of transmission is likely to play a significant role in gene regulation.

### *1.1.2 Role of Cytosine Methylation in Gene Regulation and Plant Development*

Besides having a general role in genome stability (Matassi *et al.*, 1992), it has also been shown that cytosine methylation has a role in silencing transposable elements (Bennetzen *et al.*, 1994). A clear example was provided by Miura *et al.* (2001) who demonstrated that transposons can become mobilized when hypomethylation is induced in *Arabidopsis thaliana*. Just as methylation is thought to

silence the movement of transposons, it is also known that gene methylation silences gene expression; this aspect of cytosine methylation has been extensively studied (reviewed in Suzuki and Bird, 2008; reviewed in Gehring and Henikoff, 2007). Generally, genes that are methylated are transcriptionally inactive, whereas genes that are not methylated remain active.

The first studies on the effect of cytosine methylation on gene expression were performed on mice, where methylated DNA was inserted into mice cells and RNA levels and enzyme activity were monitored (Busslinger, Hurst and Flavell, 1983; Keshet, Yisraeli and Cedar, 1985; Kruczek and Doerfler, 1983). The methylation of DNA is thought to repress transcriptional activity either by directly blocking transcription factors from binding to the DNA, or by causing chromatin condensation. The link between cytosine methylation and chromatin condensation has been studied by Jones *et al.* (1998) and Nan *et al.* (1998) who found that a protein, which specifically binds to methylated DNA (MeCP2), forms a complex with histone deacetylase, an enzyme with activity that leads to chromatin condensation. More specifically, histone deacetylation leads to a positive charge on histones, which results in bonding between histones and negatively charged phosphate groups within the DNA. Deacetylation of histones may also cause an interaction between nucleosomes that allows them to become more compact. Therefore, the interaction between histone deacetylase and MeCP2 may result in condensing of the DNA. Further evidence for a relationship between DNA methylation and chromatin structure comes from the discovery of *DDM1* (*DECREASE IN DNA METHYLATION 1*), a chromatin remodeling factor (Brzeski and Jerzmanowski, 2003) that is required for the maintenance of cytosine methylation (Vongs *et al.*, 1993). It is not clear under which circumstances methylated sites directly block transcription factors and under which

circumstances they block it indirectly via chromatin condensation. Either scenario provides an explanation for how transcription factors may be prevented from accessing the DNA.

The role of cytosine methylation in gene expression, while having many potential implications, has been linked to plant development. An excellent example of this was observed when an antisense construct of the methyltransferase *METI* was introduced into wild-type *Arabidopsis* (ecotype C24), resulting in both significant decreases in cytosine methylation at CG dinucleotides, and developmental abnormalities, such as altered flowering time, altered leaf size and shape and plant size (Finnegan, Peacock and Dennis, 1996). Studies of *FWA (FLOWERING WAGENINGEN)* in *Arabidopsis* provided further support for a developmental role of cytosine methylation because it is involved in the developmental process of flowering, and its expression is regulated by cytosine methylation at its 5' end (Soppe *et al.*, 2000). Hypomethylation of CG dinucleotides within this region of the gene leads to late flowering mutants that ectopically express *FWA* (Soppe *et al.*, 2000), which supports the idea that within-gene methylation leads to transcriptional repression.

### *1.1.3 The Process of Cytosine Methylation*

Cytosine methyltransferases are enzymes that catalyze the transfer of a methyl group from S-adenosyl methionine (a methyl carrier molecule) to the fifth carbon of the cytosine pyrimidine ring (Gold, Hurwitz and Anders, 1963). *Arabidopsis* has methyltransferases belonging to three different families: Methyltransferase 1 (MET1), Chromomethylase (CMT), and Domains Rearranged Methyltransferase (DRM) (reviewed in Goll and Bestor, 2005). The MET1 family includes proteins that may be involved in maintenance methylation at CG sites and, to a lesser extent, at CNG sites (Finnegan, Peacock and Dennis, 1996; Genger *et al.*, 1999). Members of

the CMT family are thought to be unique to plants and may encode proteins that control DNA methylation at asymmetric and CNG sites (Cao and Jacobsen, 2002; Papa *et al.*, 2001). It has also been suggested that, because CNG sequences appear to be located mostly in heterochromatic (condensed) regions of DNA, chromomethylases may act to maintain the heterochromatic state (Papa *et al.*, 2001). It remains unclear how previously unmethylated sites become methylated to generate new DNA methylation patterns, as may be the case in gene regulation (i.e. where a gene may become methylated in order to be turned "off"). Such patterns become established during a process known as *de novo* methylation, and may be controlled by members of the DRM family. DRM enzymes are putative *de novo* methylases because the proteins within this family have catalytic motifs that resemble those of the mammalian Dnmt3 family of *de novo* methyltransferases (Cao *et al.*, 2000). Furthermore, DRM's have been found to be involved in *de novo* methylation via an RNA-directed process (Cao *et al.*, 2003).

RNA-directed DNA methylation was first discovered in 1994 in viroid-infected tobacco plants (Wassenegger *et al.*, 1994). When viroid-identical transgenes were inserted into the host genome, these sequences became methylated at cytosine residues in the presence of actively replicating viroids, but not in replication-deficient controls. Since these viroids only produce RNA during their replication cycle, this provided clear evidence that the cytosine methylation was in some way being directed by homologous RNA. While this RNA-directed mode of cytosine methylation is not entirely understood, it is known to induce *de novo* methylation at all sequence contexts (i.e. CG, CNG and CNN, where N can be either A, T or C) (Pélissier *et al.*, 1999). The process is known to require a double-stranded RNA molecule, which is then cleaved into short interfering RNAs (siRNAs) (~24 nucleotides in length) by the

RNAi machinery (Mette *et al.*, 2000). The RNA molecule is then thought to direct methylation of specific sequences via a base-pairing mechanism. The ability of siRNAs to direct methylation to asymmetrical sites is of interest as it may provide an easily reversible type of methylation that can be lost simply by the lack of a siRNA source (Matzke *et al.*, 2007).

While the addition of a methyl group to a cytosine residue is important for initiating chromatin condensation, the mechanism by which these methyl groups can be removed is also of importance. It is thought that they can be removed by either passive or active demethylation. Passive demethylation happens when DNA replication is not accompanied by maintenance methylation, and is a slow-acting process. However, because genes can be turned on relatively quickly, it is likely that active demethylation also occurs. Active demethylation describes a mechanism whereby a methyl group is specifically cleaved from the cytosine pyrimidine ring because of a break in a C-C bond. Several mechanisms of active demethylation have been described, although these possibilities remain controversial (Kapoor, Agius and Zhu, 2005). Evidence has been provided that a 5-methylcytosine demethylase enzyme actively demethylates cytosines by hydrolysis (Ramchandani *et al.*, 1999), using water to generate demethylated cytosine and methanol as byproducts. Active demethylation has also been found to occur by the action of 5-methylcytosine DNA glycosylase, an enzyme that removes methylated 5-methylcytosine from the phosphodiester backbone (Jost *et al.*, 1995). One major gene family implicated in this process of active demethylation is *DEMETER* (*DME*) (Choi *et al.*, 2002). It has been found that some members of this family are responsible for the demethylation of approximately 180 loci in *Arabidopsis* (Penterman *et al.*, 2007). It is also interesting that approximately 80% of these loci were found within genes, and that the enzymes

primarily demethylate the 5' and 3' ends of these genes (Penterman *et al.*, 2007). This finding indicates that some members of the DME family may be responsible for protecting genes from cytosine methylation that could have potentially adverse effects (Penterman *et al.*, 2007).

## 1.2. Early-Flowering Lines of Flax

### *1.2.1 Early-Flowering vs. Control Lines*

Early-flowering in flax (*Linum usitatissimum* L.) has been studied using two different flax genotypes. The lines used in this study were derived from the Royal (R) genotype (an oilseed cultivar) and from the Large (L) genotroph derived from Stormont cirrus, a fiber cultivar (Durrant, 1971). The early-flowering lines of R and L were generated when germinating seedlings were treated with 5-azacytidine (Fieldes, 1994). The early-flowering phenotype was first observed in the first generation progeny of the treated plants (Fieldes, 1994). While the R and L lines are of interest, it is the differences between early flowering and control lines within a specific genotype or genotroph that are of significance. The stable, heritable changes in early-flowering lines (i.e. such as RE2 and LE2) resulted in plants that are shorter, have fewer leaves, and flower 7-13 days earlier than those of control lines (Table 1). Their DNA is also hypomethylated compared to that of their controls (Table 1; Fieldes *et al.*, 2005). The demethylation induced by azaC is thought to be random, and thus, it is not known which, if any, genes in the early-flowering lines of flax are demethylated. Methylation levels in combined cotyledon and epicotyl tissues have been found to increase from 3 days after planting until 9 days after planting (Brown, De Decker and Fieldes, 2008). Methylation levels in cotyledons from both early-

**Table 1. Differences in Morphology and Methylation Levels Between Early-Flowering and Control Lines of Flax.**

Control lines (RC and LC) were compared to early-flowering lines (RE1, RE2, RE3 and LE1, LE2) using means for all measurements of flowering age, main-stem height and leaf number from mature plants. All measurements for L lines, except methylation levels, were taken from greenhouse plants in 1996 (Measurements are means, LC: n=39, LE1 and LE2: n=20) (Amyot, 1997). The L line methylation level means are from 4-day-old seedlings grown in a growth chamber in 2004 (LC: n=4, LE1 and LE2: n=2) (Fieldes *et al.*, 2005). Measurements for R lines were taken from greenhouse populations in 2006 (n=20, except for RE3 main stem height and leaf number, where n=19). Means (n=2) for levels of methylation are taken from cotyledons and shoot tips of 8-day-old seedlings grown in a growth chamber in 2007.

| Parameter | Plant Line | | | |
|---|---|---|---|---|
| | RC | RE1 | RE2 | RE3 |
| Flowering Age (days) | 57.1 | 45.4 | 42.6 | 46.2 |
| Main Stem Height (cm)[a] | 93.7 | 53.9 | 50.9 | 63.8 |
| Leaf Number | 116.9 | 53.8 | 53.5 | 69.4 |
| Methylation Level (%)[b] | 15.1 | 13.6 | 14.5 | 13.7 |
| | | | | |
| | LC | LE1 | LE2 | |
| Flowering Age (days) | 52.0 | 46.6 | 42.0 | |
| Main Stem Height (cm)[a] | 102.3 | 84.7 | 61.1 | |
| Leaf Number | 108.0 | 70.8 | 53.3 | |
| Methylation Level (%)[b] | 14.36 | 13.45 | 12.53 | |

[a] Main stem height measured from cotyledons to the base of the inflorescence.
[b] Methylation level is for 8-day-old seedlings. DNA was sampled from cotyledons and emerging shoot tip.

flowering and control lines increase linearly with development, while levels in main shoot tips increase linearly from 24 days after planting to 34 days after planting (Brown, De Decker and Fieldes, 2008). Generally, the early-flowering lines remain hypomethylated compared to their control line during development. However, developmental profiles for methylation have revealed that this hypomethylation observed in tissues of the early-flowering lines may be absent in more mature tissues (Brown, De Decker and Fieldes, 2008). This lack of hypomethylation has also been observed in plants that were kept in the dark prior to chloroplast isolation (De Decker, 2007). While our interest in the demethylated flax lines has been on the timing of the floral transition, the vegetative growth stages and the differences in the timing of vegetative growth between the early-flowering lines and their corresponding controls have also been quantified. These studies demonstrated that the flax lines have three main vegetative phases: juvenile, transition and adult, and the early-flowering lines and controls grow and develop at the same rate until the onset of the adult phase, which is truncated in the early-flowering lines (Fieldes and Harvey, 2004). A truncated adult phase has also been observed in early-flowering *Arabidopsis* (Steynen *et al.*, 2001). This alteration in *Arabidopsis* development resulted from overexpression of *LEAFY* (*LFY*), a gene involved in both integrating the various pathways to flowering and determining floral meristem identity (Steynen *et al.*, 2001). It is, therefore, possible that *LFY* is also responsible for the shortened adult phase and associated early flowering seen in the azaC-induced early-flowering lines of flax.

### *1.2.2 Genetic Basis of Early-Flowering in Flax*

Fieldes and Amyot (1999) proposed a genetic model to explain the phenotypic ratios observed in segregating generations of crosses between early-flowering lines, RE1 and LE1, and their corresponding control lines (RC and LC). According to the

genetic model, at least three loci are involved in the early-flowering phenotype of these lines. The loci are named for the presumed effect that they have on the induced early flowering phenotype. These loci are: (1) Early flowering locus (*EF*) which is thought to control flowering age and any height effect associated with flowering age, (2) Short locus (*SH*) which is thought to control plant height, and (3) Control locus (*A*) which controls the other two loci. *EF* and *SH* interact, giving rise to a variety of phenotypic groups that can be seen in segregating progeny (Amyot, 1997); in addition, the phenotypes associated with the genotypes of the *EF* and *SH* loci are only observed in plants that are homozygous recessive for the *A* locus (Fieldes and Amyot, 1999). The possible relationship between these genes and cytosine methylation is outlined in the working concept, which is based on the idea that for the early-flowering phenotype to be seen all three loci must be demethylated (Amyot, 1997; Fieldes and Amyot, 1999).

The working concept is that, in early-flowering lines, because all three loci are demethylated as a result of the heritable effects of azaC, only a critical threshold of floral stimulus is required to induce flowering (Amyot, 1997; Fieldes and Amyot, 1999). Control plants, however, take longer to flower because the three loci need to become demethylated naturally, and only then are they able to respond to a floral stimulus (Fieldes and Harvey, 2004). It has not, however, been determined which loci in flax correspond to *EF*, *SH*, and *A* as little information is known about this aspect of the flax genome. It is likely that at least one of the proposed loci is involved in a flowering pathway of flax. Since it is not known which flowering pathway(s) operates in flax, determining the gene(s), which are likely to have been affected by the induced demethylation, is difficult.

### *1.2.3 Recent Work on Flax Early-Flowering Lines*

In *Arabidopsis* it is likely that a minimum level of *LFY* transcript is required for flowering to occur (Blázquez *et al.*, 1997); this may also be true for flax. The early-flowering flax line RE2 appears to accumulate more *LFY* transcript in the tissues of main-stem shoot-tips, at a younger age, than control line RC (De Decker, 2007). Therefore, early-flowering lines may reach a required *LFY* threshold earlier than control lines (De Decker, 2007). However, the link between increased rate of accumulation of *LFY* transcript and cytosine demethylation has not yet been demonstrated. *LFY* is known to be a flowering pathway integrator, as well as a floral meristem identity gene (reviewed in Henderson and Dean, 2004). That is, it can both receive signals from all flowering pathways and coordinate the information, but can also instruct the meristem to assume a floral fate. Therefore, it is hard to say which gene in which flowering pathway has been demethylated in early-flowering flax lines causing the altered *LFY* expression.

## 1.3. Flowering

### *1.3.1 Theories of Flowering*

The production of flowers during the life cycle of a plant marks the end point of the phase change from vegetative to reproductive growth at the shoot apical meristem (SAM). Before the apical meristem can make this switch, it must first pass through two major vegetative stages: the juvenile and adult phases (Lawson and Poethig, 1995). It is during the juvenile stage of shoot development that the true leaves, stem, and axillary buds of the plant are formed and during the adult stage that the competence to undergo sexual reproduction is gained at the SAM (Poethig, 1990).

The genes that establish reproductive competence may also play a role in other aspects of vegetative phase change (Steynen, Bolokoski and Schultz, 2001).

The timing and location of the switch away from vegetative growth is crucial to a plant's reproductive success, and includes both temporal and spatial regulation. The many different strategies that regulate flowering consist of a coordinating set of pathways. These pathways allow plants to detect and respond to environmental cues, such as photoperiod and temperature, as well as endogenous developmental cues (Bernier *et al.*, 1993). Three separate theories have been suggested for the endogenous control of this phase change: (1) Florigen, (2) Nutrient Diversion, and (3) Multifactorial Control (reviewed in Bernier, 1988).

The first theory is that a florigen and antiflorigen are responsible for floral induction. In 1934, Knott observed that in spinach, flowering was induced when the leaves were exposed to long-day conditions and the shoot tips to short-day conditions, but not when these conditions were reversed. It has since been suggested that a promoter (florigen) is produced in photoinduced leaves, and that a floral inhibitor (antiflorigen) is generated in the noninduced leaves (reviewed in Bernier, 1988). Flowering is thought to occur when the balance between the promoter and inhibitor at the apical meristem shifts in favour of the florigen which travels through the phloem from the leaves, along with the assimilates (reviewed in Bernier, 1988), ultimately initiating floral morphogenesis. Several different ideas have been proposed about the identity of 'florigen'. It has been suggested that the florigen and antiflorigen are simple and specific hormones (reviewed in Bernier *et al.*, 1993). More recently, it was proposed by Huang *et al.* (2005) that the mRNA of *FLOWERING LOCUS T* (*FT*) moves from the phloem in the leaves to the apical meristem, and is therefore the florigen. However, after this discovery was published, a retraction was issued

(Böhlenius *et al.*, 2007). Further experiments indicated that there was no evidence of the movement of *FT* mRNA from the leaves to the meristem (Böhlenius *et al.*, 2007). More recently, work on *Arabidopsis*, using various techniques, has indicated that the actual florigen compound may be the FT protein *per se* (Corbesier *et al.*, 2007; Notaguchi *et al.*, 2008). Interestingly, it is thought that *FT* may operate as a common florigen in many species, acting not only in herbaceous species, but also possibly in trees, such as aspen (Böhlenius *et al.*, 2006) and poplar (Hsu *et al.*, 2006). The identity of an antiflorigen remains elusive.

Nutrient diversion, the second theory (Sachs and Hackett, 1983), proposes the idea that a source/sink relationship within the plant must be modified in order to trigger flowering. More specifically, in order for flowering to occur the leaves (or source) must provide a higher concentration of assimilates to the apical meristem (sink) when photoinduced, than the levels produced under noninductive conditions (Sachs and Hackett, 1983). While there is support for this theory, some researchers are of the opinion that assimilates only have a metabolic role in the flowering process, providing the energy required for each stage of floral initiation and development (reviewed in Bernier, 1988).

The third theory, proposed by Bernier, Kinet and Sachs (1981), suggests that there are multiple factors, consisting of both promoters and inhibitors of flowering, responsible for the initiation of flowering. Under multifactorial control, flowering will only occur when all factors (such as various assimilates and phytohormones), are present at the apex at the appropriate time, and at the required concentration. Based on genetic differences between plants, the factors that are required and their concentrations may vary. Work done on a variety of plant species supports the idea of multifactorial control of flowering, with multiple floral inductive pathways being

utilized that produce a number of hormones and proteins that ultimately lead to flowering (reviewed in Bernier *et al.*, 1993)

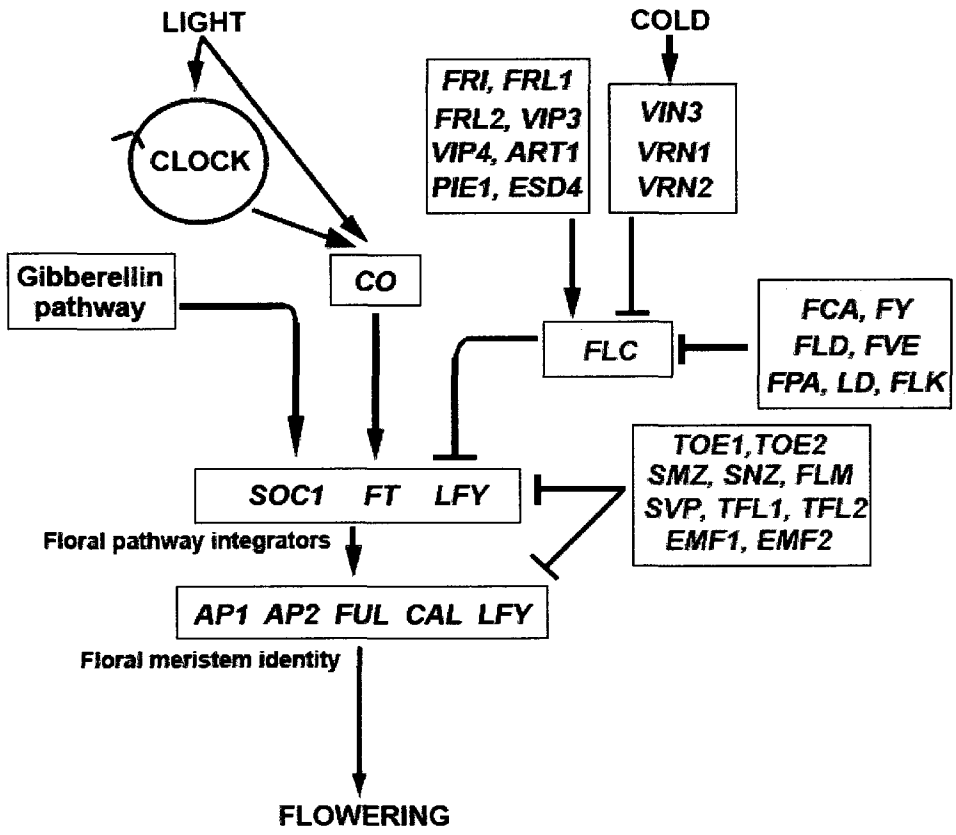
These three theories introduce a general idea of how flowering may be induced; however, in today's molecular age emphasis has shifted more towards the genetic regulation of specific flowering pathways, and the specific genes that govern phase change of the SAM.

### *1.3.2 Flowering Pathways*

Flowering time has been studied in a variety of plant species; however, it is understood most thoroughly in *Arabidopsis*. Studies have provided evidence for four main flowering pathways (Figure 1) that can be used as a model to determine the flowering pathways acting in other species, such as flax. Plants can be classified as either obligate or facultative long-day (LD) plants, or short-day (SD) plants, or as day neutral plants if they do not flower in response to photoperiodic induction. LD plants have flowering induced when the photoperiod exceeds a critical day length (CDL) and SD plants flower when the photoperiod is shorter than a CDL. *Arabidopsis thaliana* is known to be a facultative long-day plant, meaning that flowering is induced earlier in long-day conditions than in short-day conditions, and as such utilizes the photoperiodic, or light, pathway. Genes controlling this pathway promote flowering based on the length of the night, also known as the nycto-period. Photoperiod is thought to be regulated by the interaction between photoreceptors and circadian rhythm, and initiates flowering based on both day length and light quality. Photoreceptors of the light pathway are used to measure the length of the dark period, and when this period is below a critical length (as is the case for long-day plants), certain genes are activated. For short-day plants (those which require short days to flower), a nycto-period below a critical length is required to induce flowering. In

**Figure 1. Pathways to Flowering in Arabidopsis.**

Major flowering pathways are indicated as Gibberellin, photoperiod (light), vernalization (cold), and autonomous (indicated by genes in red). (Adapted from Henderson and Dean, 2004).


LIGHT
COLD
CLOCK
FRI, FRL1
FRL2, VIP3
VIP4, ART1
PIE1, ESD4
VIN3
VRN1
VRN2
Gibberellin pathway
CO
FLC
FCA, FY
FLD, FVE
FPA, LD, FLK
TOE1,TOE2
SMZ, SNZ, FLM
SVP, TFL1, TFL2
EMF1, EMF2
SOC1 FT LFY
Floral pathway integrators
AP1 AP2 FUL CAL LFY
Floral meristem identity
FLOWERING

*Arabidopsis*, particular regions of the visible spectrum of light promote flowering while others inhibit it. Specifically, red light has been observed to inhibit flowering while far-red light promotes it (reviewed in Levy and Dean, 1998). It has been determined that the photoperiod pathway, in *Arabidopsis*, acts mainly via an interaction between *CONSTANS* (*CO*), a gene with expression that is regulated by circadian rhythm (Suárez-López *et al.*, 2001), and *FT* (Samach *et al.*, 2000).

Photoperiod is not the only environmental factor that can lead to induction of flowering in plants; temperature may also be used as a cue. Using one, or both, of these environmental cues, some plants are able to respond to seasonal changes in the environment. Vernalization is the exposure of plants to cold temperatures in order to induce flowering (Figure 1). Plants that require this are found in temperate regions where seasonal changes are pronounced. The cold period occurs over winter and is used to ensure that seeds will be produced in spring or summer, when conditions are favourable. It has been determined by use of grafting experiments that the site of cold perception is the shoot apex (reviewed in Henderson, Shindo and Dean, 2003). The vernalization response is thought to be linked with cytosine methylation as vernalized plants have reduced methylation (Burn *et al.*, 1993). Because flowering in vernalized plants typically does not occur for at least a few weeks after the exposure to cold is removed, there must be some sort of memory on the part of the plant of the cold treatment that is responsible for this temporal separation. It is thought that the apex "remembers" the cold treatment by passing on any changes that have occurred through mitosis via an epigenetic mechanism, such as reduced methylation.

Similar to many other processes in plant development, flowering can also require the activity of a specific hormone (or hormones). Gibberellic Acid (GA) is one type of hormone that is thought to play a role in the initiation of flowering during

non-inductive photoperiods (Wilson, Heckman and Somerville, 1992). The role of GA in flowering was originally studied by treating plants with exogenously applied hormone (Langridge, 1957), but has more recently been studied via mutations in the biosynthesis or signaling pathways for GA (Wilson, Heckman and Somerville, 1992). In *Arabidopsis*, a facultative LDP, it is known that flowering under short-day conditions is controlled mainly by the GA pathway. Evidence for this was obtained from *Arabidopsis* mutants that had significantly reduced levels of this hormone and were unable to flower during short days (Wilson, Heckman and Somerville, 1992).

The final major pathway to flowering in *Arabidopsis* is the autonomous pathway. For many years it was thought that the genes in this pathway were not responsive to changes in environmental conditions, including photoperiod. In other words, it was thought that they are active under all day-length conditions. Plants with a mutation in genes in the autonomous pathway have been found to respond to vernalization, indicating that these two promotive pathways act redundantly. More recently, work on this flowering pathway has provided evidence that the autonomous genes may actually be involved in relaying information based on the ambient temperature (Blázquez, Ahn and Weigel, 2003). The response to ambient temperature is thought to be regulated by two genes in the autonomous pathway, *FCA* and *FVE* (Blázquez, Ahn and Weigel, 2003), providing support for the idea that the autonomous pathway is dependent on the environment and evidence against the original view that this flowering pathway acts independently of environmental conditions.

### *1.3.3 The Role of Methylation in Flowering*

Cytosine demethylation is known to result in many phenotypic changes in *Arabidopsis*, such as reduced apical dominance, altered leaf size and shape, decreased fertility, and altered flowering time (Finnegan, Peacock and Dennis, 1996). A large accumulation of work has indicated that methylation may be involved in determining flowering time though a role in the vernalization pathway (Burn *et al.*, 1993; reviewed in Finnegan *et al.*, 1998). An especially significant discovery revealed that vernalized plants have decreased cytosine methylation (Burn *et al.*, 1993). The vernalization process depends on *FLOWERING LOCUS C* (*FLC*), a known repressor of flowering (Michaels and Amasino, 1999), and it has been found that after vernalization, levels of *FLC* mRNA are significantly reduced (Michaels and Amasino, 1999). Hypomethylated lines of *A. thaliana* have also been developed by Finnegan, Peacock and Dennis (1996) using a *MET1* antisense transgene. These transgenic plants showed decreased expression of *FLC*, and a correspondingly reduced level of FLC, as well as early flowering (Finnegan *et al.*, 1998). Studies have also shown that treatment with azaC can substitute for cold treatment in vernalization-requiring ecotypes of *Arabidopsis* (Burn *et al.*, 1993). It was originally thought that *FLC*, specifically, may be demethylated, leading to the induction of flowering after vernalization (Sheldon *et al.*, 2000). However, the effects of DNA methylation on the expression of *FLC* have been shown to be indirect; bisulphite sequencing did not detect any changes in the cytosine methylation of *FLC* during vernalization (Finnegan *et al.*, 2005). Therefore, it is likely that demethylation of a gene that regulates expression of *FLC* is responsible for the vernalization response (Finnegan *et al.*, 2005).

More recently, focus has shifted away from the relationship between DNA methylation and *FLC*, towards the effects of histone modifications on *FLC* expression during vernalization (Sung and Amasino, 2004; Bastow *et al.*, 2004). Because cytosine methylation and histone modifications are known to be connected (Jackson *et al.*, 2002; Mathieu *et al.*, 2007), it is possible that future work will link these two epigenetic modifications and explain the regulation of the vernalization pathway via altered expression of *FLC*.

As previously mentioned, *FWA* is a second example of a flowering-time gene regulated by cytosine methylation. This gene has been studied extensively, and the role of methylation in its regulation is well understood. Although *FLC* and *FWA* have been studied at great length and it is clear that methylation plays a role in the timing and transition to flowering, the degree to which it is involved is not yet entirely understood.

## 1.4 Genes Involved in the Process of Flowering

### *1.4.1 Types of Genes Involved in Flowering*

The genes responsible for the transition to, and timing of, flowering have been studied in numerous species, and many commonalities in the regulation of flowering have been found; therefore, information on the genetic control of flowering in one species can often assist in understanding that of another.

