Studies of DNA Methylation and Flowering Time Genes in Early Flowering Flax (*Linum usitatissimum* L.) Lines Induced by 5-Azacytidine

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Pure-breeding, early-flowering lines of flax, derived from treatment of germinating seeds with 5-azacytdine in 1990, flower 7-13 days before controls, have fewer leaves, are shorter, and have hypomethylated total DNA, relative to control lines. This thesis examines the changes in DNA methylation levels in the cotyledons and shoot tips of early-flowering Royal flax lines (*i.e.* RE1 and RE2) and their control (RC) to determine the changes from 24 days to the onset of flowering (approximately 34 days in RE1 and RE2, and 52 days in RC). It also examines the question of whether DNA is methylated in the chloroplast genome of flax. Finally, the thesis looks at the differences in transcript abundance of the flowering genes *LEAFY* and *TERMINAL FLOWER1* in RC and RE2. Methylation levels in RE1 and RE2 were found to be lower than in RC from 24 days of age to the onset of flowering and the levels of all three lines increase with tissue age and/or differentiation. In addition, buds of RE2 were hypomethylated relative to RC. If plants were placed in the dark prior to DNA extraction, hypomethylation was not seen in the total DNA of RE2. The chloroplast DNA of flax was found to be methylated, and RE2 chloroplast DNA was hypomethylated relative to RC. Differences in transcript levels of *LFY* were seen in RC and RE2 shoot tips, where a higher accumulation of transcript seen in RE2 compared to RC may be related to its earlier flowering time. In leaves, there was no significant difference in the transcript abundance of *LFY* between RC and RE2. *TFL1* was detected in genomic DNA of RC and RE2; it was not detected in the cDNA of the two lines. In summary, compared to RC, hypomethylation was seen in the total DNA of RE2 plants grown under regular light conditions and the methylation levels in the all lines increased with age in shoot tips, cotyledons, and leaves. The chloroplast DNA of RE2 was also hypomethylated relative to that of RC. RE2 accumulated *LFY* transcript in shoot tips at flowering, which was not the case in RC. Although these ideas cannot be linked at this time, they are all likely related to the early-flowering phenotype.

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ANOVA:	Analysis of variance
azaC:	5-azacytidine
C-E1:	Comparison of the first early-flowering flax line and its corresponding control
C-E2:	Comparison of the second early-flowering flax line and its corresponding control
EtBr:	Ethidium bromide
FLIP:	Floral initiation process (a class of flowering genes)
HPLC:	High-pressure liquid chromatography
L:	Large fiber-type genotroph of flax
LC:	Control line of Large
LD:	Long day; class of flowering plants
LE1:	First early-flowering line derived from L
LE2:	Second, independently derived, early-flowering line from L
LFY:	*LEAFY*
mC:	Methylated cytosine
PCR:	Polymerase chain reaction
R:	Royal oilseed cultivar of flax
RC:	Control line of Royal
RE1:	First early-flowering line derived from R
RE2:	Second, independently derived, early-flowering line from R
RT-PCR:	Reverse-transcriptase polymerase chain reaction
SAM:	Shoot apical meristem
SD:	Short day; class of flowering plants
TBE:	Tris-borate EDTA
TFL1:	*TERMINAL FLOWER 1*

INTRODUCTION

1.1 *The Flax Lines*

1.1.1 Induction of the Early-Flowering Flax Lines

The early-flowering lines of flax were derived in 1990 by a one-time treatment of germinating seedlings with 5-azacytidine (azaC; Fieldes 1994), which is a known DNA demethylating agent (Jones 1984). Two early-flowering lines (E1 and E2) were produced from each of: i) Royal (R), which is an oilseed cultivar, ii) the genotroph Large (L), derived by Durrant from the fiber cultivar Stormont Cirrus (Durrant 1971), and iii) the fiber cultivar Mandarin (M). Four of these lines (LE1, LE2, RE1, and RE2) have been grown for eight to ten generations past the original treated generation. Because flax is an inbreeding species, self-pollination of the early-flowering plants (detected in the first generation after treatment) produced lines that were pure-breeding for early-flowering characteristics by the third generation after the treated generation (Fieldes and Amyot 1999).

Of the six early-flowering lines, those derived from Royal (*i.e.* RE1 and RE2) are the focus of this study. For comparative purposes, there are occasional references to the early-flowering L lines. The control line for RE1 and RE2, denoted RC, came from untreated Royal plants that were grown alongside the early-flowering lines throughout the generations.

1.1.2 Morphological and Developmental Characteristics of the Flax Lines

In comparison to their respective control lines, at maturity the early-flowering lines are shorter, have fewer leaves, and flower 7-13 days earlier (Table 1; Fieldes and Harvey 2004). The rates of growth and development of the early-flowering lines are relatively normal until the last (third) stage of vegetative development, which is accelerated in the early-flowering lines (Fieldes and Harvey 2004). The early-flowering characteristics vary among the early-flowering lines as well, for example, the RE2 phenotype is more pronounced than that of RE1.

Table 1: Differences in Morphology and Development of the Early Flowering and Control Lines of Flax

For the L lines, means for morphological characteristics (Amyot 1997) are from full-grown plants from 1996 greenhouse populations (LC: n=39, LE1 and LE2: n=20). For the R lines, all means are from mature greenhouse populations grown in 2005 (n=4). Means for methylation levels (Fieldes et al. 2005) are from 4-day-old seedlings grown in the growth chamber in 2004 (Controls: n=4, early-flowering lines: n=2).

Parameter:	Plant line:			Comparisons [c]:	
	RC	RE1	RE2	C-E1	C-E2
Flowering age (days)	54.4	46.1	45.1	40.2**	51.1**
Mainstem height (cm) [a]	73.46	41.24	35.89	516.56**	698.39**
Leaf number	94.5	48.6	41.1	433.1**	602.0**
Methylation level (%) [b]	14.13	12.50	13.18	14.47**	3.60ns
	LC	LE1	LE2	C-E1	C-E2
Flowering age (days)	52.0	46.6	42.0	296.2**	901.4**
Mainstem height (cm) [a]	102.3	84.7	61.1	296.5**	1620.9**
Leaf number	108.0	70.8	53.3	696.1**	1504.2**
Methylation level (%) [b]	14.36	13.45	12.53	6.30*	12.90**

** is significant at $P \leq 0.01$; * is significant at $P \leq 0.05$; ns is not significant at P=0.05.
[a] Main stem height is a measure from the cotyledons to the tip of the plant.
[b] While all other parameters are for plants at maturity, methylation level is for 4-day-old seedlings (DNA sampled from cotyledons and emerging shoot tips).
[c] Values are $F_{1/9}$ for all L parameters and $F_{1/12}$ for all R parameters, with the exception of methylation level comparisons, which are $F_{1/8}$.

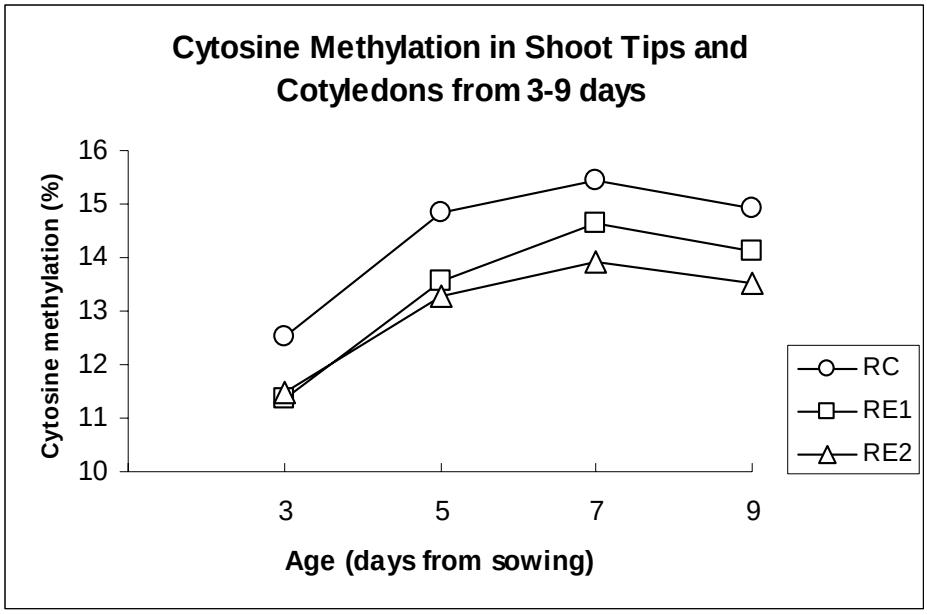
Methylation levels of total DNA are significantly decreased in the early-flowering lines when compared to control lines (Fieldes et al. 2005). In the tissues examined, approximately 14 percent of total cytosine, or 2.7 percent of total nucleotides, are methylated in flax (Fieldes et al. 2005). On average, the early-flowering flax lines have 6.2 percent less methylation in terminal clusters at 14 days of age, and 9.7 percent less methylation in four-day-old cotyledons and emerging shoot tips than corresponding control plants (Fieldes et al. 2005). This demethylation of the genome occurred because the azo-group of azaC that was used in the original treatment of the germinating seedlings is attached to the fifth carbon of the azacytidine. When the azacytidine incorporates into the DNA backbone during DNA replication, it prevents a methyl group from being added. Genetic crosses between early-flowering lines and their respective controls suggest that hypomethylation of the early-flowering lines co-segregates with the early-flowering phenotype (Fieldes et al. 2005). Hypomethylation is passed from generation to generation, suggesting that this epigenetic trait is responsible for the early-flowering characteristics and, thus, that the hypomethylation is not random and is related, in some way, to flowering time (Fieldes et al. 2005). Further, in flax seedlings, the methylation levels of the DNA increase with development (Figure 1; Fieldes unpublished).

1.1.3 Genetic Control of Early-Flowering in Flax

Segregation patterns seen in the progeny of outcrosses between the E1 lines of L, R, and M, and their respective controls suggest that changes in a minimum of three genes are required to produce the early-flowering phenotype. The genetic model suggests that there is one genetic locus called *Short* (*SH*), one called early flowering (*EF*) and a third, called *A* (Fieldes and Amyot 1999). The locus *A* must be homozygous recessive in order for the traits controlled by the other two loci, and thus the early-flowering phenotype, to be seen. The early-flowering alleles of both *SH* and *EF* are dominant or co-dominant, and they interact with each other to give variability in the early-flowering phenotype among the progeny in segregating generations of the outcrosses (Amyot 1997; Fieldes and Amyot 1999).

Figure 1: Change in Methylation Levels in Young Shoot Tips and Cotyledons of Flax

Significant differences in methylation levels in the combined shoot tip and cotyledon samples of the two early-flowering R lines, RE1 and RE2, and their control line, RC, from 3 to 9 days from sowing [$F_{1/10}$=29.9*] (Fieldes unpublished). The average standard error of the means (n=2), based on the error mean square, was 0.35.


Cytosine Methylation in Shoot Tips and Cotyledons from 3-9 days
Cytosine methylation (%)
16
15
14
13
12
11
10
3
5
7
9
Age (days from sowing)
RC
RE1
RE2

According to the working concept of the role of DNA methylation in flowering of flax (developed in the Fieldes' lab), all three of the early-flowering loci must be demethylated in order for the transition from vegetative growth to flowering to occur in flax. In the early-flowering lines, the induced demethylation from the azaC treatment is thought to have caused the three loci to be demethylated and therefore, these plants only need to reach a critical level of a floral stimulus in order to flower (Amyot 1997; Fieldes and Harvey 2004). In contrast, the transition to flowering takes longer in the control lines, since all three loci must become demethylated (naturally) before any response to cues from a floral stimulus can occur (Amyot 1997; Fieldes and Harvey 2004).

1.2 *Cytosine Methylation (mC)*

1.2.1 Global DNA and Histone Methylation

DNA methylation in plants acts as a defence against transposons, in addition to its role in regulating gene expression (reviewed in Chan, Henderson, and Jacobsen 2005). It is thought to be the most common post-replication modification in higher eukaryotes (reviewed in Li, Hall, and Holmes-Davis 2002). Most of the methylation in plants occurs at CpNpG triplets (reviewed in Chan, Henderson, and Jacobsen 2005), but 60-90 percent of all CpG pairs are also methylated, and the CpG doublets that are not methylated are generally found in the CpG islands of promoter regions (Ng and Bird 1999). DNA methylation is much more common in plants than animals, with up to 30 percent of cytosines being methylated in some plant species (Fasman 1976; reviewed in Adams 1990; Richards 1997). In plants especially, cytosine methylation may have a role in genome stabilization (Matassi et al. 1992). Although DNA methylation changes occur throughout plant development (Ruiz-Garcia, Cervera, and Martinez-Zapater 2005), permanent (epigenetic) changes can pass on lasting effects to progeny. Evidence for methylation changes with development in flax was seen in a study of cotyledons and emerging shoot tips from three to nine days of age, which showed increases in the DNA

methylation levels of RC, RE1, and RE2 over age and/or tissue development (Figure 1; Fieldes unpublished).

The methyltransferases, which are a group of proteins that regulate DNA methylation, are found in four forms in *Arabidopsis*, differing in arrangement of regulatory and methyltransferase domains. Of the four classes of transferases, two classes are responsible for *de novo* methylation, one is responsible for non-CpG methylation, where all three of these classes are thought to be directed by siRNA, and the other class has an unknown function (reviewed in Chan, Henderson, and Jacobsen 2005). Further, glycosylase enzymes can act to demethylate the genome by splicing the DNA at methylated sites and replacing methylated cytosine residues with non-methylated residues during DNA repair (reviewed in Chan, Henderson, and Jacobsen 2005). As previously mentioned, azaC can be used to induce demethylation of DNA (Jones 1984). After the removal of azaC in some plant species, such as tobacco, the genome will be re-methylated by a group of the *de novo* methyltransferases (Klaas et al. 1989). In other cases, such as rice and flax (Sano et al. 1990; Fieldes 1994), treatment with azaC can produce effects that are passed on to the progeny of treated plants.

Work on the locus *DECREASE IN DNA METHYLATION 1* (*DDM1*) in *Arabidopsis* has shown that other proteins must also be necessary for methylation to occur. Although mutations at the *DDM1* locus do not prevent the *in vitro* methylation at CpG and CpNpG locations by methyltransferases, DDM1 is required *in vivo* for methylation, where the protein is thought to mediate interactions between the methyltransferase and the DNA (Kakutani, Jeddeloh, and Richards 1995). *DDM1* encodes a SWI2/SNF2-like (switch/sucrose non-fermenting) protein that modifies the nucleosomes of chromatin and makes DNA more accessible to methyltransferases (Jeddeloh, Stokes, and Richards 1999); therefore, *ddm1* mutants have less methylation because their chromatin is more tightly packed (reviewed in Li, Hall, and Holmes-Davis 2002).

Methylation can also occur in histones, thereby modifying the structure of the chromatin and, thus, affecting gene expression. There is a complicated relationship between DNA methylation and histone methylation, where changes in histone methylation relate to changes in DNA methylation and

gene expression (He, Doyle, and Amasino 2004). Further, changes in histone acetylation are related to changes in DNA methylation and gene transcription (He, Doyle, and Amasino 2004). Specifically, DNA methylation and the rate of genome transcription tend to be inversely related; regions of the genome that have low transcriptional activity have higher levels of methylation than highly transcribed areas (reviewed in Boyes and Bird 1991).

Arabidopsis shows phenotypic changes when the methylation levels in the genome are altered. When the DNA methylation level is decreased, plants with reduced apical dominance, smaller stature, different leaf shape and size, decreased fertility and/or a change in flowering time are produced (Finnegan, Peacock, and Dennis 1996). The effects vary depending on the cultivar (Genger et al. 2003). A recent study on the short day (SD) plant *Perilla frutescens* var. *crispa* (wild basil) showed that the direct effects of azaC treatments induced demethylation of the genomic DNA and caused flowering under long day (LD) conditions, but no other phenotypic differences (Kondo et al. 2006). The phenotypic changes resulting from changes in methylation may be due to demethylation within promoter elements of the flowering genes, or transcription factors regulating them, or because of the changes in the structure of the chromatin surrounding certain flowering genes (Finnegan, Peacock, and Dennis 1996).

1.2.2 Organellar DNA Methylation

Variable results have been obtained for levels of cytosine methylation in the chloroplast of several species. In liverwort, (*Marchantia paleacea* var. *diptera*), it was determined that 0.2 percent of the chloroplast DNA is methylated, compared to 17 percent of the total genomic DNA, using HPLC and restriction enzyme techniques (Takio, Satoh, and Satoh 1994). Conversely, no chloroplast DNA methylation was detected by HPLC and restriction enzymes in *Lycopersicon esculentum (*tomato) leaves (Marano and Carrillo 1991), even though restriction digest studies had previously suggested that methylation is present in tomato leaf and fruit chloroplasts (Ngernprasirtsiri, Kobayashi, and Akazawa 1988a).

Methylation of chloroplast DNA has also been reported in *Chlamydomonas* (HPLC study; Burton, Grabowy, and Sager 1979), *Pisum sativum* (HPLC and restriction enzyme studies; Ohta et al. 1991), and the bundle sheaths of maize (restriction enzyme study; Ngernprasirtsiri et al. 1989). On the contrary, the absence of methylation has been reported in the chloroplast DNA of tobacco (Kovarik et al. 2000), and *Arabidopsis* (Godager et al. 1998). It should be noted, however, that these studies were done using restriction enzymes, which are now thought to be less reliable measures of chloroplast DNA methylation than HPLC studies (Godager et al. 1998; Fojtova, Kovarik, and Matyasek 2001).

DNA methylation levels vary between organelles as well. For example, in pea, there is more methylation in chloroplast DNA than amyloplast DNA (Ohta et al. 1991). Chloroplast DNA from *Acer pseudoplatanus* (sycamore) is not methylated (HPLC study), while amyloplast DNA contains cystosine methylation (Ngernprasirtsiri, Kobayashi, and Akazawa 1988b). Similarly, chloroplasts in leaves and seedlings of maize have no DNA methylation, while all non-photosynthetic plastids in maize have DNA methylation (restriction enzyme study, Gauly and Kossel 1989). This suggests that the methylation acts to suppress gene transcription in some, but not all, of the plastids and may be involved in conversion of plastid types (Kobayashi, Ngernprasirtsiri, and Akazawa 1990).

1.3 *Phase Change: the Transition Between Growth Stages*

The vegetative and reproductive growth stages are separated spatially and temporally (Lawson and Poethig 1995), and are controlled by the shoot apical meristem (SAM). This area, at the tip of the plant stem, produces undifferentiated cells, which later form all of the above ground plant parts. The inner (central) apex forms initials, while the outer SAM cells form organ primordia (reviewed in Evans and Barton 1997). From embryonic development, and throughout plant growth, the SAM will form initials, thus maintaining the developmental flexibility of the plant (Ma 1998). In tobacco, signals from the root maintain vegetative growth in seedlings but as plants age, the vegetative meristem responds to stimuli that promote flowering at the proper time (McDaniel 1996). The growth and shape of the SAM

differs among plant species (Evans and Barton 1997), but all are regulated by signals from external sources (Colasanti and Sundaresan 2000).

The vegetative growth of a plant is divided into juvenile and adult stages (Lawson and Poethig 1995). In the juvenile phase, leaf, stem, and axillary buds are formed, and in the adult stage, the SAM gains reproductive competence (Poethig 1990). Although SAM activity changes from the juvenile to adult phase, leaves are produced in both phases. However, different substances are sometimes produced in the vegetative tissue. For example, anthocyanin is only produced during the juvenile vegetative growth of ivy, and the leaves produced in this stage are less photosynthetic than the leaves produced during the adult stage (reviewed in Lawson and Poethig 1995). During both vegetative and reproductive development, organs are first initiated, and then identified, by switching on and off different genes, and finally, they produce their secondary components (Lawson and Poethig 1995). Reproductive growth occurs when the SAM gains competence to form sepals and petals, followed by stamens and pistils (Poethig 1990).

The genes that are involved in the floral initiation processes are referred to as FLIP genes. FLIP genes tend to up-regulate each other and work with the SAM to promote flowering. Thus far, in *Arabidopsis*, five FLIP genes have been identified: *LEAFY (LFY), APETALA1 (AP1), CAULIFLOWER (CAL), APETALA2 (AP2),* and *UNUSUAL FLORAL ORGANS (UFO)* (reviewed in Pidkowich, Kenz, and Haughn 1999). If the FLIP genes did not interact with the SAM, embryonic expression (*i.e.* the juvenile vegetative state of expression) would always occur because the plants would never become competent to flower (Pidkowich, Kenz, and Haughn 1999). The relationship between FLIP genes and the SAM needed for floral competence may be as simple as two genes producing the right amount of product at the same time.

The transition from one growth stage to another is called a phase change. It is regulated by independent and/or co-ordinated pathways of genes and other factors (Bernier et al. 1993; Lawson and Poethig 1995; reviewed in Henderson and Dean 2004). Independent regulation allows for greater plasticity for development, based on environmental changes. Pathways to flowering in plants are

redundant; if they were not organized in this way, a single mutation at any flowering gene would cause a loss of flowering in the plant. Generally, however, a mutation in a single flowering gene causes a change in floral morphology or a change in the time of flowering. Two of the most important environmental factors affecting flowering are photoperiod (i.e. day/night length) and temperature. Because so many different factors can affect flowering, some signalling pathways span the entire plant and involve substances found throughout the plant.

1.3.1 How Phase Change Occurs

The change from vegetative to floral growth is probably controlled by the balance of promoters and inhibitors in the SAM and is possibly induced when both reach a threshold level at the same time (Murfet 1985). Three different ideas have been suggested to explain this phase change. The earliest is the nutrient diversion hypothesis, which states that floral induction occurs because of a change in the relationship between products of the source and the sink. That is, plants are induced to flower when larger amounts of certain products are sent to the SAM (Sachs and Hackett 1983).

The second theory of phase change is the florigen/anti-florigen concept, where florigen promotes flowering and anti-florigen inhibits it. The promoters and inhibitors of flowering may be a combination of molecules and/or hormones (Bernier 1988). They may vary in combination among plant species. It is thought that these signals are produced in the leaves and are mobile, being sent to the SAM via the phloem (Bernier 1988). For many years, the precise nature of the compounds involved remained elusive. Recently, it was suggested that the mRNA of the floral pathway integrator *FLOWERING LOCUS T* (*FT*) might be florigen, and travel from the leaves to trigger flowering in the meristem (Huang et al. 2005). More recently, it has been suggested that it is the FT protein, and not the mRNA, in *Arabidopsis* (or its homologue Hd3a in rice) that is the florigen molecule (Corbesier et al. 2007; Tamaki et al. 2007; reviewed in Pennisi 2007). It has also recently been shown that phytochrome B in the mesophyll has a suppressive effect on *FT* in the cotyledons of *Arabidopsis* (Endo et al. 2005), which does not prove that it is the anti-florigen, but does emphasize the complexity of the system. For

example, genes affecting *FT* expression, such as *TERMINAL FLOWER 2 (TFL2),* will also likely play a role in the florigen/anti-florigen system (Kotake et al. 2003).

The third hypothesis regarding the promotion of flowering is that it is regulated by multifactorial control. This suggests that phytohormones are the main reason for the occurrence, and timing, of flowering (Bernier, Kinet, and Sachs 1981; reviewed in Bernier 1988), but that other factors, such as assimilate levels, are involved (Bernier et al. 1993). Although phytohormones are thought to be involved in floral induction, differences in genetics and growing conditions will make different phytohormones act as limiting factors for floral induction in different species. This theory incorporates the floral meristem identity genes, which switch the fate of the SAM from vegetative to reproductive growth, and the organ identity genes, which direct the formation of the reproductive organs (Bernier et al. 1993).

1.3.2 Flowering Pathway Studies in Model Plant Species

Two plant species in which flowering pathways have been extensively studied are *Pisum sativum* (pea), and *Arabidopsis*. In both species, photoperiod is important; both are LD plants (Bernier et al. 1993; Weller et al. 1997) and, therefore, they require a number of shortened dark periods between daylight in order for flowering to be induced. Important flowering time genes in pea include *Late Flowering (LF), Early initiating (E)* and *High response (HR;* Weller et al. 1997). Interactions between *LF* and *E* suggest a single flowering pathway is present in pea. In *Arabidopsis*, however, there are more flowering pathways that have been suggested (reviewed in Levy and Dean 1998; Figure 2).

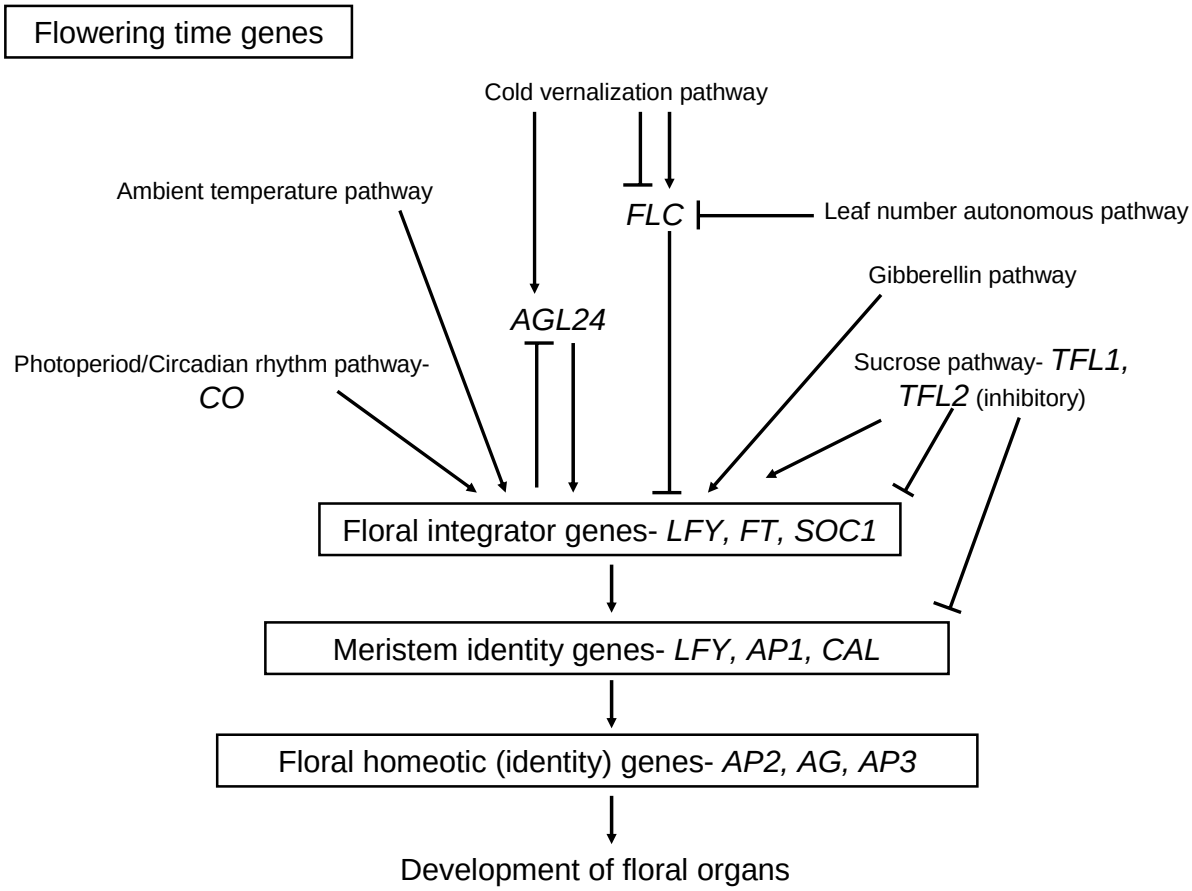
There appear to be approximately 80 different genes involved in flowering in *Arabidopsis*, most relating directly, or indirectly, to *LEAFY (LFY) (*Huala and Sussex 1992; reviewed in Levy and Dean 1998; reviewed in Araki 2001; reviewed in Ratcliffe and Riechmann 2002; Figure 2). In many cases, mutations of *LFY* have been found to change the type, or shape, of the inflorescence formed. For example, *lfy* mutants produce plants with elongated internodes and variable metamers in *Arabidopsis* (Schultz and Haughn 1991). *LFY* plays a central role in flowering time in *Arabidopsis.* It controls

Figure 2: Flowering Pathways and Classes of Genes in *Arabidopsis*.

The diagram specifies the four classes of flowering genes as well as other factors that affect flowering. Examples of the genes from each class that are mentioned in this document are indicated. Any genes that are not included in boxes are flowering time genes.

Arrows denote positive regulatory effects of genes (either those genes listed or others in the pathway) and flat lines represent inhibitory effects of genes on those functioning downstream.

(Adapted from Henderson and Dean 2004; Anthony 2006).


Flowering time genes
Cold vernalization pathway
Ambient temperature pathway
FLC
Leaf number autonomous pathway
Gibberellin pathway
AGL24
Photoperiod/Circadian rhythm pathway-
CO
Sucrose pathway- TFL1,
TFL2 (inhibitory)
Floral integrator genes- LFY, FT, SOC1
Meristem identity genes- LFY, AP1, CAL
Floral homeotic (identity) genes- AP2, AG, AP3
Development of floral organs

floral identity and integrates signals from the flowering time pathways (Koornneef et al. 1998; Pidkowich, Kenz, and Haughn 1999; reviewed in Henderson and Dean 2001).

It has been shown that DNA methylation is somehow related to flowering time in plants. For example, in the Landsberg *erecta* and Columbia ecotypes of *Arabidopsis*, *ddm1* mutant plants and transgenic antisense *METHYLTRANSFERASE1 (MET1)* plants have a reduction in DNA methylation levels and both lines have altered flowering times, as well as other phenotypic abnormalities (reviewed in Richards 1997). The hypomethylation is thought to occur in the meristem, since DNA replication and cell division are required (Burn et al. 1993), and may be passed to subsequent generations, as it is in rice (Sano et al. 1990) and in flax (Fieldes et al. 2005). In these cases, it has been proposed that a gene critical to flowering time must be demethylated and, thus, have altered expression before flowering can occur (Burn et al. 1993; Fieldes and Harvey 2004). Therefore, treated plants will flower earlier than normal, but in untreated plants, methylation levels are also likely to decrease closer to flowering as the "critical gene(s)" become demethylated in order to signal that the time has come for flowering.

Specifically, *Arabidopsis* has two flowering time genes that have been shown to be affected, directly or indirectly, by methylation. Altered methylation at the *VERNALIZATION2 (VRN2)* locus is thought to regulate expression of *FLOWERING LOCUS C (FLC)*, leading to a decrease in expression, which, in turn, causes early flowering in vernalization-sensitive ecotypes of *Arabidopsis* (Finnegan, Peacock, and Dennis 2000; Genger et al. 2003). Conversely, a direct demethylation effect at the *FWA* locus causes a delay in flowering time in the Landsberg *erecta* and Columbia ecotypes of *Arabidopsis* (Genger et al. 2003).

1.4 *Genes Involved in Flowering*

There are many genes involved in the control of flowering. These genes interact with each other, with hormones, and with the environment (Figure 2). They are divided into four functional

classes. First, there are the flowering time genes, which are involved in the timing of phase transition from vegetative growth to the onset of flowering. Second, there are the floral integrator genes, which integrate the information from all of the other factors related to flowering time. They are controlled, essentially, by flowering time genes, and encode proteins that activate the floral meristem identity genes (reviewed in Henderson and Dean 2004). The third class of flowering genes is the floral meristem identity genes, which determine what the meristem will form. If the floral meristem identity genes mutate, then the time of flowering does not change but other tissues such as leaves or leaf-like tissue replace the flowers (Poethig 1990). The final class of flowering genes is the floral identity, or floral homeotic genes. There are three sub-classes of floral identity genes, A, B, and C, which are classified based on which floral organs they are involved in developing and how they interact with each other (Parcy et al. 1998).

1.4.1 *LEAFY*

One gene that plays a critical role in the flowering time of many species is *LFY*, also known as *FLORICAULA (FLO)* in some species, such as snapdragon (*Antirrhinum majus*) where it was first reported. It is a unique transcriptional regulator classified as both a floral integrator, and a floral meristem identity gene (Henderson and Dean 2004). There is considered to be a threshold level of *LFY* transcript necessary in order for *Arabidopsis* to make the transition from vegetative to reproductive growth (Blazquez et al. 1997; Hempel et al. 1997). In addition, *LFY* is involved in floral patterning by regulating genes in all three of the A, B and C classes of floral identity genes. It affects sepal, petal, stamen and carpel development; this role of *LFY* is separate from its role in floral meristem identity, where it works in the initial floral primordia development (Parcy et al. 1998).

Flowering genes can be classified as those that respond to LFY activity, and those that affect *LFY* transcription, while some do both (Nilsson et al. 1998). There are other factors, however, that may also affect *LFY* and its proteins' function (Figure 2). For example, in *Arabidopsis*, gibberellin-deficiency in

LD species causes a reduction of *LFY* expression, and in SD species, it prevents *LFY* expression entirely (Blazquez et al. 1998).

Although the reproductive function of *LFY/FLO* is conserved among species, the developmental function is not (Kelly, Bonnlander, and Meeks-Wagner 1995; Hofer et al. 1997; Mouradov et al. 1998). In both *Arabidopsis* and *Antirrhinum*, a loss-of-function mutation in the gene (*i.e. LFY* or *FLO*) will cause indeterminant secondary shoots to form, as opposed to flowers (Weigel et al. 1992; Coen et al. 1990). In rice, the homologue to *LFY* is *RFL*. When *RFL* was put into *Arabidopsis*, the transgenic plants flowered early, but did not develop normally (Chujo et al. 2003). However, when the *RFL* gene is attached to a *LFY* promoter and put into an *Arabidopsis lfy* mutant, the gene was able to rescue, partially, the mutant so there must be some similarities between the two, even though *RFL* is somewhat divergent in function from *LFY* (Chujo et al. 2003).

Woody plant species also have *LFY* homologues. For example, *Pinus radiata* (pine) has the gene *NEEDLY*, and *Populus trichocarpa* (poplar) has *PTLF*. If over expressed, *PTLF* causes phenotypic changes after several years, and, thus, it can be concluded that negative regulation of *PTLF* only occurs at young ages (Rottmann et al. 2000). Interestingly, when *LFY* was placed into transgenic poplar trees and over expressed, development was altered, but the expected early flowering phenotype was not seen (Rottmann et al. 2000).

In *Malus sylvestris* L. var *domestica*, (apple), there is a homologue of *LFY* and *FLO*, called *AFL* that is expressed in both vegetative and reproductive organs (Kotoda et al. 2000). Other studies on citrus fruits from the family Rosaceae (subfamily Maloideae) have shown that, of the six fruits studied, all have two homologues of *LFY*, with the highest level of sequence conservation being at the C-terminus (Esumi, Tao, and Yonermori 2005). Further, *Citrus sinensis* (Washington navel orange) has a *LFY* homologue called *csLFY*.

In *Arabidopsis*, *LFY* is expressed during vegetative growth, and its expression increases with floral induction (Nilsson et al. 1998). This is not the case with the *Impatiens balsamia* homologue *FLO*, where the gene is transcribed constitutively in both vegetative tissue and the floral meristem

(Pouteau et al. 1997). Both *AFL* in apple (Kotoda et al. 2000), and *NEEDLY* in pine (Mouradov et al. 1998) have similar expression patterns to *LFY* in *Arabidopsis*, and, while *PTLF* is expressed in the young leaves of poplar, it is much more strongly expressed in inflorescences (Rottmann et al. 2000). In oranges, *csLFY* is only expressed in reproductive tissues (Pillitteri, Lovatt, and Walling 2004).

The relationships among flowering genes are very complicated. Some genes will act as inhibitors for certain genes while stimulating others and these effects will cause diverse downstream effects. *LFY*, for example, has many downstream targets such as *AP1, APETALA3 (AP3)* and *AGAMOUS (AG)* (Parcy et al. 1998; Busch, Bomblies, and Wiegel 1999; Wagner, Sablowski, and Meyerowitz 1999; Lamb et al. 2002). The interlinking of these genes is mediated by other genes. For example, *AGAMOUS-LIKE 24 (AGL24)* acts upstream of *LFY* to mediate cross talk between *FT, SOC1,* and *LFY* in *Arabidopsis* (Yu et al. 2002).

1.4.2 *TERMINAL FLOWER 1*

Another gene important to the flowering process in *Arabidopsis* is *TERMINAL FLOWER 1 (TFL1)*. This flowering time gene affects *LFY* by inhibiting *LFY*'s function in the inflorescence meristem (Shannon and Meeks-Wagner 1993; Koornneef et al. 1998), specifically in the initiation of the floral primordia (Alvarez, Guli, and Smyth 1992). *TFL1* encodes a protein that is similar to the mammalian phosphatidylethanolamine-binding protein (PBP) (Bradley et al. 1997; Ohshima et al. 1997). The *TFL1* homologue *CEN* (in *Antirrhinum*) is thought to interact with phospholipids or GTP-binding proteins on cell membranes to mediate floral signaling, preventing the signal from reaching the meristem, and it is possible that TFL1 acts similarly because the binding sites between TFL1 and CEN are highly conserved (Ohshima et al. 1997).

The down-regulation of *TFL1* expression in the shoot meristem occurs before strong expression of *LFY* and *AP1* and thus, if high levels of *TFL1* transcript are present, there could be repression of *LFY* and *AP1* expression, resulting in a later flowering time for the plant (Ratcliffe, Bradley, and Coen 1999). In *Arabidopsis*, the activity of TFL1 allows indeterminate floral meristems to form, whereas *tfl1*

mutants are determinant (Alvarez, Guli, and Smyth 1992), and are committed to flowering early (Shannon and Meeks-Wagner 1991; Bradley et al. 1997).

Although not many detailed studies have been conducted on *TFL1*, homologues to this gene have been found in other plants, such as *Impatiens balsama*, pea, oranges, *Antirrhinum (CENTRORADIALIS; CEN)*, and tomato *(SELF-PRUNING; SP)*, as well as in some fruit species, including six Maloideae species. In grapes (*Vitis vinifera* L.), the SAM produces floral and vegetative primordia at regular intervals (Boss, Sreekantan, and Thomas 2006), which is different from the activity of the SAM in many other plants, including *Arabidopsis* (Bradley et al. 1997). Expression patterns of the *TFL1* homologue in grape, called *VvTFL1*, are similar to *SP* (in tomato) in that transcripts are seen in the outer cells and primordia of the meristem until the bud opens (Boss, Sreekantan, and Thomas 2006). However, in *Arabidopsis* (Bradley et al. 1997), and tobacco (Amaya, Ratcliffe, and Bradley 1999), expression of *TFL1* (and homologues) is restricted to the central core of the SAM. Transcripts of *TFL1* are also present in the vegetative tissue of *Arabidopsis* where they work to suppress the development of the inflorescence (Bradley et al. 1997); expression is relatively weak until after commitment to flowering (Bradley et al. 1997).

TFL1 expression is thought to be involved in two separate pathways, one where *LFY* is concerned, and the other incorporating *AP1* and *AP2* (Shannon and Meeks-Wagner 1993). As with *LFY, TFL1* has an inhibitory effect on *AP1* and *AP2*; however, this may be indirect (Shannon and Meeks-Wagner 1993). Although expression patterns of *TFL1* differ among plant species, in all cases it is an inhibitor of *LFY* and a repressor of floral development.

1.4.3 Other Important Flowering Genes

The *CONSTANS (CO)* gene, which acts immediately up-stream of the proposed florigen molecule FT in *Arabidopsis*, is a flowering time gene. It has also been found in *Brassica napus*, and in the SD plant *Pharbitis nil* (Morning glory) where it is called *PnCO*. This gene is regulated by photoperiod but its circadian rhythm is unlike that of most of its *CO* homologues in that its rhythm

ensures that *PnCO* mRNA is accumulated after a long night, thereby stimulating flowering (Liu et al. 2001). In contrast, *Arabidopsis* is a LD plant and therefore accumulates *CO* transcripts following a long day period (Liu et al. 2001). This displays the evolution of *CO* as well as its importance in mediation of floral induction in photoperiod-dependent plant species.

FLOWERING LOCUS C (*FLC)* is highly conserved among plant species. This flowering time gene encodes a MADS-BOX, dosage-dependent protein that is a repressor of the downstream floral inhibitor *SUPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1)* (Sheldon et al. 2000; Tadege et al. 2001) that is indirectly controlled by methylation. In *B. napus*, there are five *FLC*-like gene sequences (Tadege et al. 2001). If constructs of any of these five *Brassica* genes are put into the *Arabidopsis* Landsberg *erecta* genotype, there is a significant delay in flowering, and vice-versa (Tadege et al. 2001).

1.5 *Study Objectives*

This study is divided into three sections: an HPLC developmental profile of DNA methylation levels, a chloroplast DNA methylation study, and an analysis of gene expression of two flowering genes. In the HPLC study, the three R lines (*i.e.* RC, RE1 and RE2) were used to compare total DNA methylation levels, examining changes with time, and development, from 24 days of age to the onset of flowering. This was done using the same extraction and HPLC methods that had been used previously for the same lines, from three to nine days of age (Fieldes unpublished; Figure 1). Therefore, a nearly completed developmental profile of total DNA methylation changes with development was obtained for the R lines. The hypotheses for this study were based on information about methylation levels that had already been determined for younger plants. They were that methylation levels would increase with tissue age and/or differentiation, and that throughout development the early-flowering R lines would remain hypomethylated relative to the control line.

Studies whereby chloroplast DNA methylation levels were measured in various plant species have produced inconsistent results. Therefore, the second part of this study was designed to determine,

first, if chloroplast DNA of flax is methylated and second, whether chloroplast DNA of an early-flowering line is hypomethylated relative to the control line, as seen in total flax DNA. If the flax chloroplast DNA was found to be methylated, the hypothesis was that methylation differences between the early-flowering line (RE2) and control line (RC) would be similar to the trend observed for the total DNA; *i.e.* the chloroplast DNA of the early-flowering line would be hypomethylated relative to the chloroplast DNA of the control line.

The third, and final, part of the study was an analysis of gene expression. These experiments were designed to compare the levels of *LFY* and *TFL1* transcripts in and between the control and early-flowering plant lines, RC and RE2. The hypotheses for this portion of the study revolve around the functions of the *TFL1* and *LFY* genes in *Arabidopsis* and the relationship between the two (*i.e.* TFL1 is an inhibitor of *LFY*). It was hypothesized that *TFL1* would be expressed in the shoot apex prior to flowering, when *LFY* is not expressed, and that after the onset of flowering, the expression pattern would be reversed. In addition, it was expected that the expression of the two genes in the early flowering and control lines would differ; the early-flowering lines should have either a greater accumulation of the *LFY* transcript in their tissues (vegetative and reproductive), or a lower accumulation of the *TFL1* transcript, in comparison to the control lines.

This thesis is organized into chapters, based on the three sections of the study. Each chapter contains the materials and methods, results, and a brief discussion of the findings from the experiments, followed by all of the figures and tables for the chapter. A more detailed discussion encompassing all three studies can be found in the final chapter.

2 DEVELOPMENTAL PROFILE OF METHYLATION LEVELS: THE HPLC STUDY

Previous research done in the Fieldes' lab has suggested that there are increases in methylation levels in early flowering and control lines of flax during early development (Fieldes unpublished). This work was done using the early-flowering R lines, and the control, RC. The current study, presented here, was performed in order to complete the developmental profile of methylation in the R lines of flax, looking at changes in DNA methylation levels in the cotyledons and shoot tips from 24 days of age to the onset of flowering.

2.1 *Materials and Methods*

2.1.1 DNA Methylation Levels from 24 days to the Onset of Flowering and Cotyledon Senescence

During the summer of 2005, plants from all three R lines (*i.e.* RC, RE1, and RE2) were grown in the greenhouse at Wilfrid Laurier University for this experiment. The RE2 line used was previously referred to as RE2', because it was the second E2 line derived from one of the original azaC treated R plants. The first RE2 line reverted in the third generation after treatment (Fieldes et al. 2005). The new derivative of RE2 is referred to here simply as RE2 and has been pure-breeding for eight generations beyond the treatment generation (Fieldes et al. 2005).

For all of the lines (RC, RE1, and RE2), plants were grown, six per pot, to 24 days of age to the onset of flowering, in 5 inch diameter round pots filled with Vermiculite® (Premier Horticulture Inc., PA). These plants were supplied with tap water, as needed. At seven-day intervals after planting, the plants were given an inorganic nutrient solution modified from that of Murashige and Skoog (1962; Appendix A). Seeds were sown on the same day (April 29th, 2005), which gave staggered sampling dates. All sampling was done in duplicate.

Shoot tips (*i.e.* all tissue above the first visible internode at the top of the mainstem) from all three lines were sampled on days 24, 27, 31 and 34. Sampling of the RC line continued through days

38, 41, 45, 48, and 52 (*i.e.* sampling stopped at the onset of flowering in all lines). For all three lines, cotyledons were sampled on days 24, 31, 38 and 52, at which time most cotyledons had visual signs of senescence. The fresh weight of the harvested tissue was taken, the tissue was ground in liquid nitrogen, and it was quickly transferred into a 1.5 mL eppendorf tube. DNA was extracted from these samples using the Qiagen® (Mississauga, ON) DNeasy minikit protocol (Appendix B) and hydrolyzed using a modification (Appendix C) of a method previously described by Matassi et al. (1992). The final volume of all samples after hydrolysis was 299 µL.

2.1.2 Shoot Tip Experiment

To test the importance of similarity in the size of the shoot tip used for DNA extraction, shoot tips were excised from 14-day-old plants and divided into an upper and lower segment. Plants for this portion of the study were planted, 10 plants per replicate (2 replicates of RC and RE2) in labelled 3.75-inch square pots, containing Vermiculite®, and germinated in an 8/16 h dark/light cycle and a temperature ranging from approximately 16 ºC to 25 ºC. Pots were covered with acetate sheets to retain humidity, and were given de-ionized water for germination. On the third day after planting, the acetate sheets were removed and the seedlings were moved to a growth chamber, where they were supplied with light from eight 40-watt cool white fluorescent tubes in a similar 8/16 h dark/light cycle with a temperature range of 16 ºC-25 ºC and humidity varying from 20 to 35 percent. These plants were supplied with nutrient solution (Appendix A) on day seven. At 14 days of age, shoot tips were removed for sampling, as previously described (2.1.1). For the upper shoot tip portion, 100 mg of leaf tissue was used, and 150 mg were used for lower shoot tip portions (combined weight from all 10 plants); the leaves in each portion were counted. DNA was extracted from this material and samples were hydrolyzed (Appendix B and Appendix C), giving a final sample volume of 299 µL.

2.1.3 Flower Bud Experiment

For the bud study, 10 plants per replicate (two) of both RC and RE2 were grown in the growth chamber in the same way as those described above. They were given nutrient solution every seven days beginning at day seven. For the extraction of bud DNA, buds were classified into three groups (small, medium, and large) based on length and width measurements of the buds, as well as general appearance of the buds (*i.e.* colour, *etc.*). Pictures were taken of the buds (Figure 3), and the procedures for extraction and hydrolysis of DNA, used above, were followed (Appendix B and Appendix C). Buds were not sampled at any specific plant age; they were sampled when plants had enough buds of all specific sizes available. The RE2 plants were approximately 47 days old (mean flowering age of 43 days in the growth chamber), and the RC plants were approximately 83 days old (mean flowering age of 62 days in the growth chamber).

2.1.4 HPLC Analysis

After DNA hydrolysis and centrifugation (7500 x g for 30 minutes), 50 µL of the supernatant of each sample was automatically injected by a Varian® Prostar 410 autosampler into a Varian® Prostar 230 HPLC. The samples were run on a 150 x 4.6 mm Supelcosil C-18S (with a LC-18C Supelguard pre-column, held at 30 °C) with an elution procedure (solutions at pH 4.0; Appendix D) that was modified from that of Matassi et al. (1992), as described in Fieldes et al. (2005). Nucleoside peaks were detected by a Varian® Prostar 350 UV-Vis Detector. The absorbance wavelength used was 260 nm, except during the elution of 5-methylcytidine peak, which was measured at 280 nm for a two-minute period (Fieldes et al. 2005). Areas of the chromatogram peaks, calculated by computer software (Star® Chromatography), were used, in combination with the appropriate extinction coefficients, to provide an estimate of the concentration of each nucleoside (in µM). These concentrations were then used to determine percentages of methylated cytidine (corresponding to the percentage of methylated cytosine) in the DNA samples, as well as the DNA concentration (per 10 plants, and per 100 mg of

fresh weight tissue). Each DNA sample was run twice on the HPLC column and averages were used for data analysis. Analyses of variance (ANOVAs) and orthogonal comparisons were used to determine the statistical differences among plant lines, the effects of plant age, and any interactions. For each parameter, the average standard errors for the means shown in the figures were derived from the error mean squares of the corresponding ANOVA.