The genes involved in the adult vegetative-to-reproductive phase change can be broken down into several classes. First, flowering time genes are those responsible for the timing of the phase transition. Typically, these genes receive the first endogenous and environmental signals influencing the timing of this transition.

Pathways of flowering time genes can fall into two distinct groups: those that promote flowering, and those that enable flowering (Boss *et al.*, 2004). The first type of flowering time pathway promotes flowering by activating the floral pathway integrators (Boss *et al.*, 2004). These integrator genes are responsible for coordinating the signals from all flowering time genes, from all pathways, in order to determine the correct timing for flowering. The main members that have been identified are *LFY*, *SOC1* and *FT*. The second, enabling, type of pathway acts to regulate repressors of the floral pathway integrators. It has been stated that these genes, "keep the meristem "blind" to promotive floral signals" (Boss *et al.*, 2004). There is ongoing competition between these two pathways that ultimately ensures the correct timing of flowering.

Once the timing signals have been passed on to the floral pathway integrator genes, they can then be coordinated and the information can be relayed to the floral meristem identity (FMI) genes. Once these FMI genes are activated, the apical meristem can assume a floral, rather than vegetative, fate. Mutant FMI genes generally lead to shoots growing in the place of flowers (reviewed in Weigel, 1995). In a publication by Nilsson *et al.* (1998), the cause of late-flowering was discussed; in general, late flowering is caused either by a failure to upregulate FMI genes, or through incompetence to respond to FMI genes. Therefore, early flowering is expected when the upregulation of FMI genes is early, or when competence to respond to FMI genes is acquired prematurely. FMI genes regulate the final class of genes involved in flowering, the floral organ identity genes, which ensure that flowers form with the correct patterning. These genes are grouped into several classes based on the particular floral organ identity (or identities) that they regulate.

### *1.4.2* LEAFY

While several meristem identity genes have been identified, *LEAFY* (*LFY*) is one of the best studied. This plant-specific transcription factor (as stated in Maizel *et al.*, 2005) has several homologues, such as *UNIFOLIATA* in *Pisum sativum* (Pea) (Hofer *et al.*, 1997), *FLORICAULA* in *Antirrhinum* (Snapdragon) (Coen *et al.*, 1990), and *PRFLL* in *Pinus radiata* (Mellerowicz *et al.*, 1998). As previously mentioned, when *LFY* is defective, the result is development of either shoot-like structures forming in the place of flowers, or inflorescences forming in the place of flowers, as is the case in snapdragon (Coen *et al.*, 1990). *LFY* is especially interesting because not only does it act as a meristem identity gene; it also functions as a floral pathway integrator (Blázquez and Weigel, 2000) where it has been found to act downstream of flowering-time genes in all floral promotion pathways (Blázquez *et al.*, 1997; Blázquez *et al.*, 1998, Blázquez and Weigel, 2000; Nilsson *et al.*, 1998). Expression of this gene has been detected in young floral meristems and flowers in numerous species (Weigel *et al.*, 1992; Blázquez *et al.*, 1997), but expression has also been found to occur in leaves during vegetative growth, with expression increasing towards the onset of flowering (Blázquez *et al.*, 1997). A high degree of *LFY* expression has also been detected during early leaf development in pea (Hofer *et al.*, 1997).

Of significance with respect to the early-flowering flax lines is the fact that *LFY* may not only act in the floral transition, but also in the transition to later vegetative stages (Steynen *et al.*, 2001). However, while the particular role of *LFY* in the reproductive phase change seems to be conserved in many species, the role of this gene in vegetative development might not be conserved (Kelly, Bonnlander and Meeks-Wagner, 1995; Mouradov *et al.*, 1998; Chujo *et al.*, 2003). For instance, when the rice homolog of *LFY*, *RFL*, was fused to a *LFY* promoter and expressed in *LFY*

mutants, the mutant phenotype was incompletely rescued, and these plants displayed features indicative of abnormal vegetative development, such as curly leaves, and stunted stem height (Chujo *et al.*, 2003).

### *1.4.3* APETALA1

*APETALA1* (*AP1*) (Koornneef *et al.*, 1983), a MADS-box gene, is not only integral in determining floral-meristem identity, but also in establishing proper floral-organ identity (Mandel *et al.*, 1992). Like *LFY*, expression of *AP1* is regulated by floral-pathway integrators, which results in promotion of the vegetative to floral transition in the apical meristem. Plants that are homozygous for *ap1-1*, a strong *AP1* mutation, do not show any alteration of vegetative growth compared to that in wild-type plants (Mandel *et al.*, 1992). However, apical meristems in these mutants do assume a partial inflorescence fate, rather than a floral fate, during the floral transition (Bowman *et al.*, 1993). It was also possible to identify mutants having leaf-like bracts in the place of sepals by distinguishing leaves and sepals based on epidermal cell characteristics and the presence of trichomes (Irish and Sussex, 1990). In addition, it has been found that *ap1-1* plants lack petals (Irish and Sussex, 1990). This discovery led to the conclusion that *AP1* is also involved in determining organ identity and, more specifically, is responsible for proper first and second whorl floral organ formation. Further work using expression analyses revealed that during flower development, *AP1* expression could be detected in the floral meristem, disappearing in the cells that will eventually give rise to the carpels and stamens, while remaining in the sepals and petals (Mandel *et al.*, 1992).

As mentioned previously, it is thought that plants need to exceed a certain threshold of *LFY* before flowering will occur; it has been proposed that a threshold of *AP1* must also be exceeded to promote flowering (Bowman *et al.*, 1993). When

either *LFY* or *AP1* is compromised, the meristem takes on partial floral identity. In some *ap1 lfy* double mutants, an inflorescence meristem is formed in the place of a floral meristem (Huala and Sussex, 1992). Therefore, neither *LFY* nor *AP1* acting alone can confer complete floral meristem identity; these two genes must act together for a proper floral transition.

### *1.4.4* SUPRESSOR OF OVEREXPRESSION OF CO 1

*SUPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), originally referred to as *AGAMOUS-LIKE 20* (*AGL20*), is known to act downstream of all major flowering pathways (GA, photoperiod, vernalization and autonomous) as a floral pathway integrator (Borner *et al.*, 2000; Lee *et al.*, 2000; Moon *et al.*, 2003). The rice *SOC1* orthologue, *OsMADS50*, has been found to have a similar expression profile to *SOC1* in *Arabidopsis* (Lee *et al.*, 2004). More specifically, expression of *OsMADS50* increases in vegetative tissues during development, with the highest expression occurring in mature leaf tissue (Lee *et al.*, 2004). The functions of *SOC1* homologues can also differ. For instance, *WSOC1*, a putative wheat *SOC1* orthologue, is not affected by vernalization (Shitsukawa *et al.*, 2007). This suggests that *SOC1* is involved in the vernalization response in *Arabidopsis*, but not in the same flowering pathway in wheat (Shitsukawa *et al.*, 2007). Phylogenetic analyses have indicated that the *SOC1* mechanism of activation for flowering was established prior to the divergence between monocots and eudicots (Lee *et al.*, 2004), but more recent phylogenetic studies based on amino acid sequence similarity have revealed that they can be separated into monocot and dicot clades (Shitsukawa *et al.*, 2007).

In *Arabidopsis*, the vernalization pathway involves *FLC*, the product of which regulates the expression of *SOC1* (Lee *et al.*, 2000). However, in rice, a monocot

species, it appears as though there is no *FLC* homologue (Goff *et al.*, 2002), and despite the report of an FLC-like factor in wheat (Rudnóy *et al.*, 2002), it has been noted that there are no *FLC* homologues outside of the Brassicaceae (Becker and Theißen, 2003). Therefore, while flowering via the vernalization and autonomous pathways in *Arabidopsis* requires *FLC*, and subsequently *SOC1*, these pathways act upon different genes in some monocots. As a result, the evolution of various flowering genes needs to be considered when attempting to predict potential functionality based on sequence similarity to known genes in other species.

### *1.4.5* CO *and* CONSTANS-LIKE

*CONSTANS-LIKE* (*COL*) genes belong to a family of genes that encode zinc finger transcription factors (Putterill *et al.*, 1995). The first member discovered, *CONSTANS* (*CO*) (Putterill *et al.*, 1995), continues to be studied, as it is an essential member of the photoperiod regulatory pathway, and is the main regulator of 'florigen'. A major point of interest for *CO* is the fact that it is regulated by circadian rhythm (Suárez-López *et al.*, 2001). Expression of *CO* has been found to peak at approximately 16 hours-after-dawn (Suárez-López *et al.*, 2001), which occurs near the end of a long day. During times of the year when short-day conditions are experienced, this peak in expression occurs during hours of dark. However, during the spring and summer when the transition to long days can occur, the peak in *CO* expression coincides with the light period; light is required for activation of CO (Valverde *et al.*, 2004).

Although *CO* is the main *COL* gene of interest, other members of the family have been characterized, with at least 17 different members of this family being identified in *Arabidopsis thaliana* (Lagercrantz and Axelsson, 2000). Members of this family have been divided into three different groups based on their composition

of conserved domains (Robson *et al.*, 2001; Griffiths *et al.*, 2003). These same three groups of *COL* genes have also been identified in rice; however, two additional groups were also discovered in rice (Griffiths *et al.*, 2003).

While *CO* has a significant role in determining flowering time, not all *COL* genes share this function (Ledger *et al.*, 2001). Some *COL* genes, such as *COL9* in *A. thaliana*, act as floral repressors (Cheng and Wang, 2005), while mutations in *COL1* and *COL2* seem to have no effect on flowering time (Ledger *et al.*, 2001). The rice gene *OsCO3* has been found to delay flowering under short-day conditions (Kim *et al.*, 2008). Constitutive expression of *CO* in *Arabidopsis* has been found to result in early-flowering (Onouchi *et al.*, 2000); conversely, constitutive expression of *COL1* and *COL2* did not have the same result (Ledger *et al.*, 2001). It is interesting, however, that like *CO*, both *COL1* and *COL2* are regulated by circadian rhythm (Ledger *et al.*, 2001). Although these *COL* genes share many similarities, it has been suggested that some may have very different roles. For instance, *COL3* has been found to regulate formation of lateral roots as well as the development of shoot elongation and branching (Datta *et al.*, 2006). The functional diversity within this gene family makes it difficult to predict functionality of similar genes in different plant species.

### *1.4.6* GIBBERELLIC ACID INSENSITIVE

Gibberellic acid (GA) is known to be involved in floral promotion in numerous plant species (Langridge, 1957; Lang, 1957; Lincoln and Hamner, 1958). Two main types of genes mediate this response: those involved in GA biosynthesis, and those involved in GA responsiveness. *ga1*, a gibberellin-deficient mutant, is involved in GA biosynthesis and can be rescued with application of GA, while *gai*,

*gibberellic acid insensitive*, is involved in GA responsiveness. The *gai* phenotype is characterized by having delayed flowering under short-day conditions (Wilson, Heckman and Somerville, 1992), as well as a reduction in height, apical dominance and seed germination (Koornneef *et al.*, 1985). The effects of these mutations are not prevalent under long-day conditions when the photoperiod pathway is actively promoting flowering.

*GAI* has been found to encode a negative regulator of the GA response, with *gai* being a gain-of-function mutation (Peng *et al.*, 1997). Previous studies on flax used exogenous applications of $GA_3$ to vegetative tissues to examine the possibility that the difference between early flowering and control lines results from a GA effect (Fieldes and Harvey, 2004). It was hypothesized that the GA treatment would preferentially accelerate flowering in the control lines compared to the early-flowering lines. The treatment of $GA_3$ alone was not found to have any effect on the plants. However, when plants were treated with a GA biosynthesis inhibitor (ancymidol) several phenotypic abnormalities were observed, such as delayed flowering and increased number of leaves in control lines, but not in the early-flowering lines (Fieldes and Harvey, 2004). This induced phenotype was rescued by treatment with $GA_3$ (Fieldes and Harvey, 2004). Therefore, it is clear that $GA_3$ can be used by flax plants, in some way, for proper vegetative development and to ensure the correct timing of the reproductive switch, but it is still not clear what role, if any, GA might play in determining the difference between control and early-flowering lines. It is possible that while the plants are able to use $GA_3$ to restore a GA-deficient phenotype, to induce early flowering in wild type plants a different GA is required.

### *1.4.7* ADP-GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1

ADP-glucose pyrophosphorylase (ADGase) is involved in starch biosynthesis in plants (Lin *et al.*, 1988a), such that plants deficient in ADGase activity have reduced starch accumulation. ADGase from leaves of *Arabidopsis* is comprised of two subunits: the large (54 kD) and the small subunits (51 kD) (Lin *et al.*, 1988a). In the *A. thaliana* mutant, *adg1-1*, there is no detectable activity of ADGase in the roots, petioles, flower stalks or flowers, which are tissues where starch would normally accumulate (Lin *et al.*, 1988a). Further work on the *ADG1* locus has indicated that it encodes the small subunit (Wang *et al.*, 1998). Interestingly, it has been found that in *adg1-1* mutants, neither the large nor the small subunit is detected by western blot analysis, and it has been proposed that this is because the stability of the large subunit is dependent on the small subunit (Wang *et al.*, 1998). A second mutant, *adg2*, has been characterized as having approximately 5 % the ADGase activity of wild type plants (Lin *et al.*, 1988b). These *adg2* mutants result from a single base pair mutation that affects the large subunit (Lin *et al.*, 1988b). Despite having only 5 % activity levels for ADGase, the amount of starch accumulated is still 40 % that in wild type plants (Lin *et al.*, 1988b). This indicates that the small subunit alone may have some level of activity (Li and Preiss, 1992).

It has been postulated numerous times that starch metabolism and floral initiation are linked (Bernier *et al.*, 1993; Friend, Bodson and Bernier, 1984; reviewed in Levy and Dean, 1998), though direct evidence for this is scarce. Several starch-deficient mutants have been examined, and under long-day conditions, flowering of these plants occurs at the same time as in wild-type plants (Yu *et al.*, 2000). Under short-day conditions, flowering in the mutants was delayed compared to the time at which flowering occurred in wild type plants (Yu *et al.*, 2000). It is thought that this

delay in the onset of flowering may be a direct result of the lack of starch accumulation (Yu *et al.*, 2000). In other words, it has been proposed that the small carbohydrate metabolites provided as a result of starch degradation act as a signal for floral initiation (Yu *et al.*, 2000). In the starch-deficient mutant *pgi1-1*, which shows a similar flowering phenotype as *adg1* (i.e. normal flowering under long-day conditions but delayed flowering under short-day conditions where GA normally triggers flowering), the late-flowering observed under short-day conditions can be reversed by an application of either 1% sucrose, glucose or fructose (Yu *et al.*, 2000). While similar studies have not yet been conducted with *adg1*, it is possible that application of simple sugars could reverse its late-flowering phenotype under short-day conditions as well.

This thesis involved three studies, the general objective of which was to characterize the differences between 5-azacytidine-induced early-flowering lines of flax and their corresponding control lines. The first study investigated levels of cytosine methylation in DNA from main-stem shoot-tips, leaves and cotyledons of RC and RE2 plants sampled at different times of the day, using an HPLC method. The aim of a second experiment was to confirm that flax is a facultative long-day plant by comparing growth and development of plants from the L and R lines grown in long-day conditions to those grown in short-day conditions. A second objective of this day-length study was to determine whether the early-flowering lines have a differential response to short-day conditions compared to their corresponding control lines. The third experiment examined levels of gene expression in five putative flowering genes throughout development in leaves, main-stem shoot-tips and buds of RC and RE2, using a semi-quantitative RT-PCR method.

# 2. 24-HOUR PROFILE OF DNA METHYLATION

## 2.1 Introduction

Through previous work it has been determined that levels of cytosine methylation in flax are dependent on both tissue type and plant development (Fieldes *et al.*, 2005; Brown, De Decker and Fieldes, 2008). In general, levels of methylation are lower in RE2 relative to those levels in RC (Fieldes *et al.*, 2005; Brown, De Decker and Fieldes, 2008). Interestingly, however, this hypomethylation can be absent in more mature plants and tissues (Brown, De Decker and Fieldes, 2008).

A study was performed recently to determine whether or not the chloroplast DNA of flax is methylated, and if so, to compare the levels in RE2 to those in RC (De Decker, 2007). In order to optimize chloroplast isolation for these experiments, plants (~ 60 plants per sample) were placed in the dark for various lengths of time (16 h - 68 h) prior to isolation (placing plants in the dark reduces the starch content and can limit bursting of the chloroplasts). For each dark period, approximately one plant per sample was used for total DNA extraction. A combination of main-stem shoot-tips, leaves of the main stem, and cotyledons (referred to as "all green tissues") from 21-day-old plants were used for both chloroplast and total DNA extraction. Results from this experiment indicated that the chloroplast DNA of flax is methylated, and that it is hypomethylated in RE2 compared to RC (De Decker, 2007). The unexpected observation was that unlike most previous findings (Brown, De Decker and Fieldes, 2008), the total DNA of RE2 was not hypomethylated relative to that of RC. Two possible explanations for this unexpected observation were proposed.

First, because the effects of light and dark on methylation levels had not been previously examined, it was possible that methylation levels in RC and RE2 are

always similar during dark periods. That is, that the difference in methylation level is always absent, or reduced, during the night. Since the transcription of many genes follows a circadian rhythm, it was postulated that changes in cytosine methylation regulate the expression of some genes that follow a daily pattern.

Second, the plants grown in the dark were exposed to higher than normal levels of humidity, and were also grown at a higher plant density than usual (both of these conditions were a side-effect of a dark box used to house the plants). Therefore, it is possible that the absence of hypomethylation in RE2 was a stress response and that the levels of methylation in RE2 increased in response to stress, thereby eliminating the difference in the levels normally seen between the two genotypes.

The study described here was performed to determine if the absence of hypomethylation observed in RE2 plants grown in the dark prior to harvesting was related to a time-of-day effect. More specifically, the study was done to determine whether the lack of hypomethylation in RE2 occurs on a nightly basis.

## 2.2 Materials & Methods

### *2.2.1 Growth Conditions*

Plants from two R lines (RC and RE2) were sown in plastic pots (8.5 x 8.5 inch square pots) filled with Vermiculite® (Holiday ®, Montreal, Ontario), and were grown in a growth chamber, using a 16/8 h light/dark; 24 °C/18 °C day/night cycle. The dark period was the normal 8-hour-night period set in the growth chamber. Plants were fertilized weekly using an inorganic nutrient solution modified from a recipe by Murashige and Skoog (1962; Appendix A), and were watered as needed with tap water.

### *2.2.2 Methylation levels in shoot tips, leaves and cotyledons of 24-day-old plants.*

Main-stem shoot-tip (tissue above the youngest visible internode), leaf (first four leaves below the main-shoot tip) and cotyledon tissues were harvested from 14-day-old plants at 0, 4, 8, 12, 16, 20 and 24 hours-after-dawn. The experiment was set up and sampled as two replicates. Fresh weight of harvested tissue was measured and samples were immediately ground in liquid nitrogen and transferred to 1.5 mL eppendorf tubes. DNA was extracted from each tissue using the Qiagen® (Mississauga, ON) DNeasy minikit protocol with some slight modifications (Appendix B) and was then hydrolyzed using a modification (Appendix C) of a method described by Matassi *et al.* (1992), which has been previously used for hydrolysis of flax DNA (Brown, De Decker and Fieldes, 2008).

### *2.2.3 HPLC Analysis*

In order to quantify levels of 5-methylcytosine (5mC), an established HPLC method was used (Brown, De Decker and Fieldes, 2008). All hydrolyzed DNA samples (299 µl after hydrolysis) were centrifuged (7500 x g, 30 minutes), and 50 µl of each sample was then automatically injected by a Varian® Prostar 410 autosampler into a Varian® Prostar 230 HPLC. Samples were run on a 150 x 4.6 mm column (Supelcosil C-18S), with a LC-18C Supelguard pre-column, held at 30 °C. Samples were eluted using a gradient of methanol and 50 mM $KH_2PO_4$ as described previously (Brown, De Decker and Fieldes, 2008) (Appendix D). The chromatography was monitored by a Varian® Prostar 350 UV-Vis Detector at an absorbance wavelength of 260 nm, except during a 2 min period where the 5mC deoxyriboside was detected at 280 nm. Star® Chromatography was used to calculate the area of each nucleoside peak, which was then used, in conjunction with the corresponding extinction

coefficients, to calculate an estimate of the concentration of each nucleoside (μM). These concentrations were used to calculate percentages of methylated cytidine (which correspond to the percentages of methylated cytosine) for each DNA sample, and the DNA concentration per 10 plants, as well as per 100 mg of fresh weight tissue.

Two runs of each DNA sample on the HPLC column were performed, and averages were used for data analysis. Analyses of variance (ANOVAs) and orthogonal comparisons were used to examine line and time-of-day components, as well as any interactions between the lines and the time of day.

## 2.3 Results

### *2.3.1 Comparisons of Cytosine Methylation Levels*

Previous work has shown that levels of cytosine methylation (5mC) differ significantly between the early-flowering line RE2 and its corresponding control line, RC, in tissues from main-stem shoot-tips and cotyledons (Brown, De Decker and Fieldes, 2008). The methylation patterns observed here were consistent with the previous observations; significant hypomethylation was seen in RE2 compared to RC in DNA extracted from main-stem shoot-tips ($F_{1/13}$=30.4**) (Figure 2a) and cotyledons ($F_{1/13}$=5.34*) (Figure 2c), but not in DNA from the top four leaves of the stem ($F_{1/13}$=2.42 ns) (Figure 2b).

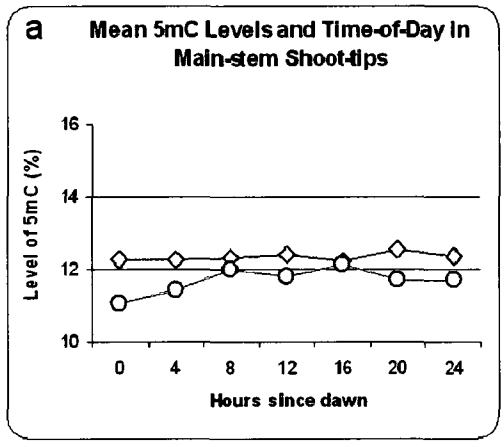
While no significant trends in the levels of cytosine methylation were observed in either the leaves or the cotyledons, the level of 5mC in the main-stem shoot-tips approached significance for a linear increase as the day progressed ($F_{1/13}$ = 4.30 ns) (Figure 2a).

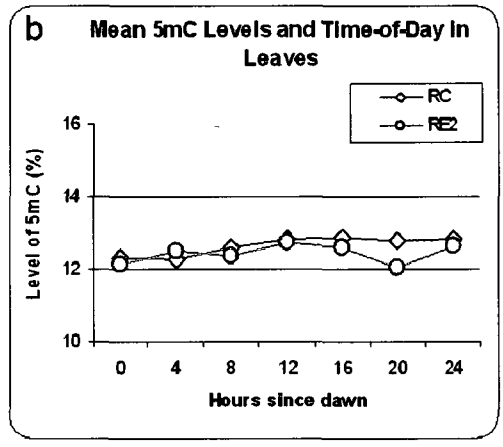
### *2.3.2 Comparisons of Other DNA and Tissue Characteristics*

In main-stem shoot-tips, no differences in weight (mg per 10 tips) were found between RC and RE2, or over the course of the 24-hour day (Figure 3a), but in the leaf and cotyledon samples, RC was found to have a higher overall weight compared to RE2 (leaves $F_{1/13}$=38.1**; cotyledons $F_{1/13}$=68.0**) (Figures 4a and 5a). Non-orthogonal comparisons revealed that the weight increased linearly in cotyledons from RC ($F_{1/13}$=9.50**), but that there was no significant linear trend in RE2 ($F_{1/13}$=3.49 ns) (Figure 5a). DNA concentration (μM) per 10 plants was found to be higher in RC when compared to RE2 in all three tissues (shoot tips $F_{1/13}$=13.7**; leaves $F_{1/13}$=38.2**; cotyledons $F_{1/13}$=9.30**) (Figures 3b, 4b and 5b). DNA concentration (μM) per 100 mg was also found to be higher in RC compared to RE2 in main-stem shoot-tips and leaves (shoot tips $F_{1/13}$=7.17*; leaves $F_{1/13}$=15.4**) (Figures 3c and 4c). Non-orthogonal comparisons showed that the DNA content increased linearly in cotyledons from RE2 ($F_{1/13}$=10.2**), but that there was no similar trend in RC (Figure 5c).

**Figure 2. Levels of Methylation in Shoot Tips, Leaves and Cotyledons Over a 24-hr Day.**

a) Hypomethylation of RE2 relative to RC was observed in main-stem shoot-tips (SE=0.2). b) No difference in methylation levels was detected between RC and RE2 in leaves (SE=0.27). c) Hypomethylation of RE2 relative to RC was detected in cotyledons (SE=0.61). Data plotted are means (n=2).


a
Mean 5mC Levels and Time-of-Day in Main-stem Shoot-tips
Level of 5mC (%)
16
14
12
10
0
4
8
12
16
20
24
Hours since dawn


b
Mean 5mC Levels and Time-of-Day in Leaves
RC
RE2
Level of 5mC (%)
16
14
12
10
0
4
8
12
16
20
24
Hours since dawn

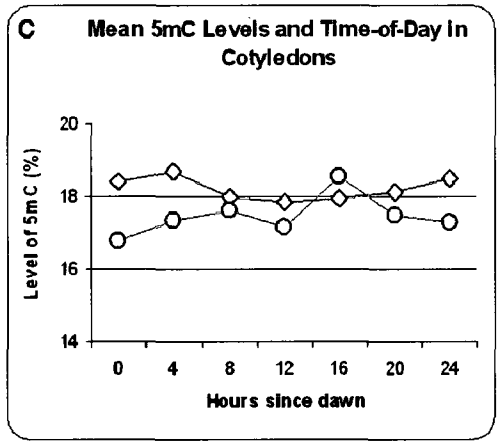

c
Mean 5mC Levels and Time-of-Day in Cotyledons
Level of 5mC (%)
20
18
16
14
0
4
8
12
16
20
24
Hours since dawn

**Figure 3. Characteristics of Shoot Tips from 14-day-old Plants Over a 24-hr Day.**

a) No significant trends over the 24-hour-period or differences between plant lines were detected for fresh weight (mg per 10 shoot tips) (SE=23.8). b) DNA recovered (μM per 10 shoot tips) was significantly higher in RC compared to RE2 (SE=11.9). c) DNA recovered (μM per 100 mg fresh weight) was significantly higher in RC compared to RE2 (SE=5.7). Data plotted are means (n=2).

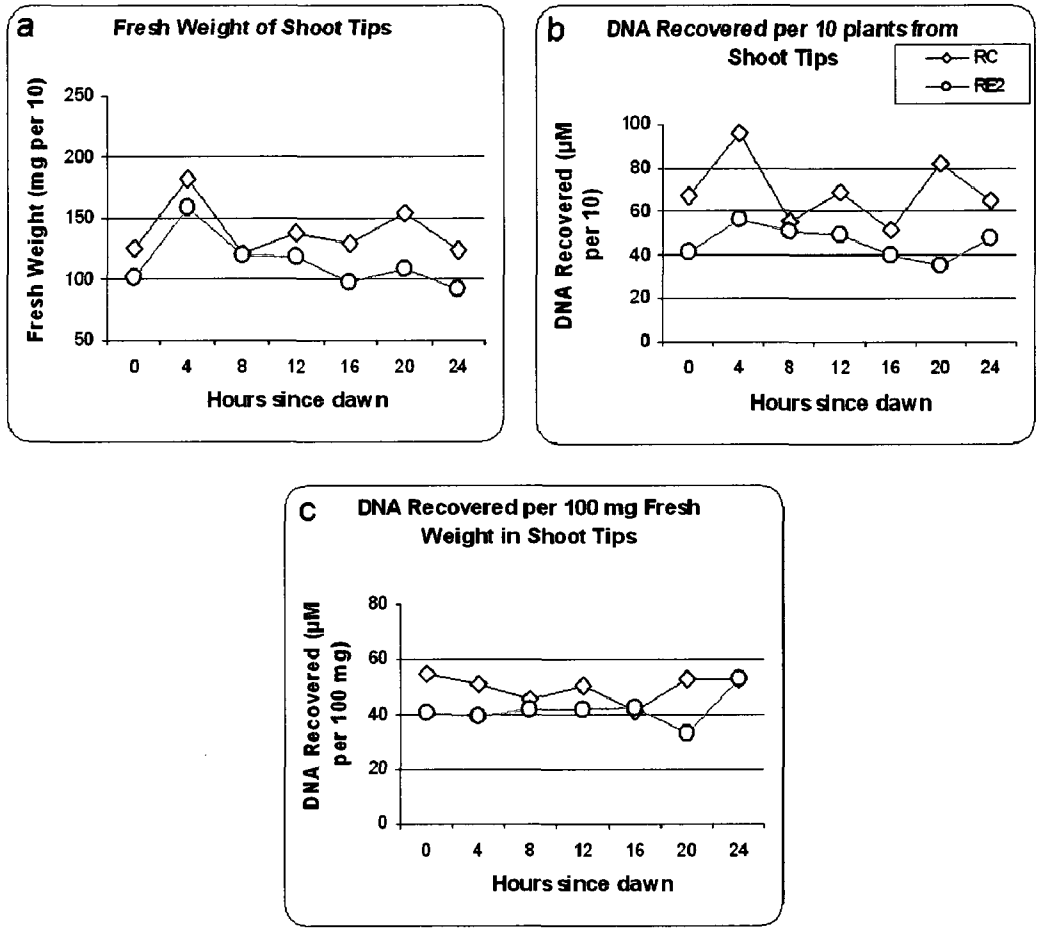

a
Fresh Weight of Shoot Tips
Fresh Weight (mg per 10)
250
200
150
100
50
0 4 8 12 16 20 24
Hours since dawn
b
DNA Recovered per 10 plants from Shoot Tips
RC
RE2
DNA Recovered (µM per 10)
100
80
60
40
20
0
0 4 8 12 16 20 24
Hours since dawn
c
DNA Recovered per 100 mg Fresh Weight in Shoot Tips
DNA Recovered (µM per 100 mg)
80
60
40
20
0
0 4 8 12 16 20 24
Hours since dawn

**Figure 4. Characteristics of Leaves from 14-day-old Plants Over a 24-hr Day.** a) Fresh weight (mg per 10 plants) of RC was found to be significantly higher than that of RE2 (SE=12.2). b) DNA recovered (μM per 10 plants) was significantly higher in RC compared to RE2 (SE=3.6). c) DNA recovered (μM per 100 mg fresh weight) was significantly higher in RC compared to RE2 (SE=1.4). Data plotted are means (n=2).

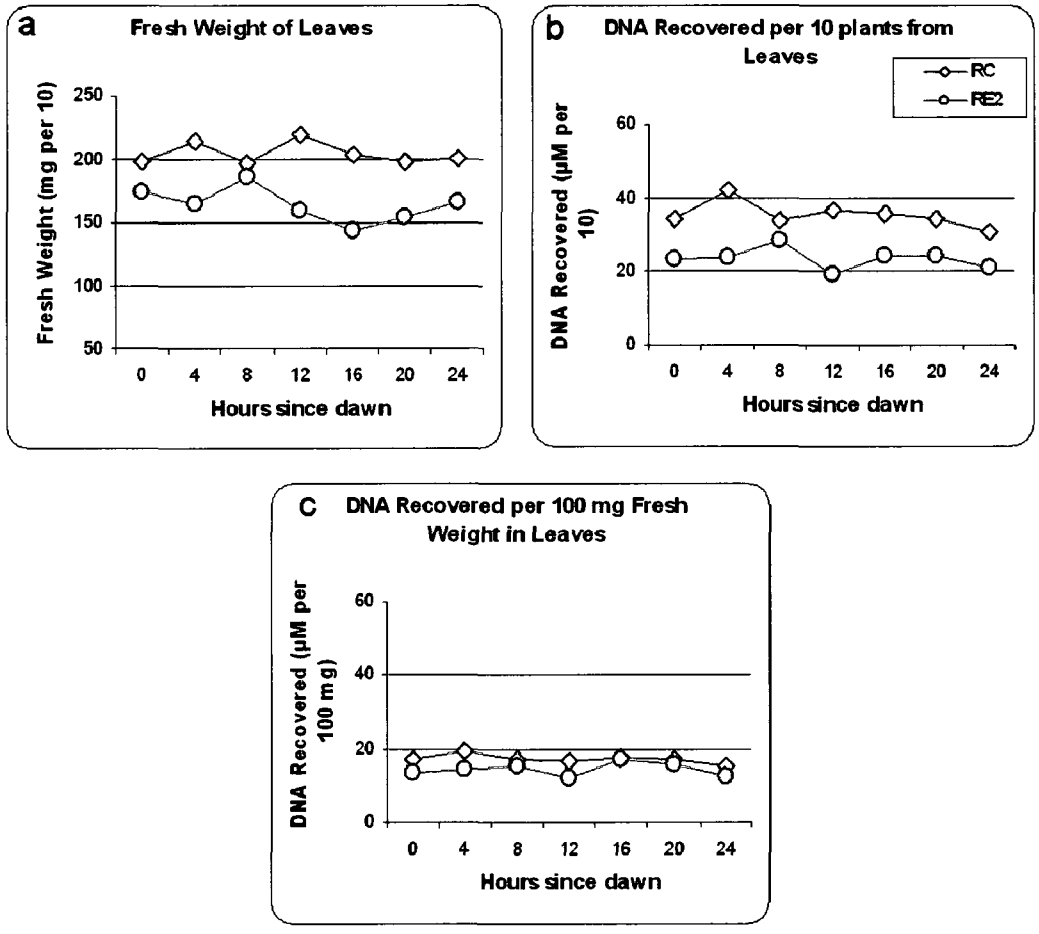
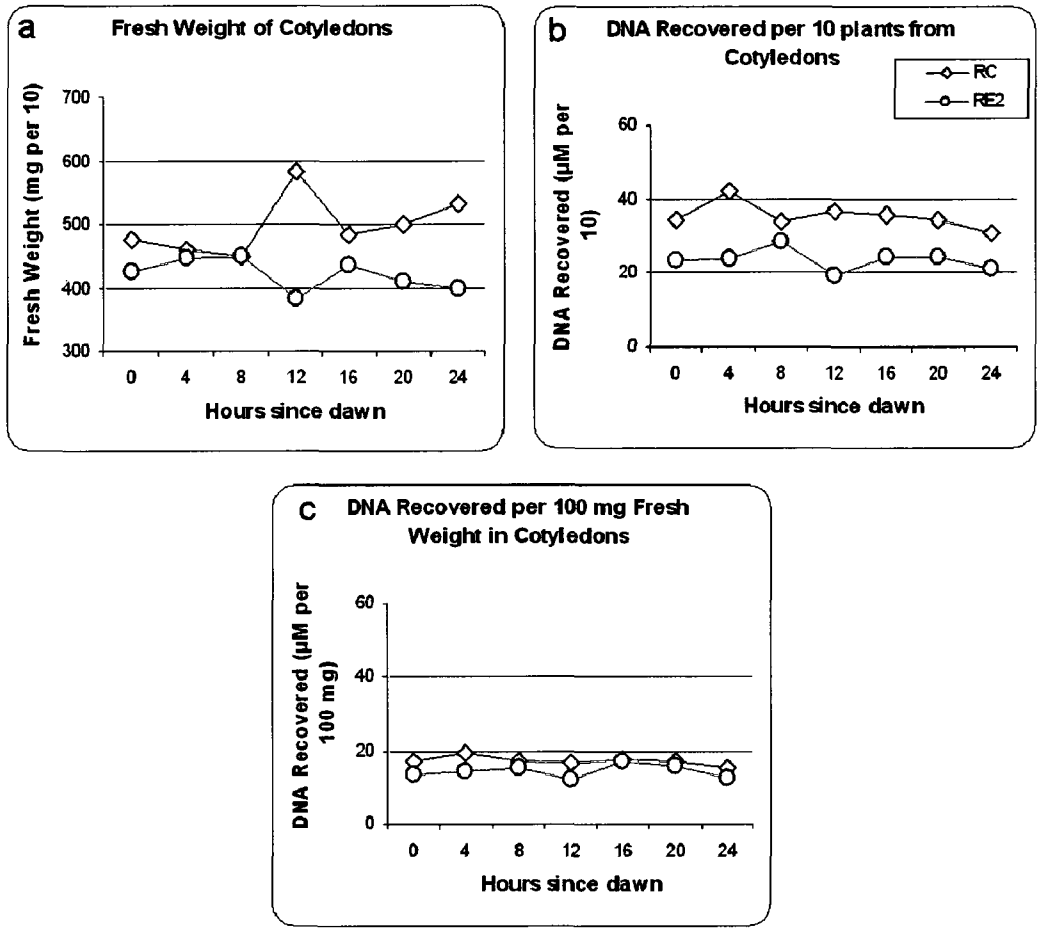

a
Fresh Weight of Leaves
Fresh Weight (mg per 10)
250
200
150
100
50
0
4
8
12
16
20
24
Hours since dawn
b
DNA Recovered per 10 plants from Leaves
RC
RE2
DNA Recovered (µM per 10)
60
40
20
0
0
4
8
12
16
20
24
Hours since dawn
C
DNA Recovered per 100 mg Fresh Weight in Leaves
DNA Recovered (µM per 100 mg)
60
40
20
0
0
4
8
12
16
20
24
Hours since dawn

**Figure 5. Characteristics of Cotyledons from 14-day-old Plants Over a 24-hr Day.**

a) Fresh weight (mg per 10 pairs of cotyledons) of RC was found to be significantly higher than that of RE2, with RC increasing linearly over the 24-hour-period (SE=17.4). b) DNA recovered (μM per 10 pairs of cotyledons) was significantly higher in RC compared to RE2 (SE=1.4). c) DNA recovered (μM per 100 mg fresh weight) increased linearly in RE2 (SE=0.3). Data plotted are means (n=2).


a
Fresh Weight of Cotyledons
Fresh Weight (mg per 10)
700
600
500
400
300
0 4 8 12 16 20 24
Hours since dawn
b
DNA Recovered per 10 plants from Cotyledons
RC
RE2
DNA Recovered (µM per 10)
60
40
20
0
0 4 8 12 16 20 24
Hours since dawn
c
DNA Recovered per 100 mg Fresh Weight in Cotyledons
DNA Recovered (µM per 100 mg)
60
40
20
0
0 4 8 12 16 20 24
Hours since dawn

## 2.4 Conclusions

In the previous experiment, the absence of a difference in the level of 5mC between RC and RE2 in total DNA samples was reported (De Decker, 2007). It was originally hypothesized that this lack of a difference in the methylation level might be due to the time of day at which the DNA was extracted. In other words, it was thought that there might be time-of-day effects on the level of cytosine methylation in green tissues from flax plants. However, the data from the study reported here did not support this idea.

As originally proposed, time-of-day effects were not the only possible explanation for the lack of hypomethylation of DNA in RE2 relative to that in RC. A connection between changes in the level of cytosine methylation and stress has previously been reported (Labra *et al.*, 2002). That is, the time spent in the dark may have led to a stress response that altered the levels of methylation.

Interestingly, it appears that over the 24-hour-period, the cells of the cotyledons of RC were expanding, while those of RE2 were actively dividing. This would explain why the fresh weight of cotyledons from RC was increasing, while that of RE2 remained relatively constant, and why the DNA content per 100 mg fresh weight increased in RE2, but remained relatively constant in RC. Previous findings have indicated that after 14 days-of-age the DNA content in the cotyledons (per 10 plants) begins to decrease (Brown, De Decker and Fieldes, 2008). Therefore, the data presented here may represent the final stages of cell division in cotyledons of RC.

Main-stem shoot-tips from both genotypes were approximately the same weight throughout the entire 24-hour-period; however, RC tip samples were found to contain more DNA per 10 tips, as well as more DNA per 100 mg fresh weight. These results are consistent with previous findings (Brown, De Decker and Fieldes, 2008),

and can be interpreted to mean that the tips of RC at 14 days-after-planting contain a higher number of less expanded cells than the tips of RE2.

Leaf samples of RC were found to weigh more, and contain more DNA per 10 plants and per 100 mg fresh weight than leaves from RE2. Essentially, this means that leaves sampled from RC contained a higher number of cells in general, as well as a higher number of less expanded cells.

# 3. SHORT-DAY EXPERIMENT

## 3.1 Introduction

Flowering in angiosperms is controlled by a complex network of regulatory pathways. Flowering-time genes in some of these pathways depend on endogenous cues, while others rely on input from the environment to determine the appropriate time for floral initiation. One such pathway is the photoperiod pathway, which initiates flowering based on the length of the night (nycto period). Flax is reportedly a long-day facultative species (Thomas, Carre and Jackson, 2006), meaning that it flowers earlier when exposed to long-day conditions, but will still flower if exposed only to short-day conditions.

Early evidence suggested that the flax lines differed in their photoperiodic response (Fieldes and Harvey, 2004). When both control and early-flowering lines were grown under partially 'shaded' conditions (i.e. lower than normal light levels), the control lines showed a greater amount of variation in the time at which they flowered, while the early-flowering lines showed less variation (Fieldes and Harvey, 2004). Essentially, flowering time was less affected by light intensity in the early-flowering lines than in the controls. This suggested that the regulation of flowering time in the early-flowering lines may be based on some intrinsic factor (such as the number of nodes produced), while flowering time in the control lines may be based on an extrinsic factor (Fieldes and Harvey, 2004).

Experiments were performed to confirm that flax is a long-day facultative plant, and to determine whether RC and RE2 have a differential response to short-day conditions. It was expected that plants grown under short-day conditions would show delayed flowering relative to the plants grown under long-day conditions, but that the

early-flowering lines would continue to flower early relative to their corresponding controls. If the early-flowering lines did not show delayed flowering in short-day conditions, it could indicate that a gene in the photoperiod pathway was affected by the azaC treatment. In other words, a gene may have been 'turned on' in this flowering pathway, specifically, that allows flax to flower early despite being grown under non-inductive short days.

## 3.2 Materials & Methods

### *3.2.1 Growth Conditions*

An experiment with two replicates was grown in the greenhouse to examine the responses of the three L lines (LC, LE1 and LE2) and the three R lines (RC, RE1 and RE2) to altered day length. In treatment 1, plants were exposed to the ambient long-day conditions (i.e. the natural light conditions in the greenhouse at Wilfrid Laurier University between May and August 2008) and in treatment 2 the plants were exposed to short-day conditions (8/16 h day/night cycle). The plants in the short-day exposure-group were kept in the greenhouse between the hours of 8:30 am to 4:30 pm each day, and were then placed in a "dark room" between the hours of 4:30 pm to 8:30 am where they received an artificial 16-h period of darkness. All plants were grown, six plants were pot, in 5" diameter, round pots, filled with Vermiculite (Holiday ®, Montreal, Ontario). Plants were watered with tap water as needed, and were fertilized on a weekly basis using a modified inorganic nutrient solution from Murashige and Skoog (1962; Appendix A).

A second, similar experiment was grown in the Wilfrid Laurier University greenhouse during the spring and summer of 2009, using the same experimental

design and the same six flax lines. It is important to mention that the growing conditions in the spring and summer of 2009 were quite different from those in 2008. For instance, in 2009 temperatures at the seedling stage of development were higher than usual, and the summer was relatively cool and damp.

### *3.3.2 Data Collection & Analysis*

The time required to flower was measured for all plants as the number of days between sowing and first anthesis. The total height (measured as the height between the cotyledons and the tallest part of the plant) was measured at 26, 33 and 40 days-after-planting for all plants. Total height was also measured at maturity, along with main-stem height (measured as the height between the cotyledons and the base of the inflorescence) and the number of leaves on the main stem.

Seeds were collected from all plants, and total seed weight per plant along with above ground biomass (not including the leaves) per plant (as dry weight), were used to calculate estimates of harvest index:

$$\% \text{ HI} = (\text{seed weight} / \text{aerial biomass}) \times 100$$

Measurements of short-wave infrared radiation that were recorded by the University of Waterloo Weather Station using a horizontally-mounted pyronometer, at 15-minute intervals between the months of May and September 2008, were used to calculate an estimate of the total amount of light energy that the plants were exposed to in the greenhouse. All of these measurements were performed on the plants grown in 2008.

The plants grown in 2009 were used to examine possible effects of the treatment on various leaf and stem characteristics. The number of leaves on each

plant was counted on a weekly basis from 14 to 42 days after planting. At maturity, several leaf characteristics were measured at the node closest to 70% of the total number of nodes produced. These characteristics included leaf width, leaf length, and stem diameter.

ANOVAs were used to analyze the data for the various growth parameters and examined the differences between lines, as well as between treated and untreated plants.

## 3.3 Results

### *3.3.1 Effects of Short-Day Treatment on Height, Leaf Number, and Flowering Age*

Measurements of total plant height (the height between the cotyledons and the tallest point of the plant) at 26, 33 and 40 days-after-planting (dap) indicated that compared to ambient conditions, the reduced day length significantly reduced stem elongation during vegetative growth of all of the genotypes examined (For 26 days $F_{1/11}$=43.3**, 33 days $F_{1/11}$=89.1**, and for 40 days $F_{1/11}$=168 **) (Figures 6 and 7). No significant interactions were detected.

The majority of plants grown in short-day conditions flowered while being exposed to 16-hour nights; however, several RC plants that had not flowered yet, but showed evidence of bud development, were moved back to ambient light conditions, ultimately flowering in late August. In both R and L lines, the short-day treatment also significantly delayed flowering ($F_{1/11}$=817 **) (Figures 8a and 9a) and increased the number of leaves produced on the main stem ($F_{1/11}$=317 **) (Figures 8b and 9b). Both of these treatment effects were more extreme in the control lines than in the corresponding early-flowering line (interactions for flowering, in R: $F_{1/11}$=162 **, in L: $F_{1/11}$=67.6**; interactions for leaf number, in R: $F_{1/11}$=42.5**, in L: $F_{1/11}$=90.4**).

Significant interactions were detected between the short-day treatment effect and the R lines for total plant height at maturity ($F_{1/11}$=5.11*), and non-orthogonal comparisons indicated that plant height was reduced by the treatment in the early-flowering R lines ($F_{1/11}$= 24.4**), but was not altered in RC ($F_{1/11}$<1.0 ns) (Figure 8c). A significant interaction showed that the R and L lines responded differently to the treatment in terms of total height ($F_{1/11}$=13.7**). The treatment had no effect on the total plant height in any of the L lines (non-orthogonal $F_{1/11}$<1.0 ns) (Figure 9c). A significant interaction ($F_{1/11}$=18.9**) indicated that it significantly increased main-stem height in RC (non-orthogonal $F_{1/11}$=5.17*), but significantly decreased main-stem height in RE1 and RE2 (non-orthogonal $F_{1/11}$=18.6**) (Figure 8d). It was also found that the response of LC to the treatment differed from that of the early-flowering L lines in terms of main stem height (Interaction $F_{1/11}$=40.6**), the treatment increased height in LC (non-orthogonal $F_{1/11}$=48.0**), but had no effect on either LE1 or LE2 (non-orthogonal $F_{1/11}$<1.53 ns) (Figure 9d).

### *3.3.2 Effects of Short-Day Treatment on Seed Yield*

The short-day treatment decreased inflorescence height in all three R lines (Figure 10a), and interactions between the treatment effect and the L lines ($F_{1/11}$=53.5**) indicated that the response of LC differed from that of LE1 and LE2 and that the responses of the two early-flowering lines also differed ($F_{1/11}$=11.7**). More specifically, non-orthogonal comparisons showed that the treatment decreased inflorescence height in LC ($F_{1/11}$= 53.9**), increased inflorescence height in LE2 ($F_{1/11}$=16.3**), and had no effect on the inflorescence height in LE1 ($F_{1/11}$<1.0 ns) (Figure 11a).

The number of seeds produced per capsule was reduced as a result of the treatment in all plant lines examined ($F_{1/11}$=37.7**) (Figures 10b and 11b). The

number of filled seeds per plant was decreased in all lines ($F_{1/11}$=49.6**), with all L lines being effected equally (no interactions) (Figure 11c), and with RC being more affected than RE1 and RE2 (interaction $F_{1/11}$=8.86*) (Figure 10c). Significant interactions ($F_{1/11}$=12.0**) revealed that the treatment decreased seed weight per 100 seeds in RC (non-orthogonal $F_{1/11}$=5.78*) and increased it in both early-flowering R lines (non-orthogonal $F_{1/11}$=6.70*) (Figure 12a). Seed weight per 100 seeds was significantly decreased in all of the L lines ($F_{1/11}$=6.42*) (Figure 13a). Harvest index was reduced in response to the treatment in all plant lines ($F_{1/11}$=252**), but the reduction was less severe in LE1 and RE2 than in LE2 and RE1, respectively (interactions for R: $F_{1/11}$=13.4**, L: $F_{1/11}$=4.98*) (Figures 12b and 13b).

Although the amount of incoming shortwave radiation received by the early-flowering R-line plants, by the time of first anthesis, was the same regardless of whether the plants were treated, or untreated (non-orthogonal $F_{1/11}$=4.21 ns), by flowering the RC plants exposed to short-day conditions had in fact received significantly more radiation than those grown under ambient conditions (non-orthogonal $F_{1/11}$=92.1**) (Figure 12c). All treated L line plants had received significantly more radiation by the time flowering occurred than the corresponding untreated L line plants ($F_{1/11}$=69.9**); however, the difference in radiation exposure between treated and untreated plants was more extreme in LC than in LE1 and LE2 (Interaction $F_{1/11}$=18.6**) (Figure 13c).

### 3.3.3 *Effects of Short-Day Treatment on Leaf Characteristics*

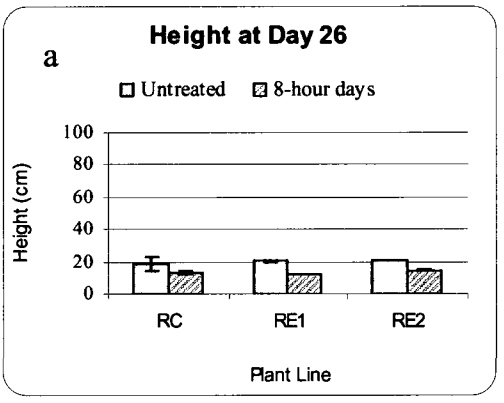
The data collected from the original day-length experiment (2008) did not include leaf and stem measurements. For this reason when the 2009 experiment was conducted, measurements for maximum leaf width and leaf length, as well as stem diameter were recorded. In addition, the same characteristics were examined that had

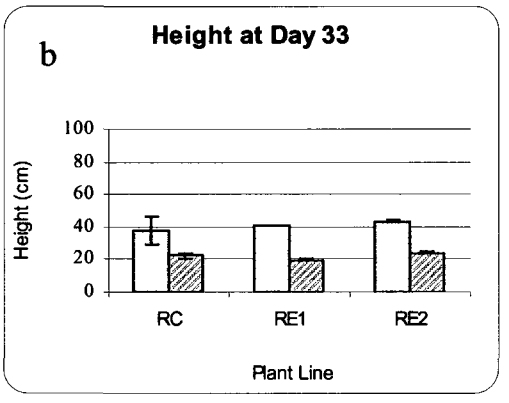
been examined during the 2008 experiment, and the data were found to be fairly consistent between the two experiments.

All data for leaf characteristics were obtained from the node closest to 70 % of the total number of nodes produced by the plant. Interestingly, in terms of leaf width, while there was an overall treatment effect for both R and L lines ($F_{1/11}$=19.8**) (Figures 14a and 15a), but interactions between the R lines and treatment effects ($F_{1/11}$=12.3**) revealed that the only plant line affected was RC (non-orthogonal $F_{1/11}$= 30.8**) (Figure 14a). On the contrary, the treatment significantly decreased leaf length in all plant lines examined ($F_{1/11}$=56.6**) (Figures 14b and 15b). It was also found that the treatment significantly decreased stem diameter at the node at 70 % in RC (non-orthogonal $F_{1/11}$=22.2**), but not in any other plant line ($F_{1/11}$=2.44 ns) (Figures 14c and 15c).

**Figure 6. Effects of 8-hr Days on Plant Height during Vegetative Growth in the R lines.**

Significantly reduced total plant height at a) 26 days after planting (dap), b) 33 dap, and c) 40 dap. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Height at Day 26
Untreated
8-hour days
Height (cm)
100
80
60
40
20
0
RC
RE1
RE2
Plant Line


b
Height at Day 33
Height (cm)
100
80
60
40
20
0
RC
RE1
RE2
Plant Line

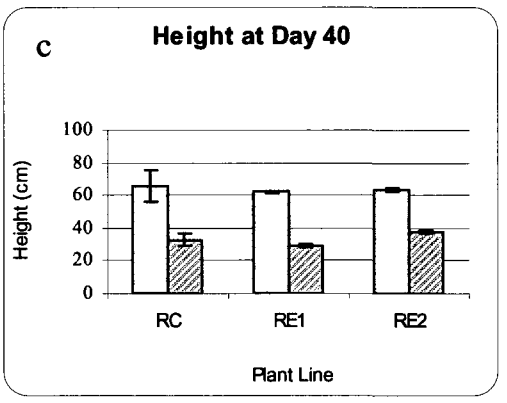
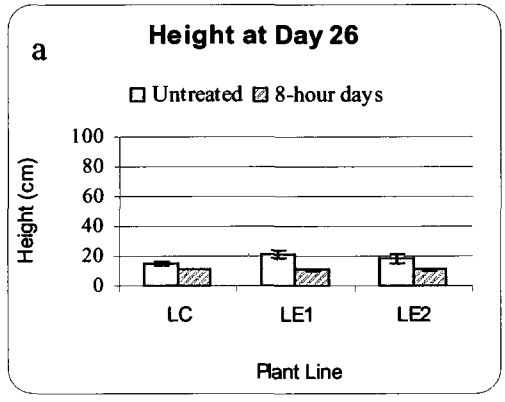
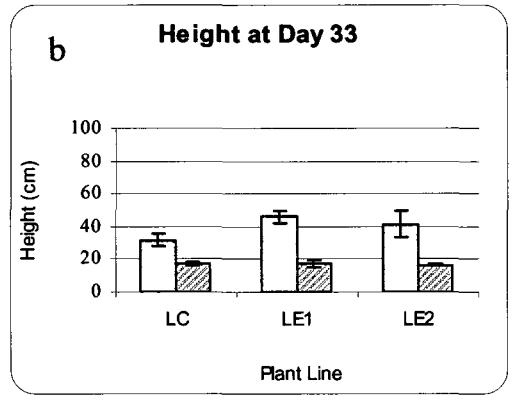

c
Height at Day 40
Height (cm)
100
80
60
40
20
0
RC
RE1
RE2
Plant Line

**Figure 7. Effects of 8-hr Days on Plant Height during Vegetative Growth in the L lines.**

Significantly reduced total plant height at a) 26 days after planting (dap), b) 33 dap, and c) 40 dap. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Height at Day 26
Untreated
8-hour days
Height (cm)
100
80
60
40
20
0
LC
LE1
LE2
Plant Line


b
Height at Day 33
Height (cm)
100
80
60
40
20
0
LC
LE1
LE2
Plant Line

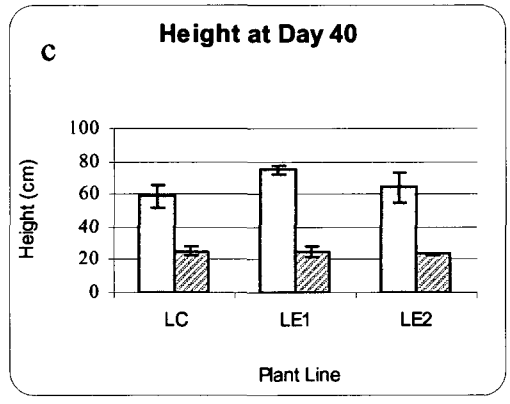
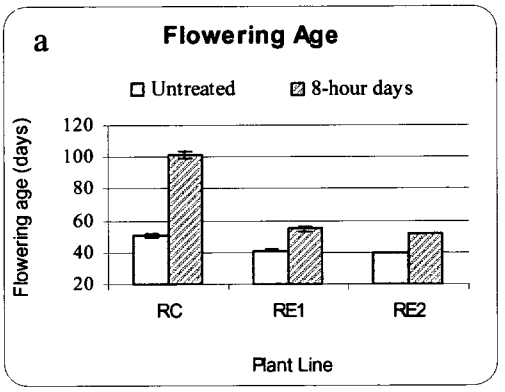
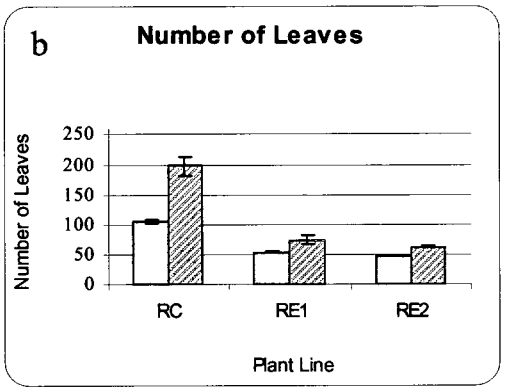
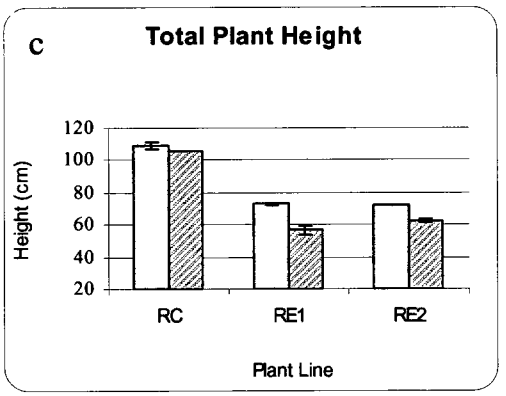

c
Height at Day 40
Height (cm)
100
80
60
40
20
0
LC
LE1
LE2
Plant Line

**Figure 8. Effects of 8-hr Days on Various Growth Parameters in R lines.**

a) A significant delay in flowering and (b) a significant increase in the number of leaves produced during vegetative growth were observed in all three R lines. (c) Total plant height at maturity was reduced in RE1 and RE2, but not in RC, while (d) main-stem height was increased in RC, but decreased in RE1 and RE2. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Flowering Age
Untreated
8-hour days
Flowering age (days)
120
100
80
60
40
20
RC
RE1
RE2
Plant Line


b
Number of Leaves
Number of Leaves
250
200
150
100
50
0
RC
RE1
RE2
Plant Line


c
Total Plant Height
Height (cm)
120
100
80
60
40
20
RC
RE1
RE2
Plant Line

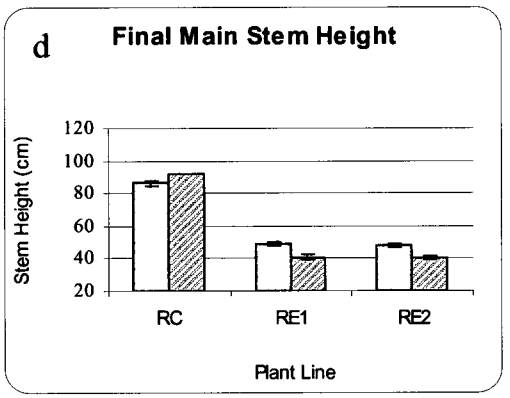

d
Final Main Stem Height
Stem Height (cm)
120
100
80
60
40
20
RC
RE1
RE2
Plant Line

**Figure 9. Effects of 8-hr Days on Various Growth Parameters in L lines.**

a) A significant delay in flowering and (b) a significant increase in the number of leaves produced during vegetative growth were observed in all three L lines. (c) Total plant height at maturity was unaffected by the treatment in all L lines, while (d) main stem height was increased in LC, but was unaffected in LE1 and LE2. The error bars shown on plots are ± SE for the means plotted (n=2).