2.2 *Results*

2.2.1 Comparisons of DNA Methylation Levels

In the shoot tips, the methylation levels increased significantly [$F_{1/11}$=9.15*] from day 24 to day 34 (Figure 4a; Table 2a). Previous work had determined that there are three vegetative growth phases in these flax lines, called the early- , mid-, and late-vegetative phases (Fieldes and Harvey 2004). The increase in DNA methylation seen, here, began approximately at the transition between the mid- and late-vegetative growth stages. Non-orthogonal comparisons showed that the methylation value of RE2 was significantly [$F_{1/11}$=7.24*] lower than that of RC at 24 and 27 days of age, however, this was not the case [$F_{1/11}$ <1.0] with RE1.

Methylation levels also increased significantly [$F_{1/14}$=30.9**] in the cotyledons from day 24 to 52 (Figure 5a; Table 2b) and the hypomethylation of the early-flowering lines was only seen at the first three sampling ages [$F_{1/14}$=6.06**].

2.2.2 Comparisons of Other DNA and Tissue Characteristics

In the shoot tips, linear increases were seen in the tissue weight [$F_{1/11}$= 34.5**], DNA concentration (µM) per 10 shoot tips [$F_{1/11}$= 23.3**], and DNA concentration (µM) per mg fresh weight [$F_{1/11}$= 103**] (Figure 4b-d; Table 2a) with increasing age. The increase in DNA concentration per unit fresh weight did not continue in the RC line after sampling ended in the early-flowering lines (*i.e.* after

the transition from mid- to late- vegetative growth), however, the increases in the other above parameters did continue.

In the cotyledons, there were no significant differences between the early flowering and control lines for any of the parameters, except methylation levels, as previously discussed (2.2.1; Figure 5b-d; Table 2b). Yellowing and abscission of the cotyledons had begun in all lines by day 52. The data for DNA concentrations obtained on day 52 were anomalous and were not included in the analyses.

2.2.3 Cytosine Methylation Differences in Divided Shoot Tips

It was somewhat surprising that as the plants aged, the shoot tips did not retain the hypomethylation in RE2 seen in the other tissues. The increases in tissue weight and DNA concentration per tip in older tissues suggested that the size of the tip was a possible explanation. To test this possibility, the upper and lower regions of tips from 14 day-old plants were examined. In all three lines, the upper shoot tip regions were hypomethylated, relative to the lower regions [$F_{1/5}$= 9.86*]. The early-flowering lines had a lower methylation value in both regions of the shoot tips than the RC line [$F_{1/5=}$ 11.62*] (Figure 6a; Table 3a). In all three of the R lines, a higher DNA concentration (µM per 10 seedlings [$F_{1/5}$=25.4**] and µM per 100 mg fresh weight [$F_{1/5}$=33.6**]) was seen in the upper region of the shoot tips versus the lower region (Figure 6b; Table 3a).

2.2.4 Cytosine Methylation Levels in Flower Buds

Although methylation levels in the shoot tips of all three lines became similar as flowering approached (Figure 4a), hypomethylation was seen in RE2 flower buds [$F_{1/5}$= 7.40*] compared to the buds of RC (Figure 6c; Table 3b). There was no significant difference in methylation levels of the buds relating to size (therefore age) [$F_{1/5}$ <1.0]. The differences in the bud sizes (Table 3b) can be seen in Figure 3. There was no significant [$F_{1/5}$=3.84ns] difference in DNA recovered (µM per 10 buds) in the different sized buds; however, there was a significant [$F_{1/5}$=65.72**] linear decrease in DNA recovery (µM per 100 mg tissue) as bud size increased (Figure 6d; Table 3b).

2.3 *Conclusions*

From the data presented in this chapter, it appears that the methylation levels in both early-flowering and control lines are increasing with tissue development. This was seen in the cotyledons and shoot tips. In support of this observation, there is less DNA methylation in the upper portions of shoot tips, where the primordia are younger, than in the lower portion of the shoot tips. In the cotyledons, the methylation differences between the early-flowering lines and RC are seen until cotyledon senescence. In the shoot tips, the differences are seen until the onset of flowering. Nevertheless, RE2 buds are hypomethylated, relative to RC, suggesting that hypomethylation of RE2 is maintained in the meristematic regions to be passed onto progeny.

High levels of DNA recovery at younger ages in shoot tips and cotyledons are likely related to cell division. Further, this could be related to cells becoming polyploid. When the DNA recovery is no longer increasing, the cells are likely to be fully differentiated (*i.e.* in the cotyledons). In cotyledons, the increase in DNA recovery would cease when the cotyledons are no longer growing by cell division and could be making the transition from growth to senescence. The buds do not show a difference in the DNA recovery with increasing size (*i.e.* per bud). However, they do show a decrease in DNA recovery per unit weight, which may be because small buds are differentiated to the same degree as large buds and the size difference is due, mainly, to cell expansion in the flower buds as floral organs expand.

In must be considered that the changes in the physical state of the tissue being extracted were unlikely to be causing changes in DNA recovery in the shoot tips because the recovery levels are increasing with age. However, in the cotyledons, the decrease in DNA recovery with age could potentially be related to continuing development of cell walls and increases in secondary metabolites.

Figure 3: Differences in Bud Sizes of RC and RE2 Flower Buds

Photographs of the different bud sizes (in all pictures: left to right are large, medium, small) for two replicates of both RC and RE2. Pictures were taken separately, as sampling occurred on different days. The differences in size are based on length, width, and colour.

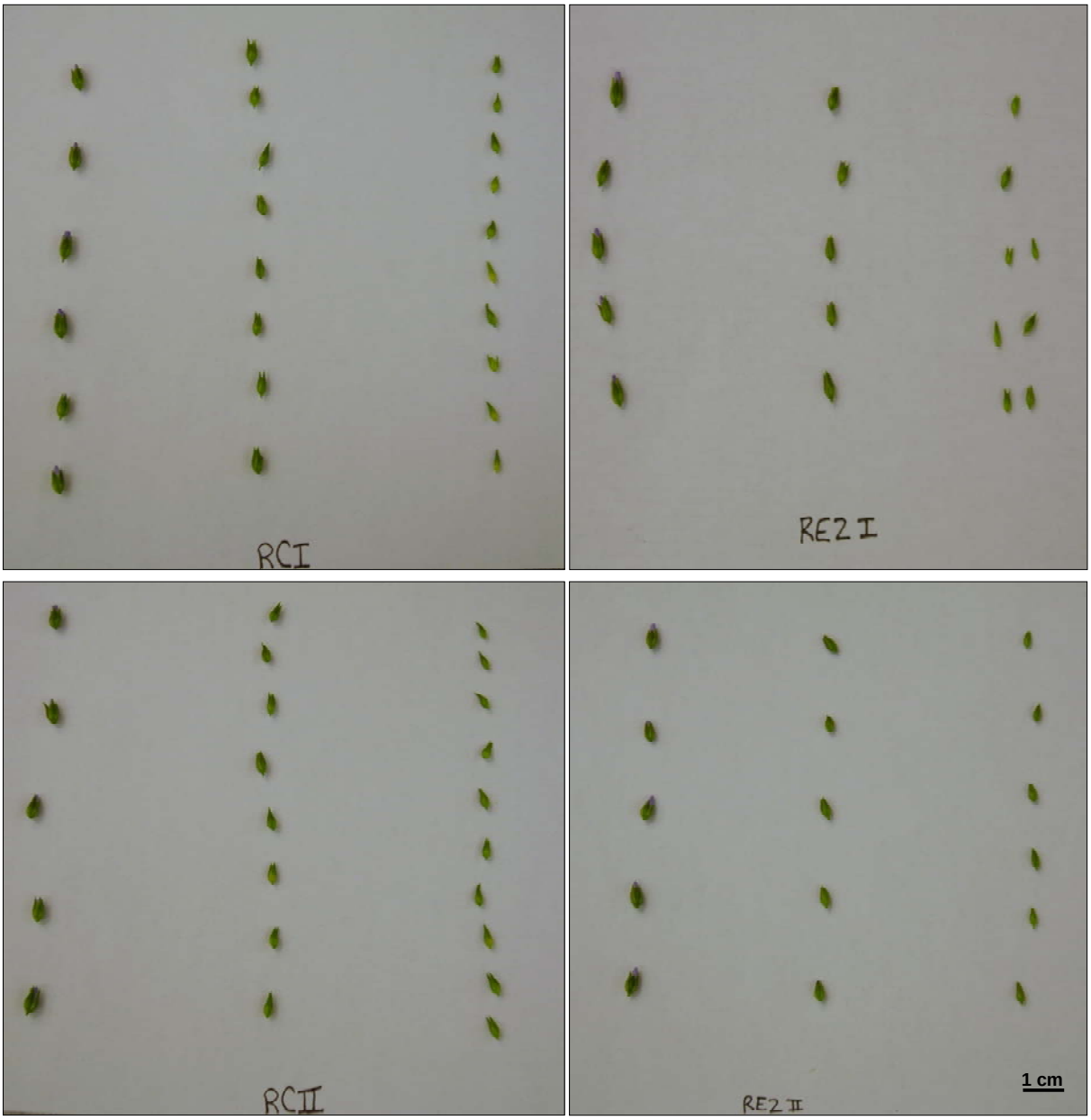

RCI
RE2 I
RCII
RE2 II
1 cm

Figure 4: Characteristics of Shoot Tips from 24 days of Growth to Flowering in R Lines

a) Linear increase in cytosine methylation from day 24 to day 34 (SE= 0.70). b) Linear increases in fresh weight (mg per 10 shoot tips) (SE= 61). c) Linear increase in DNA recovered (µM per 10 shoot tips) (SE= 9.1). d) Linear increase in DNA recovered per unit weight (µM per 10 shoot tips/mg per 10 shoot tips) (SE= 3.7). Data plotted are means (n=2).

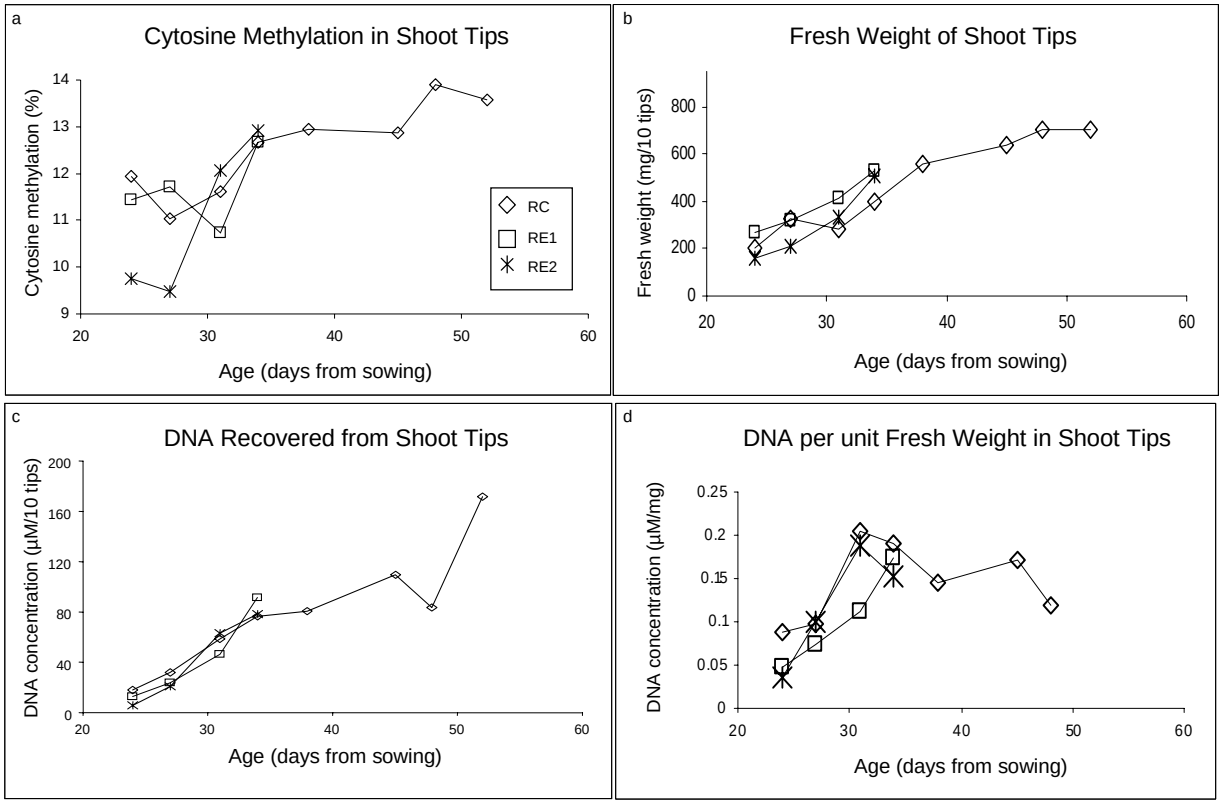

a
Cytosine Methylation in Shoot Tips
Cytosine methylation (%)
14
13
12
11
10
9
20
30
40
50
60
Age (days from sowing)
RC
RE1
RE2
b
Fresh Weight of Shoot Tips
Fresh weight (mg/10 tips)
800
600
400
200
0
20
30
40
50
60
Age (days from sowing)
c
DNA Recovered from Shoot Tips
DNA concentration (µM/10 tips)
200
160
120
80
40
0
20
30
40
50
60
Age (days from sowing)
d
DNA per unit Fresh Weight in Shoot Tips
DNA concentration (µM/mg)
0.25
0.2
0.15
0.1
0.05
0
20
30
40
50
60
Age (days from sowing)

Figure 5: Characteristics of Cotyledons from 24 to 52 days of Growth in R Lines

Significant differences in control and early-flowering lines and between early-flowering lines were seen in all parameters: a) Linear increase in cytosine methylation from day 24 to day 52 with differences among plant lines from 24 to 38 days of age (SE= 1.43). b) Quadratic change in fresh weight (mg per 10 pairs; SE= 57). c) Quadratic change in DNA recovered (μM per 10 pairs; SE= 0.97) d) No significant change in DNA recovered per unit weight (μM per mg) over development (SE= 0.225). Data plotted are means (n=2).

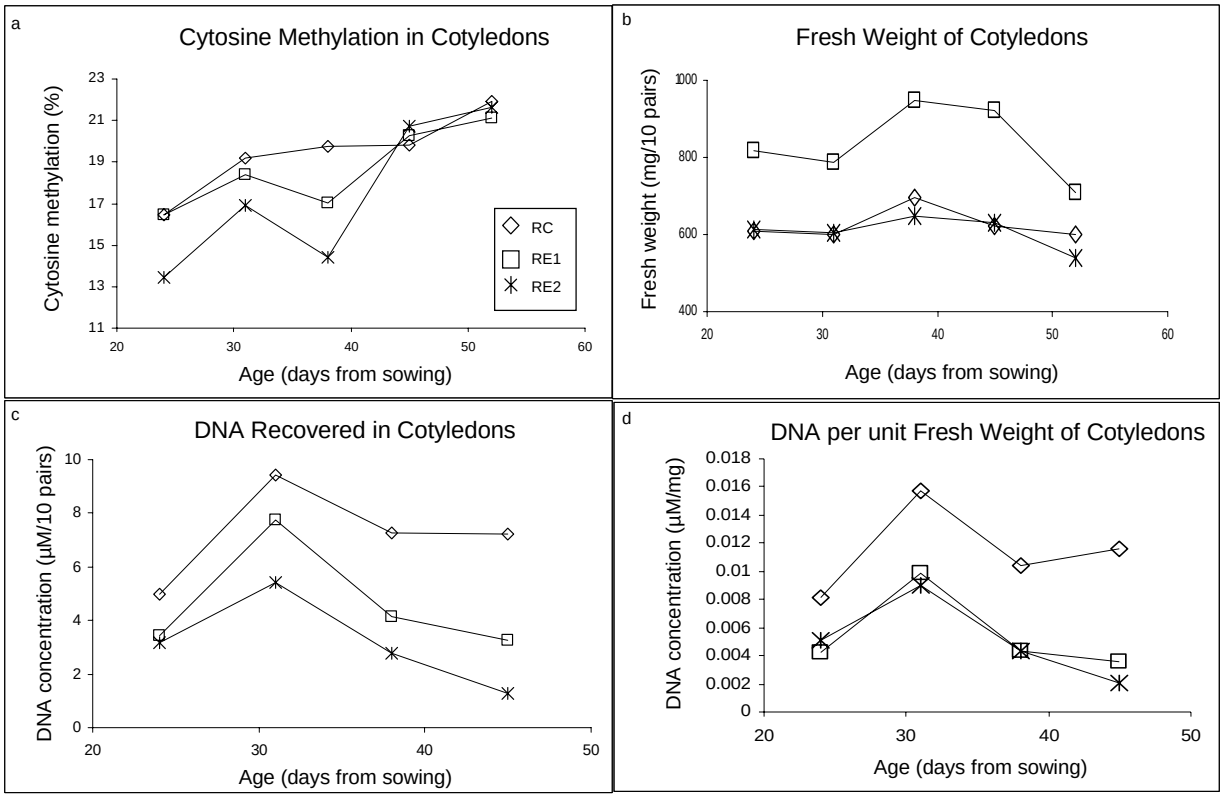

a
Cytosine Methylation in Cotyledons
Cytosine methylation (%)
23
21
19
17
15
13
11
20
30
40
50
60
Age (days from sowing)
RC
RE1
RE2
b
Fresh Weight of Cotyledons
Fresh weight (mg/10 pairs)
1000
800
600
400
20
30
40
50
60
Age (days from sowing)
c
DNA Recovered in Cotyledons
DNA concentration (µM/10 pairs)
10
8
6
4
2
0
20
30
40
50
Age (days from sowing)
d
DNA per unit Fresh Weight of Cotyledons
DNA concentration (µM/mg)
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
20
30
40
50
Age (days from sowing)

Figure 6: DNA Methylation Levels and DNA Recovery for Shoot Tip Regions and Flower Buds

a) Significant hypomethylation in the upper regions of all lines, as well as in the early-flowering lines, compared to RC (SE=0.38). b) Significantly higher concentration of DNA recovered (µM per 100 mg tissue) in the upper shoot tip regions when compared to the lower shoot tip regions (SE=4.24). c) Significant difference in DNA methylation levels between RC and RE2 was seen, but there was no differences in methylation levels with bud size (SE= 0.48). d) Significant linear decrease in DNA recovery (µM per 100 mg tissue) with increased bud size (SE= 1.52). Data plotted are means (n=2).

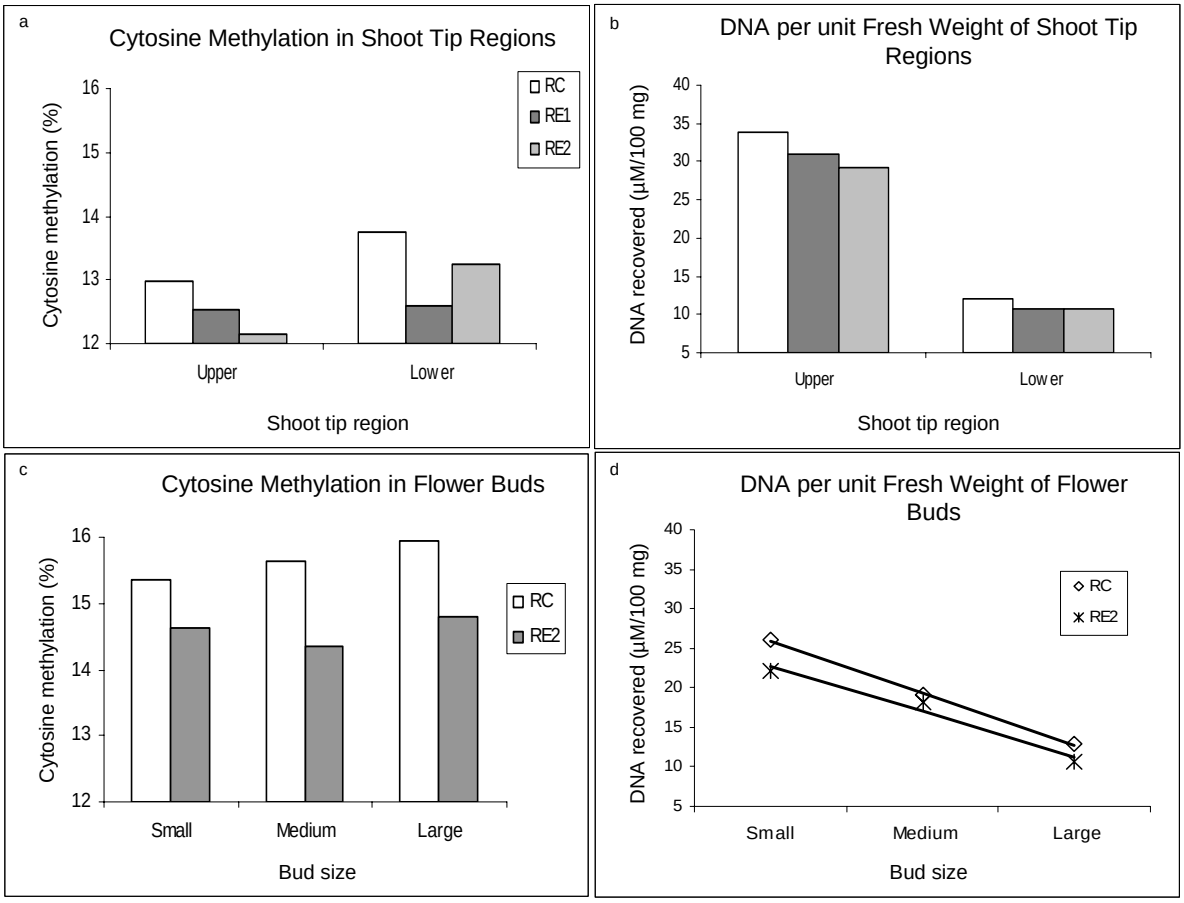

a
Cytosine Methylation in Shoot Tip Regions
Cytosine methylation (%)
16
15
14
13
12
RC
RE1
RE2
Upper
Lower
Shoot tip region
b
DNA per unit Fresh Weight of Shoot Tip Regions
DNA recovered (µM/100 mg)
40
35
30
25
20
15
10
5
Upper
Lower
Shoot tip region
c
Cytosine Methylation in Flower Buds
Cytosine methylation (%)
16
15
14
13
12
RC
RE2
Small
Medium
Large
Bud size
d
DNA per unit Fresh Weight of Flower Buds
DNA recovered (µM/100 mg)
40
35
30
25
20
15
10
5
RC
RE2
Small
Medium
Large
Bud size

Table 2: Means and F-values from the ANOVAs for Data from Shoot Tips and Cotyledons

a) Shoot tip means (n=8) are from days 24 to 34, with sampling twice per week, while b) cotyledon means (n=10) are from days 24 to 52, sampling once per week. DNA/unit tissue and DNA/unit weight analyses did not include day 52 data (means are n=8). No significant interactions were seen between sampling ages and plant lines (not shown).

Parameter	Plant Line			Comparisons			
				Lines		Ages	
	RC	RE1	RE2	C-E	E1-E2	Linear	Quadratic
a) Shoot tips:					$F_{1/11}$ values		
Tissue weight [a]	340	382	304	<1.0	3.18ns	34.5**	<1.0
DNA (μM)/unit tissue*10	46	44	42	<1.0	<1.0	103**	1.45ns
DNA (μM)/unit weight*100	12.64	10.53	11.89	<1.0	<1.0	23.3**	<1.0
mC level (%)	11.82	11.64	11.05	1.20	1.51ns	9.15*	3.34ns
b) Cotyledons:					$F_{1/14}$ values [b]		
Tissue weight [a]	625	837	607	9.54**	40.1**	<1.0	6.82*
DNA (μM)/unit tissue*10	7.2	4.64	3.13	46.6**	7.18*	1.67ns	23.4*
DNA (μM)/unit weight*100	1.14	0.58	0.55	16.07**	<1.0	<1.0	4.33ns
mC level (%)	19.42	18.66	17.43	3.45 ns	2.26ns	30.9**	<1.0

** is significant at $P \leq 0.01$; * is significant at $P \leq 0.05$; ns is not significant at P=0.05.
[a] Tissue weight is per 10 unit tissue (*i.e.* mg per 10 shoot tips, 10 pairs of cotyledons, or 10 buds).
[b] Values are $F_{1/11}$ for DNA/unit tissue and DNA/unit weight because 52 day data points were excluded from analysis.

Table 3: Means and F-values from the ANOVAs for Data from Divided Shoot Tips and Flower Buds

a) Means for the lines (n=2) for upper and lower stem regions b) Bud means (n=6) are from three different sizes of buds, small, medium and large. No significant interactions were seen between sampling ages and plant lines (not shown).

Parameter	Region	Plant Line			Comparisons		
					Lines		Region
		RC	RE1	RE2	C-E	E1-E2	Region
a) Shoot tips:					$F_{1/5}$ values		
Tissue weight [a]	Upper	120.9	74.5	89.7	13.91*	<1.0	28.2**
	Lower	184.9	137.9	136.7			
DNA (μM)/unit tissue*10	Upper	39.1	23.0	26.1	22.0**	<1.0	25.4**
	Lower	23.5	14.8	14.6			
DNA (μM)/unit weight*100	Upper	33.8	30.9	29.3	<1.0	<1.0	33.6**
	Lower	12.0	10.8	10.7			
mC level (%)	Upper	12.98	12.53	12.16	11.62*	<1.0	9.86*
	Lower	13.76	12.60	13.25			

Parameter	Plant Line		Comparisons		
	RC	RE2	Lines	Bud size	
			C-E	Linear	Quadratic
b) Flower buds:				$F_{1/5}$ values	
Tissue weight [a]	12.91	13.62	<1.0	128.10**	1.32ns
DNA (μM)/unit tissue*10	19.35	17.00	<1.0	3.84ns	1.09ns
DNA (μM)/unit weight*100	21.82	20.30	3.57ns	65.71**	<1.0
mC level (%)	15.56	14.58	7.40*	<1.0	<1.0

** is significant at $P \leq 0.01$; * is significant at $P \leq 0.05$; ns is not significant at P=0.05.
[a] Tissue weight is per unit tissue (*i.e.* mg per upper, or lower, segment of shoot tip or flower bud).

3 CHLOROPLAST DNA METHYLATION STUDY

The focus of the chloroplast DNA methylation study was to examine the primary question of whether there is cytosine methylation in the chloroplast DNA of flax and, if so, whether RE2 is hypomethylated relative to RC, as was previously seen in total DNA. Starch levels in the leaves were also examined in an attempt to determine optimal chloroplast isolation conditions. In addition, the possibility of contamination of the chloroplast DNA by nuclear and mitochondrial DNA was examined. Results of these experiments led to subsequent work which was done in order to examine methylation levels in total DNA of leaves from different segments of the stem, methylation levels in green tissue (*i.e.* shoot tips, cotyledons, and leaves) grown in different light conditions, and the fresh and dry weights of green tissue grown under different light conditions.

3.1 *Materials and Methods*

3.1.1 Comparison of Chloroplast and Total DNA

In the chloroplast methylation study, 60 plants per replicate (two) of both RC and RE2 were planted (12 seeds per pot) and grown in the same manner as the plants used for the division of the tip material and bud DNA, as described above (2.1). Alongside these plants, 5-10 plants per replicate, per line, were grown in the same conditions for total DNA extracts (where total DNA refers to all DNA extracted from the plant, including nuclear, mitochondrial, and chloroplast DNA). At seven and fourteen days after planting, all of the plants were supplied with nutrient solution (Appendix A). In this part of the study, the plants used for chloroplast isolation and for total DNA extractions were kept in the dark for variable amounts of time immediately prior to chloroplast isolation and/or DNA extraction, in order to reduce starch content (Coates and Cullis 1982). The times in the dark were 16 h, 29 h, 42 h, 55 h, and 68 h. At the appropriate time before extraction, the plants were placed in the dark, with increased humidity (100 percent vs. 25 to 35 percent as in regular light conditions) but the same temperature conditions. DNA was extracted from the plants at 21 days of age.

Shoot tips, cotyledons, and leaves from each of the two lines were collected and weighed before the chloroplast isolation. Chloroplasts were isolated using a modification of the protocol (Appendix E and Appendix F) described by Smith et al. (2002). Immediately following the chloroplast isolation, DNA was extracted and hydrolyzed from the chloroplast samples, and from the plants grown for total DNA. HPLC analysis was done on the chloroplast and total DNA samples, using the method outlined previously (2.1). The statistical analyses that were done to compare the chloroplast DNA methylation to the total DNA methylation, the methylation levels of the two lines, and the methylation levels of plants left in the dark for different lengths of time, were performed as described earlier (2.1).

3.1.2 Starch Levels in 21 day old Leaves

Starch levels were examined qualitatively to determine if the amount of starch in the leaves decreased due to the dark treatment, and to determine the optimal time in the dark for chloroplast isolation. The plants used for this were grown after the main experiment, but under the same conditions, including the range of dark intervals. Whole leaves were taken from the plants, at 21 days of age, and stained with iodine-potassium iodide (0.3 g I_2 and 1.0 g KI in 100 mL H_2O; as described in Schneider and Phillips 1981). The leaves were not cleared to remove pigments before staining.

3.1.3 Contamination of Chloroplast DNA by Nuclear and Mitochondrial DNA

To test the chloroplast DNA samples for contamination by mitochondrial and/or nuclear DNA, two replicates of RC and RE2 plants (five plants per replicate) were grown for DNA isolation, using the techniques and growing procedures as described above (2.1). Total DNA and chloroplast DNA were extracted, after a 42 h period in the dark, when the plants were 21 days old. When the DNA was extracted, instead of using Tris as an elution buffer in the final step, the AE buffer supplied in the DNeasy® kit was used (see Appendix B). Primers were needed in order to compare the levels of mitochondrial and nuclear DNA in total and chloroplast DNA samples by semi-quantitative PCR on genomic DNA. The primers for a portion of the mitochondrial gene *Maturase R (matR)* of *Linum*

arboretum (AY674533; sense- CCGCTCGGGTCGAGGCTGCC and antisense-CCCCAGCTCTATCAGCAGACC) were used to amplify the total DNA and the chloroplast DNA samples to check for mitochondrial DNA contamination in the chloroplast DNA samples (24 cycles of: 95°C for 30 s., 63°C for 1.5 min., 72°C for 1.5 min.). The template DNA for the PCR was diluted to a plant-to-plant ratio of 1:1 (chloroplast samples vs. total samples). Similarly, primers for a portion of the nuclear gene actin (AY857865; sense- GGTGGTGGCTCCCCCGGAG and antisense-CAAAGCAGCCAATCCCCCC) were designed for PCR of total and chloroplast DNA (31 cycles of: 95°C for 30 s., 67°C for 1.5 min., 72°C for 1 min.); however, in this case, the plant-to-plant ratio for the sources of DNA used as PCR template was (chloroplast:total) 60:1.

For both the mitochondrial and nuclear genes, the optimal number of PCR cycles was determined before the study was started, making this a semi-quantitative genomic DNA study. The gels were two percent agarose, run in 0.5X Tris-borate EDTA (TBE) buffer at 110V for 1.5 h using a PowerPac Basic power supply (BioRad, Mississauga, ON). They were run in a MiniCell EC370M (E-C Apparatus Co., Florida). The gels were stained in 0.5 µg/mL ethidium bromide (EtBr) for 15 minutes. Pictures of the gels were taken using the GelDocXR® Photosystem (BioRad) and band intensity was calculated using QuantityOne® software. Comparisons of band intensity between the total and chloroplast samples for each line (and each replicate) were done using Quantity One® software in order to determine the percentages of mitochondrial and nuclear DNA contamination in the chloroplast samples. The average band intensity for each DNA type was used to calculate the percentage ratio of amount of amplified material in the chloroplast DNA samples to the total DNA samples (*i.e.* cp/tot *100 for each of the two lines and two replicates). All four sample pairs (*i.e.* RC and RE2, replicates II and I) were then averaged for the final percentage contamination.

3.1.4 Subsequent Cytosine Methylation and Plant Weight Studies

After the main comparisons of the chloroplast and total DNA were completed, three subsequent methylation level studies were performed. The first was to determine the methylation differences

between RC and RE2 leaves sampled from the entire stem and from plants grown under regular light conditions. For this experiment, plants from both lines (two replicates) were grown to 21 days of age, in regular light conditions, as described previously (2.1). The plant stems were then divided into three equal sections, based on leaf number, and the DNA from the top, middle, and bottom leaves (relating to leaf location on the stem) was extracted, hydrolyzed and run on HPLC for analysis, using methods previously described (2.1). Secondly, a similar study was done, at 22 days of age, using plants grown under regular light conditions, as well as plants that had been grown in the dark for 42 h prior to DNA extraction. Thirdly, DNA methylation levels were examined using RC and RE2 (two replicates) plants, grown to 21 days, and extracting the total DNA from all plants grown in regular light conditions and from the same tissues from plants grown for 42 h in the dark before DNA extraction. This was also done using methods previously described (2.1).

Because of the possibility that both fresh and dry plant weight changed with time spent in the dark, plants of both the RC and RE2 lines grown in: i) regular light conditions, ii) for 42 h in the dark before DNA extraction, and iii) for 68 h in the dark before extraction, were used to determine how fresh and dry weight measurements were affected by the time in the dark. All green tissues were removed from the stems at 22 days of age, weighed, placed into oven-dried envelopes at 35 °C for seven days, and re-weighed. Data were converted into a percentage of dry to fresh weight. All data sets were analyzed statistically by ANOVA and orthogonal comparisons. The average standard errors for means, given in the figure legends, were derived from the error mean square values of the appropriate analyses.

3.2 *Results*

3.2.1 Comparisons of Cytosine Methylation and DNA Recovery in Total and Chloroplast Samples

The chloroplast DNA contained methylated cytosines and there was a significant [$F_{1/9}$= 8.63*] difference between the lines in the level of chloroplast DNA methylation; the RE2 chloroplast DNA was hypomethylated relative to RC (Figure 7a; Table 4). Unexpectedly, the level of methylation in

total DNA from dark grown plants was not significantly different in RC and RE2 [$F_{1/9}$<1.0ns]. This lack of hypomethylation in the total DNA from the green tissues of dark-grown RE2 plants was in contrast to the hypomethylation seen previously in the shoot tips (Figure 4a) and cotyledons of plants grown in regular light conditions (Figure 5a).

There was a significant [$F_{1/9}$= 11.21**] linear decrease in the DNA recovery (per 100 mg of tissue), in the total DNA samples of the plants grown alongside those for chloroplast isolation, as the time in the dark increased (Figure 7b). There was no significant [$F_{1/9}$= 4.17ns] trend in the recovery of DNA (per 100 mg of tissue) from the isolated chloroplasts (Figure 7c; Table 4).

3.2.2 Levels of Starch in Leaves

Leaves were stained to examine the starch levels in both lines after the various times in the dark, however pigments were not removed before the qualitative staining was done. The results (Figure 8) showed that leaves from dark-grown plants contained reduced starch levels, but that the length of time in the dark did not have any marked effect on the starch content. There was no gradient in starch content evident from leaves of plants grown in regular light conditions to leaves of plants placed in the dark for 68 h prior to staining.

3.2.3 Chloroplast DNA Contamination by Nuclear and Mitochondrial DNA

Using a semi-quantitative PCR approach on genomic DNA and by comparing band intensities of the amplified products in the total and chloroplast samples, it was determined that there was approximately ten percent mitochondrial contamination (Figure 9) and three percent nuclear contamination (Figure 10) in the chloroplast samples.

3.2.4 Levels of Cytosine Methylation in Leaves

The lack of hypomethylation in the total DNA of RE2 vs. RC in the green tissues of dark grown plants was of interest because this result had not been seen previously under regular light conditions. However, previous studies had only examined methylation levels in shoot tips and cotyledons, but not

the leaves down the stems of the flax plants. Since the leaves of plants grown in the light had not been previously examined, one possibility was that the hypomethylation of RE2 was not seen in the leaves of light grown plants, explaining the similarities in methylation between RC and RE2 for green tissue studies. Total DNA extracted from leaves at three separate stem positions of light grown plants revealed an overall significant [$F_{1/4}$=24.27**] methylation difference between RC and RE2, and between leaf samples taken from the three stem positions [quadratic; $F_{1/4}$ =18.86*] (Figure 11a; Table 5). The methylation level was higher in the bottom leaves than in the top leaves but the increase occurred predominantly in RC and, thus, the hypomethylation of RE2 occurred exclusively in the bottom leaves.

The next question was whether this stem location difference also occurred in dark grown plants. Looking at leaves from the three regions of the stem in RC and RE2 in normal light conditions, as well as 42 h in the dark prior to DNA extraction, there was no difference [$F_{1/11}$=3.98ns] between RC and RE2, however there was a significant [$F_{1/11}$=4.90*] interaction between the light conditions and the stem position. While a quadratic relationship of the leaf samples at different stem positions was not seen in this second study, there was a significant [$F_{1/11}$=34.79**] linear relationship for the leaves at different stem positions (Figure 11b; Table 5). Again, methylation levels were higher in leaves from the bottom of the stem and the hypomethylation of RE2 was more pronounced in these leaves.

3.2.5 Levels of Cytosine Methylation in Green Tissue of Plants Grown in Regular Light vs. 42 h Dark Conditions

While the methylation levels in the total DNA samples from plants grown in the dark alongside those used for chloroplast isolation had not shown significant differences between RC and RE2 (Figure 7a), unexpectedly, the total DNA from regular light grown and 42 h dark-grown plants showed a significant difference [$F_{1/3}$=14.85*] in methylation level between RC and RE2 (Figure 11c). The methylation level for the regular light grown plants was lower than that for the dark grown plants [$F_{1/3}$=18.09*]; however there was no interaction between the plant lines and the light conditions.

3.2.6 Plant Weight Analysis: Fresh and Dry Weights

To take into account the possible effect of growing plants in the dark on plant weight, the fresh and dry weights of plants that had been grown: i) in normal light conditions, ii) for 42 h in the dark prior to weight measurements, and iii) for 68 h in the dark prior to weight measurements, were compared. When the ratios of dry weight:fresh weight were analyzed (Figure 12a), it was found that there was a significant [$F_{1/5}$=19.84**] increase in the ratio between 42 h and 68 h. There was no significant [$F_{1/5}$=1.62ns] difference between RC and RE2 in the dry:fresh weight ratio, nor were there any significant interactions. There was a significant [$F_{1/5}$= 17.88**] linear decrease in fresh weight across the three conditions (Figure 12b) and a significant [$F_{1/5}$=19.03**] decrease in dry weight after 42 h in the dark (Figure 12c), with a significant [$F_{1/5}$= 7.69*] difference between RC and RE2 for fresh weight, but not dry weight [$F_{1/5}$= 5.71ns].

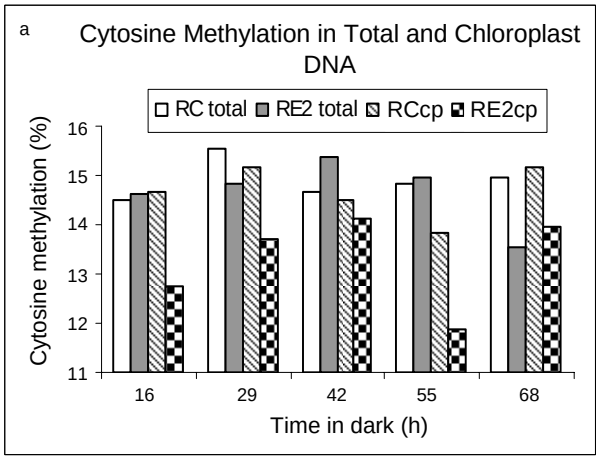
3.3 *Conclusions*

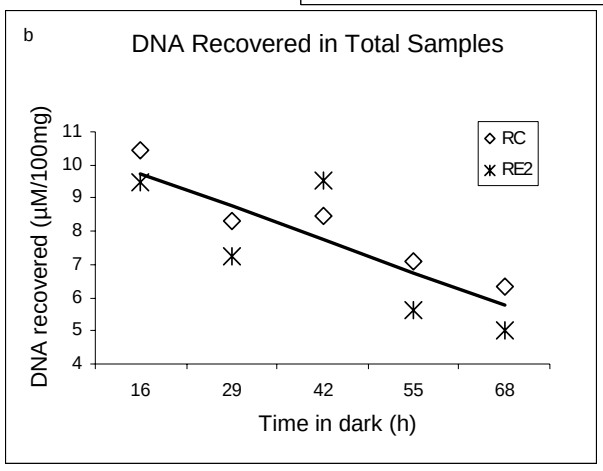
In this study, it was found that there was a substantial amount of DNA methylation in the chloroplast genome of both RC and RE2, and that RE2 has chloroplast DNA that is hypomethylated relative to RC. Interestingly, there was no significant difference in the methylation level between the total DNA in RC and RE2 plants grown under dark conditions. Previous studies have suggested that DNA of shoot tips and cotyledons of RE2 is hypomethylated at 21 days (the time of this study) relative to RC. The current study showed that leaves could also have hypomethylated DNA in RE2 compared to RC but that this is more likely to be seen in leaves from the bottom of the stem than leaves at the top. Thus, the similarity in the total DNA methylation between the two lines in the dark grown plants is probably a function of time in the dark on the shoot tips and cotyledons. The additional experiments partially explained the overall effects of dark on methylation levels seen in the data from the main chloroplast experiment. Nevertheless, results from these experiments did not attempt to explain why there were marked differences seen across the different times in the dark (Figure 7a).

Further, while there was no linear decrease in the recovery of chloroplast DNA over time in the dark, there was a decrease in the total DNA recovery. Because both fresh and dry weight also decrease with increasing time in the dark, it seems probable that plant growth stalled when the plants were placed in the dark. Hence, the plants with longer periods in the dark were effectively younger. However, the chloroplast and total DNA samples were subjected to the same dark treatments and, therefore, they should display similar trends in DNA recovery. The fact that there was no linear decrease in chloroplast DNA recovery from plants grown under similar conditions as those used for total DNA, suggests that starch content was a factor in chloroplast isolation. It is possible that shorter times in the dark were not sufficient to reduce starch content for optimal chloroplast isolation. That is, shorter times in the dark would have a loss in chloroplast DNA recovery due to chloroplasts rupturing, which would counteract the fact that a shorter time in the dark leads to plants that are effectively older and would potentially have more DNA recovery.

Figure 7: Methylation Levels and DNA Recovery for Chloroplast and Total DNA Samples

a) The DNA methylation level was lower in RE2 than in RC in the chloroplast (cp) DNA, but not in the total DNA (SE= 0.65). b) There was a linear decrease in DNA recovery (μM) per 100 mg of tissue in RC and RE2 total DNA (SE= 1.33). c) The time in the dark had no significant effect on the DNA recovered (μM) per 100 mg for RC and RE2 chloroplast DNA (SE= 0.10). Data plotted are means (n=2).


a
Cytosine Methylation in Total and Chloroplast DNA
RC total
RE2 total
RCcp
RE2cp
Cytosine methylation (%)
16
15
14
13
12
11
16
29
42
55
68
Time in dark (h)


b
DNA Recovered in Total Samples
DNA recovered (µM/100mg)
11
10
9
8
7
6
5
4
RC
RE2
16
29
42
55
68
Time in dark (h)

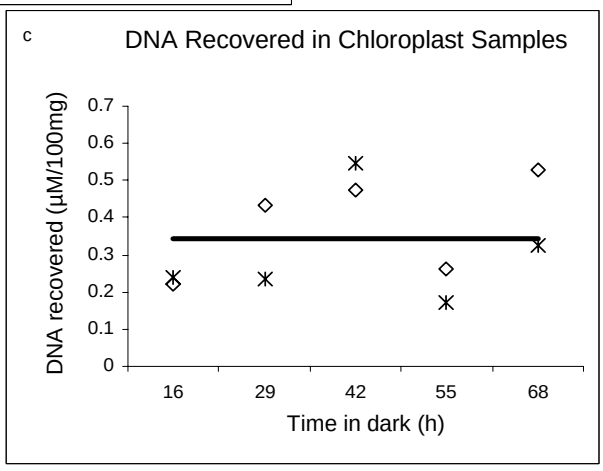
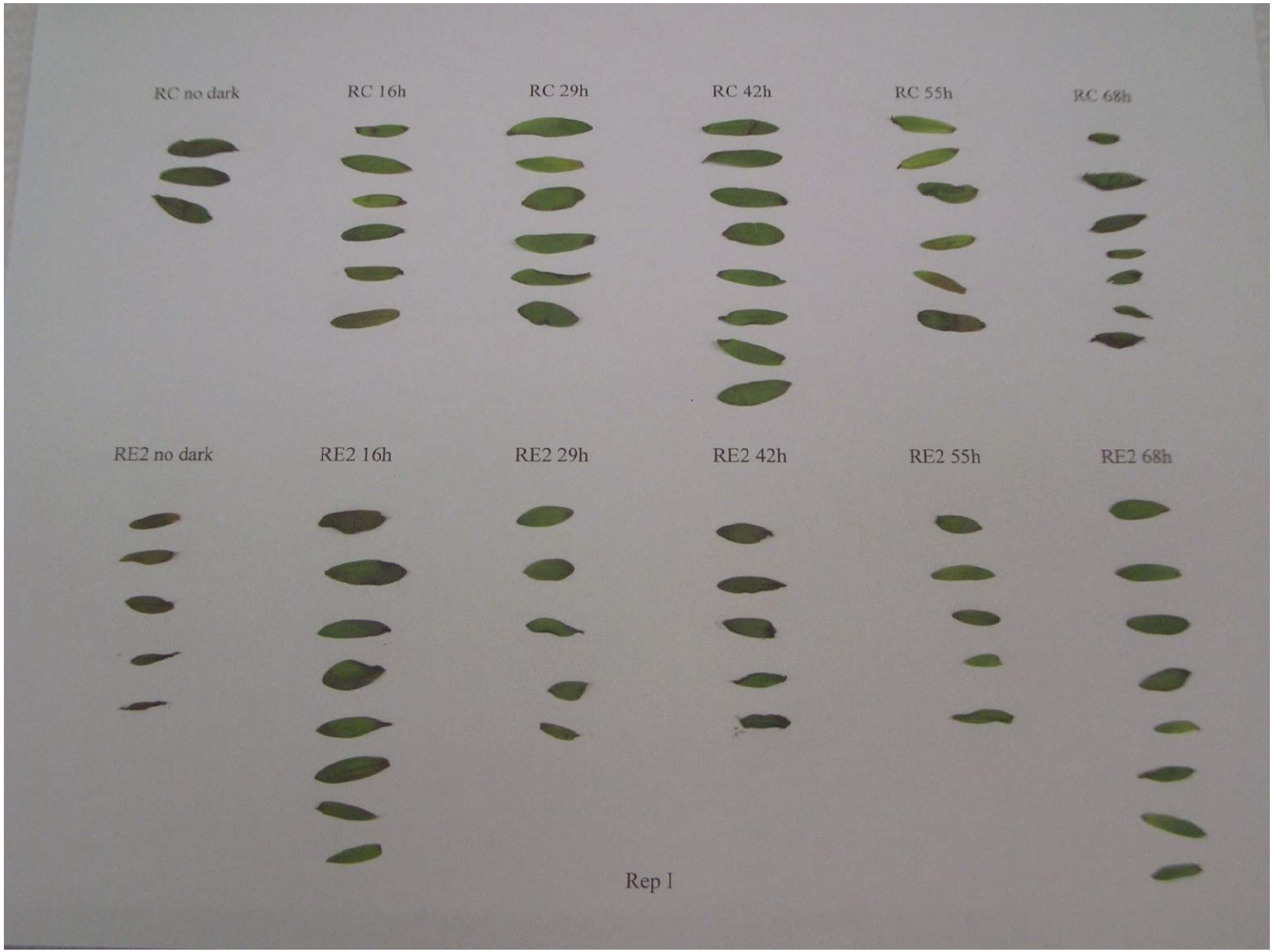

c
DNA Recovered in Chloroplast Samples
DNA recovered (µM/100mg)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
16
29
42
55
68
Time in dark (h)

Figure 8: Starch Levels in Leaves Sampled after Various Times in the Dark

Qualitative results of starch levels in the leaves of both RC and RE2 at all times in the dark. Leaves were taken from various locations down the stem. Both lines, and both replicates, have been displayed.