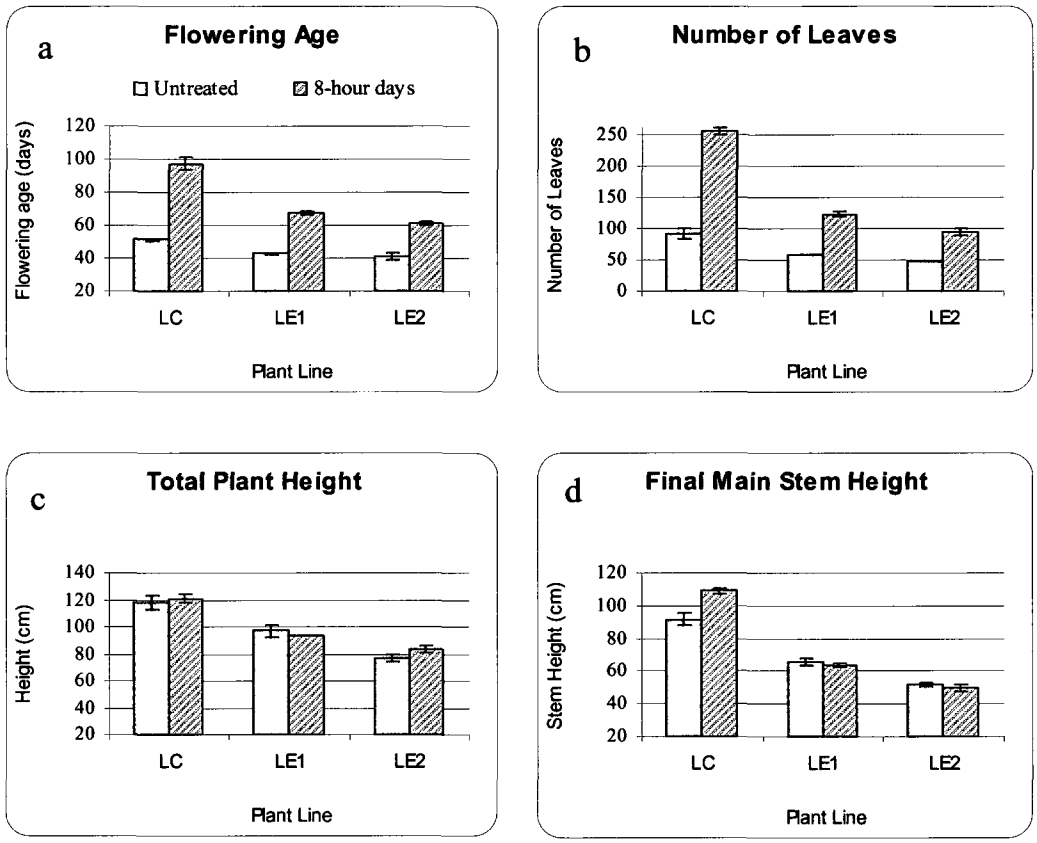
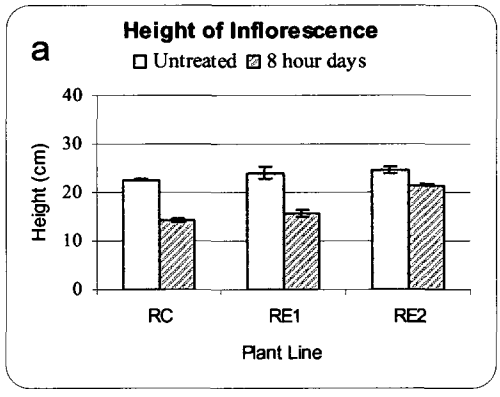
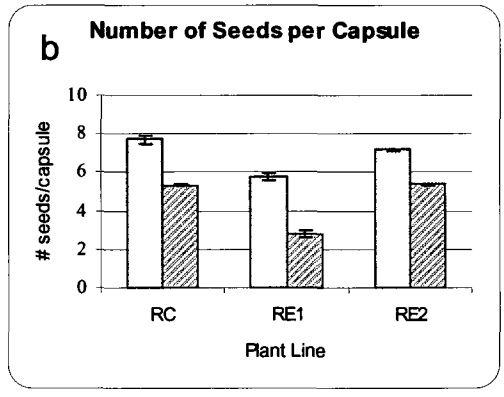

a
Flowering Age
Untreated
8-hour days
Flowering age (days)
120
100
80
60
40
20
LC
LE1
LE2
Plant Line
b
Number of Leaves
Number of Leaves
250
200
150
100
50
0
LC
LE1
LE2
Plant Line
c
Total Plant Height
Height (cm)
140
120
100
80
60
40
20
LC
LE1
LE2
Plant Line
d
Final Main Stem Height
Stem Height (cm)
120
100
80
60
40
20
LC
LE1
LE2
Plant Line

**Figure 10. Effects of 8-hr Days on Seed Yield and Inflorescence Height in R lines** a) Inflorescence height was decreased in all three R lines, b) seed number per capsule was decreased in all three R lines, and c) the number of filled seeds per plant was reduced in all lines, however, to a greater degree in RC than RE1 and RE2. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Height of Inflorescence
Untreated
8 hour days
Height (cm)
40
30
20
10
0
RC
RE1
RE2
Plant Line


b
Number of Seeds per Capsule
# seeds/capsule
10
8
6
4
2
0
RC
RE1
RE2
Plant Line

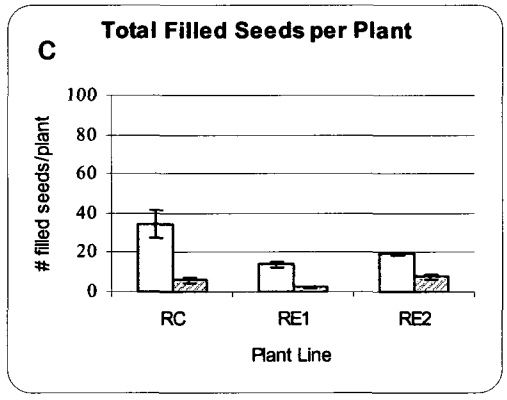
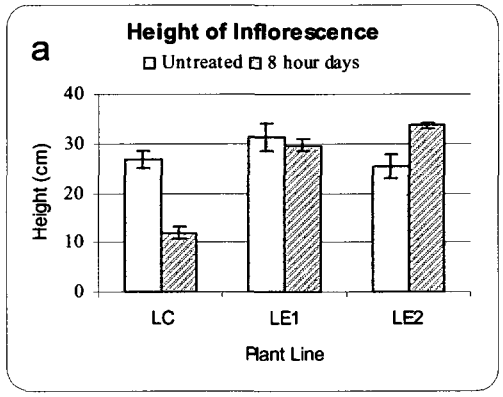
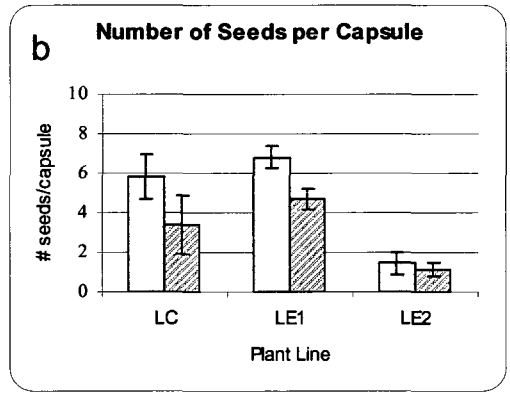

c
Total Filled Seeds per Plant
# filled seeds/plant
100
80
60
40
20
0
RC
RE1
RE2
Plant Line

**Figure 11. Effects of 8-hr Days on Seed Yield and Inflorescence Height in L lines.**

a) Inflorescence height was decreased LC, increased in LE2, and there was no effect on LE1. b) Seed number per capsule and c) the number of filled seeds per plant were decreased in all three L lines. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Height of Inflorescence
Untreated 8 hour days
Height (cm)
40
30
20
10
0
LC
LE1
LE2
Plant Line


b
Number of Seeds per Capsule
# seeds/capsule
10
8
6
4
2
0
LC
LE1
LE2
Plant Line

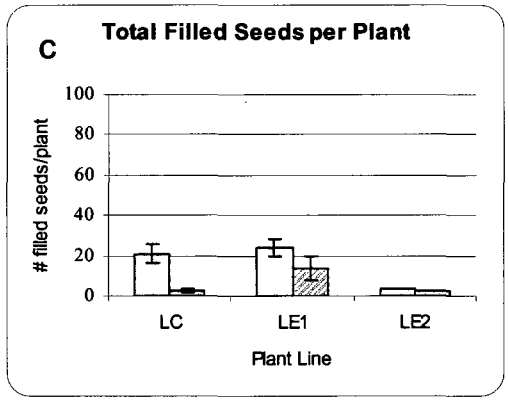
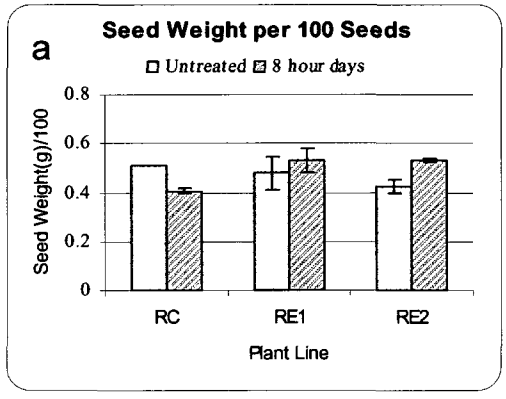
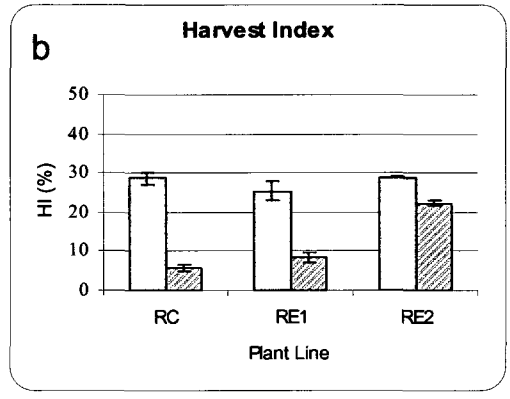

c
Total Filled Seeds per Plant
# filled seeds/plant
100
80
60
40
20
0
LC
LE1
LE2
Plant Line

**Figure 12. Effects of 8-hr Days on Seed Weight, Harvest Index and Radiation Received in R lines.**

a) Seed weight per 100 seeds decreased in RC, and increased in RE1 and RE2. b) Harvest was decreased in all three R lines; however, to a greater degree in RC and RE1 than in RE2. c) The amount of incoming shortwave radiation received was greater in treated RC plants than in the untreated RC plants. There was no significant difference in the amount of radiation received between treated and untreated early-flowering R lines. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Seed Weight per 100 Seeds
Untreated 8 hour days
Seed Weight(g)/100
0.8
0.6
0.4
0.2
0
RC
RE1
RE2
Plant Line


b
Harvest Index
HI (%)
50
40
30
20
10
0
RC
RE1
RE2
Plant Line

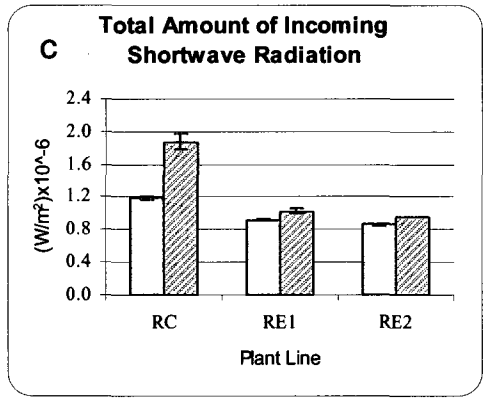
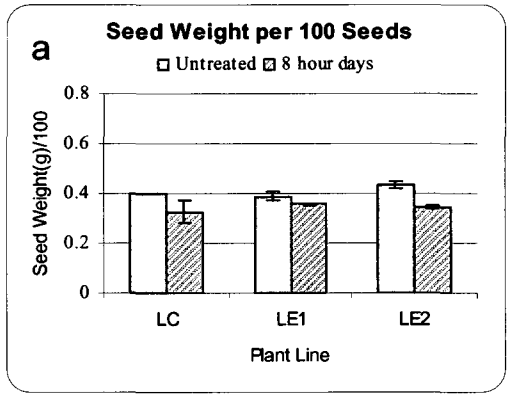
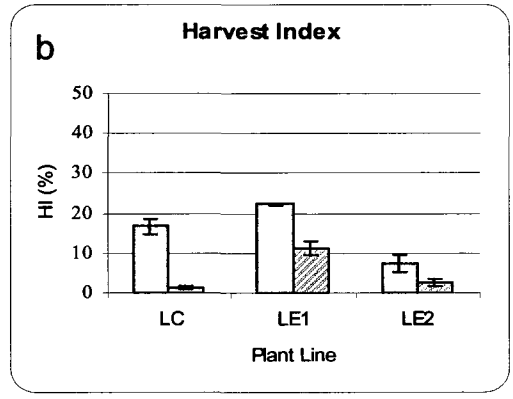

c
Total Amount of Incoming Shortwave Radiation
(W/m²)x10^-6
2.4
2.0
1.6
1.2
0.8
0.4
0.0
RC
RE1
RE2
Plant Line

**Figure 13. Effects of 8-hr-days on Seed Weight, Harvest Index and Radiation Received in L lines.**

a) The short day treatment decreased the seed weight per 100 seeds in all three L lines. b) Harvest was decreased in all three L lines; however, to a greater degree in LC and LE1 than in LE2. c) The amount of incoming shortwave radiation received was greater in all treated L plants than in the untreated L plants. The difference for radiation received between treated and untreated plants was more extreme in LC than in either early-flowering L line. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Seed Weight per 100 Seeds
Untreated 8 hour days
Seed Weight(g)/100
0.8
0.6
0.4
0.2
0
LC
LE1
LE2
Plant Line


b
Harvest Index
HI (%)
50
40
30
20
10
0
LC
LE1
LE2
Plant Line

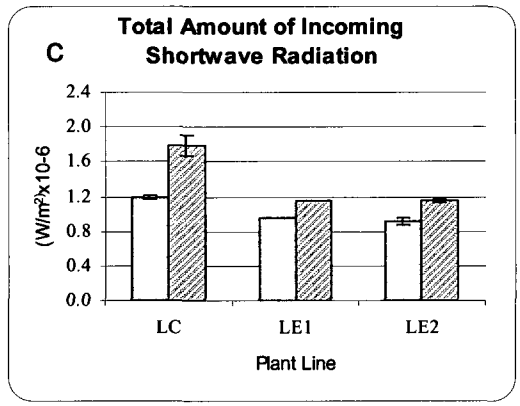
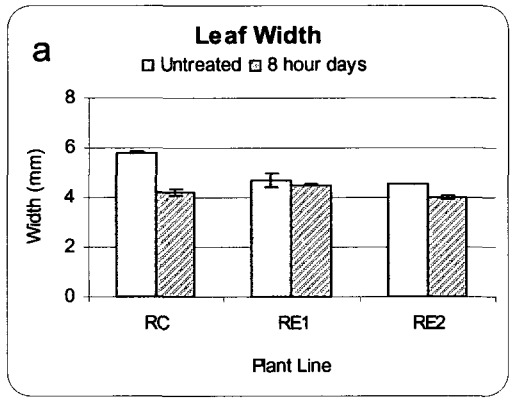
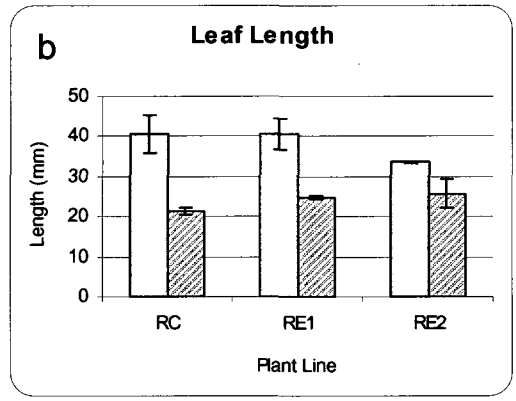

c
Total Amount of Incoming Shortwave Radiation
(W/m²x10-6
2.4
2.0
1.6
1.2
0.8
0.4
0.0
LC
LE1
LE2
Plant Line

**Figure 14. Effects of 8-hr-days on Various Leaf and Stem Characteristics in R lines.**

Various characteristics were measured at the node at 70% the total number of nodes. a) The treatment significantly decreased leaf width in RC and (b) significantly decreased leaf length in all three R lines. (c) Stem diameter decreased in RC only. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Leaf Width
Untreated 8 hour days
Width (mm)
8
6
4
2
0
RC
RE1
RE2
Plant Line


b
Leaf Length
Length (mm)
50
40
30
20
10
0
RC
RE1
RE2
Plant Line

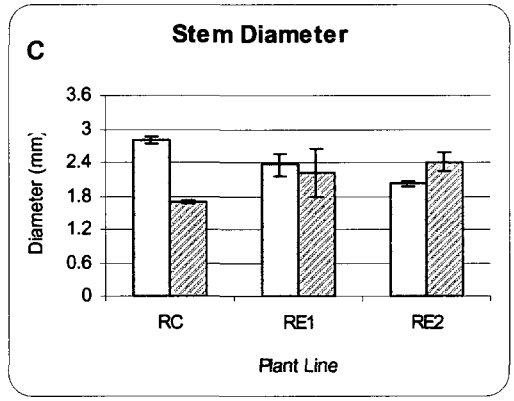
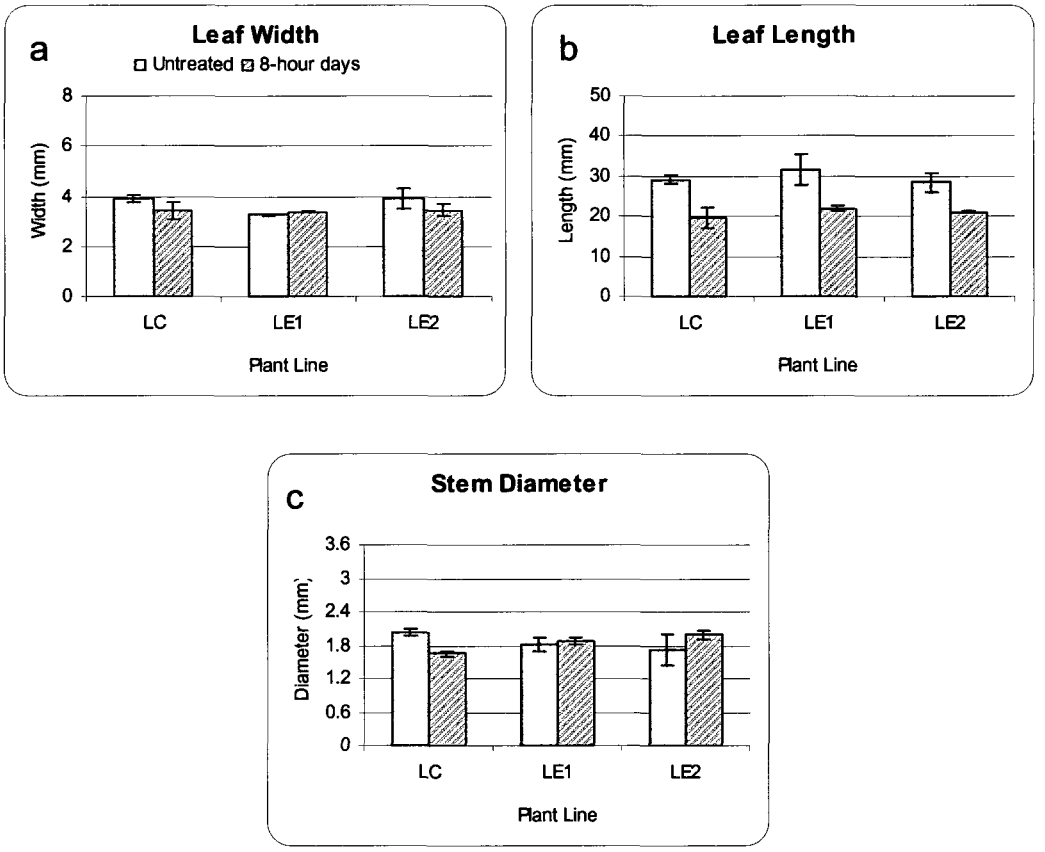

c
Stem Diameter
Diameter (mm)
3.6
3
2.4
1.8
1.2
0.6
0
RC
RE1
RE2
Plant Line

**Figure 15. Effects of 8-hr-days on Various Leaf and Stem Characteristics in L lines.**

Various characteristics were measured at the node at 70% the total number of nodes. a) The treatment did not have any effect on leaf width in any L line and (b) significantly decreased leaf length in all three L lines. (c) The treatment did not have any effect on stem diameter in the L lines. The error bars shown on plots are ± SE for the means plotted (n=2).


a
Leaf Width
Untreated 8-hour days
Width (mm)
8
6
4
2
0
LC
LE1
LE2
Plant Line
b
Leaf Length
Length (mm)
50
40
30
20
10
0
LC
LE1
LE2
Plant Line
c
Stem Diameter
Diameter (mm)
3.6
3
2.4
1.8
1.2
0.6
0
LC
LE1
LE2
Plant Line

### 3.4 Conclusion

It was clear from the data presented here that flax is indeed a long-day facultative plant, as it flowered even when grown in short-day conditions, but flowered earlier when grown under long-day conditions. It is also clear that growing flax plants in short-day conditions has a marked affect on their growth and development, and on their ability to produce viable seed. The short-day treatment delayed the onset of flowering and increased the number of leaves produced on the main stem in all six lines examined. Most interestingly though is the fact that both of these parameters were less affected in the early-flowering lines than in their corresponding controls. This provides valuable information that may contribute to a better understanding of the genetic, and epigenetic, differences between the control and early-flowering lines.

Flax is an agriculturally important plant, and for that reason the potential influence of various environmental conditions on both plant growth and seed production are of interest. In general, seed yield and harvest index were reduced as a result of the short-day treatment. However, there were some exceptions. For example, RE2 responded to the treatment better than RE1 and RC, in terms of seed production. While RE2 did show a decrease in the number of seeds per plant produced and a decreased harvest index, these decreases were not as great as they were for other plant lines. In addition, the seed weight per 100 seeds actually increased in RE2 in short days, whereas it decreased in most other lines examined. Because RE2 was derived from Royal, an oilseed cultivar, this is especially interesting because the increased seed weight could potentially mean that there is more oil available for extraction. These observations may mean that RE2 has increased tolerance to suboptimal light

conditions. Crops with the ability to perform optimally even when conditions are poor are useful and important.

# 4. GENE EXPRESSION STUDY

## 4.1 Introduction

Studies on flax have shown that the early-flowering lines are, in general, hypomethylated compared to their corresponding control lines (Fieldes *et al.*, 2005; Brown, De Decker and Fieldes, 2008). It is also known that DNA demethylation can be linked to transcriptional activation (reviewed in Suzuki and Bird, 2008). It has been proposed that the DNA demethylation of the early-flowering flax lines has led to upregulation of at least one gene that plays a role in the transition to the reproductive phase (Amyot, 1997; Fieldes and Amyot, 1999). However, little is known about the genes involved in the regulation of flowering time in flax. Therefore, it is not known which gene(s), if any, in the flax genome were affected by the DNA demethylation treatment. In order to understand the potential role of cytosine methylation in the regulation of flowering time in flax, it is first necessary to determine the genes involved. Recent work has identified *LEAFY* (*LFY*) in the flax genome (De Decker, 2007). Analysis of *LFY* expression in main-stem shoot-tips indicated that its expression in RE2 increased prior to the onset of flowering, while the same trend was not observed in RC (Figure 16) (De Decker, 2007). Based on this finding it would be easy to presume that either *LFY*, or a gene upstream of *LFY*, was affected by the azaC treatment, but until further work is done this hypothesis can not be confirmed with any certainty.

For the purposes of this study, five different genes of interest were used in an analysis of gene expression. Two of these genes are known to be involved in the regulation of flowering time in other plant species: *ADG1*, and *GAI* (Yu *et al.*, 2000; Wilson, Heckman and Somerville, 1992). The three other genes selected were *SOC1*,

a known floral pathway integrator, *AP1*, a floral-meristem identity gene, as well as a floral-organ identity gene, and a *COL* (*CONSTANS-LIKE*) gene. This combination of genes was selected because it spans a wide-variety of flowering pathways, including the photoperiod, autonomous, and gibberellin pathways. Since we do not know specifically which flowering pathway was altered in the early-flowering lines, covering a variety of pathways is of importance. There were originally several other candidate genes, but the final selection process was based on the success of designing suitable primers for detection in flax.

Until it is clear which flowering pathways are involved in the timing of the floral transition in flax, it is not clear which genes might have been affected by demethylation. This being said, genes with demethylated DNA tend to show increased expression. Because the floral transition occurs earlier in the early-flowering lines, it is expected that expression of most genes that increase with the onset of flowering will do so earlier in these lines. Any genes with higher levels of expression in the early-flowering lines than in the controls are also of interest. *SOC1* and *AP1* have been studied thoroughly, and for this reason we have a good idea of what can be expected in terms of their expression in flax. *SOC1* in *Arabidopsis* has been found to be expressed in most tissues but is mostly expressed in leaves and shoot tips, with levels increasing towards the onset of flowering (Borner *et al.*, 2000). It can therefore be expected that expression of *SOC1* will be detected in both leaves and shoot tips in flax, and that an increase in expression will occur earlier in the early-flowering lines than in the controls. If a gene involved in the regulation of *SOC1* has been affected by the demethylation treatment, then the expression of *SOC1* may reach higher levels in the early-flowering lines than in control lines.

Previous findings have determined that *AP1* can be detected in young flowers immediately after the transition to flowering in *Arabidopsis* (Mandel *et al.*, 1992). Mandel *et al.* (1992) did not detect expression of *AP1* in leaves, and for this reason, it can be expected that expression might not be detected in the leaves of flax, but should still be detected in the shoot tips of plants during the floral transition. Of the various floral organs, *AP1* expression was detected specifically in the sepals and petals (Mandel *et al.*, 1992), meaning that some expression should be detected in flower buds as well.

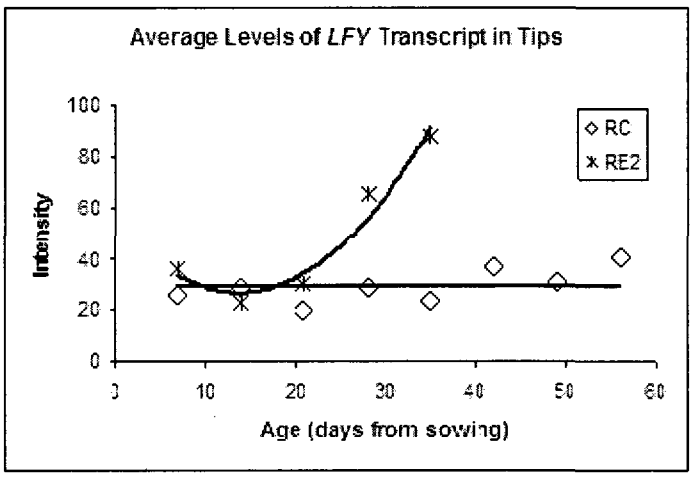
There are numerous *COL* genes that have been studied in a variety of plant species. Most of these genes appear to be involved in the process of flowering, and transcription of some of these genes is regulated by circadian rhythm (Suárez-López *et al.*, 2001; Ledger *et al.*, 2001). The flax DNA sequence with similarity to a *COL* gene, *COL1*, was also similar to a number of other *COL* genes. Therefore, without being sure which *COL* gene the flax sequence belongs to, it is hard to predict the expression profile of this gene. It was, however, possible that its expression profile would clarify this question.

Expression of *GAI* has also been studied, although not to the same extent as the previous genes discussed. Tyler *et al.* (2004) found that in *Arabidopsis*, *GAI* is moderately expressed in most plant tissues, which is what one would expect since GA is a hormone involved in many processes. The highest levels of *GAI* expression were found to occur in seeds, 5-day-old seedlings, roots, 33-day-old rosette leaves, and siliques. Based on these findings, it can be expected that expression will be detected in both shoot tips and leaves, with peak expression occurring in leaves after the onset of flowering. Because GAI is known to function, in some species, in responsiveness

to GA (a promoter of flowering), it was also predicted that the early-flowering line would have higher expression of this gene than the corresponding control line.

**Figure 16. Comparison of *LFY* Transcript Levels of RC and RE2 in Shoot Tips.**

Average levels of *LFY* transcript plotted against plant age (in days) (SE=7.0) (De Decker, 2007).


Average Levels of *LFY* Transcript in Tips
Intensity
100
80
60
40
20
0
0
10
20
30
40
50
60
Age (days from sowing)
RC
RE2

## 4.2 Materials & Methods

### *4.2.1 Primer Design and PCR Program Specifications*

The focus for this particular project was on five genes known to be involved in the flowering process in a variety of species. These genes were *SUPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*), *CONSTANS-LIKE* (*COL*), *ADP-GLUCOSE PYROPHOSPHORYLASE SMALL SUBUNIT 1* (*ADG1*), *GIBBERELLIC ACID INSENSITIVE* (*GAI*), and *APETALA1* (*AP1*). Except for *SOC1*, cDNA sequences for these genes from other plant species were located using GenBank and were compared, using a BLAST search, to flax (*L. usitatissimum*) Expressed Sequence Tags (ESTs) from the NCBI website (http://www.ncbi.nlm.nih.gov/). The flax ESTs used as the basis for primer design were the following: *COL*: EX720159.1, *ADG1*: EH792002.1, *GAI*: CV478922.1 and *AP1*: EU830923.1.

Degenerate primers were originally designed in an attempt to amplify a *SOC1* fragment in flax. These primers were designed based on sequence similarities found between several known *SOC1* genes. When tested on cDNA, these primers amplified a band of approximately 300 base pairs (bp) (final sequences are in Appendix J). The sequence from the amplified fragment showed a high degree of sequence similarity (Expect value = $4e^{-27}$) to known *SOC1* in *A. thaliana*, so it was used to design specific primers for flax in the same way that the flax EST's were used.

Flax EST's showing a high sequence similarity to known sequences for the genes of interest were used to design specific primers. Free software, Primer3 (http://frodo.wi.mit.edu/primer3/), was used to design these primers (Table 2).

These primers were tested on cDNA made by a previous student (De Decker,

2007). The cDNA samples were made from RNA extracted from RC and RE2 at a variety of ages, and from either main-stem shoot-tips or leaves.

All PCRs were performed on an MJ Mini® Thermal Cycler. Each PCR was set up as outlined in Table 3. Each PCR program involved an initial hot-start activation step at 95 °C for 15 minutes, and a final extension step at 72 °C for 10 min. The individual denaturation, annealing and extension conditions, as well as the number of cycles, are outlined in Table 4 for each primer set, with the size of the fragment amplified indicated in bp. The number of cycles used was based on the linear range determined for each specific reaction (Appendix H).

Once a set of primers had been used for PCR, the amplification products were visualized using gel electrophoresis. Samples were run on a mini (10 cm x 7 cm) 1 % agarose gel in 0.5 x Tris-borate-EDTA (TBE) buffer using a PowerPac Basic power supply (BioRad, Missisauga, ON), and a MiniCell EC370M (E-C Apparatus Co., Florida). Gels were run at a voltage between 110 v and 120 v, for between 30 min to 60 min. All gels were stained for 20 minutes in a 1 μg/mL ethidium bromide solution.

Once amplified products were assessed via gel electrophoresis, a GenElute™ PCR Clean-Up Kit from Sigma (Appendix E) was used to purify amplified products prior to sequencing. Sequencing was performed on all amplified products by Mobix (Hamilton, ON). The sequences for the various amplified products were then compared to known sequences in the NCBI database using a BLAST search. An expect (e) value was used to evaluate homology of the sequence of interest to other known sequences, where a value of $e^{-10}$ was used as a cut-off point. Table 5 outlines the e values and percentage sequence identity of the flax sequences compared to known genes.

**Table 2. Primers Used for Amplification of Various Putative Flowering Genes in Flax.**

| Gene of Interest | Primer Orientation | Primer Sequence (5'-3') |
|---|---|---|
| *SOC1* | Sense | GTTTGAGCTCTCTGTTCTCTGTGAT |
| | Antisense | ATATCCTTATGCTTCTTCTCGTACC |
| *COL* | Sense | TATCATCATCGTCAATAGACGTGGGAGTC |
| | Antisense | TACCGAATCGTCTTCTCGAACTTCCTATTC |
| *ADG1* | Sense | GCCAGTAGAAGTGTTCTAGGCATAA |
| | Antisense | TACAAAGCCTTCGTTCTTGTAGC |
| *GAI* | Sense | CATGTGATGTCGGAGCTGTATC |
| | Antisense | TAGTATGCCAGCCAAGCATCAA |
| *AP1* | Sense | AAGAGGATCGAGAACAAAATCAAC |
| | Antisense | ATGTTTAAGTCCAGAATCAAGTTGC |

**Table 3. PCR Reaction Components**

| Component | Volume/Reaction | Final Concentration |
|---|---|---|
| 10 x PCR Buffer* | 2 ul | 1 x |
| dNTP (10 mM of each) | 0.4 ul | 200 μM of each dNTP |
| Primer A | 0.6 ul | 0.3 uM |
| Primer B | 0.6 ul | 0.3 uM |
| HotStarTaq DNA Polymerase* | 0.3 ul | 1.5 units/reaction |
| Water | 14.1 | - |
| Template cDNA | 2 ul | Variable |
| Total Volume | 20 ul | - |

*These components are from the HotStarTaq® DNA Polymerase Kit from Qiagen.

**Table 4. PCR Program Used for Semi-Quantitative Transcript Analysis**

| Primers | # of cycles | Denaturation | Annealing | Extension | Size (bp) |
|---|---|---|---|---|---|
| *SOC1* | 27 | 95 °C, 1 min. | 54 °C, 1 min. | 72 °C, 1 min. | 136 |
| *COL* | 24 | 95 °C, 1 min. | 64 °C, 1 min. | 72 °C, 1 min. | 185 |
| *ADG1* | 26 | 94 °C, 1 min. | 56 °C, 1 min. | 72 °C, 1 min. | 260 |
| *GAI* | 26 | 94 °C, 1 min. | 56 °C, 1 min. | 72 °C, 1 min. | 253 |
| *AP1* | 27 | 94 °C, 1 min. | 55 °C, 1 min. | 72 °C, 1 min. | 393 |

*Cycle number was worked out by finding the linear range of each reaction (Appendix H).

**Table 5. Expect Values and Percentage Sequence Identity between Flax Sequences and Known Genes.**

| Gene of Interest | Expect Value | % Identity | Species |
|---|---|---|---|
| *SOC1* | $5\ e^{-19}$ | 86 | *P. sativum* |
| *COL* | $9\ e^{-24}$ | 75 | *P. sativum* |
| *ADG1* | $3\ e^{-70}$ | 83 | *A. thaliana* |
| *GAI* | $1\ e^{-50}$ | 78 | *Malus x domestica* |
| *AP1* | $3\ e^{-78}$ | 77 | *E. globulus* |

* "species" refers to the species containing the gene of interest that the flax sequence is most similar to.

### *4.2.2 Semi-Quantitative Analyses of Transcript Levels*

The plants from RC and RE2 to be used for RNA extraction were grown as outlined previously (2.2.1). The only modification was that the plants were grown in 5-inch diameter round pots, with the number of plants per pot varying depending on the age at which the plants were to be used for extraction.

Total RNA was extracted from main-stem shoot-tips, leaves (top four on the stem), and cotyledons from plants every seven days, from 14 dap to 56 dap for RC, and from 14 to 35 dap, as well as from plants at 56 dap for RE2, using the Qiagen RNeasy® Mini Kit (Appendix F). For plants that had flower buds (instead of tips) at the time of extraction, buds were harvested and used for extraction. Using diluted RNA samples (to a final volume of 1 mL) in BrandTech X-treme Range UV semi-micro cuvettes (Ultident, QC), RNA concentrations were determined using a Cary-UV Vis spectrophotometer. Two readings at 260 nm were taken for each diluted samples, as well as two readings at 320 nm for background correction.

For the 49 d and 56 d RNA extractions, plants grown in the greenhouse at Wilfrid Laurier University during the spring and summer of 2009 were used instead of those grown in the growth chamber. The greenhouse plants were grown five plants per pot in 5-inch diameter round pots, were fertilized on a weekly basis, and were watered as needed with tap water. These plants were exposed to the natural, ambient light and temperature conditions in the greenhouse. Plants grown in the greenhouse have accelerated development compared to plants grown in the growth chamber. Therefore, 42-day-old plants from the greenhouse are effectively equivalent to 49-day old-plants grown in the growth chamber. That is, the samples designated as 49-d and 56-d samples actually came from 42-day and 49-day-old plants from the greenhouse.

First strand cDNA synthesis was performed using 2 μg of each RNA sample and the Qiagen Omniscript® Reverse Transcriptase kit (Appendix G). PCR was then completed on the resulting cDNA samples using an MJ Mini® thermal cycler (BioRad). All experimental samples were run in triplicate (except when amplifying *AP1*, in which case samples were run in duplicate). A set of primers was used to amplify an *ACTIN* fragment from each of the cDNA samples used for the genes of interest. The primers and program used were those designed by De Decker (2007), and the reactions were set up as outlined in Table 3. *ACTIN* acted as a standard to test for the efficacy of each cDNA reaction, as well as the quality of the gel staining. Each PCR sample was visualized twice using gel electrophoresis, with the samples for the genes of interest being run on the same gels as their corresponding *ACTIN* standards.

Samples were run on 1.5% agarose gels in 0.5 x Tris-borate-EDTA (TBE) buffer using a Sub-Cell GT. Gels were run at 120 V, for 90 min. All gels were stained for 20 minutes in a 1 μg/mL ethidium bromide solution. In order to quantify the transcript level for each sample, band intensity was determined using photos taken by a GelDocXR® system (BioRad) and calculations performed by QuantityOne® software.

### *4.2.3 Statistical Analyses*

ANOVAs and orthogonal comparisons were used to make four major comparisons for the data obtained from the gel imaging software. Firstly, RC and RE2 were compared to determine whether there were any overall differences in transcript levels, for any given gene, between the two plant lines. Secondly, expression levels from 14 to 35 dap were compared to determine whether there were any trends occurring with vegetative development (increasing plant age). Thirdly,

expression levels at 56 dap were compared to the average of expression from 14 to 35 dap to determine whether there were any differences in expression in the mature tissues, as the plants were flowering, compared to expression in tissues when plants were growing vegetatively. Fourthly, interactions between the plant lines and the above mentioned trends were examined to determine whether any of the trends identified differed between RC and RE2. Although graphs include data points for RC at 42 and 49 dap, the corresponding data were not used for analyses because there were no equivalent RE2 samples.

For a given gene, the data used for analysis were means for two complete replicates of tissue samples, for each of the different sampling ages and plant line combinations. In most cases, the transcript levels for each sample within a replicate were obtained by average results from 6 separate electrophoresis gels (or in the case of *AP1*, 4 separate electrophoresis gels). But, in a few instances, there was an obvious anomaly in the gel electrophoresis and the data for that gel were not used.

## 4.3 Results

### *4.3.1 Putative Flowering Genes in Flax*

Sequences sharing a high degree of sequence similarity with genes involved in the floral transition in other plant species were identified in flax. However, it is important to note that although these sequences share sequence identity with known genes, further functional analyses are needed in order to determine their identity with a higher degree of confidence. In addition, while it was predicted that not all of the genes examined would be detected in both the leaf and shoot tip samples, it is important to mention that all were expressed in both.

Graphs shown are comprised of averages of data from six PCRs (three PCRs for each of the two environmental replicates). There is an exception in the case of the graphs corresponding to *AP1* because only two PCRs were performed for each environmental replicate.

Photographs of the original gels used can be found in appendix I.

### *4.3.2* SOC1

Expression of *SOC1* in leaves was found to be higher in RC than RE2 ($F_{1/9}$=19.2**), and both genotypes showed a quadratic expression profile from 14 to 35 dap ($F_{1/9}$=14.5**) (Figure 17a). Between 14 and 35 days (i.e. during vegetative growth) expression of *SOC1* in RC increased linearly ($F_{1/9}$=7.55*), while no significant linear trend was observed for expression of *SOC1* in RE2 ($F_{1/9}$=3.45 ns). However, in both genotypes, expression was significantly reduced in leaves of 56-day-old plants compared to the average at 14 to 35 days ($F_{1/9}$=76.1**).

*SOC1* expression in both the control and early-flowering line was found to be significantly lower in buds than in tips of younger plants ($F_{1/9}$=21.0**) (Figure 17b).

### *4.3.3* COL

Expression of the flax *COL* gene did not change significantly in leaves (Figure 18a) or shoot tips (Figure 18b) between the ages of 14 and 35 dap, in RC and RE2. However, both lines showed a decreased level of expression in the leaves ($F_{1/9}$=63.8**) and shoot tips ($F_{1/9}$=7.99*) at 56 dap.

### *4.3.4* ADG1

Expression of *ADG1* remained relatively constant in both tissues until 35 dap; after this point a significant decrease was detected in leaves of 56-day-old plants ($F_{1/9}$=5.76*) (Figure 19a), and in flower buds ($F_{1/9}$=70.1**) (Figure 19b) relative to the average for days 14 to 35. However, a significant interaction ($F_{1/9}$=11.2**) occurred between these two components (plant age and plant lines) because the decrease in the level of expression of *ADG1* was greater in RE2 than in RC (non-orthogonal for RE2: $F_{1/9}$=68.7**; non-orthogonal for RC: $F_{1/9}$=12.6**).

### *4.3.5* GAI

Between 14 and 35 dap, expression of *GAI* in the leaves of RC and RE2 increased linearly ($F_{1/9}$=11.4**) (Figure 20a). A significant interaction ($F_{1/9}$=6.42*) occurred between plant age and plant line, and non-orthogonal comparisons revealed that expression of this gene in RC increased in leaves of 56-day-old plants ($F_{1/9}$=6.57*), while there was no change in the expression in leaves of the same age in RE2 ($F_{1/9}$=1.04 ns).

Expression of *GAI* in shoot tips was similar to that in the leaves (Figure 20b). A significant interaction ($F_{1/9}$=12.3**) occurred between plant line and plant age, because expression increased in the buds of RC relative to the expression at younger ages (non-orthogonal $F_{1/9}$=10.3*), but did not in RE2 (non-orthogonal $F_{1/9}$=3.08 ns). In contrast to the leaves, expression was constant between 14 and 35 dap in both genotypes ($F_{1/9}$=4.14 ns). Therefore, it appears that expression of *GAI* increases in shoot tips of RC at the onset of flowering, while a similar increase does not occur in RE2.

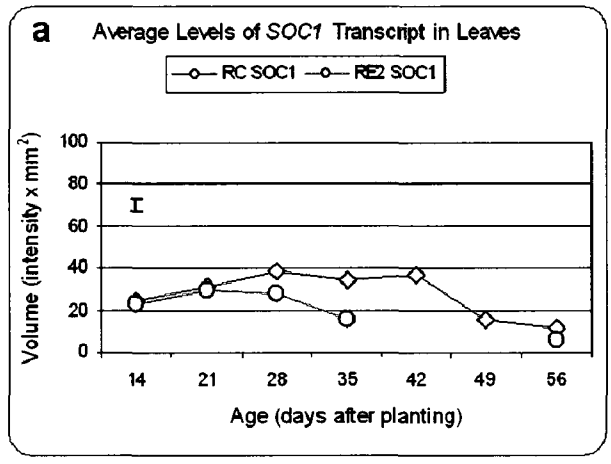
### *4.3.6* AP1

While the majority of genes examined did not show significant overall differences in expression between genotypes in either tissue, *AP1* expression was found to differ significantly between RC and RE2 in the leaves ($F_{1/9}$=36.8**), with expression being higher in RE2 than in RC (Figure 21a). Interestingly, non-orthogonal comparisons revealed that the expression in leaves of RE2 increased linearly between 14 and 35 dap ($F_{1/9}$=42.0**), but did not change in RC ($F_{1/9}$=1.96 ns). In addition, in both genotypes the expression was significantly higher in leaves from 56-day-old plants than in leaves from younger plants ($F_{1/9}$=142**), and non-orthogonal comparisons showed that this increase occurred to a greater degree in RE2 ($F_{1/9}$=104**) than in RC ($F_{1/9}$=44.3**).

In main-stem shoot-tips, there was no significant difference in expression between genotypes ($F_{1/9}$ < 1.0 ns), and there were no significant trends between 14 and 35 dap in either tissue of either genotype (Figure 21b). However, expression in both genotypes was found to be significantly increased in the buds relative to the average level from 14 to 35 dap ($F_{1/9}$=7.93*).

**Figure 17. Comparison of *SOC1* Transcript Levels in RC and RE2.**

a) Transcript levels for *SOC1* in leaves plotted against plant age. b) Transcript levels for *SOC1* in main-stem shoot-tips plotted against plant age.


a
Average Levels of SOC1 Transcript in Leaves
RC SOC1
RE2 SOC1
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
56
Age (days after planting)

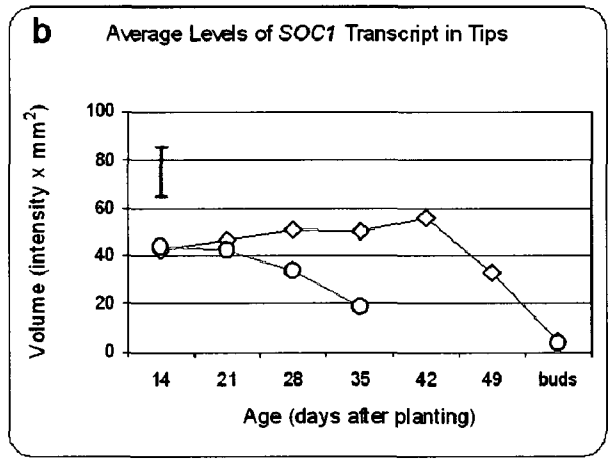
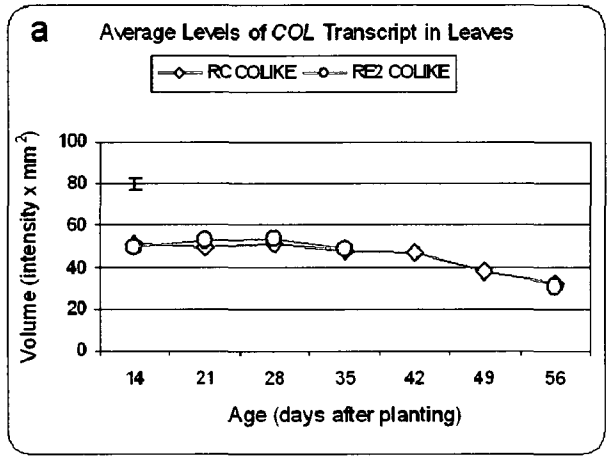

b
Average Levels of SOC1 Transcript in Tips
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
buds
Age (days after planting)

**Figure 18. Comparison of *COL* Transcript Levels in RC and RE2.**

a) Transcript levels for *COL* in leaves plotted against plant age. b) Transcript levels for *COL* in main-stem shoot-tips plotted against plant age.


a
Average Levels of COL Transcript in Leaves
RC COLIKE
RE2 COLIKE
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
56
Age (days after planting)

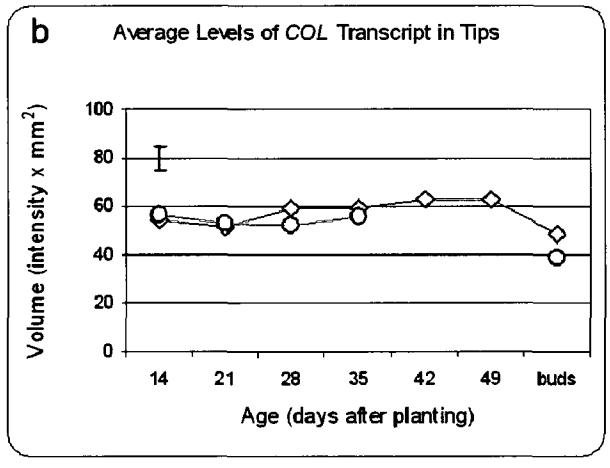
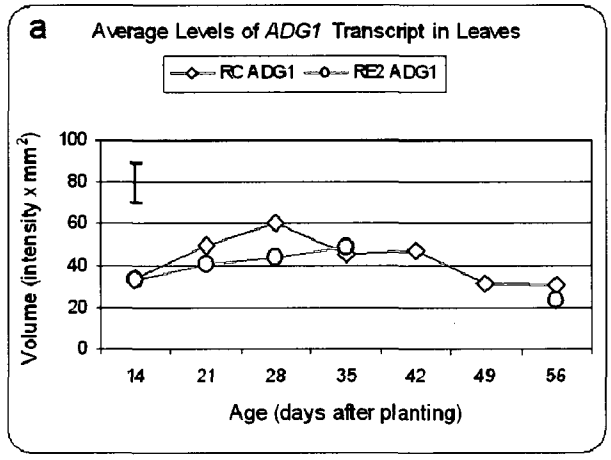

b
Average Levels of COL Transcript in Tips
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
buds
Age (days after planting)

**Figure 19. Comparison of *ADG1* Transcript Levels in RC and RE2.**

a) Transcript levels for *ADG1* in leaves plotted against plant age. B) Transcript levels for *ADG1* in main-stem shoot-tips plotted against plant age.


a
Average Levels of ADG1 Transcript in Leaves
RC ADG1
RE2 ADG1
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
56
Age (days after planting)

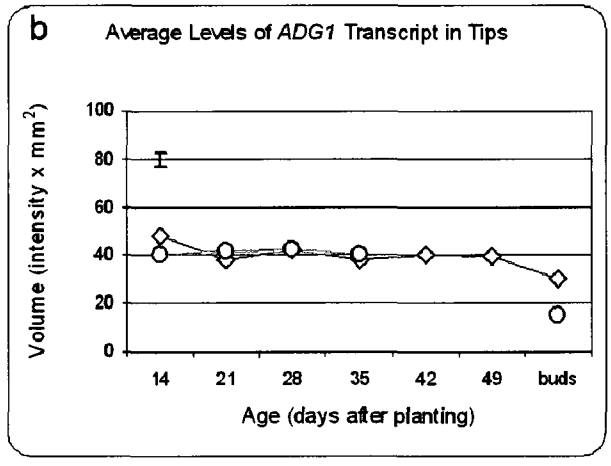
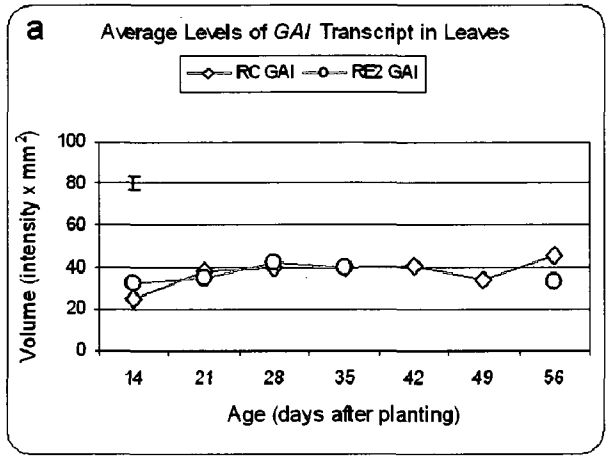

b
Average Levels of ADG1 Transcript in Tips
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
buds
Age (days after planting)

**Figure 20. Comparison of *GAI* Transcript Levels in RC and RE2.**

a) Transcript levels for *GAI* in leaves plotted against plant age. b) Transcript levels for *GAI* in main-stem shoot-tips plotted against plant age.


a
Average Levels of GAI Transcript in Leaves
RC GAI
RE2 GAI
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
56
Age (days after planting)

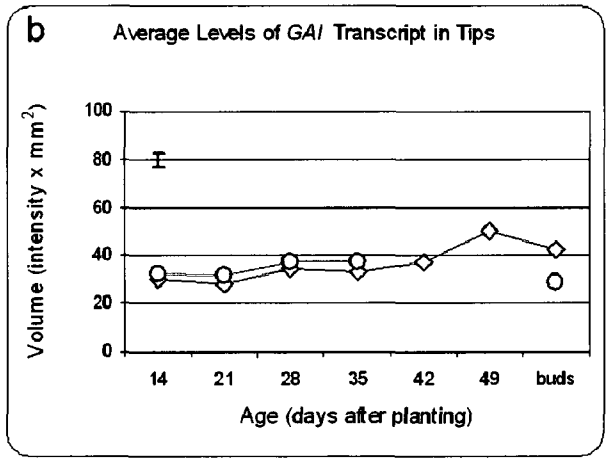
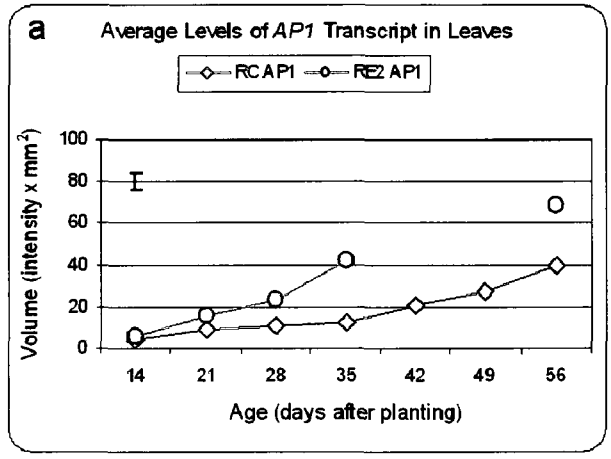
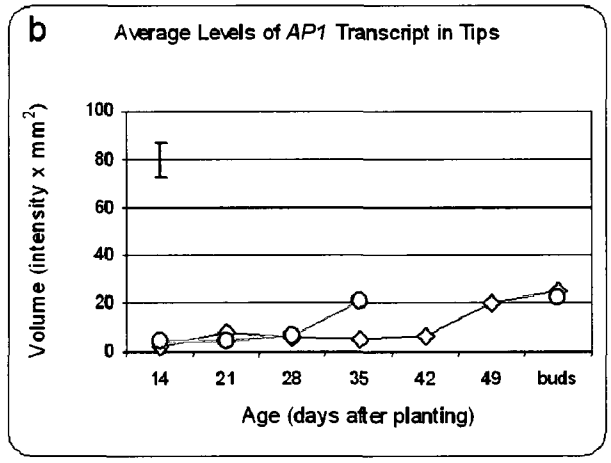

b
Average Levels of GAI Transcript in Tips
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
buds
Age (days after planting)

**Figure 21. Comparison of *AP1* Transcript Levels in RC and RE2.**

a) Transcript levels for *AP1* in leaves plotted against plant age. b) Transcript levels for *AP1* in main-stem shoot-tips plotted against plant age.


a
Average Levels of AP1 Transcript in Leaves
RC AP1
RE2 AP1
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
56
Age (days after planting)


b
Average Levels of AP1 Transcript in Tips
Volume (intensity x mm2)
100
80
60
40
20
0
14
21
28
35
42
49
buds
Age (days after planting)

### 4.4 Gene Study Conclusion

Primers for five different putative flowering genes in flax were designed, and were used to detect expression levels in main-stem shoot-tips and in the top four leaves on the stem. Expression of *SOC1*, a floral pathway integrator, was expected to increase at the onset of floral initiation. While this increase, specifically, was not detected, it was found that expression levels of this gene were significantly lower in the buds and leaves at 56 dap. It was also observed, although not statistically tested, that levels of expression appeared to decrease after 21 dap in the early-flowering lines, and after 42 dap in the control line. This may represent the time at which floral initiation has begun, and where SOC1 is no longer required for the process of flowering to continue.

Expression of the putative flax *COL* gene was found to be constant in both leaves and tips, except at 56 dap where it decreased in both tissues, and in both plant lines. This expression profile may help us to identify the specific identity of this gene. It appears that this *COL* gene may be involved in the initiation of flowering as its expression drops off after the floral transition; however, this description is likely to fit a number of different *COL* genes.

*ADG1* was found to remain constant in expression throughout vegetative growth, and was then found to decrease at 56 dap in both tissues. Based on this information alone, it seems unlikely that this change in expression relates to a role of *ADG1* in flowering in flax.