RC no dark
RC 16h
RC 29h
RC 42h
RC 55h
RC 68h
RE2 no dark
RE2 16h
RE2 29h
RE2 42h
RE2 55h
RE2 68h
Rep I

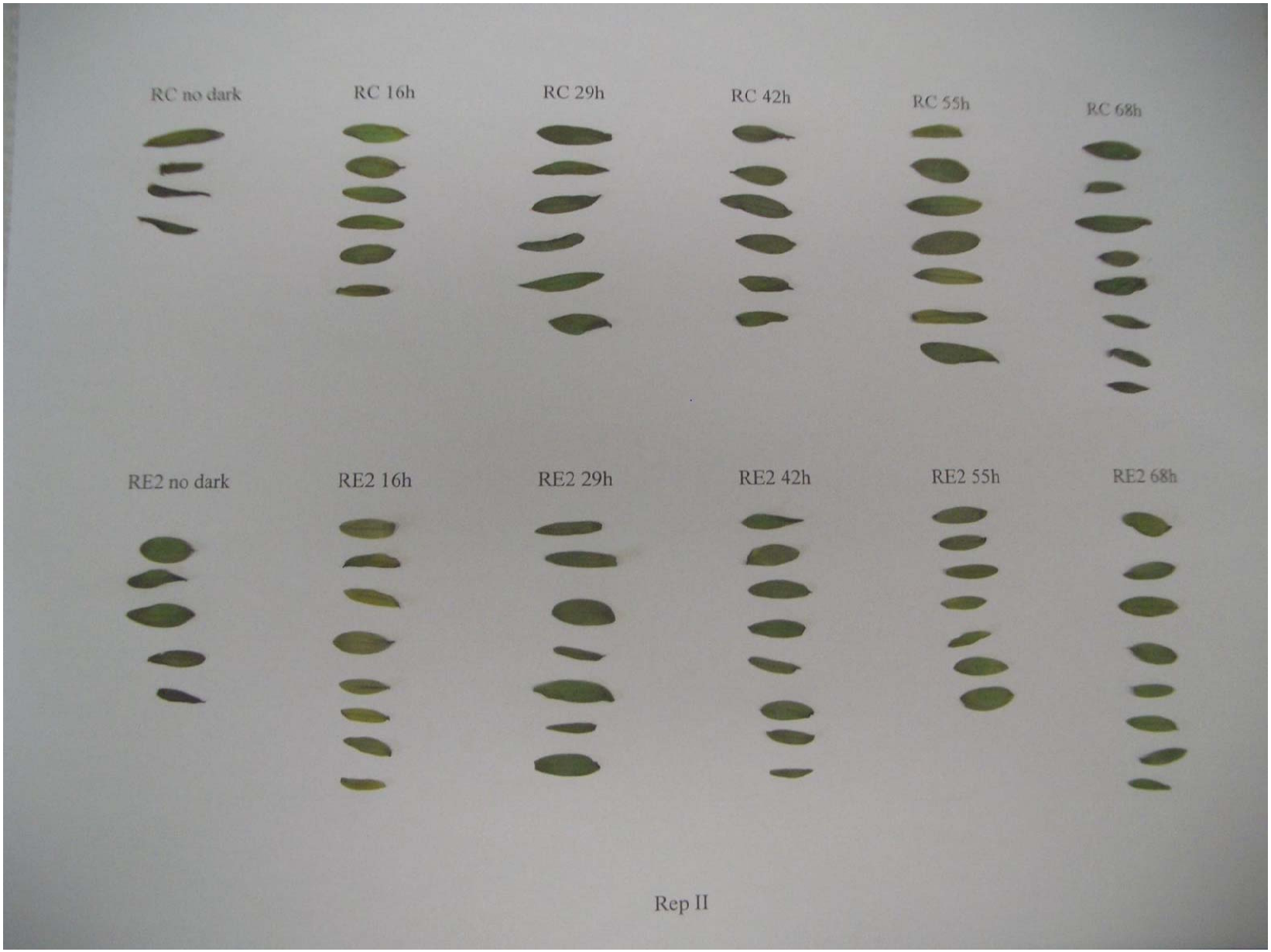

RC no dark
RC 16h
RC 29h
RC 42h
RC 55h
RC 68h
RE2 no dark
RE2 16h
RE2 29h
RE2 42h
RE2 55h
RE2 68h
Rep II

Figure 9: Analysis of Chloroplast DNA Contamination by Mitochondrial DNA

The pictures show three PCR runs (with *matR* primers) on two replicates of chloroplast and total DNA from both RC and RE2 grown for 42 h in the dark prior to DNA extraction.

Note: The DNA was diluted so that the ratio of chloroplast:total is 1:1.

tot= total DNA; cp= chloroplast DNA

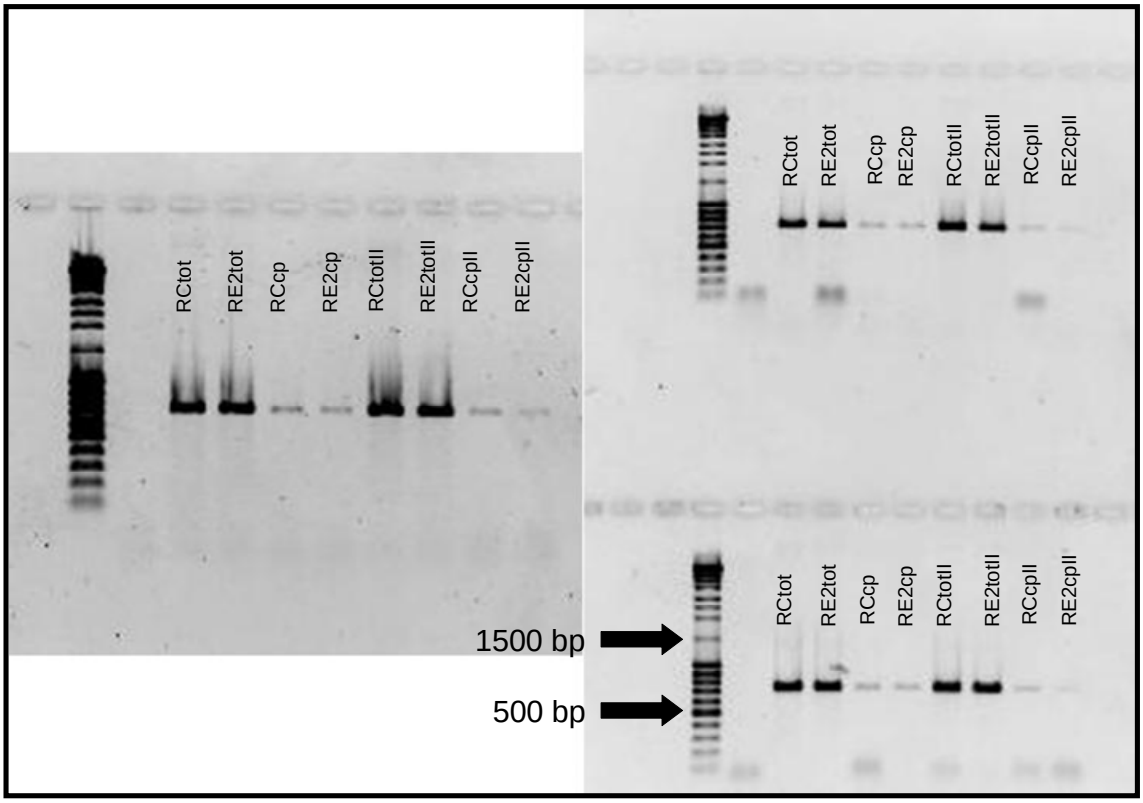

RCtot
RE2tot
RCcp
RE2cp
RCtotII
RE2totII
RCcpII
RE2cpII
1500 bp
500 bp

Figure 10: Analysis of Chloroplast DNA Contamination by Nuclear DNA

The pictures show three PCR runs (with actin primers) on two replicates of chloroplast and total DNA from both RC and RE2 grown for 42 h in the dark prior to DNA extraction.

Note: The DNA samples were not diluted so the ratio of chloroplast:total is 60:1.

tot= total DNA; cp= chloroplast DNA

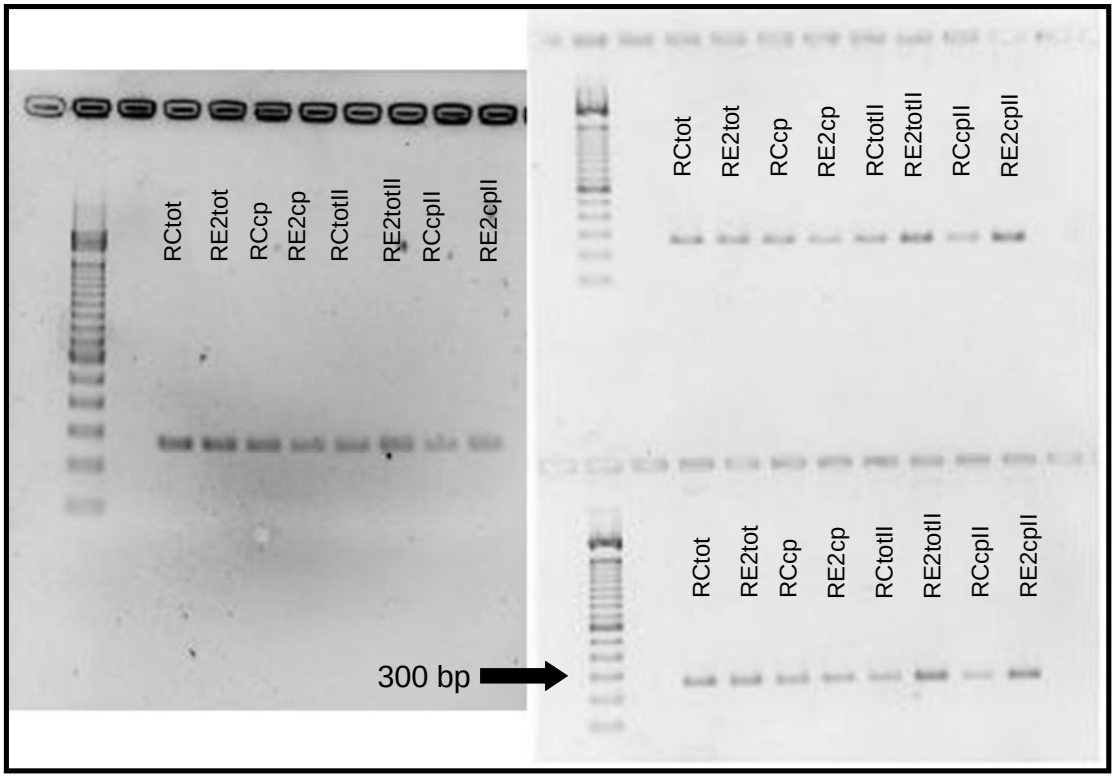

RCtot
RE2tot
RCcp
RE2cp
RCtotII
RE2totII
RCcpII
RE2cpII
300 bp

Figure 11: Comparisons of Methylation Levels for Leaf and Total Green Tissue DNA

a) Difference in cytosine methylation between the RC and RE2 in leaves and in leaf location of plants grown in regular light conditions (SE=0.28). b) Significant difference in methylation level between the top and the bottom leaves of RC and RE2, and, as a result, no significant difference between RC and RE2 in regular light and 42 h dark growing conditions (SE=0.45). c) For green tissues from stems of 22-day-old plants, the methylation level in total DNA from plants grown in regular light conditions, compared to 42 h in the dark prior to extraction there was a significant difference in methylation levels between the two growing conditions, as well as between the two lines (SE=0.11). Data plotted are means (n=2).

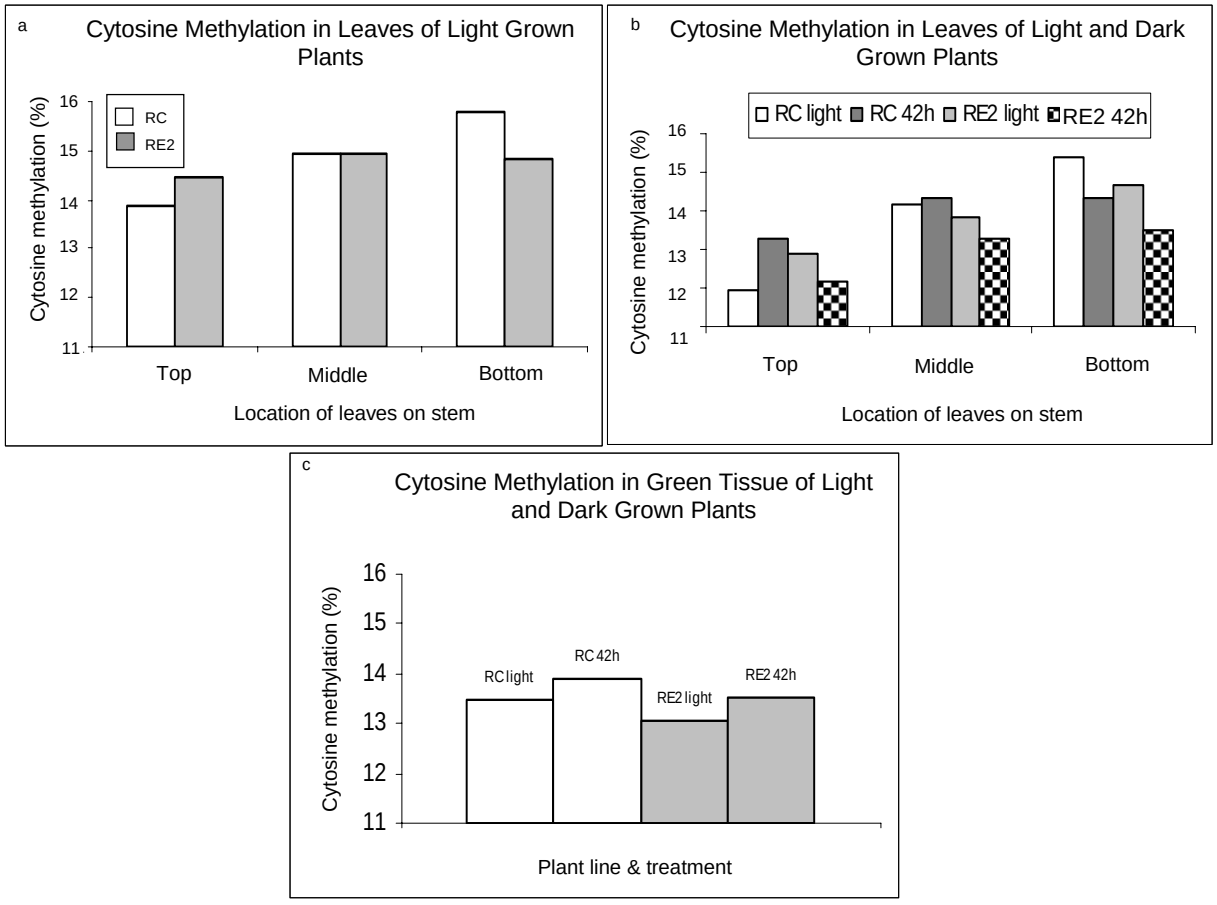
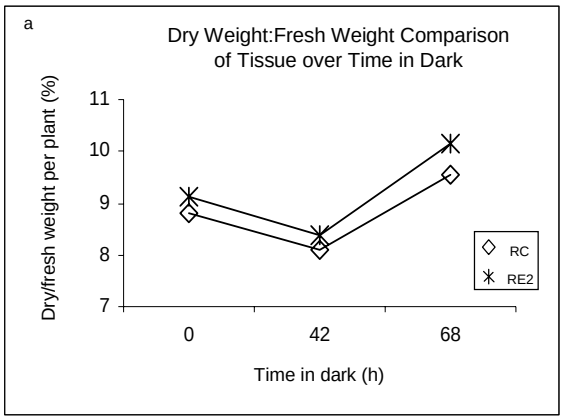
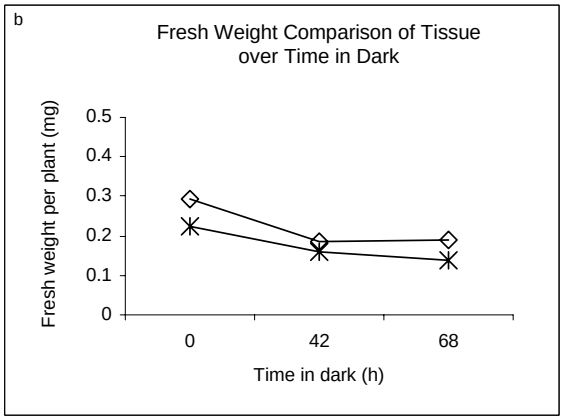

a
Cytosine Methylation in Leaves of Light Grown Plants
Cytosine methylation (%)
16
15
14
13
12
11
RC
RE2
Top
Middle
Bottom
Location of leaves on stem
b
Cytosine Methylation in Leaves of Light and Dark Grown Plants
RC light
RC 42h
RE2 light
RE2 42h
Cytosine methylation (%)
16
15
14
13
12
11
Top
Middle
Bottom
Location of leaves on stem
c
Cytosine Methylation in Green Tissue of Light and Dark Grown Plants
Cytosine methylation (%)
16
15
14
13
12
11
RC light
RC 42h
RE2 light
RE2 42h
Plant line & treatment

Figure 12: Comparisons of Weight for Light and Dark Grown Tissue

a) Ratios of dry weight as a percentage of fresh weight showed no significant difference between RC and RE2; however, there was a significant difference between the three times, relating to the highly significant difference between the ratios of 42h and 68h dark-grown plants (SE=0.36). b) Linear relationship of fresh weight (mg) and growing condition, with a significant difference between RC and RE2 (SE= 0.02). c) Linear relationship of dry weight and growing condition in RC and RE2, however there was no significant difference between the two lines for this parameter (SE= 0.002). Data plotted are means (n=2).


a
Dry Weight:Fresh Weight Comparison of Tissue over Time in Dark
Dry/fresh weight per plant (%)
11
10
9
8
7
0
42
68
Time in dark (h)
RC
RE2


b
Fresh Weight Comparison of Tissue over Time in Dark
Fresh weight per plant (mg)
0.5
0.4
0.3
0.2
0.1
0
0
42
68
Time in dark (h)

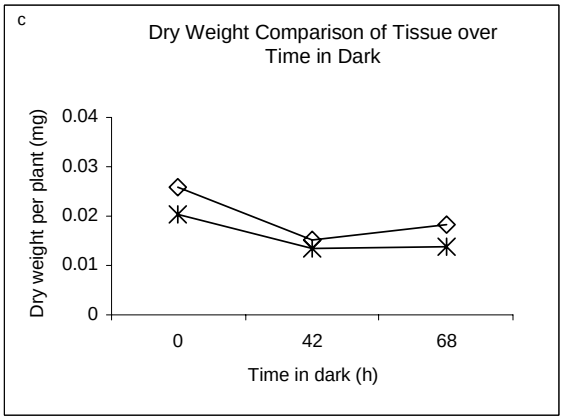

c
Dry Weight Comparison of Tissue over Time in Dark
Dry weight per plant (mg)
0.04
0.03
0.02
0.01
0
0
42
68
Time in dark (h)

Table 4: Means and F-values from the ANOVAs for Data from the Chloroplast and Total DNA Experiment

Means for chloroplast and total DNA (n=10) are from green tissue samples (combined shoot tips, leaves, and cotyledons) harvested after 16, 29, 42, 55, and 68 h in the dark prior to chloroplast isolation and DNA extraction from 21-day-old plants.

Parameter	Plant Line		Comparison			
			Lines	Times in the Dark		
	RC	RE2	C-E	Linear	Quadratic	Cubic
Chloroplast DNA:				$F_{1/9}$ values		
Tissue weight [a]	141.92	117.64	5.41*	<1.0	4.62ns	4.11ns
DNA (µM)/unit tissue*10	4.50	4.07	1.74ns	1.67ns	1.01ns	4.17ns
DNA (µM)/unit weight*100	0.38	0.30	7.44*	5.41*	<1.0	10.67**
mC level (%)	14.67	13.28	8.63*	<1.0	<1.0	5.79*
Total DNA:				$F_{1/9}$ values		
Tissue weight [a]	152.25	119.07	3.15ns	1.91ns	<1.0	<1.0
DNA (µM)/unit tissue*10	118.97	85.87	<1.0	11.21**	<1.0	<1.0
DNA (µM)/unit weight*100	8.14	7.37	6.19*	13.55**	<1.0	<1.0
mC level (%)	14.90	14.67	<1.0	<1.0	3.04ns	<1.0

** is significant at $P \leq 0.01$; * is significant at $P \leq 0.05$; ns is not significant at P=0.05.
[a] Tissue weight is per unit tissue (*i.e.* mg per plant, or segment of plant).

Table 5: Means and F-values from the ANOVAs for Data from Leaves at Different Stem Regions

Means for leaf DNA samples from plants grown under regular light conditions (n=6), and from plants grown under regular light compared to 42 h dark conditions (n=12). Leaves were sampled from the top, middle, and bottom portions of the stem. Significant interactions were detected.

Parameter	Plant Line		Comparison			
			Lines	Location		Interaction
	RC	RE2	C-E	Linear	Quadratic	Linear x Line
Leaf DNA for Regular Light Conditions:				$F_{1/4}$ values		
Tissue weight [a]	101.13	80.18	6.18ns	8.57*	35.42**	<1.0
DNA (μM)/unit tissue*10	9.13	7.62	4.53ns	281.80**	169.31**	7.42ns
DNA (μM)/unit weight*100	74.83	53.94	8.38*	89.89**	13.81*	6.28ns
mC level (%)	15.14	14.44	24.27**	121.26**	18.86*	7.96*
			Lines	Location	Light Cond.	Interaction
	RC	RE2	C-E	Linear	Light-Dark	Linear x Line
Leaf DNA from Regular Light and 42h Dark Conditions:				$F_{1/11}$ values		
Tissue weight [a]	69.60	45.69	8.72*	10.58**	4.48ns	<1.0
DNA (μM)/unit tissue*10	66.45	43.38	8.12*	23.99**	11.54**	1.57ns
DNA (μM)/unit weight*100 [b]	116.45	104.59	<1.0	116.92**	7.11*	1.65ns
mC level (%)	13.91	13.39	3.98ns	34.79**	1.39ns	4.90*

** is significant at $P \leq 0.01$; * is significant at $P \leq 0.05$; ns is not significant at P=0.05.
[a] Tissue weight is per unit tissue (*i.e.* mg per segment of plant).
[b] Also displayed a quadratic difference between the lines [$F_{1/11}$=9.75**].

4 ANALYSIS OF GENE EXPRESSION

This study was a semi-quantitative analysis of mRNA transcript abundance for the floral integrator/floral meristem gene *LFY* and its inhibitor, *TFL1*, during development in RC and RE2. The approach taken was to use shoot tips and leaves, sampled over the developmental period from 7 days of age to the onset of flowering. The hypothesis was that the early-flowering line RE2 would have either an increased amount of *LFY* transcript in its tissues, compared to RC, or a decreased amount of *TFL1* transcript, when compared to RC. Although the original plan involved using actin as an experimental control to ensure accurate loading and concentration of RNA, systematic changes in actin transcript levels during development and differences in actin transcript levels between RC and RE2 that were seen made this impossible.

4.1 *Materials and Methods*

4.1.1 Preliminary Work: Degenerate Primer Design

In designing the primers to be used in the semi-quantitative study, genomic DNA sequences were used. This DNA came from either shoot tips or leaves of plants grown in the growth chamber, as described previously (2.1). The age of plants at extraction was variable. DNA was extracted using the Qiagen DNeasy® Mini Kit (Appendix B). All primers used in this experiment, both degenerate and specific were from Invitrogen, Canada (Hamilton, ON). The location of the primers in DNA sequences took into account the presence of introns in the DNA sequences and allowed for an EPIC (exon priming intron crossing) approach to PCR.