*GAI* increased in the buds and leaves at 56 dap in RC, but not in RE2, and while expression increased in leaves of RC and RE2 between 14 and 35 dap, there was no similar trend in the tips. These findings could again be explained by a possible role of *GAI* in flowering. However, these findings may also be related to a

role of *GAI* in many other biological processes. Directly measuring levels of GA, as opposed to a gene involved in the plant's responsiveness to it, maybe more useful for determining whether there is a role of GA in flowering in flax.

The only gene examined that was thought to have a potential role in floral morphogenesis was *AP1*. Interestingly, expression of this gene corresponded to what was expected, for the most part, with increases being detected in the buds and leaves of plants at 56 dap, and with these increases being greater in RE2 than RC. The fact that RE2 expressed *AP1* to a higher degree in reproductive tissue may result from the altered level in DNA methylation. Because *AP1* is downstream of many other genes involved in flowering, including *LFY*, it is also possible that *AP1* itself was not directly affected, but that another gene upstream of *AP1* was affected, and has in turn affected the expression of *AP1*. An observation that was not expected was that expression of *AP1* was detected in the leaves. This may be explained by the fact that it was the top four leaves on the stem that were examined, as opposed to those located further down the stem, and further from the site of floral initiation. It is possible that floral inductive signals are produced in the top four leaves as they are in close proximity to the apical meristem.

# 5. DISCUSSION

## 5.1 Patterns of Methylation Over a 24-Hour-Period

The HPLC method used in this study to measure levels of methylated cytosine in DNA from main-stem shoot tips, leaves and cotyledons, during development, has been used in a number of previous studies. Results of these studies have included determining that the early-flowering lines are generally hypomethylated compared to their corresponding controls (Fieldes *et al.*, 2005; Brown, De Decker and Fieldes, 2008), producing developmental profiles for two early-flowering R lines and their control line (Brown, De Decker and Fieldes, 2008), and determining that the chloroplast DNA of flax is methylated, and most interestingly, that it is hypomethylated in RE2 relative to RC (De Decker, 2007). Therefore, the use of this HPLC technique as a way to measure global levels of cytosine methylation is undoubtedly useful. However, for the purposes of detecting differences in levels of cytosine methylation over a 24-hour-period, it is possible that a more sensitive method may be required.

While there is no evidence that cytosine methylation levels vary depending on the time of the day, it seems likely that both increases and decreases might be occurring at the same time and can simply not be detected by examining overall changes in methylation, using an HPLC method that only detects global levels. Perhaps measuring methylation levels at, and surrounding, specific genes that are thought to be regulated by circadian rhythm would reveal differences in methylation levels.

It is interesting to note that the increase in the overall levels of cytosine methylation over the 24-hour period in the main-stem shoot-tips approached

significance. The fact that the levels of methylation at 0 and 24 hours-after-dawn do not match each other could indicate that the increase is not the result of a daily pattern, and that the increase may be a result of the plant's developmental profile for methylation.

### 5.2 Possible Causes of Changes in Methylation Levels

Previous research has shown that levels of cytosine methylation in plants can change in response to stress (Labra *et al.*, 2002). More specifically, Labra *et al.* (2002) were able to determine that hypermethylation is induced in pea plants when the root tips of these plants are exposed to water stress. In addition, it has been shown that some transposable elements can be mobilized by both abiotic and biotic stress in plants (reviewed in Feschotte, Jiang, and Wessler, 2002), and since methylation can act to keep transposons inactive, it is possible that methylation may increase as a result of stress in an attempt to prevent movement of transposons. Thus, for the plants grown in the dark prior to chloroplast isolation, it is possible that a stress response connected to the time spent in the dark, caused the absence of hypomethylation in the DNA of RE2 relative to that of RC.

Aside from the extended period of growth in the dark, there are also two other possible sources of stress that may have been caused by the use of a dark box; first, it is known that the humidity was higher than normal in the dark box, and second, because the volume in the box was reduced, a higher-than-normal plant density was used and atmospheric concentrations of oxygen and carbon dioxide were probably affected. If the plant line similarity in methylation level is the result of a stress effect, then it is clear that the stress has a differential affect on RE2 relative to RC (i.e. either more of an affect on RE2 or less of an affect on RC). It is possible, then, that one of the two lines is less susceptible to stress than the other. Therefore, for several

reasons, it would be interesting to examine how RC and RE2 respond to a variety of potential stresses, such as increased or decreased temperature, salinity, and also to include the two other early-flowering R lines, and the early-flowering L lines and their control, to see if it is a generalized effect in the azaC–induced early-flowering flax lines. The ability to produce viable seed early, and being more stress-tolerant, would make the early-flowering flax lines interesting in terms of agricultural significance, as these plants could probably be grown in regions typically deemed unsuitable for flax.

### 5.3 Flax, a Facultative Long-day Plant

The finding that flax can flower when exposed to short-day conditions, but that the time required to flower is shorter in plants grown under long-day conditions demonstrates that flax is a facultative long-day plant. Aside from the age of the plant at which the first flower opened on each plant, a number of other parameters were measured, and it was found that exposing a variety of flax lines, including both early flowering and control lines, to short-day conditions has an obvious effect on their growth and development. The total height of each plant was measured several times during vegetative growth, and the treated plants (i.e. those exposed to short-day conditions) were consistently shorter than untreated plants. Therefore, the effects of short-day conditions on flax are not limited to adult, reproductive development. Both the late juvenile and transition phases of vegetative growth were both affected.

Flowering age and the number of leaves produced on the main-stem were affected in the same way by the short-day treatment. More specifically, flowering was delayed and the number of leaves produced on the main stem increased in all plant lines examined. Interestingly, the treatment had less affect on the early-flowering lines than it did on either the L or the R line controls. In RC, the time required to

flower was increased by 100.6 %, whereas it was only increased by 34.15 % and 32.25 % in RE1 and RE2, respectively. It is as though the early-flowering lines respond to some other stimulus, as opposed to photoperiod, to initiate the process of flowering when grown in short-day conditions.

### 5.4 Regulation of Flowering in the Early-Flowering Lines

Based on the differential response between the early-flowering and control lines to the short-day treatment, it seems as though the early-flowering lines may use an alternative flowering pathway compared to the control lines. It has been proposed that the early-flowering lines use an intrinsic, developmental "node counting" method to determine the appropriate time for flowering (Fieldes and Harvey, 2004). However, results of the short-day experiment indicate that this is unlikely. If the early-flowering lines were flowering when a specific number of nodes were produced, then the final leaf count in the early-flowering lines grown under short and long-day conditions should be the same. However, the number of leaves produced on the main stem (below the inflorescence) was significantly increased in the early-flowering lines grown in the short-day treatment.

As a facultative long-day plant, flax should flower under long-day conditions once it has become competent to respond to inductive cues. As explained previously, under long-day conditions peak expression of *CONSTANS* (a flowering-time gene) coincides with a light period (Suárez-López *et al.*, 2001), where light is required for activation of CO protein (Valverde *et al.*, 2004). Under short-day conditions, peak *CO* expression coincides with a dark period and, consequently, CO protein cannot be activated. However, facultative long-day plants will still flower even when long-day conditions are not present; an alternative pathway must be used. It is likely that all flax plants use some alternative 'default' pathway in short days, such as the GA or

autonomous pathways. It is possible that it is a gene in this default pathway of flax that has been affected in the early-flowering lines. If an intrinsic default pathway induces the early-flowering observed in the early-flowering lines, then even in the absence of an optimal photoperiod, the early-flowering lines should continue to flower earlier than controls; this is exactly what has been found to occur. The question then is how this might explain why the early-flowering lines differ in their response to normal, long-day conditions, compared to their corresponding controls. It is important to note that even though the early-flowering lines flowered considerably earlier than their controls in short-day conditions, there was still a delay compared to the time at which they begin flowering under more optimal light conditions. Under normal, long-day conditions then, the early-flowering lines may flower early because this default pathway initiates flowering early, and also because they flower in response to a combination of inductive signals from both the photoperiod pathway and the default pathway.

Some facultative long-day plants use the GA pathway to regulate flowering as an alternate to the photoperiod pathway (i.e. under short-day conditions). It is known that *LFY*, as a floral pathway integrator, acts downstream of both the photoperiod and GA flowering pathways (Simon, Igeño and Coupland, 1996; Blázquez *et al.*, 1998). Therefore, even in the absence of a photoperiodic inductive signal, flowering can still be initiated via GA. Without knowing more about the genetics behind flowering in flax, it is hard to predict exactly how the process of flowering is regulated. This being said, it seems plausible that the alternative pathway in flax is the GA pathway, and that, in the early-flowering lines, a gene in this pathway has been demethylated.

Previous work indicated that the difference between the control and early-flowering lines may not occur in the GA flowering pathway (Fieldes and Harvey,

2004), and for this reason it would be easy to rule out the GA pathway as the potential default pathway in flax. However, the earlier work was done using $GA_3$, which is one of many forms of the hormone. It is possible that the 'wrong' GA was applied. For instance, in some species the active GA is $GA_4$ (Eriksson *et al.*, 2006), while in some, such as *Lolium temulentum*, it is $GA_5$ and $GA_6$ (King *et al.*, 2006). Therefore, at this point, and until further studies are completed assessing the role of GA in the flowering process in flax, the GA pathway as the possible default pathway in flax can not be ruled out.

Another possibility that has been considered is that it is actually a gene in the photoperiod pathway itself that has been demethylated. A flowering-time gene in this pathway with higher-than-normal, or early, expression, would explain why the early-flowering lines are not as delayed in their flowering under short-day conditions as the control lines, and would certainly explain the early-flowering observed under long-day conditions. However, it might be expected that if this were the case, there would be no delay in flowering at all in short-day conditions. Since there is a significant delay, it seems more likely that the gene with altered expression will be found in the 'default' flowering pathway of flax.

### 5.5 Seed Yield is Reduced in Plants Grown in Short-Day Conditions

Flax is an agriculturally and economically important crop plant. It is used for both linseed oil, which can be extracted from the seeds, and for the fibres, which can be removed from the stem; the Royal cultivar is an oilseed type. When examining the growth of flax in environmentally relevant conditions, it is important to understand how these agriculturally pertinent traits might be affected. In general, the short-day treatment resulted in decreased seed yield. However, there are a few exceptions to this generalization. Even though the number of filled seeds per plant and the harvest

index were reduced in all R lines, they were reduced to a lesser degree in RE2. In addition, seed weight per 100 seeds was increased in RE2. There was also one L line that was slightly less affected in terms of seed yield. More specifically, the reduction observed for harvest index was not as great for LE1 as it was for LC and LE2. Therefore, it seems as though, compared to the other lines examined, LE1, and especially RE2, are able to flower, and ultimately produce viable seed, most effectively when grown in less-than-optimal conditions. This observation of RE2 is most likely due to a combination of its genetic background and the demethylation treatment.

### 5.6 The Role of Light Energy in Flowering in Flax

The amount of light energy received by the plants exposed to short-day conditions, and those exposed to the natural light conditions between May and September of 2008, was measured. For the R line plants, controls that were exposed to short days received more energy by first anthesis than those exposed to ambient long days, while all early-flowering R line plants, regardless of treatment, received the same amount of light energy. At first glance, it may seem as though the early-flowering plants initiate the flowering process when they have received some critical amount of energy, whereas the control lines flower based on a cue from a different source. While it is likely that the amount of energy received plays some metabolic role in the flowering process, it is unlikely that the specific amount of energy received acts as a direct trigger for initiating floral meristem identity or competence. These results can be interpreted in another way. The early-flowering R lines were delayed in the time at which they flowered; however, the delay was not as great as it was for RC. Although there was no significant difference in the amount of energy received by the treated and untreated early-flowering R lines, the increase in energy received

by the early-flowering lines approached significance. It is more likely that some other genetic factor is responsible for the specific time at which flowering is initiated, and the amount of energy received is simply a consequence of the length of time required to flower.

Leaf number was increased as a result of the short-day treatment. Two possible explanations for this observation are as follows. First, when the days are short, the amount of light energy received by the plants is lower, and therefore they may grow more leaves in an attempt to receive more light energy. Second, the signals telling the plant when to flower were probably not produced at the same time as usual in short days, so the switch from vegetative to reproductive growth was not triggered, resulting in the plants continuing to make leaves even after the point in development where the floral transition would normally occur. In the summer of 2009 the short-day experiment was repeated, primarily so that the number of leaves on the plants could be counted at various times during vegetative growth. If it was true that the plants grew normally until the point where the vegetative to reproductive switch normally occurs, but then continued producing leaves in the absence of a floral trigger, then the number of leaves produced between treated and control plants should have been the same until phase transition. This is not what was observed. The number of leaves increased in plants grown under short days throughout vegetative growth. At 14 dap when the first counts of leaf number were taken, there was a significant increase in the number of leaves produced on the main stem in all L (LC, LE1 and LE2) and R (RC, RE1 and RE2) lines used. This evidence supports the earlier idea that the increase in the number of leaves produced was in response to the change in either quality or quantity, of the light energy being received. However, it is not clear what role, if any, this had on the actual initiation of flowering. Because

energy is required for limitless processes in the plant, the increase in leaf number was likely a generic response to the decrease in the amount of energy received.

## 5.7 Gene Expression of Putative Flowering Genes in Flax

While a large amount of work on flax has focused on various aspects of its development, including the timing at which various phase changes occur, not much is known about the genetic regulation of these phase changes. However, some recent work on deciphering the regulatory pathways controlling the process of flowering in flax has led to the discovery of the *LEAFY* gene in the flax genome, and that its regulation differs in the early-flowering lines compared to the controls (De Decker, 2007). This difference in expression may be the result of demethylation of a gene in RE2 that is upstream of *LFY*. However, because *LFY* is a floral pathway integrator and participates, in some way, in a variety of regulatory pathways, it is hard to know which pathway might have been affected. Therefore, it is important to observe the expression of genes in a number of flowering pathways that are known to have an influence on the expression of *LFY*, as well as other genes so that an overall picture of flowering in flax can be deciphered.

### *5.7.1 Expression of Two Putative Flowering-Time Genes in Flax*

Two putative flax flowering time genes were examined: *ADG1* and *GAI*. The expression profiles for these genes indicated that they did not correspond to the expression profile observed for *LFY* in flax. In other words, it does not appear that the demethylation occurred in either of these genes, or genes upstream of them. It was proposed that these genes may be involved in determining flowering time in flax, and while levels of *ADG1* were found to decrease at 56 dap, this does not necessarily mean that this gene plays a role in the timing of flowering. Since ADG1 has a role in

starch biosynthesis, its expression is not expected to be specifically related to the floral transition. This gene was of interest because a difference in its expression levels between RC and RE2 would indicate that not only does it play a role in flowering, but also that its expression shows the effects of the demethylation treatment. Because flowering in some starch mutants is only affected when plants are grown in short-day conditions (Yu *et al.*, 2000), it may also be possible that expression differences are only detected during these particular light conditions.

*GAI*, another gene that is thought to play a role in many processes aside from flowering, does not appear to be involved in the flowering-time difference between control and early-flowering flax lines. Levels of expression of *GAI* were found to increase from 14 to 35 dap leaves, and to increase in buds and leaves in plants that are at 56 dap, but it is difficult to know whether the trends observed are in any way related to flowering time, or whether they correspond to other functions of GA-mediated responses.

### *5.7.2 Identity of a Putative* COL *Gene in Flax*

Although a set of primers has been designed that detect a putative *COL* gene in flax, it is not known which *COL* gene it is. The sequence for a fragment of this gene shows similarity to *COL1*, among other genes. It has been determined that some *COL* genes, such as *COL9* in *A. thaliana*, act as repressors of flowering (Cheng and Wang, 2005), while some, such as *COL1* and *COL2*, appear to have no affect on the flowering process (Ledger *et al.*, 2001). It was thought that the expression profile of the putative flax *COL* gene might indicate its true identity. The only change in expression was found to be a decrease in expression of the *COL* gene in buds and leaves of both RC and RE2 at 56 dap. This decrease could be interpreted in two ways. On one hand, if the flax *COL* is an activator of flowering, a decrease in its

expression is expected in tissues where the protein is no longer required (such as the flowers, or leaves of plants that have already initiated flowering). On the other hand, if the *COL* gene is a repressor of flowering, its levels would be expected to decrease in floral tissues where the process of flowering is clearly no longer being repressed. Therefore, the specific identity of the putative flax *COL* gene has not yet been determined, but in combination with future work these tip and leaf expression profiles may be useful.

### *5.7.3 Expression of a Putative Floral Pathway Integrator Gene in Flax*

While it appears as though the expression of *LFY*, a floral pathway integrator, has been affected by the demethylation treatment, it is also important to look at other floral pathway integrator genes in order to identify a common gene upstream that may have been directly affected. If a gene that was demethylated affects multiple floral pathway integrators, then we should see a similar difference to that seen in *LFY* in the other floral pathway integrators. However, if a gene that affects only *LFY* has been demethylated, then we might not see a difference in expression of other floral pathway integrators. For this purpose, the expression of *SOC1* was examined. In other species, because this gene activates FMI genes, it has been found to increase at the onset of the floral transition. In flax, no such increase was detected. However, a sharp decrease in floral buds, and in leaves, was detected in plants approaching flowering. So, while the expected increase in expression of the putative *SOC1* gene was not detected, the decrease observed provides evidence that this gene may in fact be *SOC1*. A decrease in expression of this gene in floral tissues is expected since it is no longer required once FMI has been established. Interestingly, although it was not found to be significant, it appears that the levels of *SOC1* in tips of RE2 began to drop after 21 dap, whereas in tips of RC they began to drop off after 42 dap. This is

interesting because in both genotypes, the drop occurred approximately two weeks before flower buds typically become visible. This decrease may indicate the time at which FMI is first established in flax. It is possible that if more sensitive techniques were used for detecting expression levels, the expected increase at the onset of floral initiation would be detected for *SOC1*.

### *5.7.4 Expression of a Putative Floral Meristem and Organ Identity Gene in Flax*

Of the five genes examined, *AP1* showed some of the most interesting results. It was not expected that expression of *AP1* would be detected in the leaves of flax because, in previous studies on *Arabidopsis*, its expression was not found in the leaves (Mandel *et al.*, 1992); this, however, was not the case in flax. Expression of *AP1* was detected in the leaves of both RC and RE2, and even more interestingly, was found to be higher in RE2 than in RC. It is possible its expression in the leaves occurred because only the top four leaves were examined, as opposed to leaves occurring lower on the stem. It was not clear which leaves (rosette or cauline) in *Arabidopsis* were examined in the study by Mandel *et al.* (1992); it is possible that expression in the rosette leaves was assessed. The expression of *AP1* in the leaves also increased linearly in plants between 14 and 35 dap, which was unexpected because LFY is a known activator of *AP1* (Wagner, Sablowski and Meyerowitz, 1999), and, in the leaves of flax, *LFY* expression was stable in both RC and RE2 (De Decker, 2007). However, *AP1* is not only regulated by LFY, so it is possible that another regulatory protein is responsible for this increase.

The highest levels of *AP1* expression were found in tissues from 56 dap, and although it was not tested, it appeared that the levels in shoot tips began to increase in RE2 at 35 dap. This timing coincides with the beginning of flower formation in RE2, so the increase may be indicative of the role of *AP1* in floral organ identity in flax.

### 5.8 Use of *ACTIN* as a Standard for Gene Expression Studies in Flax

When using the semi-quantitative method of measuring gene expression that was used in this study (section 4), it is important to understand the potential sources of variation that can lead to what appear to be differences in expression levels. One way to control the variation generated when making cDNA is to have a standard gene. This gene is typically a housekeeping gene that has constitutive levels of expression in all tissues, and at all developmental stages being examined. For the purposes of this study, this gene was *ACTIN*. In previous work, this gene has been found to be differentially expressed in RC and RE2, and to show various trends with development in shoot tips (De Decker, 2007). During the studies presented here, expression of *ACTIN* in tips was found to be relatively even in both RC and RE2 and throughout development. In leaves, however, its expression appeared to change with development. For this reason, the *ACTIN* standards were used by inspection only; when unexpected levels of expression were observed for the gene-of-interest, the *ACTIN* standard would be used to determine whether there was any problem with the cDNA itself. For instance, in one *COL* sample (RC 49d shoot tip, Appendix I13, top left gel) it was found that expression was unexpectedly low compared to that in other replicates. When this particular sample was compared to its corresponding *ACTIN* sample (Appendix I14, top left gel), it was found that the expression was also lower than expected for *ACTIN*, so the problem with this particular data point could be attributed to a problem with the original cDNA.

The *ACTIN* standards have also been useful when interpreting any trends observed in the genes of interest. For instance, if a linear trend is observed for a gene of interest, and this same trend is also observed for the corresponding *ACTIN* samples, then it is possible that the trend originated from a problem with the cDNA, rather than

being a genuine increase in gene expression. However, this situation was not observed with the any of the results presented in the gene study.

Ideally, to eliminate some of the human error associated with this type of work, a different method could be used that would reduce this error. For instance, multiplex PCR would remove the pipetting and PCR-related error between the gene-of-interest and its associated control sample. Taking this one step further would be to use multiplex Real Time PCR. Using this method, a gene of interest and its corresponding control can be amplified in the same reaction, and the expression could actually be measured quantitatively, allowing for a greater degree of accuracy in the results. In addition, if a standard can not be identified in flax that shows relatively constitutive expression in the leaves of both RC and RE2, then perhaps an alternative form of control should be considered. Other genes, such as *GAPDH*, have been found to make suitable standards in other plant species. In a study by King *et al.* (2006), commercially available mouse liver RNA was added to experimental RNA samples prior to cDNA synthesis and was subsequently used as a standard, so this is also another alternative to using *ACTIN*.

### 5.9 Future work

There are now several indications that the early-flowering flax lines may respond differently from their corresponding controls to stress (De Decker, 2007; unpublished). For this reason, it would be interesting to examine a variety of growth and developmental parameters, as well as levels of cytosine methylation, upon exposure to different stresses to determine whether there is a differential response between RC and the early-flowering R lines. This type of experiment could also be performed on the L lines. A few different types of stress that could be examined are metal stress (such as zinc), salt stress (or water stress), high (or low) temperatures,

and high humidity. It is possible that no global differences in the levels of methylation will be induced following these stress treatments, but is thought that differences in methylation at specific sites within the genome could lead to a differential response between control and early-flowering lines.

Earlier work indicated that GA may not be involved in the regulation of flowering in flax (Fieldes and Harvey, 2004). However, some of the work presented here indicates that the GA pathway may be involved in flowering by acting as a default pathway under short-day conditions. It would be interesting to repeat the experiments GA experiments performed by Fieldes and Harvey (2004), but to also add a short and long-day treatment. Perhaps the only response of the plants to GA, in terms of flowering, will be observed when they are grown in short-day conditions. Also, because the only GA used in the earlier study was $GA_3$, it would be important to repeat the experiments using different forms of GA.

It was predicted that the putative flax *COL* gene would be identified based on its developmental expression profile. However, it seems as though this profile is not enough to determine, with any certainty, which *COL* gene it is most likely to be. However, several *COL* genes are known to have regulation that follows a circadian rhythm. Therefore, examining the expression of the flax *COL* gene over a 24-hour-period may provide more insight as to its true identity. In addition, expression of the *COL* gene, as well as the other genes, should be examined in other tissues, in order to have a better understanding their role in development in other parts of the plant. Along these same lines, in order to know the true identity of any of the putative flax genes, their functionality should be examined. For this reason, transgenic flax lines with knockout mutations for the various genes of interest could be developed so that the role of these genes could be examined. This type of experiment would also help

to determine whether there is a differential role in the early-flowering and control lines.

The question of how, specifically, the demethylation treatment might have affected expression of a gene involved in the regulation of flowering has yet to be answered. It does not appear as though any of the genes examined to date have been affected directly by the demethylation treatment, although *LFY* is a possible candidate. For this reason, it would be interesting to perform bisulphite sequencing on *LFY*, as well as any future flowering genes identified in flax. This technique would determine whether any cytosine nucleotides at specific loci differ in their methylation between the early-flowering and control lines of flax. A difference would indicate that the specific gene being examined was directly affected by the demethylation treatment. Prior to performing bisulphite sequencing, RACE-PCR could be performed so that full-length sequences could be used for identifying methylation difference.

**APPENDIX A: Murashige and Skoog Nutrient Solution**

*(Modified from Murashige and Skoog, 1962)*

The nutrient solution was made by mixing stock solutions, as listed below, with deionized water:

| Solution | Ingredients (per L) |
|---|---|
| A | 82.50 g $NH_4NO_3$ |
| B | 95.00 g $KNO_3$ |
| $C_1$ | 1.24 g $H_3BO_3$ |
| $C_2$ | 0.166 g KI<br>0.05 g $NaMoO_4 \cdot 2H_2O$<br>0.005 g $CoCl_2 \cdot 6H_2O$ |
| D | 88.00 g $CaCl_2 \cdot 2H_2O$ |
| $E_1$ | 74.00 g $MgSO_4 \cdot 7H_2O$<br>3.38 g $MnSO_4 \cdot H_2O$<br>1.72 g $ZnSO_4 \cdot 7H_2O$ |
| E2 | 0.005 g $CuSO_4 \cdot 5H_2O$ |
| F* | 8.25 g $Na_2EDTA \cdot 2H_2O$<br>5.57 g $FeSO_4 \cdot 7H_2O$ |

*Note: The $Na_2EDTA \cdot 2H_2O$ was dissolved in 200 mL of water, and the $FeSO_4 \cdot 7H_2O$ was dissolved in 200 mL of water and heated to 80 °C. When this temperature was reached, the two solutions were mixed and allowed to cool. The final volume was brought up to 1 L with deionized water.

For experiments grown in the greenhouse (*i.e.* as opposed to the growth chamber), tap water was used instead of deionized water to dilute the stock solutions to their proper final concentrations.

## APPENDIX B: Protocol for Extraction of DNA

*(Derived from DNeasy® Plant Mini Kit manual from Qiagen)*

1. Approximately 200 mg fresh weight of plant tissue (per sample) was ground in liquid nitrogen using a mortar and pestle and immediately transferred into a chilled 1.5 mL eppenedorf tube.
2. 400 µL of buffer AP1 and 5 µL RNase A were added to the samples.
3. Samples were vortexed and allowed to stand for 10 minutes at room temperature.
4. Samples were incubated for 15 minutes at 65 °C and were mixed by vortexing every 5 minutes.
5. 130 µL of Buffer AP2 were added to each sample. The samples were then mixed by vortexing and incubated on ice for 5 minutes.
6. Samples were centrifuged for 5 minutes at 8000 x g.
7. The liquid lysate was added to a QIAshredder spin column and was centrifuged at 8000 x g for 2 minutes.
8. The samples were transferred to new 1.5 mL tubes and 675 µL of Buffer AP3/E were added. The lysate and buffer were mixed by pipetting.
9. 650 µL of the mixture were put into a DNeasy mini spin column and centrifuged for 1 minute at 6000 x g.
10. The flow-through was discarded and step 9 was repeated with the remainder of the sample.
11. A new collection tube was added to the column and 500 µL of Buffer AW were added to the DNeasy column and centrifuged for 1 minute at 6000 x g. The waste was discarded.
12. 500 µL of Buffer AW were again added to the DNeasy column and centrifuged for 2 minutes at 8000 x g to dry the membrane.
13. The columns were transferred to new collection tubes.
14. 100 µL of preheated 10mM Tris were added to the membrane.
15. The columns were incubated at 65 °C for 5 minutes before centrifugation at 6000 x g for 1 minute.
16. Steps 14 and 15 were repeated once and the new eluate was added to that already stored in the 1.5 mL tubes.
17. Samples were stored at 4 °C until used for HPLC analysis.

## APPENDIX C: Protocol for DNA Hydrolysis for HPLC Analysis

1. Following DNA extraction, all samples (200 ul) were acidified with 2 uL of 0.1 M HCl, boiled for 2 min, and then cooled on ice for 5 min.
2. 30 ul of 8.3x incubation buffer (0.3 M sodium acetate, 0.4 M NaCl, 2.7 mM $ZnSO_4$, pH 4.5), 2 ul of 0.1 M HCl and 3 uL (300 U) of S1 nuclease (Fermentas), 2 uL 8.3x incubation buffer and 10 uL $H_2O$ were added to each sample.
3. Samples were then incubated at 37 °C for 17 h.
4. 28 ul of 0.67 M Tris, 10 ul of 1.0 N NaOH, and 12 ul (0.167 U) of alkaline phosphatase (Sigma P-5521) were added to each sample.
5. Samples were incubated at 37 °C for 3 hours.
6. Samples were stored at 4 °C until they were used for HPLC analysis.
7. Samples were further prepared for HPLC analysis by centrifugation at 7500 g for 30 min.
8. 125 ul of supernatant were transferred to glass inserts in septa vials for sample injection.