When this study began (October 2005), there were 42 species reported to have *LFY*, or a *LFY* homologues in their genome, however, only three had published genomic sequences. These genomic sequences (Figure 13) were compared to mRNA sequences (in order to avoid primers being located in introns) and they were used to design degenerate primers for *LFY*. The code for degenerate primers is given in Appendix G. Although many primer sets were tested (see Appendix Ha), one set, sense-

AARKCTGGDGGAAGYTACAT and antisense-AGYTTBGTWGGMACRTACC (shown in green on Figure 13), was used to design specific primers (PCR program: 95 °C for 3 min., 35 cycles of: 95 °C for 30 s., 51 °C for 1.5 min., 72 °C for 1 min., and a final extension of 72 °C for 10 min.).

For primers to amplify a segment of the *TFL1* gene, only one genomic DNA sequence was available (from *Citrus sinensis*), however, an alignment of other *TFL1* mRNA and cDNA sequences was done to find coding region similarities between *TFL1* homologues (Figure 14). All of the primers that were designed based on these sequences were unsuccessful (see Appendix Hb). Thus, the degenerate primers, sense-AATGGCCATGAGCTCTTTCCTTC and anti-sense-CTYCTGGCAGCRGTYTCKCKCTG, reported by Amaya, Ratcliffe, and Bradley (1999) were used. These successfully amplified bands by PCR (35 cycles of 95 °C for 30 s., 63 °C for 1.5 min., 72 °C for 1.5 min.; final extension of 72 °C for 10 min.).

In testing the degenerate primers, PCR products were examined using gel electrophoresis, and all of the gels were agarose run in Tris-borate-EDTA (TBE) buffer (0.5 X) using a PowerPac Basic power supply (BioRad, Mississauga, ON). The mini (10 cm x 7 cm) cell used was a MiniCell EC370M (E-C Apparatus Co., Florida) while the large gel rig (15 cm x 20 cm) was a DNA Sub-Cell GT (BioRad). For gels run to examine the products of degenerate primers, the voltage range was from 110 V to 120 V. The time that the gel ran, varying from 0.75 h to 2 h, depended on the size of the gel (MiniCell or Sub-Cell GT), and the percentage of agarose being used. Percentages of gels ranged from 1.0-2.0, depending on the size of the band being examined. Gels were stained in 0.5 µg/mL EtBr solution for 30 min. Gel pictures were taken using the GelDocXR® Photosystem (BioRad).

4.1.2 Designing Specific Primers

The promising band amplified using the degenerate *LFY* primers was excised from the gel using the QIAquick® Spin kit from Qiagen (Appendix I) and sent to Mobix (McMaster University, Hamilton, ON) for sequencing. It was approximately 100 base pairs long. In the case of *TFL1*, three bands of interest were amplified using the degenerate primers. All were approximately 1.5 kilobase pairs in size

and the high concentration of DNA required for sequencing these bands could not be obtained by gel extraction. As an alternate approach, the TOPO TA Cloning Kit (Invitrogen; Appendix Ja) was used in an attempt to clone the three bands produced with the degenerate *TFL1* primers. Plasmids were isolated using PureLink Quick Plasmid MiniPrep Kit (Invitrogen; Appendix Jb) and sequenced (Mobix, McMaster, Hamilton ON) using the M13 primers provided with the MiniPrep kit.

The sequences obtained for the various PCR products were entered into the BLAST program of the NCBI website (http://www.ncbi.nlm.nih.gov/), and compared with known sequences. A cut-off value of e^{-10} was used to evaluate homology. The *LFY* band provided a value of $3e^{-27}$ when compared to the *LFY* homologue from *Lotus corniculatus* var. japonicus (Bird's foot trefoil; AY770393). From this sequence, specific *LFY* primers were obtained using the OligoPerfect® custom primer design tool from Invitrogen (sense, CTCCTACGTTCTCCCCTCTTTCCTTGA, and anti-sense, AGGCACTACGTGCATTGCTACGCG). They amplified a band of approximately 100 base pairs (95 °C for 3 min., 35 cycles of: 95 °C for 30 s., 67 °C for 1.5 min., 72 °C for 1 min., and a final extension of 72 °C for 10 min.), using genomic DNA from RC or RE2.

A homology of $5e^{-12}$ was found between one of the *TFL1* sequences and *CEN*, which is the *TFL1* homologue in *Antirrhinum* (AJ251994). Specific *TFL1* primers, to be used for the semi-quantitative study, were designed from this sequence using the OligoPerfect® program (Invitrogen) (sense-GGTTGAAGTTCTTGGTGGTGA and anti-sense-CGGCATCTCATAGCTCAACC).

The specific primers for actin, which was meant to be used as the positive loading control in this experiment, came directly from the published *Linum usitatissimum* actin sequence (AY857865, see 3.1.3). The band amplified by the actin primers (approximately 300 base pairs in flax cDNA) was extracted (Appendix I) and sent to Mobix for sequencing to verify that it was actin (the agreement of the sequence to AY857865 was $7e^{-98}$).

4.1.3 Semi-Quantitative Analyses of Transcript Levels

RNA was extracted from the shoot tips of the main stem, and from the top four leaves just below the shoot tip, of plants grown under the growth chamber conditions described in section 2.1. This was done, in replicate, every seven days from day seven until day 35 for RE2 and day 56 for RC. These final sampling ages are thought to coincide with the onset of flowering. In the growth chamber, the average flowering age (first anthesis) in RE2 is 43 days, and in RC, it is 62 days. The Qiagen RNeasy® Mini Kit (Appendix K) was used to extract RNA. RNA concentration was determined using a Cary-UV Vis spectrophotometer. This was done using readings for diluted volumes of RNA (to a volume of 1 mL) in BrandTech X-treme Range UV semi-micro cuvettes (Ultident, QC). Each concentration was estimated twice at 260 nm readings correcting for background (at 320 nm). Concentrations were only deemed accurate if the 260 nm was in the range of 0.1-1.0. If the readings were not in this range, samples were concentrated, or further diluted, until readings were in the indicated range.

First strand cDNA synthesis was done, using 2 µg of RNA template, with the Qiagen Omniscript® Reverse Transcriptase kit (Appendix L). RT-PCR was done with the Qiagen HotStarTaq® kit using the MJ Mini® thermal cycler (BioRad). For actin and *LFY*, the PCR program was: 95 °C 15 min., 25 cycles (actin) or 31 cycles (*LFY*) of: 95 °C for 30 s., 67 °C for 1.5min., 72 °C for 1 min.; final extension of 72 °C for 10 min. For *TLF1*, the PCR cycle was*:* 95 °C 15 min., 41 cycles of: 95 °C for 30 s., 58 °C for 1.5 min., 72 °C for 1.5 min.; final extension of 72 °C for 10 min. The number of cycles used for each gene was determined using linear standardization of cycles (Appendix M; *TFL1* not included), in triplicate. All experimental samples were also run in triplicate. Actin standards were run to correspond with both *LFY* and *TFL1*. The PCR products of the two flowering genes were run on separate gels; however, each was run on a gel along with their corresponding actin samples.

The gels used to examine the PCR products were 2.0 percent agarose, run for 2 h (unless otherwise specified in figure legend). They were run at 110 V using the Sub-Cell GT. For the

transcript level study, gels were run using two combs, with either *LFY* or *TFL1* in the top wells, and the corresponding actin samples in the bottom wells. Gels were stained in 1 µg/mL EtBr for 30 min., with a 10 min. wash in 0.5 X TBE. Band intensity was determined using pictures taken with the GelDocXR® system (BioRad) and QuantityOne® software. The intensities were used for analysis as described in 3.1.3.

Statistical analyses were done on the averages of the three runs using ANOVAs and Orthogonal comparisons. The standard errors (SE) shown are for means (n=2) and were derived from the error terms of the corresponding ANOVAs. The ANOVAs examined the developmental changes in RC over seven (leaf) or eight (shoot tip) ages, in RE2 over four (leaf) or five (shoot tip) ages, and compared the developmental change in RC and RE2 over four (leaf) or five (shoot tip) ages.

4.2 *Results*

4.2.1 *LFY* and Actin Transcript Levels in Shoot Tips

In the RE2 line, there was a significant [$F_{1/4}$=13.89*] quadratic increase of *LFY* transcript level in the shoot tips as the onset of flowering approached, while this was not the case [$F_{1/7}$=1.11ns] in RC (Figure 15a and Figure 16). As a result, there was a significant [$F_{1/9}$=26.74**] increase in the amount of *LFY* transcript accumulated in the shoot tips of RE2, compared to RC. These results are for comparisons made between plants of the same age. However, if the results are shifted, so that the accelerated developmental scale of RE2 is compensated for, then the same conclusions can be drawn (Figure 15b). It is important to note that, as the plants aged, there was a significant [$F_{1/7}$=6.45*] linear increase in the level of actin transcript in the shoot tips of RC, but not in RE2 [$F_{1/3}$<1.0]. Thus, actin could not be used as a standard for this *LFY* shoot tip data (Figure 15c and Figure 17).

4.2.2 *LFY* and Actin Transcript Levels in Leaves

Comparing the transcript levels of *LFY* in the top four leaves just below the shoot tip of RC and RE2, there was no significant [$F_{1/7}$=5.04ns] difference between the two lines from 14-35 days (Figure

15d and Figure 18). In addition, there was no significant [$F_{1/3}$<1.0] change in the level of transcript in the leaves of RE2 during development; however, in RC, there was a highly significant [$F_{1/6}$=14.32**] linear decrease in *LFY* transcript from 14-56 days of age (Figure 15d). There was a significantly [$F_{1/7}$=23.69**] higher level of actin transcript in the leaves of RC compared to RE2 in the leaves and (Figure 15e and Figure 19); thus, actin could not be used as a loading standard for the leaves because comparisons could not be made between the two lines.

4.2.3 Searching for *TFL1:* Genomic versus cDNA

While the specific primers determined for the *TFL1* gene amplified a region of genomic DNA from RC, RE2 and also from LC (Figure 20a), these primers did not produce any bands using the cDNA of RC or RE2 for either shoot tips or leaves of various ages (from 7-35 days in RE2 and 7-56 days in RC). DNA smears did appear in each of the lanes, at approximately 100 base pairs (Figure 20b), negative controls (*i.e.* PCR runs with no cDNA template) run for different numbers of PCR cycles, from 35-55, demonstrated that the DNA at 100bp in each PCR lane was an artefact (Figure 20c).

4.3 *Conclusions*

The data from the shoot tips showed that there was an increase in the *LFY* transcript level in RE2 as flowering approached, while in RC, there was no substantial increase in transcript level. Thus, at the onset of flowering, the level *LFY* transcript in RE2 was considerably higher than the level in RC. In the leaves, there was a constant low baseline level of *LFY* expression. Clear differences in actin transcript level between RC and RE2 leaves, as well as the increasing transcript abundance in RC during development in the shoot tips, meant that actin could not be used as the standard in this experiment. Since it had been expected that actin would be expressed at similar levels in both lines, at all ages, the differences in actin transcript levels were intrinsically interesting and may indicate altered expression of at least some housekeeping genes.

TFL1 transcripts were not detected in leaves or shoot tips, at any age; however, the gene was clearly present in genomic DNA from LC, RC and RE2. This suggests that *TFL1* is not being expressed in flax; however, more detailed and specific studies would have to be done in order to confirm this result. Further, if *TFL1* is not expressed in flax, other studies need to be conducted in order to determine the regulators of *LFY*.

Figure 13: Alignment of Genomic *LFY* Sequences for the Determination of Primers

The three genomic *LFY* sequences used to design primers for use in flax. From top to bottom, these are: *Citrus sinensis* (AY338976), *Titanotrichum oldhamii* (AY526319), and *Chrysanthemum lavendulifolium* (AY672542). Included on the diagram are the locations of: introns (black), coding regions (blue), and the degenerate primers used (green).

Figure 14: Alignment of mRNA *TFL1* Sequences for Determination of Primers

The three mRNA *TFL1* sequences used to design primers for use in this study were (from top to bottom): *Metrosideros excelsa* (AY170872), *Citrus sinensis* (AY344244), and *Lolium perenne* (AF316419).

Figure 15: Comparison of Transcript Levels in the Shoot Tips and Leaves of RC and RE2

a) Transcript levels plotted against plant age, comparing RC and RE2 (SE=7.0). b) Comparison of RC and RE2 *LFY* transcript levels on a developmental scale (*i.e.* RE2 points have been shifted so that they are aligned with the time of flowering in RC). c) Differences in the transcript level for actin during shoot tip development in RC and RE2, with an increase in actin transcription in RC but not RE2 (SE=14.4). d) Decrease in the transcript level of *LFY* during leaf development in RC but not in RE2 (SE=1.83). e) Increase in the transcript level of actin in the leaves of RC compared to those of RE2 (SE=7.09). Data plotted are means (n=2).

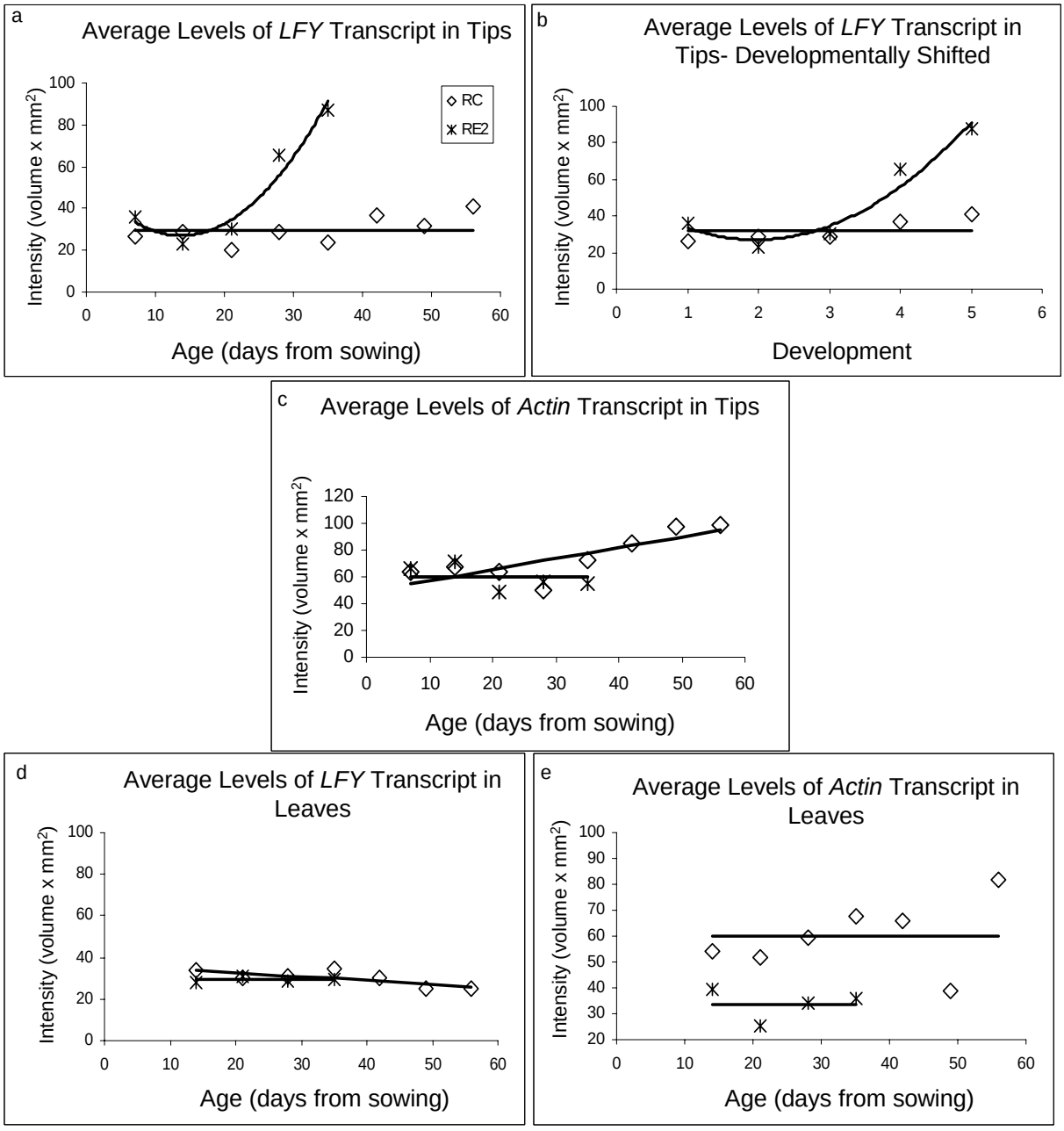

a
Average Levels of *LFY* Transcript in Tips
Intensity (volume x mm2)
RC
RE2
Age (days from sowing)
b
Average Levels of *LFY* Transcript in Tips- Developmentally Shifted
Intensity (volume x mm2)
Development
c
Average Levels of *Actin* Transcript in Tips
Intensity (volume x mm2)
Age (days from sowing)
d
Average Levels of *LFY* Transcript in Leaves
Intensity (volume x mm2)
Age (days from sowing)
e
Average Levels of *Actin* Transcript in Leaves
Intensity (volume x mm2)
Age (days from sowing)

Figure 16: *LFY* Transcript Levels in Shoot Tips

The three gels on the left are replicate I, whereas the three gels on the right are replicate II. The primers used were those described above (4.1.2). The gels were all loaded in the same order, as described on the top left gel. All gels were run as described in section 4.1.3, with the exception of the top left gel, which was only run for 1 hour.

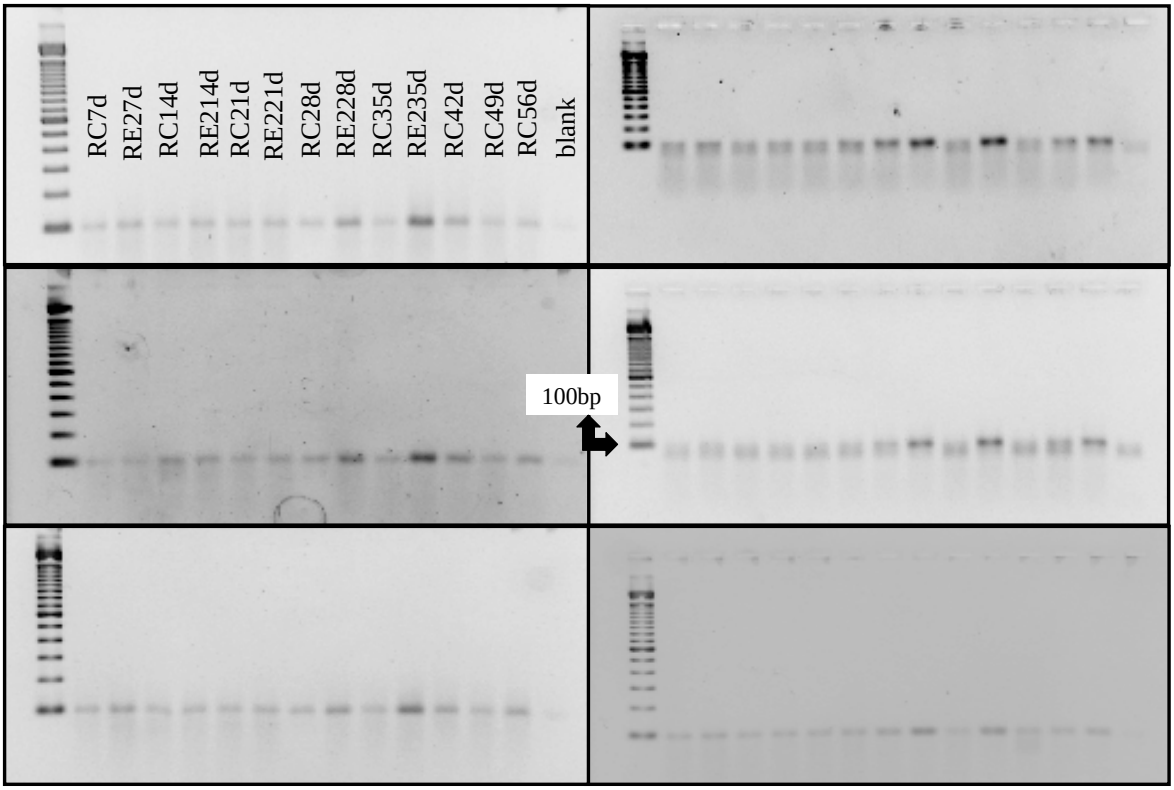

RC7d
RE27d
RC14d
RE214d
RC21d
RE221d
RC28d
RE228d
RC35d
RE235d
RC42d
RC49d
RC56d
blank
100bp

Figure 17: *Actin* Transcript Levels in Shoot Tips

The three gels on the left are replicate I, whereas the three gels on the right are replicate II. The primers used were those described above (4.1.2). The gels were all loaded in the same order, as described on the top left gel (with one exception, noted on the middle gel of replicate I). All gels were run following the protocol described in 4.1.3, with the exception of the top left gel, which was only run for 1 hour.

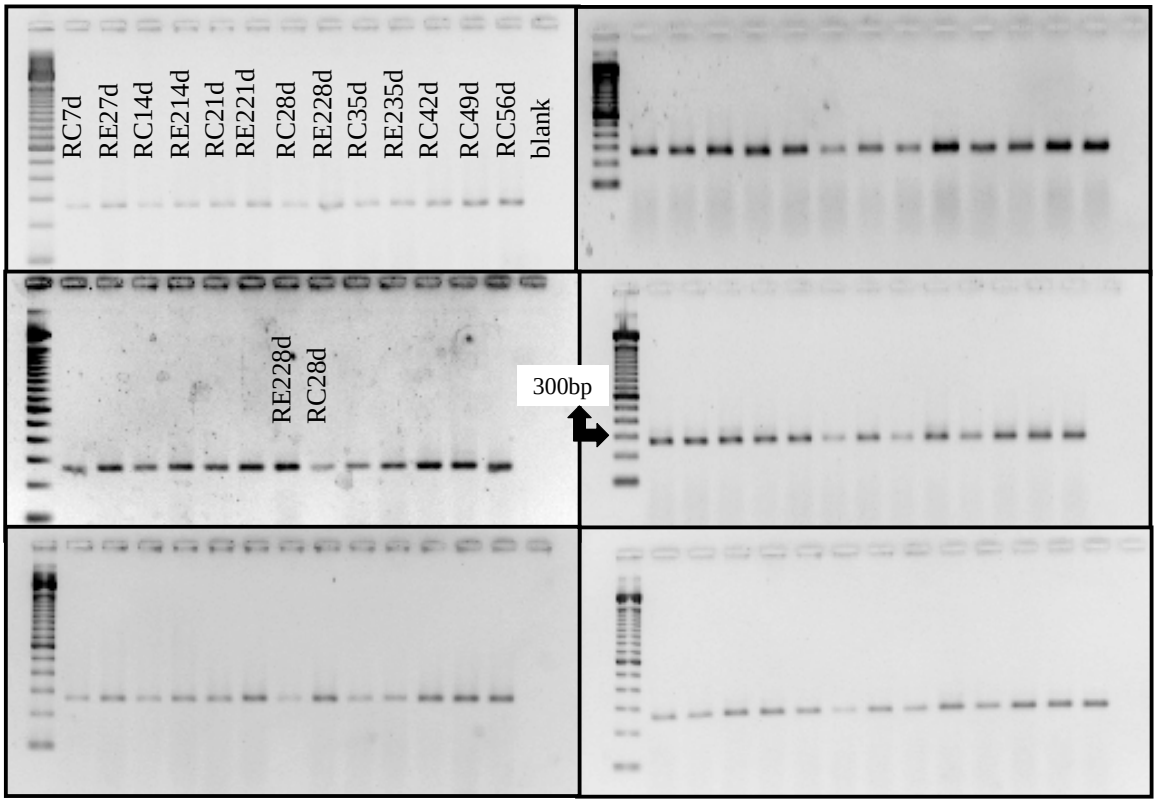

RC7d
RE27d
RC14d
RE214d
RC21d
RE221d
RC28d
RE228d
RC35d
RE235d
RC42d
RC49d
RC56d
blank
RE228d
RC28d
300bp

Figure 18: *LFY* Transcript Levels in Leaves

The three gels on the left are replicate I, whereas the three gels on the right are replicate II. The primers used were those described above (4.1.2). The gels were all loaded in the same order, as described on the top left gel.

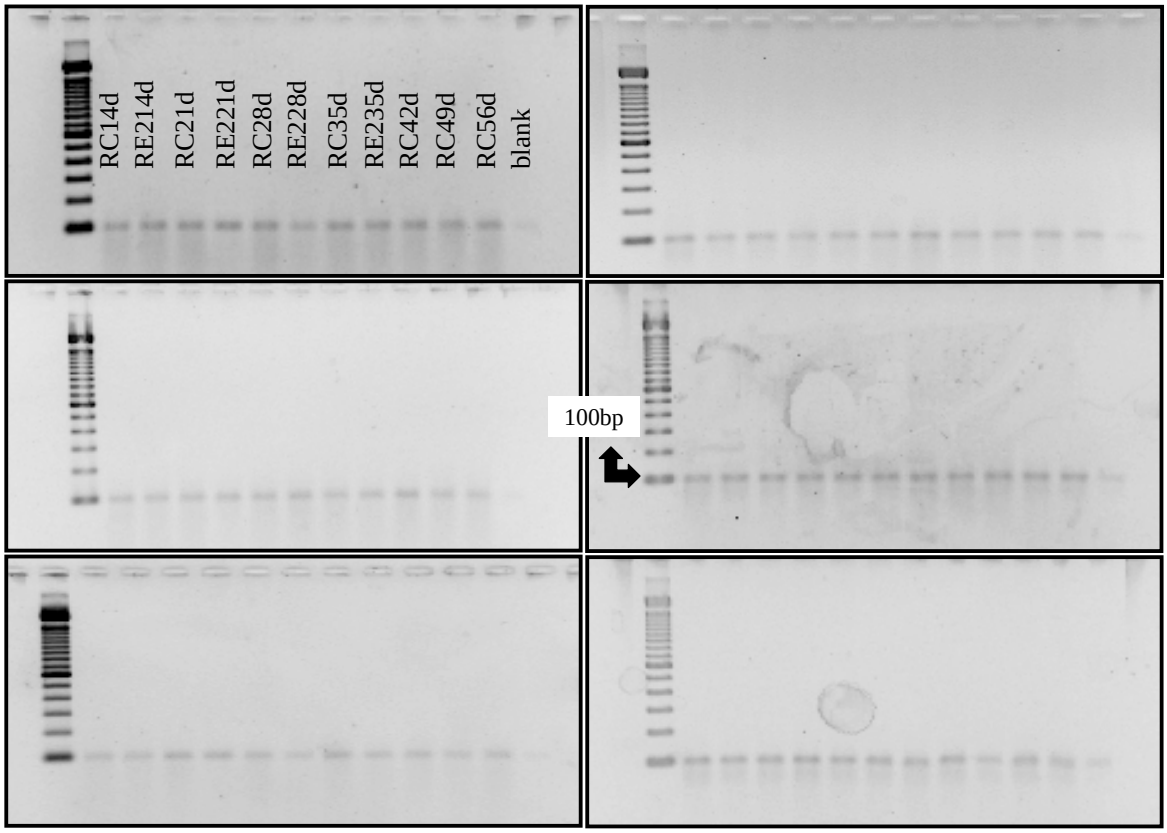

RC14d
RE214d
RC21d
RE221d
RC28d
RE228d
RC35d
RE235d
RC42d
RC49d
RC56d
blank
100bp

Figure 19: *Actin* Transcript Levels in Leaves

The three gels on the left are replicate I, whereas the three gels on the right are replicate II. The primers used were those described above (4.1.2). The gels were all loaded in the same order, as described on the top left gel.

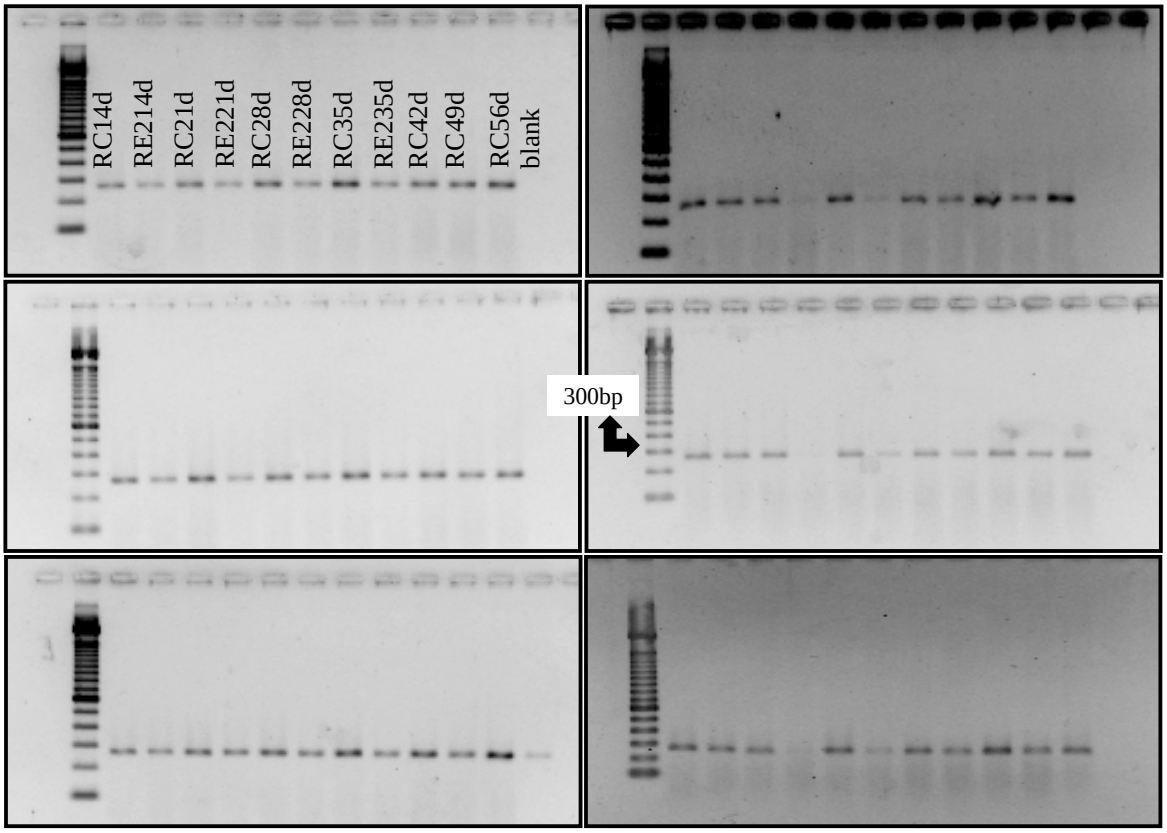
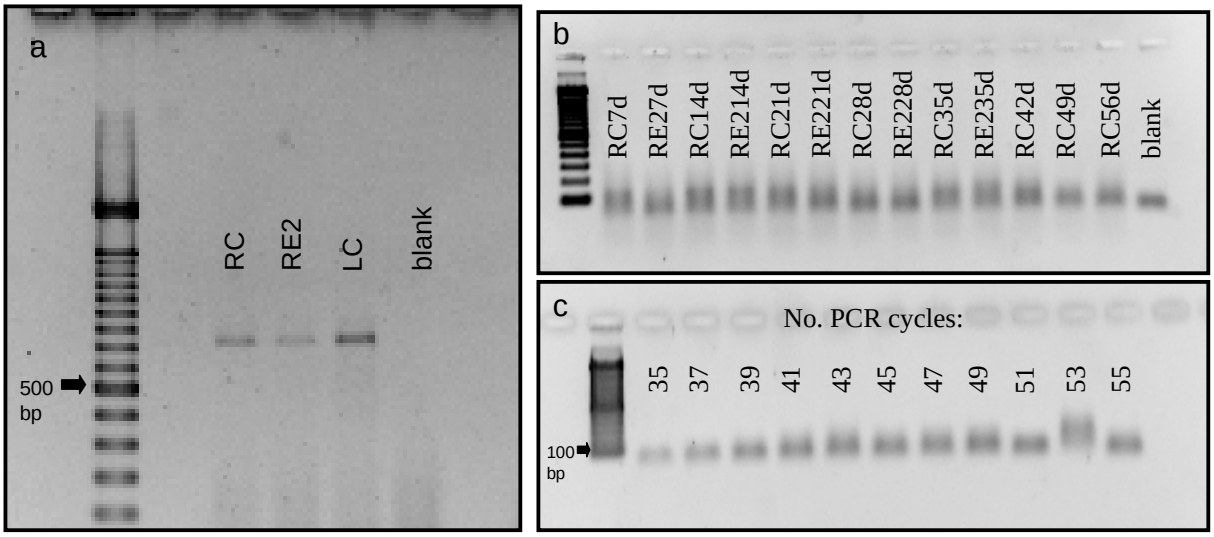

RC14d
RE214d
RC21d
RE221d
RC28d
RE228d
RC35d
RE235d
RC42d
RC49d
RC56d
blank
300bp

Figure 20: *TFL1* in Genomic DNA and cDNA

a) Products of specific *TFL1* primers (see section 4.1.2) in the genomic DNA of different lines of flax. b) Specific *TFL1* primers run on cDNA, with a blank. c) Only water as a template, at different numbers of PCR cycles, ranging from 35-55.


a
RC
RE2
LC
blank
500
bp
b
RC7d
RE27d
RC14d
RE214d
RC21d
RE221d
RC28d
RE228d
RC35d
RE235d
RC42d
RC49d
RC56d
blank
c
No. PCR cycles:
35
37
39
41
43
45
47
49
51
53
55
100
bp

5 DISCUSSION

5.1 *Amounts of DNA Recovered Relate to Tissue Differentiation and Cotyledon Senescence*

Estimates of tissue weight, DNA recovery per plant, and DNA recovery per tissue weight provide insights into the physiological and/or developmental status of the flax plants. In the shoot tips of flax, there were increases in DNA recovery and sample weight per 10 shoot tips from early vegetative growth to the onset of flowering (Figure 4b-d). This suggests that there were more leaves in the older shoot tips, or that the leaves in the older tips were larger. Interestingly, the quadratic changes in DNA recovery and fresh weight with development in cotyledon samples (Figure 5c) from the same plants suggest that there is a point in cotyledon growth in flax where cell division ceases. The timing of this point (between 32 and 38 days) coincides with the transition from mid- to late-vegetative growth, which was established using other parameters (Fieldes and Harvey 2004). In flax, cotyledons are the first tissues that emerge from the soil (*i.e.* it is an epigeal plant) and, therefore they are photosynthetic tissue. Over time, the structure of the cotyledons may be changing (*i.e.* cell wall development) which, at later ages could be leading to a decrease in DNA recovery due to difficulty mechanically extracting it from the tissue. This is not the case in the shoot tips, where DNA recovery is increasing with age.

As expected, plant growth stalled when the plants were placed in the dark, resulting in lower fresh weights and less DNA recovery with greater effects on plants that had longer periods in the dark (plants kept in the dark would, essentially, only be 18 days-of-age; Figure 7b), and resulted in a linear decrease in fresh weight and a quadratic change in dry weight with time in the dark (Figure 12b-c). Conversely, there did not appear to be a relationship between DNA recovery and time spent in the dark for the chloroplast samples (Figure 7c). The reason for putting the plants in the dark was to decrease the starch content of the leaf tissue to an optimal level for chloroplast isolation because past studies on flax chloroplast have suggested that dark treatment is required prior to chloroplast isolation (Coates and Cullis 1982). Therefore, even though the leaves stained for starch did not show marked trends of

changing starch content with time in the dark (Figure 8), it is possible that there was a decrease in starch content with time in the dark, which resulted in increased chloroplast recovery (hence, chloroplast DNA recovery at 68 h vs. 16 h), and that the stalled plant growth at 68 h vs. 16 h offset this so that no change in DNA recovery was seen in the chloroplast samples with time in the dark.

5.2 *Cytosine Methylation Levels Increase with Plant Age*

In the total DNA samples of flax, cytosine methylation in the shoot tips (Figure 4a), cotyledons (Figure 5a) and leaves (Figure 11a-b) was found to increase with age and/or tissue development in both early flowering and control lines. Increases of this type have been reported in other plants, such as in *Arabidopsis* (Ruiz-Garcia, Cervera, and Martinez-Zapater 2005), and in the SAM of *Prunus persica* L. Batsch (peach; Bitonti et al. 2002). Further, in all three flax tissue types grown under regular light conditions, total DNA from RE2 was hypomethylated relative to RC.

In the shoot tips (Figure 4a), the hypomethylation of RE2 continued until close to the onset of flowering (34 days) when it became similar to the DNA methylation seen in RC at the same age. However, this comparison was made between samples of DNA extracted from plants of the same age. If the methylation levels in the shoot tips of early-flowering and control lines were compared on the same developmental scale (*i.e.* developmentally, 31 and 34 days of age in the early-flowering lines compared approximately to 45 and 52 days of age in RC), the early-flowering lines would show consistent hypomethylation in shoot tips, for all of the ages that have been examined (except for RE1 at 24 days). Strengthening this argument is the fact that DNA methylation levels in the flower buds are higher than the levels in the shoot tips at the onset of flowering but the DNA from the buds of RE2 is hypomethylated relative to RC (Figure 6c). Thus, although the methylation level of RE2 is increasing with age in the shoot tip, the meristematic cells are probably retaining the hypomethylation to be passed from generation to generation.

As the cotyledons begin to senesce, the methylation levels increase and the levels of RE2 and RE1 become like those of RC (Figure 5a). With senescence, the similarities in methylation levels

between the cotyledons of early-flowering and control lines may relate to the fact that methylation functions to regulate the sequence of events that take place during senescence (reviewed in Chan, Henderson, and Jacobsen 2005). Other DNA characteristics (*i.e.* tissue weight, DNA recovery; Figure 5b-d) suggest that senescence of the cotyledons has begun before 52 days. It has been shown that internal cues for senescence in plant tissues occur before visual symptoms, and that DNA fragmentation is part of this process (Caccia et al. 2001). Thus, the cotyledons may begin to senesce as early as day 30 but the yellowing and abscission may only occur later (approximately day 52 in all three lines). Therefore, the increases in methylation levels may be related to the protection of DNA from degradation.

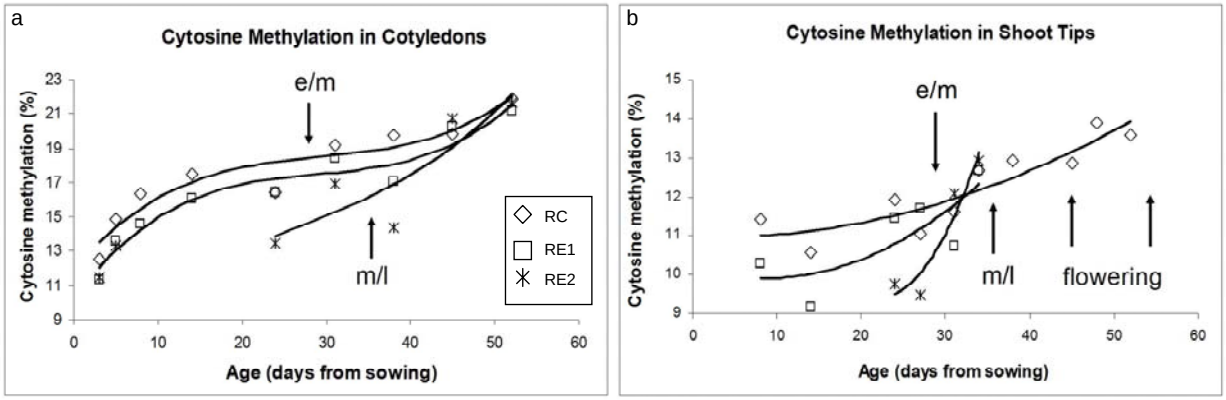
The patchwork of HPLC studies provided developmental profiles of DNA methylation changes in flax cotyledons and shoot tips. When earlier data on methylation levels in seedlings (from 3-14 days of age, Fieldes unpublished) is combined with the work presented here, it can be seen that there is an increase in DNA methylation levels in both cotyledons and shoot tips over the entire vegetative growth of the plants (Figure 21). Subsequently, similar results have also been obtained for the two early-flowering L lines (LE1 and LE2) and their control, LC (Fieldes unpublished). Measurements of DNA methylation during the early part (3-24 days) of development in RE2 remain to be completed; however, hypomethylation in both RE1 and RE2 has been seen in shoot tip and cotyledon samples at young ages (Figure 1).

In the work reported here, the total DNA samples from plants subjected to dark treatments that were grown alongside those used for chloroplast DNA methylation studies did not show a difference in methylation between RC and RE2 (Figure 7a). However, all three tissue types examined (*i.e.* shoot tips, cotyledons and leaves from various stem locations) showed methylation levels that were lower in RE2 than RC when the plants were grown under normal growth conditions (Figure 4a, Figure 5a, and Figure 11a-b). Therefore, it seems likely that the dark has an effect on the methylation levels in the nuclear DNA of flax. Strengthening this argument, there was a significant difference in the methylation levels seen in DNA extracted from green tissues of plants grown under regular light conditions versus

Figure 21: Developmental Profiles for Methylation Levels in the R Lines of Flax

a) In the cotyledons, the methylation level increased from day 3 to day 5, and from day 8 to day 14 in RC and RE1 (Fieldes unpublished). There was also a linear increase from day 24 to day 52 in all three lines. b) In the shoot tips, there was a linear increase in methylation level from day 24 to day 34 in all three lines, but no significant change in methylation between days 8 and 14 in RC and RE1 (Fieldes unpublished).

Note: Shifts from early- to mid- vegetative phase (e/m) and mid- to late- vegetative phase (m/l) are indicated. The mean flowering ages for these greenhouse grown plants were at 45 days for RE1 and RE2, and 56 days for RC and have been indicated.


a
Cytosine Methylation in Cotyledons
e/m
m/l
RC
RE1
RE2
Cytosine methylation (%)
Age (days from sowing)
b
Cytosine Methylation in Shoot Tips
e/m
m/l
flowering
Cytosine methylation (%)
Age (days from sowing)

from plants grown in the dark for 42 h before DNA extraction, where the leaves from dark grown plants had higher methylation levels than those grown in regular light conditions (Figure 11c).

The changes in DNA methylation levels in total DNA extracts from plants grown for different times in the dark lead to questions about how regular light conditions affect the methylation levels in plants. It is possible that even the eight hours of dark in a regular light/dark cycles changes the methylation level of RE2 and/or RC. Comparing methylation levels of plants kept in the dark to those grown in regular light conditions, the effects of the dark treatment seem to cause an increase in RE2 DNA methylation, as opposed to a decrease in RC DNA methylation values. The increased methylation of RE2 DNA from plants kept in the dark may be related to gene regulation, or may be a potential stress response mechanism induced by the dark conditions.

In all of the HPLC studies presented here, it is important to take into account the fact that there may be day-to-day variation in the samples. It was not always possible to design the experiments so that DNA from all samples was extracted on the same day. Thus, there may be some variability in the samples related to differences in extraction days. However, day-to-day variability cannot explain the statistically significant trends that are seen in the data points, such as linear and quadratic effects.

5.3 *Cytosine Methylation Detected in the Chloroplast DNA of Flax*

In this study, it was found that the chloroplast DNA of flax is methylated, and, in contrast to the total DNA (from plants grown alongside the chloroplast DNA samples), the chloroplast DNA of RE2 was significantly hypomethylated relative to that of RC. This difference was seen in all of the different times in the dark, including the most extreme, 68 h period in the dark prior to chloroplast isolation. The hypomethylation of RE2 could be related to different effects of photoperiodism on RC and RE2 (see 5.4 for further discussion).

Previous work on chloroplasts in tobacco, pea, maize, and *Medicago truncatula* suggests that chloroplast DNA is degraded as the leaves age, especially in maize (Shaver, Oldenburg, and Bendich 2006). When the chloroplast DNA recovery was compared to total DNA recovery, per 10 plants or per

100 mg, (Table 4) as a percentage, the percentages were similar in RC and RE2. Hence at 21 days, RE2 chloroplasts are not aging faster than RC chloroplasts, even though there are potential differences in nuclear gene expression.

No methyltransferase genes have been detected in chloroplast genomes. Further, when the sequences of the nuclear-encoded methyltransferases of *Arabidopsis* were tested for prediction of targeting into the chloroplasts (*i.e.* using the Xpasi® website), results were negative. However, in *Chlamydomonas,* it has been predicted that the methyltransferase crMET1 is transferred into chloroplasts (Nishiyama et al. 2002) and in this species, the chloroplast DNA is known to be methylated (Burton, Grabowy, and Sager 1979). Thus, it is possible that plant species with methylated chloroplast DNA have nuclear-encoded methyltransferases that are translocated to the chloroplast but that their identities have not yet been elucidated.

Interestingly, the hypomethylated state of the chloroplast DNA in early-flowering lines of flax indicates that the azaC treatment applied to germinating seeds not only affected the nuclear DNA of flax, but also the chloroplast DNA. Thus, it is possible that mitochondrial DNA was also affected and that RE2 mitochondrial DNA is also hypomethylated compared to that of RC. While chloroplast inheritance in plants can be uni- or bi- parental, mitochondrial DNA is usually transmitted maternally (Fairbanks and Anderson 1999). For total DNA, the general trend is that the hypomethylation and early flowering characteristics co-segregate in the segregating generations of outcrosses, with no indication of uni-parental inheritance (Fieldes et al. 2005). Thus, if one or both of the chloroplast and mitochondrial genomes are uni-parental, the genes that they carry are probably not affecting flowering time. However, if the inheritance of the chloroplast genome is bi-parental, then some of the chloroplast genes may be involved in flowering time. In which case, cytoplasmic segregation and recombination could explain the rare anomalous plants (*i.e.* those which are hypomethylated but do not have the early-flowering phenotype) that have been noted in segregating generations of outcrosses (Fieldes et al. 2005).

5.4 *Circadian Rhythm and its Effects on Gene Expression and Flowering Time*

Plants that respond to day length in order to flower have a circadian rhythm of responsiveness to the changes in length of day/night periods. Flax is a LD quantitative (or facultative) plant, meaning that long days are not essential for flowering but their presence will speed up the process (Thomas, Carre, and Jackson 2006). While SD plants are generally governed by how much dark the plant receives, LD plants, such as flax and *Arabidopsis*, are regulated by how much light they are subjected to as well as the composition of the light, especially in the later part of the day (Thomas, Carre, and Jackson 2006). With respect to flowering, perception of day length is done by the leaves, and signals are then sent to the SAM for floral evocation. In some species, once flowering has been initiated, appropriate day length is no longer necessary, but in others, the appropriate day length must continue until the floral meristem has developed, at least partially, into floral organs (Thomas, Carre, and Jackson 2006).

In *Arabidopsis*, six percent of transcribed genes are regulated by circadian rhythm (Harmer et al. 2000), including the flowering pathway genes *CO*, *FT* and *GIGANTEA (GI)*. The function of *CO* is upstream of that of FT in *Arabidopsis* and, after a short night, there is an accumulation of *CO* mRNA, which stimulates *FT* expression (Thomas, Carre, and Jackson 2006). It is likely that *FT* is present in the flax genome, because one of the bands excised and sequenced in the effort to locate *TFL1* in flax was found to be partially homologous, albeit at a low level (e-value: $1e^{-6}$), to *FT*.

There have been no reported studies linking methylation to *CO* or *FT*; however, if present in flax, it is possible for these genes to be affected by the hypomethylation of RE2, which could affect flowering time. If as many as six percent of genes in the transcriptome are controlled by circadian rhythm, the demethylation of RE2, which is thought to have been induced randomly by the azaC treatment, could have affected circadian rhythm genes, either in the nuclear or chloroplast genome, that affect flowering time.

In its role as a floral integrator in *Arabidopsis*, *LFY* is thought to be involved in integrating signals from multiple flowering pathways, including the photoperiod pathway. This pathway includes

CO, for which transcription and abundance of mRNA are regulated by circadian rhythm. Thus, *LFY* is indirectly affected by circadian rhythm. If light conditions become irregular, the time of flowering becomes regulated by the level of *CO* transcript accumulated in the light conditions (Thomas, Carre, and Jackson 2006). It has been suggested that *CO* regulates a plant's ability to flower, based on responses to the floral-meristem identity genes, but it is thought to affect *LFY* indirectly via *TFL1* (Simon, Igeno, and Coupland 1996). However, even if *TFL1* is not transcribed in flax, as may be the case, it is possible that *CO* could still affect floral competence mediated by LD light conditions.