## APPENDIX D: Recipes for HPLC Solutions

1. 13.6 g of potassium phosphate monobasic were added to 1 L of water in a 1 L Erlenmeyer flask. The solution was mixed until the contents were completely dissolved.
2. The mixture was poured into a 2 L volumetric flask and MilliQ water was added to give a final volume of 2 L (final concentration was 0.05 M).
3. For solution A: In a new 1 L flask, 25 mL of 100 % ethanol were made up to 1 L with the potassium phosphate solution. The flask was covered and inverted to mix.
4. For solution B: Separately, 200 mL of 100 % ethanol were topped up in a 1 L volumetric flask, covered, and inverted to mix.
5. Solutions A and B were both stored in amber bottles and the pH was corrected to 4.0 by addition of 1.0 M $H_3PO_4$.
6. For solution C: 150 mL of 100 % ethanol were put into an Erlenmeyer flask with 100 mL MilliQ water, covered, and inverted to mix.
7. All three solutions were water-suction filtered through a 0.45 μm Millipore filter.
8. Solutions were stored at 4 °C until needed and kept no more than two days before use.

## APPENDIX E: PCR Clean-Up Procedure

*(Derived from the GenElute™ PCR Clean-Up Kit Protocol)*

1. A GenElute Miniprep Binding Column was inserted into a collection tube (1 set per sample), and 0.5 mL of the Column Preparation Solution was added to each column and centrifuged at 12,000 x g (maximum speed) for 1 minute. The eluate was discarded.
2. 5 volumes of Binding Solution were added per 1 volume of the PCR reaction and mixed. The solution was then transferred to the binding column and was centrifuged at max. speed for 1 minute. The eluate was discarded.
3. 0.5 mL of diluted Wash Solution was applied to the column and was centrifuged at max. speed for 1 minute. The eluate was discarded.
4. The column was centrifuged at max. speed for 2 minutes.
5. The column was transferred to a new 2 mL collection tube. 50 μL of Elution Solution was applied to the centre of the column and was incubated at room temperature for 1 minute.
6. The column was centrifuged at max. speed for 1 minute to elute the DNA.
7. If sequences were not immediately sent away for sequencing, then they were stored at -20 °C.

## APPENDIX F: Procedure for Extraction of RNA

*(Derived from the RNeasy® Mini Handbook from Qiagen)*

1. A maximum of 100 mg fresh weight of flax plant material were ground in liquid nitrogen using a mortar and pestle and placed quickly into a pre-chilled 1.5 mL eppendorf tube.
2. 450 µL of the Buffer RLT/B-meracaptoethanol solution were added to the tissue powder and were vortexed vigorously.
3. The lysate was transferred onto the QIAshredder spin column in a collection tube and centrifuged for 2 minutes at 8000 x g.
4. The supernatant was put into a new eppendorf tube and 225 µL of 100 % ethanol were added and mixed by pipetting.
5. The sample was transferred onto an RNeasy mini column in a collection tube and centrifuged for 15 seconds at 8000 x g. The flow-through was discarded.
6. 700 µL of Buffer RW1 was added to the RNeasy column, centrifuged for 15 seconds at 8000 x g. The flow-through was discarded.
7. The RNeasy column was put into a new collection tube. 500 µL of Buffer RPE was added onto the column.
8. The column was centrifuged for 15 seconds at 8000 x g and the flow-through was discarded.
9. 500 µL of Buffer RPE were added to the column and centrifuged at 8000 x g for 2 minutes.
10. The column was transferred to a new collection tube and centrifuged for 1 minute at 8000 x g.
11. The column was transferred to a new collection tube and 30-50 µL of molecular grade water were added to the membrane.
12. The sample was centrifuged for 1 minute at 8000 x g and the flow-through was transferred to a 1.5 mL eppendorf tube for storage at –80 °C until it was ready to be used.

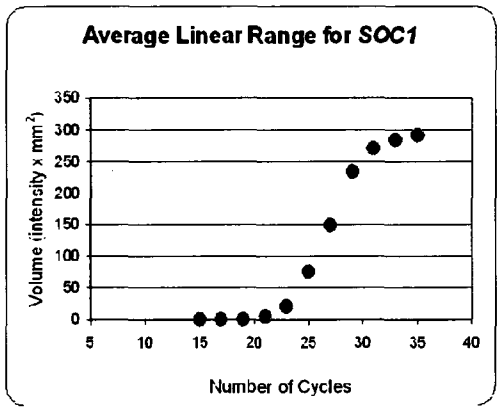
## APPENDIX G: RT-PCR Procedure

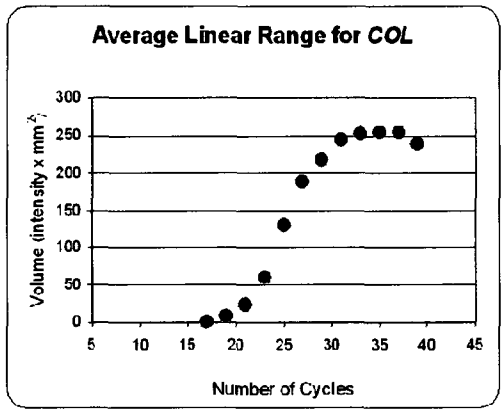
*(Derived from the Omniscript® Reverse Transcriptase Handbook from Qiagen)*

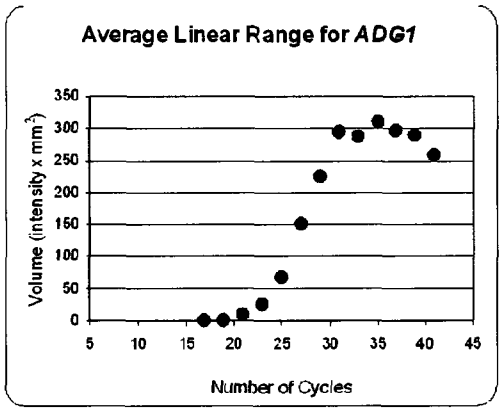
1. All reagents (*i.e.* oligo-dT, 10 X buffer, RT, dNTP mix and water) were thawed on ice, briefly vortexed, and then stored on ice until ready to use. RNA was thawed on ice.
2. RNase inhibitor (Promega) was diluted to a concentration of 10 units/uL using 1 X Buffer (diluted from the 10 X buffer supplied) and mixed by briefly vortexing.
3. Combined into a 1.5 mL eppendorf tube were 2 μL of 10 X buffer, 2 μL dNTP mix, 0.2 μL oligo-dT (Invitrogen), 1 μL (RNase inhibitor), and 1 μL Omniscript Reverse Transcriptase. Also added were 2 μg RNA. The volume of each sample was topped up to 20 μL with DNA/RNA-free water (volume of water added to each sample was dependent on the volume of RNA added).
4. Each tube was briefly centrifuged and incubated for 1 hour at 37 °C.
5. cDNA was stored at -80 °C.
6. Samples were used for PCR amplification using HotStartTaq® provided by Qiagen.

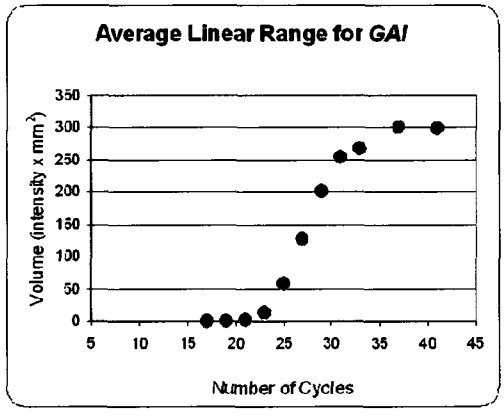
## APPENDIX H: Determining the Optimal Number of PCR cycles

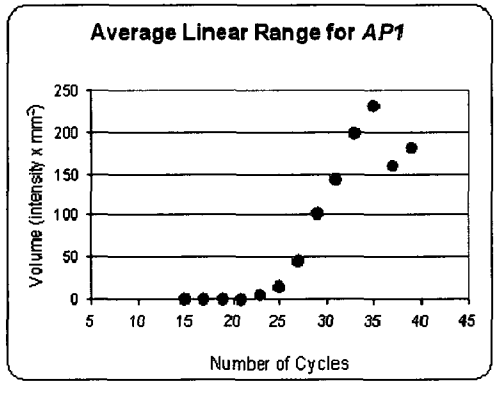
These graphs represent the average of at least three PCR runs, and were used to find the optimal cycle number for each gene.



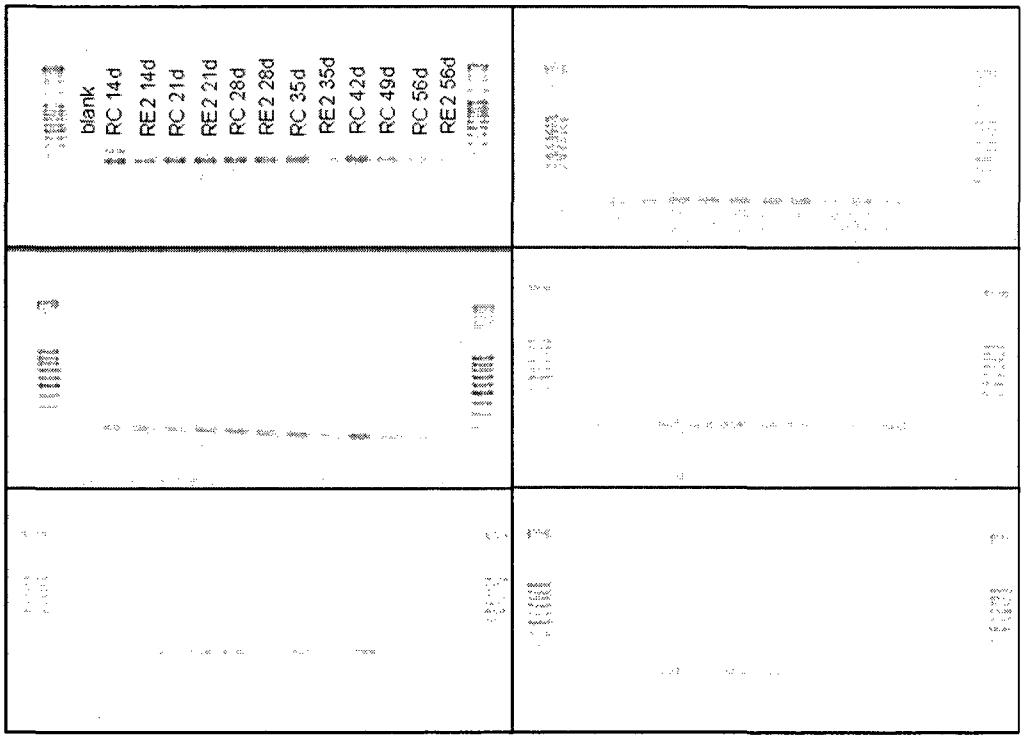
## APPENDIX I: Original Gel Images Used for the Gene Expression Study

PCR was performed in triplicate for each environmental replicate, and for each PCR, two gel replicates were run. However, this appendix only includes one gel image per PCR performed. This combination of images was chosen to show the variation between environmental replicates, as well as the PCR variation. Although not every gel is shown here, all gels were examined for analyses of expression levels

.The gels shown are in two columns, where the left column includes images corresponding to the first environmental replicate, and the right column to the second environmental replicate. Each row includes an image from a separate PCR.

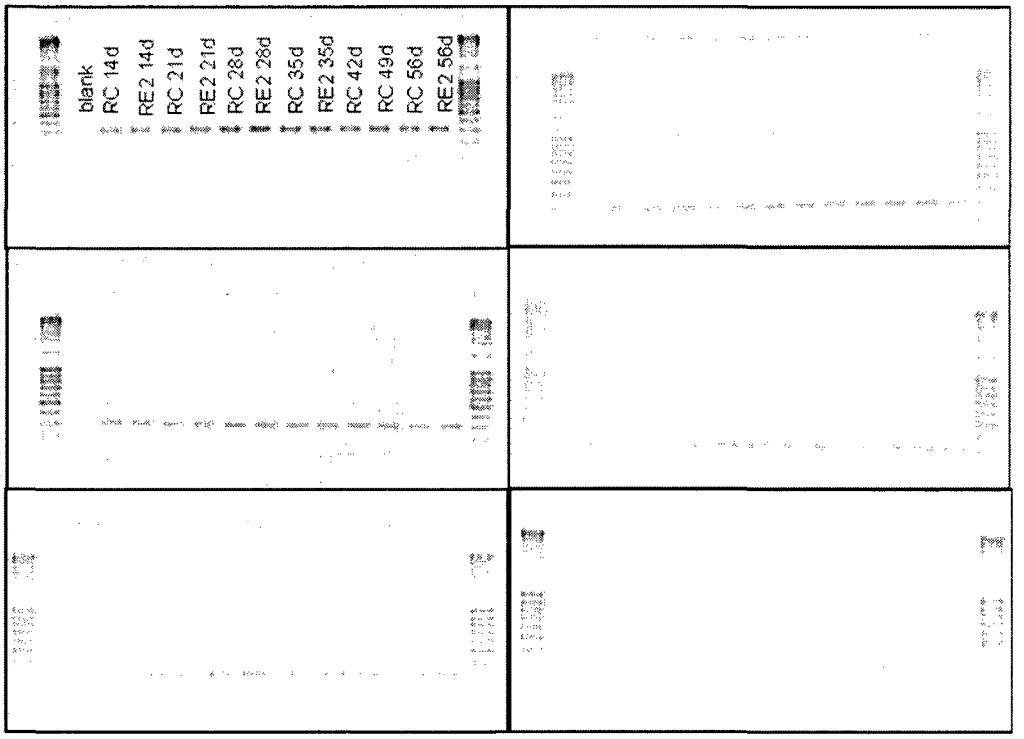
**APPENDIX I1: *SOC1* Transcript Levels in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



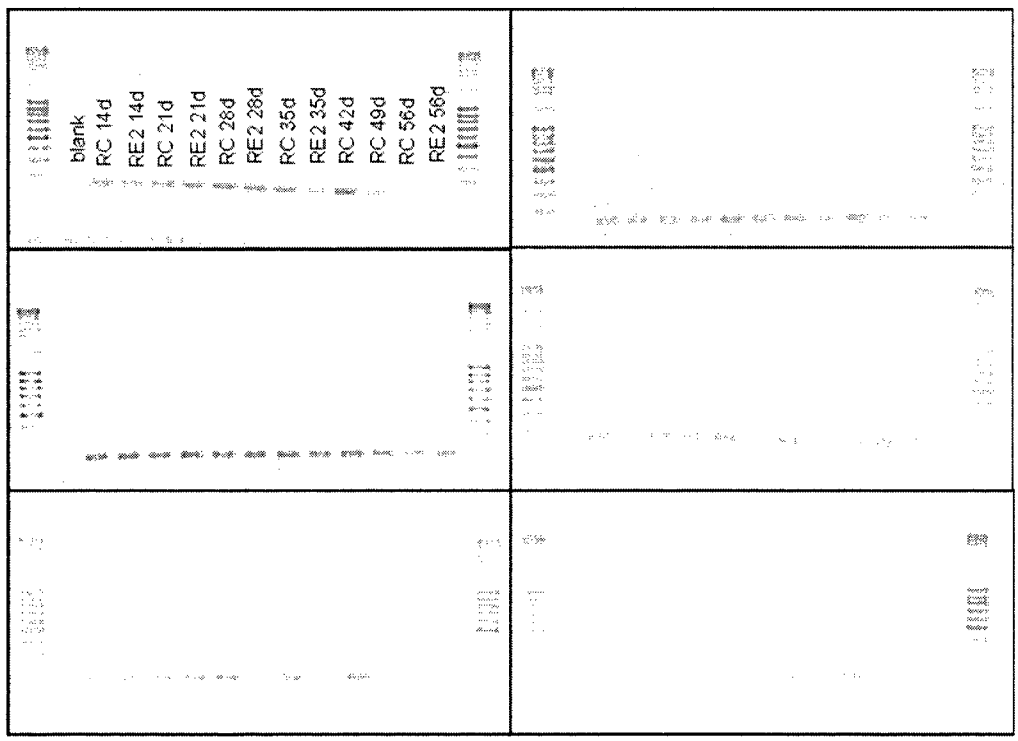
**APPENDIX I2: *ACTIN* Standards Corresponding to *SOC1* in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



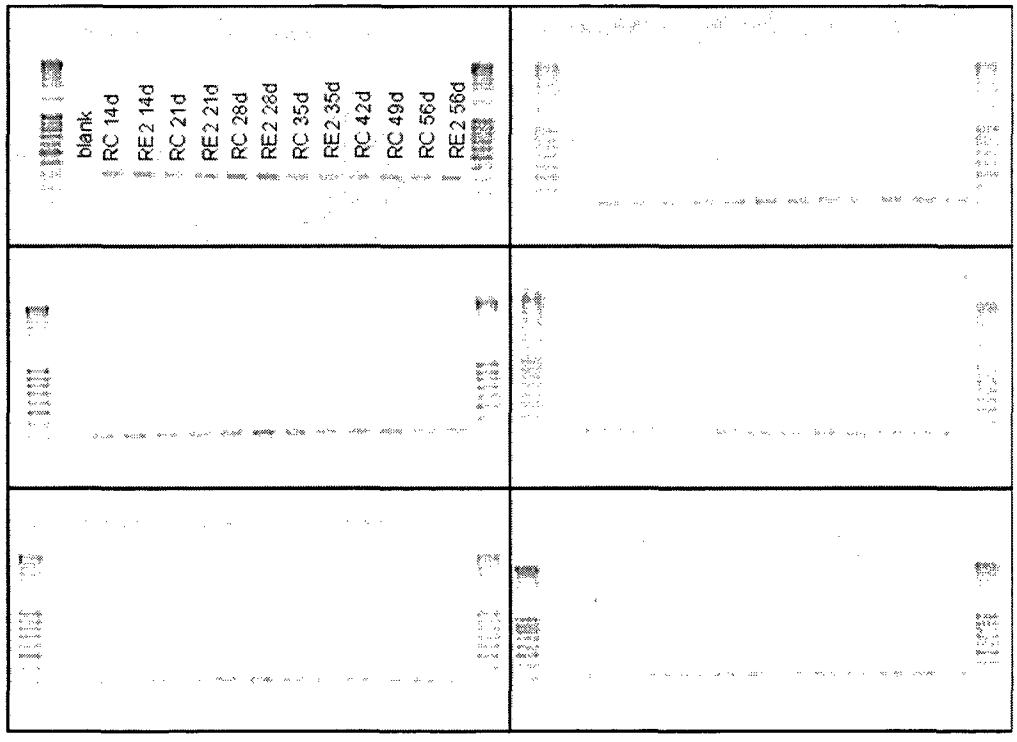
**APPENDIX I3: *COL* Transcript Levels in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



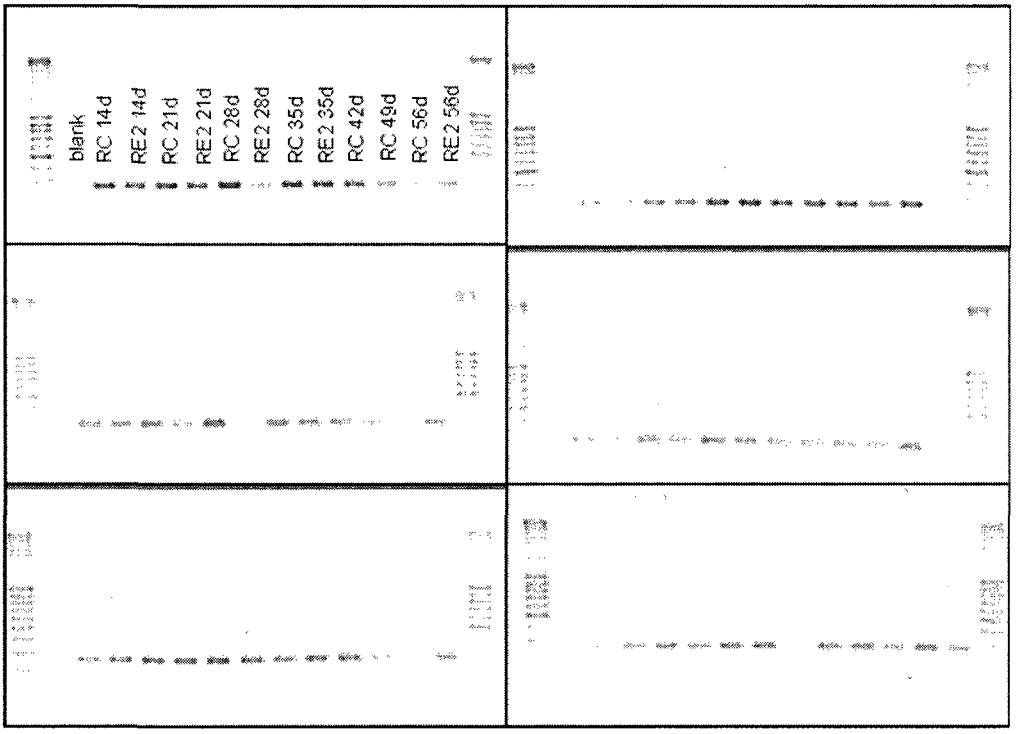
**APPENDIX I4: *ACTIN* Standards Corresponding to *COL* in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



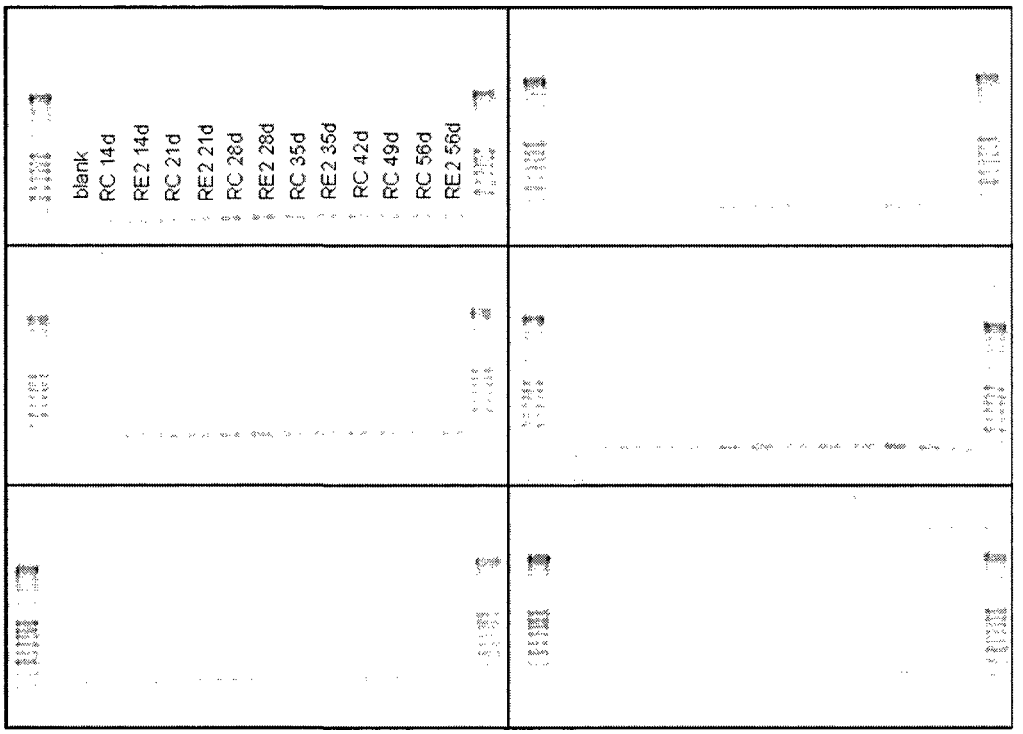
**APPENDIX I5: *ADG1* Transcript Levels in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



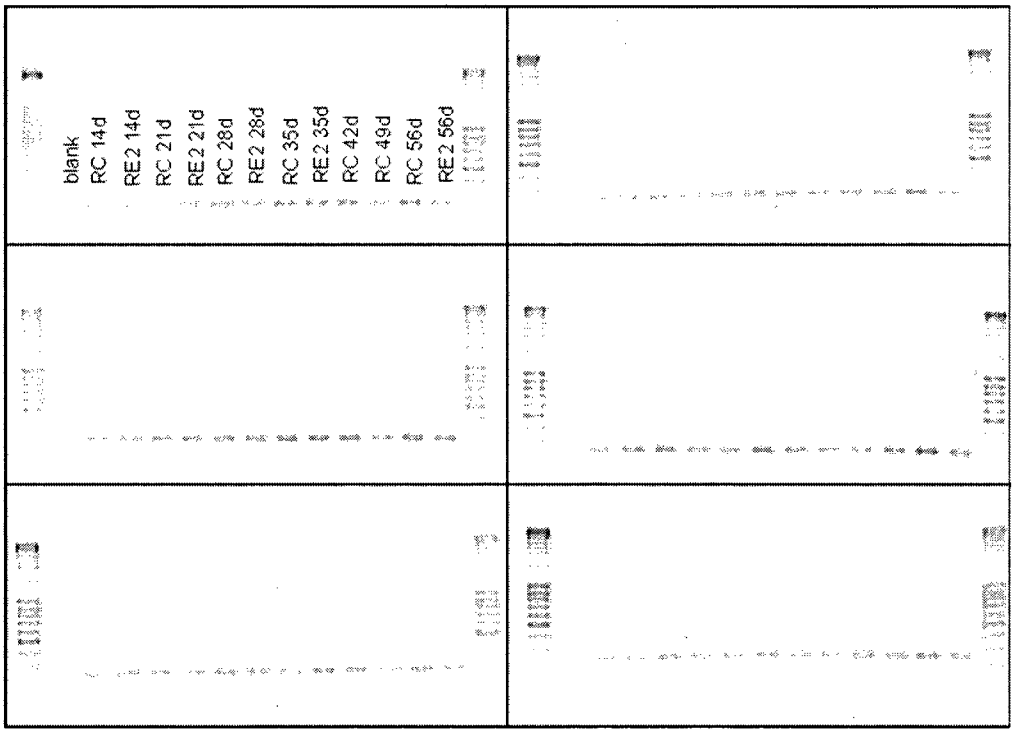
**APPENDIX I6: *ACTIN* Standards Corresponding to *ADG1* in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



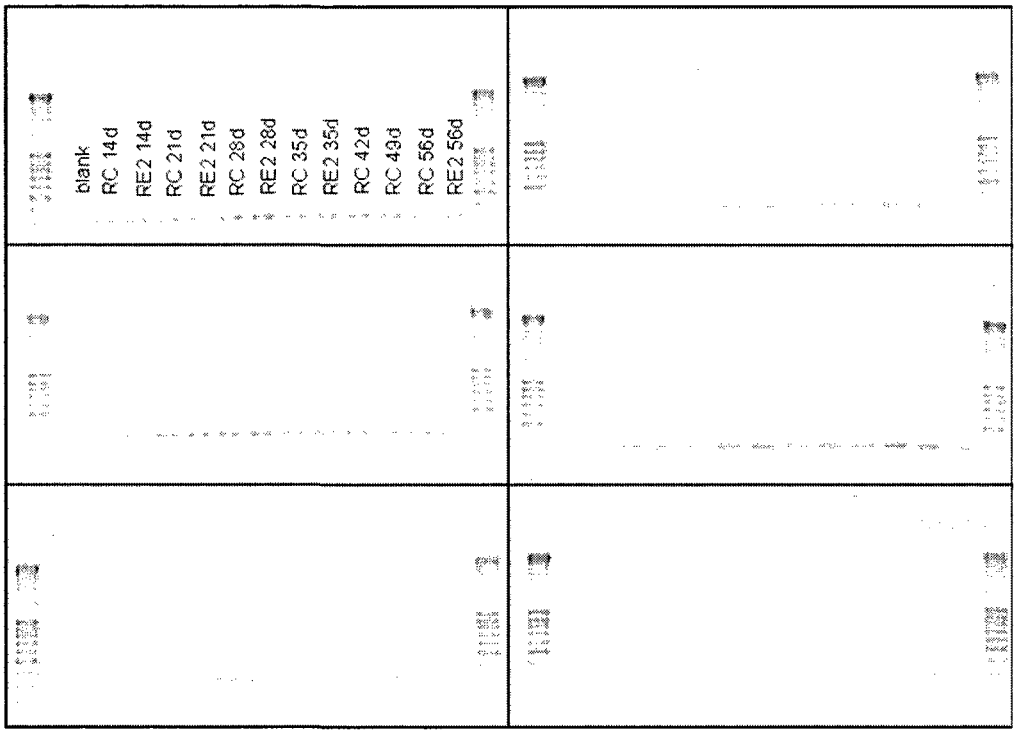
**APPENDIX I7: *GAI* Transcript Levels in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



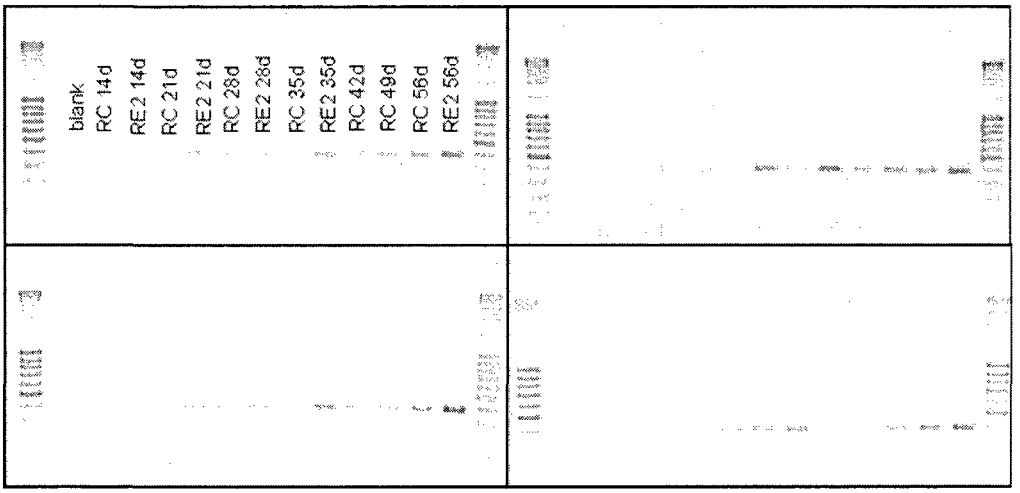
**APPENDIX I8: *ACTIN* Standards Corresponding to *GAI* in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



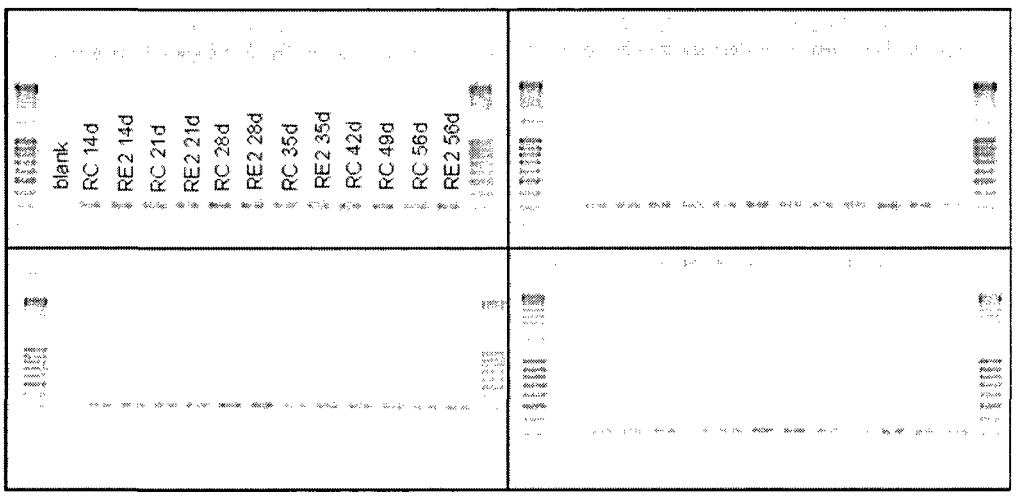
**APPENDIX I9: *AP1* Transcript Levels in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



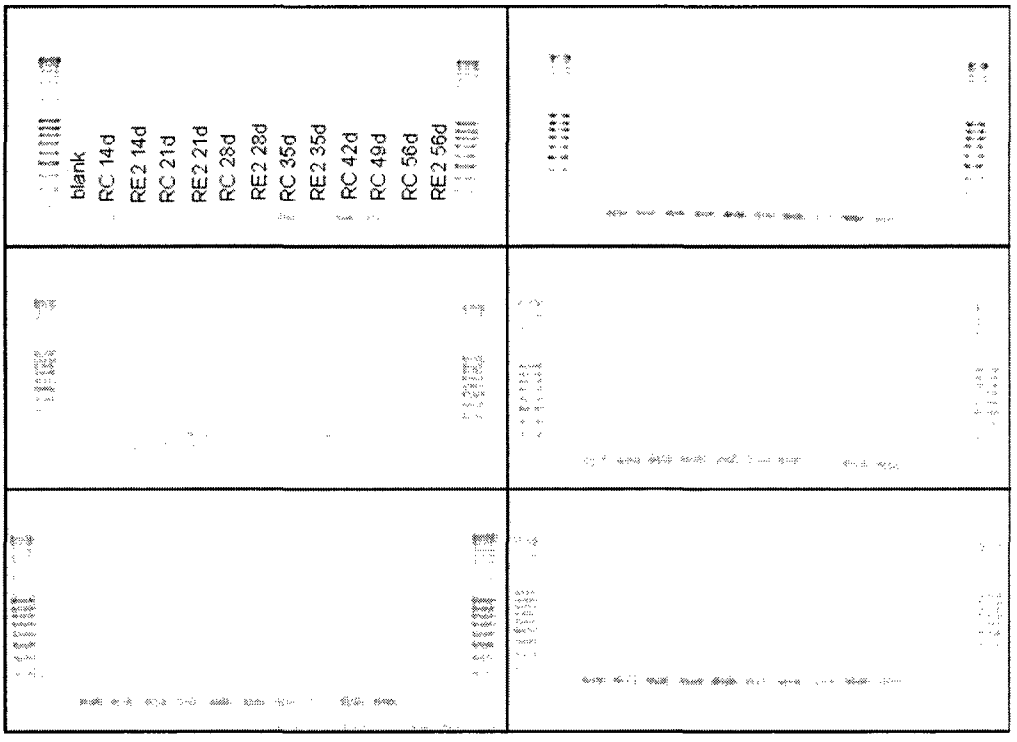
**APPENDIX I10: *ACTIN* Standards Corresponding to *API* in Leaves.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



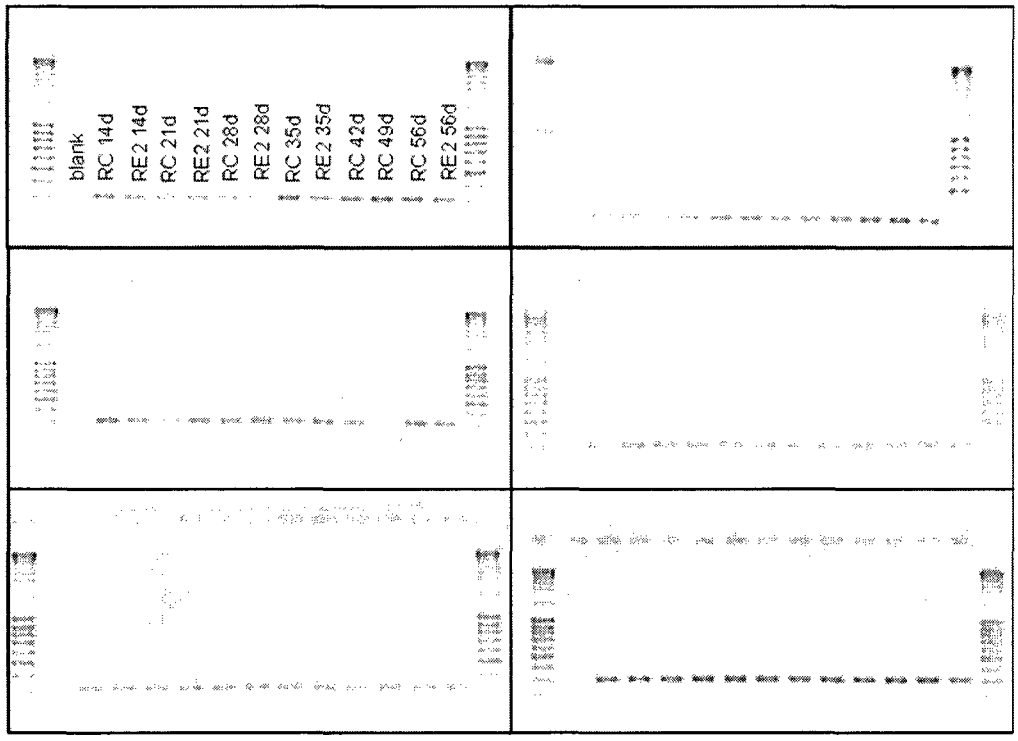
**APPENDIX I11: *SOC1* Transcript Levels in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



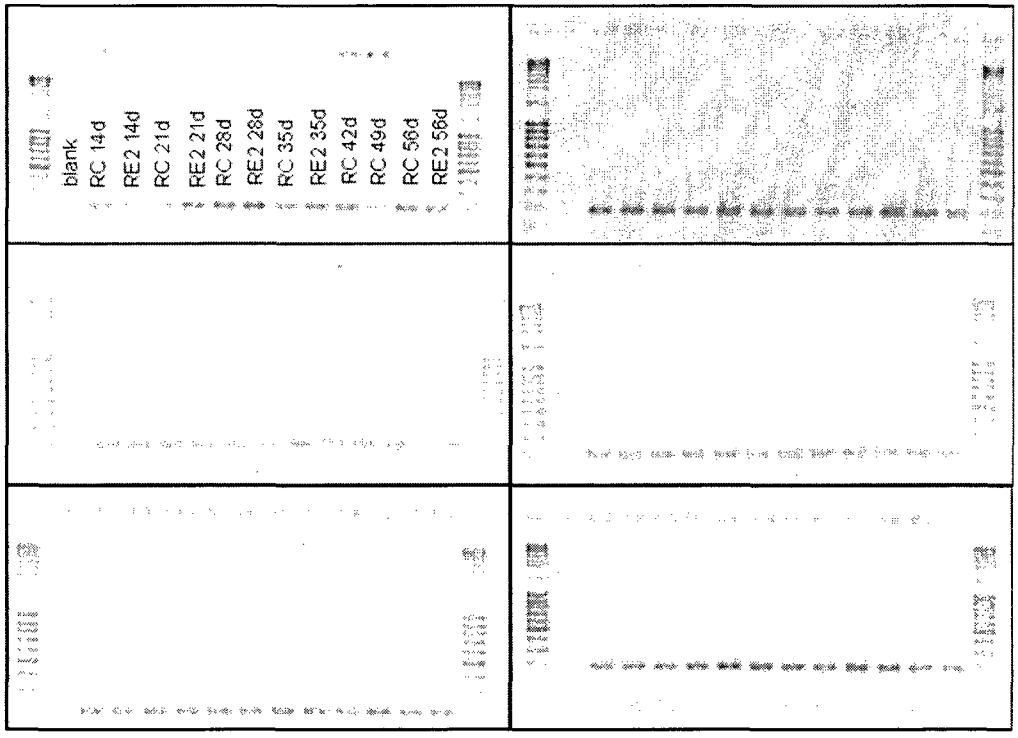
**APPENDIX I12: *ACTIN* Standards Corresponding to *SOC1* in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



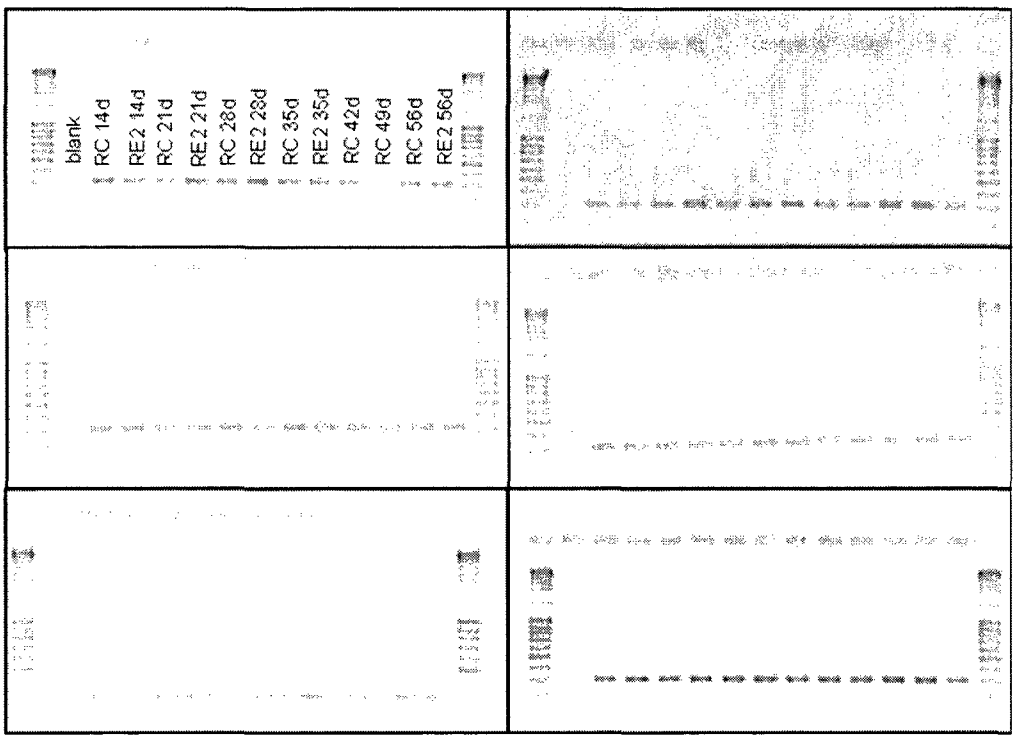
**APPENDIX I13: *COL* Transcript Levels in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



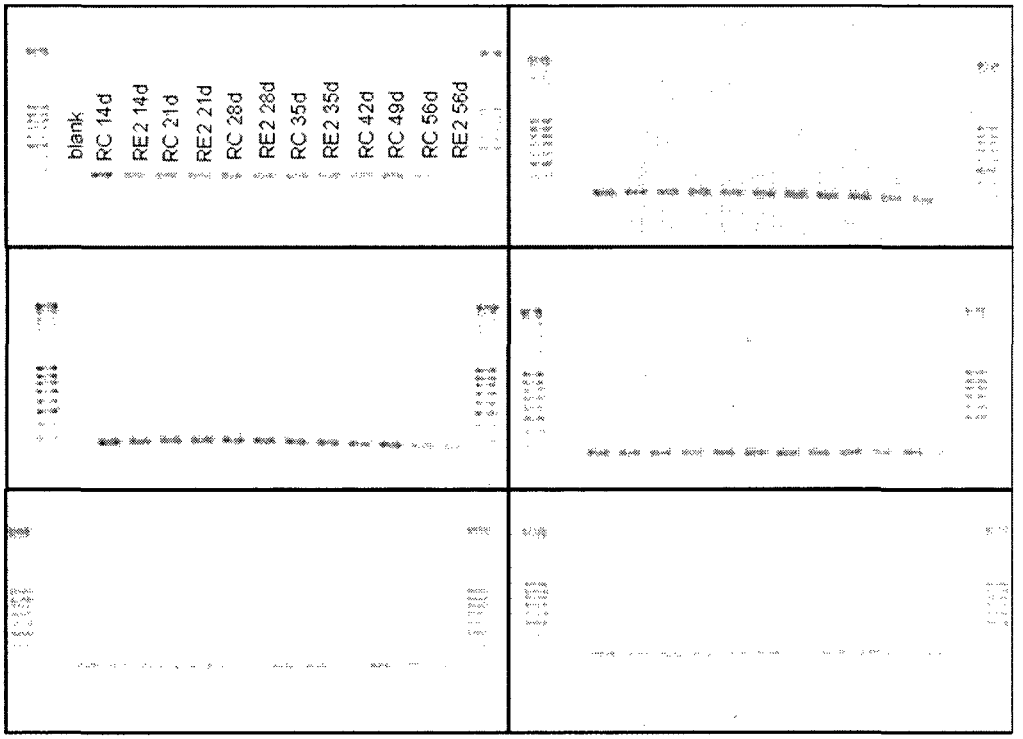
**APPENDIX I14: *ACTIN* Standards Corresponding to *COL* in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



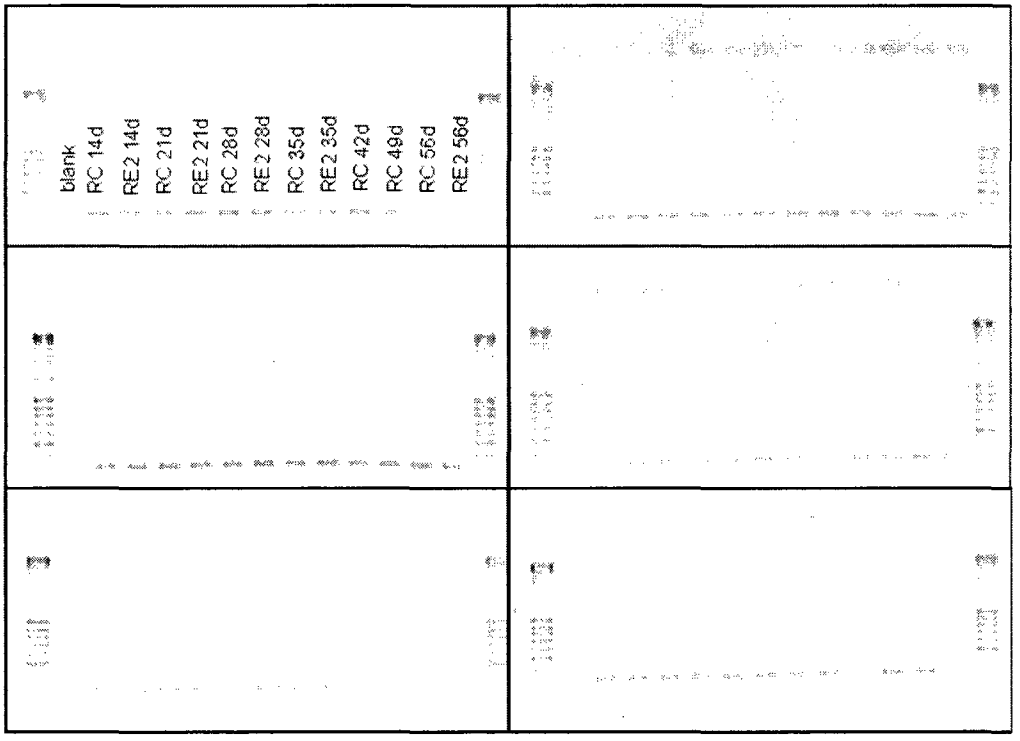
**APPENDIX I15: *ADG1* Transcript Levels in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



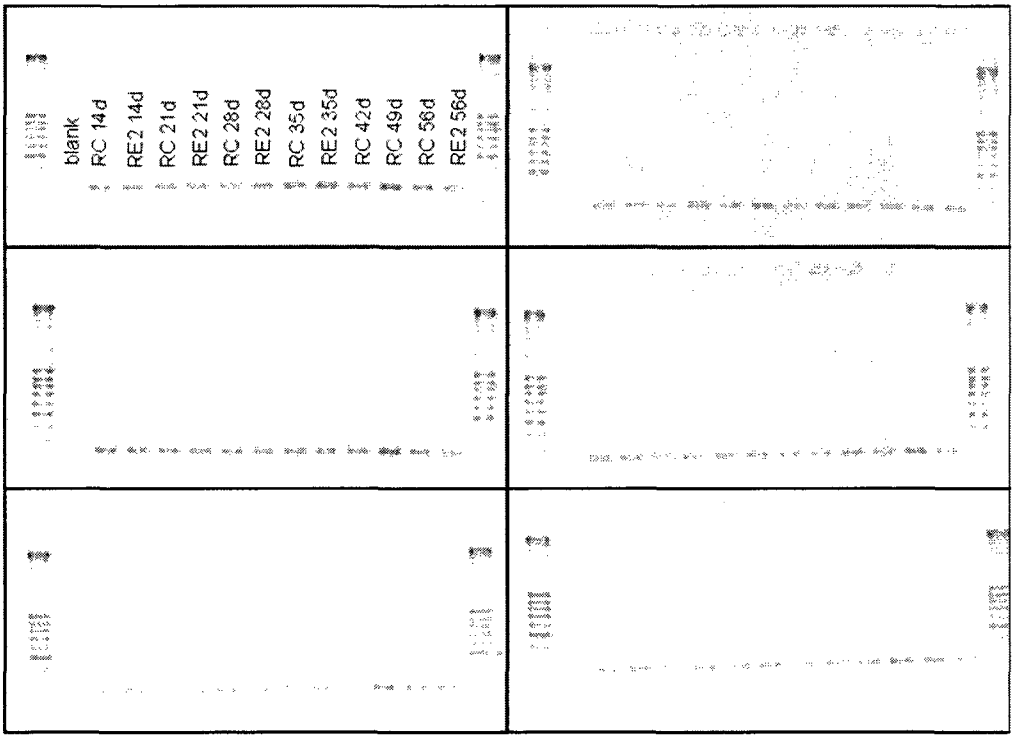
**APPENDIX I16: *ACTIN* Standards Corresponding to *ADG1* in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



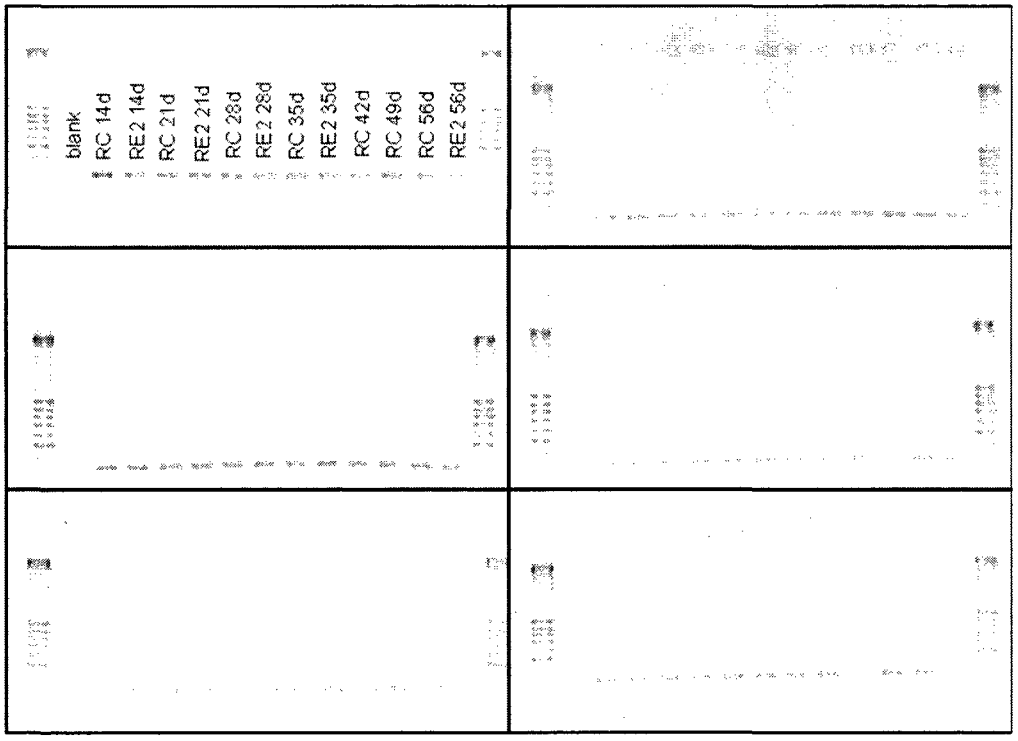
**APPENDIX I17: *GAI* Transcript Levels in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



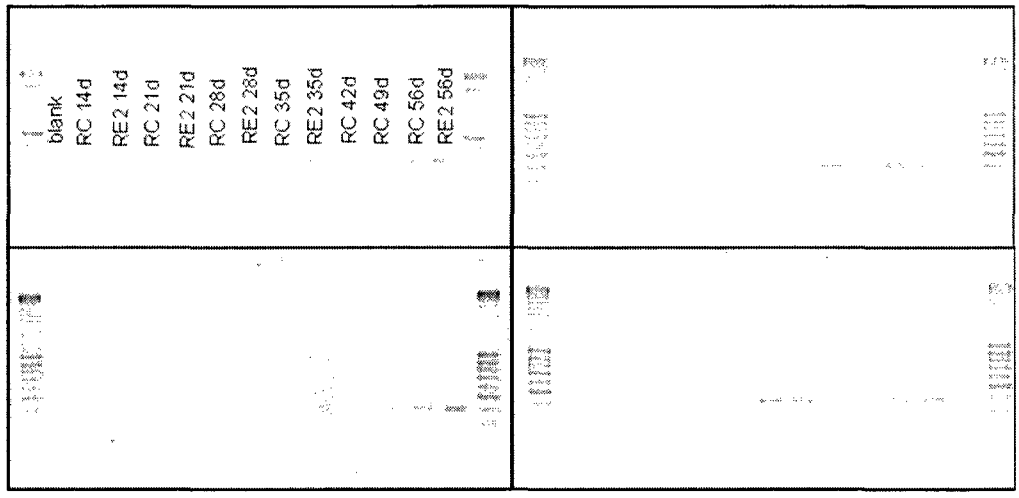
**APPENDIX I18: *ACTIN* Standards Corresponding to *GAI* in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



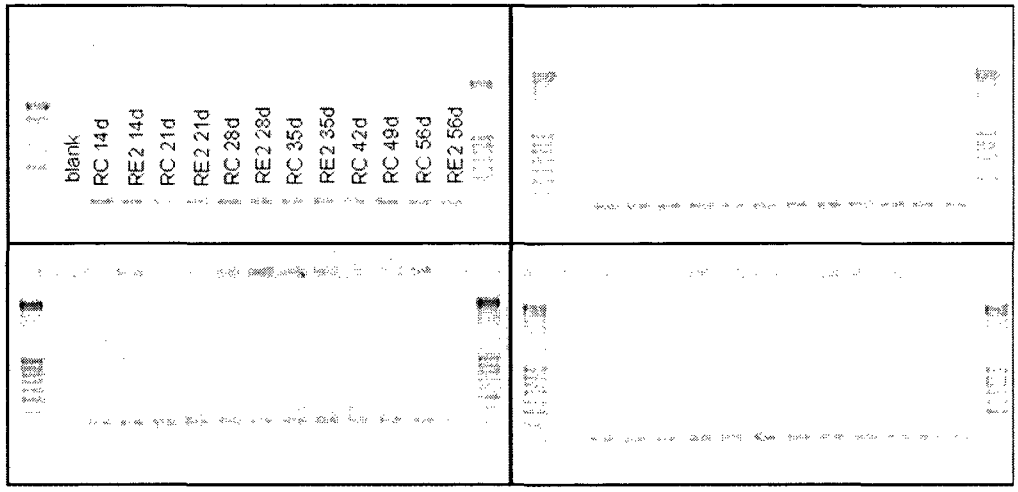
**APPENDIX I19: *API* Transcript Levels in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



**APPENDIX I20: *ACTIN* Standards Corresponding to *API* in Shoot Tips.**

Those gels from the first replicate are in the left column, and those from the second replicate are in the right column. All samples were run in the order shown for the top left gel, and were run using the conditions outlined in section 4.2.2.



**APPENDIX J.: cDNA Sequences for Five Genes in Flax**

"N" indicates that the specific nucleotide could not be determined by sequencing, and a lower case letter indicates that there was low resolution for that specific nucleotide.

***SOC1***:

gtgtttgAGCTCtCTGTTctCTGTgATGCtGAAGTTGCNcTNATCATTTtctcNCCCAA
GAggNAAACTTTATGAGTTCTCCaGCTCTagNatCANCAAAacnATnGAGCGGT
ACGAGaAGAAGCATAAGGaTaTa

***COL***:

TTATCATCATCGTCAATAGACGTGGGAGTCGTACCGGAAGGATGCGCAAT
GACGGAGATGTCGAATCCGGCGGCGGATTCGGCAGTGAGCCAGGCGGTTC
CGTTGTCGGCGGCGGATAGGGAGGCAAGGGTAATGAGGTACAGGAGAAG
AGGAAGAATAGGAAGTTCGAGAAGACGATTCGGTAA

***ADG1***:

TGCCAGTAGAAGTGTTCTAGGCATAATCCTTGGAGGTGGCGCTGGAACTC
GnCTTTACCCACTGACCAAGAAGAGGGCAAAACCTGCnGTGCCTCTGGGA
GCAAACTACAGGCTCATCGATATTCCTGTCAGCAATTGTTTGAACAGCAA
CATATCTAAGATTTATGTTCTTACTCAATTCAATTCTGCATCCCTGAATCGT
CACCTTTCTCGAGCTTATGCAAGCAACATGGGCGGCTACAAGAACGAAGG
CTTTGTAA

***GAI***:

TCATGTGATGTnCGGAGCTGTATCTCGGnAGGCAGATCTGCAACGTGGTGG
CGTGCGAAGGTGGTGACCGAGTTGAGCGGCACGAGACGTCGACTCAGTGG
AGGAGTAGnATGGAATCGGCTGGGTTCGACTCGGTTCACCTGGGATCGAA
CGCGTACAAGCAGGCGAGTATGTTGCtGGCCTTGTTCGCCGGCGGCGATGG
GTACaGAGTGGAGGAGAAGGAnGGGTCGTTGATGCTTGGCTggcAtACTAA

***AP1***:

TAAGAGGGATCGAGAACAAAATCAACAGGCAGGTCACTTTCTCCAAGCGG
AGGTCTGGTCTTCTCAAGAAGGCTCATGAGATCTCTGTCCTCTGCGACGCT
GAAGTCGCCCTCATCGTCTTCTCCCCCAAGGGCAAGCTCTTTGATTACTCC
ACCGACTCCTGCATGGAGAGCATCCTGGAACGCCACGAGAGACACTCATA
TGCTGACAGGAACCTTGTTCTCAATGACACTCAAACATGCGGTAGCTGGA
CTCTAGAATATGCAAAACTGAAAGCCAGGATTGAAGTGTTACAGAAGAAC
CTGAGGCACTACAACGGGGAAGAAATTGATACCTTAAACATTAGGGAGCT
TCAAAACCTGGAGCAGCAACTTGATTCTGGacttaaACATA

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