Because phytochromes are related to light detection, if their effects are altered, regulation via photoperiod in the plant will be altered as well. Generally, plants that cannot create functional phytochromes (*i.e.* they cannot synthesize the chromophores of the phytochromes) will flower earlier than control plants (Montgomery et al. 1999, 2001; Sawers et al. 2002). Similarly, the expression of the phytochrome genes, *PHYTOCHROME A* and *PHYTOCHROME B*, changes over the course of the day (Toth et al. 2001) and thus could lead to differences in flowering time. PHYB is also known to be an inhibitor of FT production (Endo et al. 2005) and, since FT is, perhaps, the florigen molecule, PHYB is likely to be a key regulator of flowering time.

As mentioned previously, the dark treatment had an apparently greater effect on methylation levels in the total DNA of RE2 than RC. Therefore, it is possible that, even over the course of a regular day, the circadian cycle of RE2 causes changes in photosynthetic capacity and energy usage at a different rate (likely faster) than in RC. It has been previously shown that in some other lines of flax (RE2 was not included in the study), the control and early-flowering lines respond differently to their growing conditions, especially light conditions (Fieldes and Harvey 2004). It is possible that RE2 is using light more effectively, or storing materials that are needed for flowering at a faster rate than RC. Because flax is a qualitative LD plant, it is possible that early-flowering plants are working more efficiently than RC at harnessing light energy while it is available.

In the chloroplast genome, the genes *psbA* and *psbD* encode the chlorophyll binding proteins, D1 and D2 respectively, which are part of the reaction system for photosystem II (Thum et al. 2001a).

These genes are differentially expressed, based on differences in light activation (Klein and Mullet 1990; Chun et al. 2001; Gamble and Mullet, 1989; Sexton, Christopher, and Mullet 1990). The *psbD* gene has three different promoters, one of which, the light-responsive promoter (*psbD*-LRP), is transcribed by the plastid-encoded RNA polymerase (PEP) (Thum et al. 2001b). In order for *psbD* to be transcribed, a number of nuclear-encoded transcription factors must be bound, including nuclear-encoded AGF and PGTF, the second of which governs the activity of *psbD*-LRP in response to light and dark cycling (Kim, Christopher, and Mullet 1999), thus implying that *psbD* functions under circadian rhythm (Nakahira et al. 1998; Thum et al. 2001b).

Because methylation of the chloroplast DNA of flax has not been studied previously, and because very little research has been done on the chloroplast genome in flax it is only possible to speculate what relationships and interactions exist between genes (nuclear or chloroplast) and methylation levels, based on work done in other plants, be it LD or SD species. Although in *Hordeum vulgare* L. (barley), no differences in DNA methylation of the chloroplast were seen between 5 and 8 day-old seedlings (Krupinska 1992), this does not exclude the possibility of differences in methylation level at the *psbD* promoter region in early flowering and control lines of flax. This could explain, at least partly, differences in flowering time between the two lines of flax. It has also been shown that phosphorylation of specific protein kinases and protein phosphatases in the nucleus and cytoplasm of *Triticum aestivum* (wheat) is responsible for transcriptional changes in *psbD* (Christopher et al. 1997). It is possible that differences in methylation levels in the nuclear DNA of RE2 and RC, specifically at sites affecting the transcription factors regulating *psbD*, are affecting the circadian rhythmic cycling of D2 (the protein encoded by *psbD*), thereby affecting flowering time in flax.

Although Langdale, Taylor, and Nelson (1991) were unable to identify differentially methylated regions of the *RbcL* (Rubisco large sub-unit) gene in the chloroplast of maize leaves, it would not be unrealistic to suggest that there is a difference in methylation (and therefore expression) of this gene in the chloroplast of flax. The up-regulation of *RbcL* in RE2 would potentially allow it to make better use of light energy for photosynthesis and flowering purposes. Further, this consideration needs to be made

for the Rubisco small sub-unit (*RbcS*), which would likely be transcribed at a similar level, however it is a nuclear-encoded gene.

5.5 *Differences in* LFY *Transcript Levels: RC vs. RE2*

Studies of transgenic *Arabidopsis* plants have suggested that a minimum threshold level of *LFY* transcript must be present in order for the plants to flower (Blazquez et al. 1997). This situation could also occur in flax. RE2 plants appear to be reaching this threshold level sooner than RC plants, which may contribute to the early-flowering phenotype. From the data presented here (Figure 15a), however, it appears that if there is a minimum level of *LFY* transcript required, RE2 may far exceed it (*i.e.* there is much more transcript accumulation in this line than in RC). Levels of *LFY* transcript in excess of the minimum threshold appear to cause, simply, early flowering, with no further effects (Blazquez et al. 1997). It has been suggested that younger vegetative tissue would require a higher threshold of *LFY* transcript in order to flower, as younger plants are otherwise less competent to flower (Molinero-Rosales et al. 1999). This theory would do well in explaining the significantly higher levels of *LFY* transcript in RE2 compared to RC. Further, because there is no real linear increase in the level of *LFY* transcript in RC over time (Figure 15a), it is possible that because the late vegetative phase of growth is prolonged in RC, the concentration of *LFY* transcript does not have to increase to as high of a level as it does in RE2. From 38-48 days in RC, there was no observed increase in DNA recovery per shoot tip or per unit fresh weight (Figure 4c-d), suggesting that the SAM is prepared for the transition from vegetative to reproductive growth at this time and is waiting for cues to flower, possibly waiting for the level of *LFY* transcript to increase to a minimum threshold. Methylation levels are not increasing at this point in RC either (Figure 4a), further indicating that the plants are involved in a "waiting period".

Blazquez et al. (1997) also determined that there is up-regulation of *LFY* transcript in leaf primordia, not just floral primordia. This may be why there are low levels of *LFY* transcript in the four leaves immediately below the shoot tip (Figure 15d and Figure 18) of flax, as well as in the young shoot tips (Figure 15a and Figure 16). The transcripts are regulated at a constitutively low level, keeping the

shoot tips from forming flowers too early, and preventing the shoot apices from becoming flowers. Blazquez et al. (1997) show that there is no real need for regulation of expression of *LFY* in advanced leaf primordia because, once the leaves are initiated, *LFY* expression will not cause them to flower. This could explain why there is a decrease in *LFY* transcription in the leaves of RC over time. The same decrease in *LFY* transcription in leaves over time is not seen in RE2 because the decrease is in the late vegetative phase of RC and this phase is truncated in RE2 (Fieldes and Harvey, 2004). Interestingly, it has also been shown in *Arabidopsis* that plants that over-express *LFY* and under-express *TFL1* have accelerated progression through the later stages of rosette growth (*i.e.* later vegetative stages) and therefore, flower earlier than wild-type plants (Steynen, Boloski, and Schultz 2001).

While it is tempting to suggest that *LFY* could be one of the genes in the genetic model proposed for the early flowering phenotype of flax (Amyot 1999; Fieldes and Amyot 1999a), more direct relationships between methylation and *LFY* transcript accumulation would have to be established before making this claim.

5.6 *Lack of* TFL1 *Transcript Accumulation in Flax*

Although *TFL1* was shown to be present in the genomic DNA of three different lines of flax (*i.e.* RC, RE2 and LC) (Figure 20a), transcripts were not detected in the cDNA of RC or RE2 at various ages, in various tissues, with these primers (Figure 20b-c). Since finding a set of degenerate primers, as well as specific primers, proved very difficult for this gene, and because the primers work in genomic DNA to produce a band of the expected size, it is tempting to suggest that *TFL1* is not transcribed in flax. However, because *TFL1* is not expressed in vegetative tissues of all species (Bradley et al. 1997), its transcripts may not be present in flax leaves. The absence of *TFL1* transcripts in the young shoot tips is more difficult to explain. One possibility is that other factors are regulating the expression of *LFY*. Alternatively, *TFL1* could be transcribed at very low levels, or with a very high turnover rate. It is also possible, however, that the expression patterns of *TFL1* are quite limited (Bradley et al. 1997;

Amaya, Ratcliffe, and Bradley 1999; Boss, Sreekantan, and Thomas 2006), and that a PCR study is not the optimal method of finding *TFL1* transcripts.

5.7 *Differences in Actin Transcript Levels: RC vs. RE2*

It is apparent, from the attempt to use the actin gene as a standard in the *LFY* transcript study, that the levels of actin are not the same in the leaves of RC and RE2 and that they change during development in RC shoot tips (Figure 15c and Figure 17). In the shoot tips of RC, actin transcript levels may increase during the prolonged third vegetative phase because in this phase there is a higher proportion of leaf tissue in the older versus the younger shoot tips.

Studies on levels of the actin binding protein profilin (PFN) in *Arabidposis*, found that plants which under-express the protein had a decreased height, number of leaves, and flowering time (Ramachandran et al. 2000). In more primitive species (*i.e.* yeast and amoeba), altered expression of PFN has been shown to alter actin cytoskeleton arrangements (Balasubramanian et al. 1994; Finkel et al. 1994). However, in *Arabidopsis*, no differences in cytoskeleton arrangement were found between plants with wild-type levels of PFN and those with lower PFN levels (Ramachandran et al. 2000). It would be interesting to look for differences in PFN levels between RC and RE2 lines and see if, like more primitive species, differences in the expression levels of this protein relate to differences in actin cytoskeletal arrangements, perhaps to the point of differences in mRNA transcript levels in the plants.

Over-expression of the actin-depolymerizing factor (ADF) in *Arabidopsis* reduced the formation of actin cables in some tissues with no significant change in flowering time, while the under-expression of ADF produced plants with delayed flowering time and increased actin cables (Dong et al. 2000). It has been suggested that, because of actins' involvement in intracellular signalling via cytoplasmic streaming and plasmodesmata (McLean, Hempel, and Zambryski 1997), a reduction in the expression of ADF, as well as other actin binding proteins, may be necessary for flowering to occur (Dong et al. 2000). In the case of flax, the decrease in actin expression in RE2 tissue was seen most predominantly in the leaves. However, if a florigen protein (*i.e.* FT) moves from other areas in the plant to the

meristem, actin binding protein levels may need to be decreased throughout RE2 in order for this to occur more efficiently.

5.8 *Searching the Flax Genome: Genomic vs. cDNA*

While it may have been simpler, and more efficient, to carry out the gene hunt for *LFY* and *TFL1* using cDNA, there were reasons for initially using genomic DNA. Had the project worked as it expected, it would have been much faster to use genomic DNA instead of cDNA. In fact, it took a very short time to determine the primers for *LFY*. Because *TFL1* is not as well documented in plants as *LFY*, the location of its expression in flax was not certain. In fact, evidence from this study suggests that while the gene is found in the flax genome (Figure 20a), it is not being expressed in either leaves or shoot tips (Figure 20b-c). It had been anticipated that *TFL1* would be found at moderately low levels in leaves, to counteract *LFY* expression. In shoot tips, it was assumed that *TFL1* would be present at high levels in young shoots, and that there would be a decrease in its expression with the onset of flowering. Although the results reported here suggest that it is not being expressed, more primer sets, or another method of detection, would need to be used to fully confirm whether *TFL1* is expressed in flax leaves and shoots or not.

5.9 *Methylation Levels and Transcript Levels: Cause and Effect*

It is tempting to suggest that the differences in methylation levels and transcript levels of *LFY* in RC versus RE2 are directly related but this statement would be inappropriate at this time. Although both are likely related to the early flowering of RE2, it would only be speculative to suggest that the hypomethylation of RE2 is causing the increased levels of *LFY* transcript in RE2.

In *Arabidopsis*, methylation is known to directly affect *FWA* expression (Genger et al. 2003), and to indirectly affect *FLC* expression, both of which then affect the time of flowering (Finnegan, Peacock, and Dennis 2000; Genger et al. 2003). Further, *FLC* is known to be an inhibitor of floral pathway integrators, the class of genes that includes *LFY* (Figure 2; Henderson and Dean 2004). Thus, it is

possible that methylation affects the expression of *LFY* by a number of indirect pathways. For example: i) the methylation affecting *FLC* could be indirectly affecting *LFY* transcription, and ii) the hypomethylation of RE2 could affect genes controlling circadian rhythm, including those in the photoperiod pathway, such as *CO* (Figure 2). If this is the case, the changes in the photoperiod pathway could be causing the changes in *LFY* transcript levels, because this pathway has a direct affect on floral pathway integrators (Henderson and Dean 2004).

The chloroplast genomes of RC and RE2 have substantial levels of DNA methylation, and because the chloroplast genome of RE2 is significantly hypomethylated, relative to RC, it is possible that this methylation is somehow related to the early flowering phenotype of RE2. Further research needs to be done on the genes in the flowering time pathways in flax, as well as on the effects of methylation on all flowering time genes, in order to determine whether the demethylation of RE2 is related, either directly or indirectly, to the increased amount of *LFY* transcript in RE2 shoot tips.

5.10 *Future Research*

It would be interesting to examine DNA methylation levels over the course of the day (*i.e.* with photoperiodism), especially because dark treatments appear to alter the total DNA methylation levels in flax. It is likely that RE2 remains constantly hypomethylated relative to RC in the chloroplast DNA, but it seems plausible that at night and early in the morning nuclear DNA methylation levels are similar in early flowering and control lines. In the chloroplast, it is possible that the differences in DNA methylation are related to gene expression, leading to differences in protein levels, where there could be a higher accumulation of photosynthetic proteins (such as RbcL) in RE2, relative to RC. It would also be interesting to examine the accumulation of D2 (the protein encoded by *psbD*) in RE2 and RC, as *psbD* is transcribed based on circadian rhythm, and to relate this to flowering time. Because there are high levels of DNA methylation in the chloroplast genomes of RC and RE2, it would be interesting to consider the mechanisms that methylate this DNA, that is, the possible presence of methyltransferases in the chloroplasts of flax, and how they arrive there.

In order to look at the inheritance of methylation levels from generation to generation, an immunohistochemical approach could be used to examine methylation levels more precisely in shoot tips and to study methylation at the central core of the SAM in order to see if RE2 is always hypomethylated in the meristematic tissue.

Although *TFL1* is an inhibitor of *LFY*, because, as mentioned earlier (1.4.2), it has a very precise expression in the SAM, it was probably not the most suitable choice for this PCR-based semi-quantitative study. With the primers for *TLF1* that gave the band in the genomic DNA of RC and RE2, probes for *TFL1* could be produced and used to detect the mRNA of *TFL1*. More specifically, these probes could be used to study the expression of *TFL1* in the SAM, with the necessary precision.

Actin, which was to serve as a standard in the transcript level experiment proved interesting because the transcript abundance differed between the two lines studied. It would be interesting to see which other seemingly constitutively expressed structural (or other) genes are being transcribed at an altered rate in RE2. A parameter of this kind might be useful in determining the exact time at which the switch from vegetative to reproductive growth is occurring in early-flowering lines of flax. However, the question remains as to what structural gene could be used as a standard for a semi-quantitative study. Ribosomal subunit genes are being considered.

The work presented here is beneficial in understanding some of the details of flowering time in flax, although there is still a wealth of information to obtain. It has also opened new avenues for flax research in the field of chloroplast development/metabolism in early flowering versus control lines. With continued research, it is possible that methylation will be found to be a common thread that links together many of the developmental differences between RC and RE2.

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APPENDIX A: Murashige and Skoog Nutrient Solution

(Modified from: Murashige and Skoog, 1962)

The nutrient solution was made by mixing stock solutions of specific chemicals, 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 0.05 g $NaMoO_4 \cdot 2H_2O$ 0.005 g $CoCl_2 \cdot 6H_2O$
D	88.00 g $CaCl_2 \cdot 2H_2O$
E_1	74.00 g $MgSO_4 \cdot 7H_2O$ 3.38 g $MnSO_4 \cdot H_2O$ 1.72 g $ZnSO_4 \cdot 7H_2O$
E2	0.005 g $CuSO_4 \cdot 5H_2O$
F*	8.25 g $Na_2EDTA \cdot 2H_2O$ 5.57 g $FeSO_4 \cdot 7H_2O$

*Note: The $FeSO_4 \cdot 7H_2O$ was dissolved in 200 mL of water and heated to 80 ºC and the $Na_2EDTA \cdot 2H_2O$ was then added and dissolved at this temperature and the volume brought up, with deionized water, to 1 L.

For experiments grown in the greenhouse (*i.e.* as opposed to the growth chamber), 100 mL of each of solution C_1, D, E_1 and F, and 200 mL of each of A, B, C_2 and E_2 were put into a 20 L carboy and water (tap) was added to make a total volume of 20 L. Each tray (approximately 30 plants) received 750 mL of nutrient solution each week, while smaller trays (approximately 20 plants) received 500 mL each week. For plants grown in the growth chamber to more than 7 days, nutrient solution was given each week; the volumes used were calculated based on the number of plants per tray being grown.

APPENDIX B: Protocol for Extraction of DNA

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

1. Approximately 200 mg of plant tissue (per sample) was ground in liquid nitrogen using a mortar and pestle and rapidly transferred into a 1.5 mL eppenedorf tube.
2. 400 µL of buffer AP1 and 4 µL RNase A were added to the samples.
3. Samples were vortexed vigorously and allowed to stand 10 min. at room temperature.
4. Samples were incubated for 15 min. at 65 ºC, mixing by vortexing, to lyse the cells, every 5 min.
5. 130 µL of Buffer AP2 were added to each sample; the samples were mixed by vortexing and incubated in an ice bath for 5 min.
6. Samples were centrifuged for 5 min. at 8000 g.
7. Liquid lysate was put onto QIAshredder spin columns and centrifuged at 8000 g for two min.
8. The samples were transferred to new eppendorf tubes at 675 µL of Buffer AP3/E was added. Mixing of lysate and buffer was done by pipetting.
9. 650 µL of the mixture was put into a DNeasy mini spin column and centrifuged for 1 min. at 6000 g.
10. The flow-through was discarded and step 9 was repeated with the rest of the sample.
11. A new collection tube was used and 500 µL of Buffer AW was added to the DNeasy column and centrifuged for 1 minute at 6000 g. Waste was discarded.
12. 500 µL of Buffer AW was again added to the DNeasy column but this was centrifuged for 2 min. at maximum speed to dry the membrane.
13. The columns were transferred to new collection tubes.
14. 100 µL of preheated 10mM Tris* was pipette onto the membrane.
15. The columns were incubated at 65 ºC* for 5 min. before centrifugation at 6000 g for 1 min.
16. Steps 14 and 15 were repeated and the new eluate was added to that already stored in the eppendorfs.

Note: The asterisks (*) indicated procedure used for DNA that was then hydrolyzed for HPLC analysis. For DNA used in PCR/electrophoresis procedures, Buffer AE was used in place of 10 mM Tris and the incubation period was carried out at room temperature, not 65 ºC, as suggested in the DNeasy® Plant Mini Kit manual.

APPENDIX C: Protocol for DNA Hydrolysis for HPLC Analysis

1. DNA was extracted (see Appendix B and its footnote).
2. Immediately following the extraction, 2 µL of 0.1M HCl were added to each sample and they were boiled for two minutes and placed immediately into an ice bath for five minutes. The rest of the procedure was carried out on ice.
3. 30 µL of 8.3X S_1 nuclease incubation buffer (0.27 M NaAc, 0.42 M NaCl, 2.7 mM $ZnSO_4$, pH 4.5 at room temperature), 2 µL of 0.1M HCl and 15 µL (240U) of S_1 nuclease (Roche) were added to each sample.
4. Samples were incubated for 17 h at 37 ºC.
5. 28 µL of 0.67 M Tris, 10 µL of 1.0N NaOH and 12 µL of alkaline phosphatase (Sigma) were added to the samples.
6. Samples were incubated for three more h at 37 ºC.
7. Samples were stored at 4 ºC until HPLC analysis.
8. Immediately preceding HPLC, samples were centrifuged at 7500 g for 30 min.
9. 125 µL (*i.e.* enough for two HPLC runs) of the supernatant were then transferred to glass inserts in septa vials to be used for sample injection into the HPLC system.

APPENDIX D: Recipes for HPLC Solutions

1. 13.6 g potassium phosphate monobasic were put into a 2 L volumetric flask and made up with MilliQ water (to give 0.05 M).
2. Water and potassium phosphate were mixed until the potassium phosphate was completely dissolved.
3. For solution A: Into a separate 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 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 ºC until needed and kept no more than two days before use (to ensure proper pH).

APPENDIX E: Protocol for Chloroplast Isolation

(Derived from Smith et al. *2002)*

1. Continuous Percoll® gradients (2 per sample) were created by spinning 12.5 mL of Percoll® and 12.5 mL of water at 39 000 g for 30 min. with slow deceleration.
2. Sixty flax plants per sample per line (both RC and RE2) at 21 days of age were used, and all green tissue (*i.e.* leaves, shoot tip and cotyledons) was harvested.
3. Tissue was homogenized in approximately 200 mL of cold 1 X GB using a PowerGen700® homogenizer (Fisher Scientific).
4. The homogenate was filtered through two layers of Miracloth® into pre-chilled 500 mL centrifuge bottles and spun for 8 min. at 1000 g at 4 ºC
5. Supernatant was discarded and the pellet was re-suspended in 8 mL of fresh 1 X GB.
6. The re-suspended material was divided evenly between the two-Percoll® gradients (4 mL per gradient).
7. Gradients were spun in the centrifuge at 8000 g at 4 ºC for 15 min. in a swinging bucket rotor with slow acceleration and deceleration.
8. Broken chloroplasts, and the entire Percoll® gradient above the intact chloroplasts, were aspirated off.
9. The intact chloroplasts were collected and HS buffer was added to a volume of approximately 50 mL.
10. Chloroplasts were centrifuged for 6 min. at 1000 g at 4 ºC.
11. Supernatant was discarded and the pellet was re-suspended in 300 µL of HS buffer.
12. The chloroplasts and HS buffer were transferred into a micro centrifuge tube and the volume was estimated.
13. For chlorophyll concentration calculations, 10 µL of chloroplast and 990 µL of 80 % acetone were combined in a microcentrifuge tube. In a separate tube, a blank of 990 µL of 80 % acetone and 10 µL of HS buffer were combined.
14. Tubes were vortexed and then centrifuged for 1 min. at 8000 g.
15. The absorbance was measured by spectrophotometry at 652 nm (twice and averaged), and the concentration of chlorophyll was calculated in mg/mL ([CP] $=A_{652}$*(1000/10)/36).
16. DNA from chloroplast samples was extracted using the same protocol as total DNA.

APPENDIX F: Recipes for Chloroplast Isolation

(Derived from Smith et al. *2002)*

2 X Grinding Buffer

30 mL 1 M Hepes-KOH pH 7.5
2.4 mL 0.5 M EDTA pH 8.0
6 mL 1 M $MnCl_2$
300 µL 2 M $MgCl_2$
36.07 g Sorbitol
pH to 7.5 (with 1 M KOH)
Adjust volume to 300 mL with MilliQ water
(Final concentrations: 100 mM Hepes, 4 mM EDTA, 20 mM $MnCl_2$, 2 mM $MgCl_2$, 660 mM Sorbitol)

1 X Grinding Buffer

125 mL 2 X grinding buffer
4.95 g ascorbic acid
0.625 g BSA (do NOT mix on a stir plate)
Adjust volume to 250 mL with MilliQ water
(Final concentrations: 50 mM Hepes, 2 mM EDTA, 10 mM $MnCl_2$, 1 mM $MgCl_2$, 330 mM Sorbitol, 100 mM ascorbic acid, $2.5x10^{-3}$ % BSA)

HS Buffer

10 mL 1 M Hepes-KOH pH 7.5
12.02 g Sorbitol
pH to 7.5 (with 1 M KOH)
Adjust volume to 200 mL with MilliQ water
(Final concentrations: 50 mM Hepes, 330 mM Sorbitol)

Note: 2 X grinding buffer and HS buffer were made beforehand and stored at 4 ºC; however, both the 1 X grinding buffer and the Percoll® gradients were made immediately prior to the experiment.

APPENDIX G: Code of Degenerate Bases
(*Obtained from www.invitrogen.com*)

Base	Code
A, C, G	V
A, C, G, T	N
A, T, G	D
T, C, G	B
A, T, C	H
A, T	W
C, G	S
T, G	K
A, C	M
C, T	Y
A, G	R

APPENDIX H: Primer Sets used for Gene Search

Appendix Ha: *LFY* primers

Primer Combinations	Sequence	Direction	Melting Temperature (°C)
1	AGYTTBGTWGGMACRTACC	Reverse	53.00
1	AARKCTGGDGGAAGYTACAT	Forward	56.00
1	GCYTGTCTCCAWGMNCCMAC	Reverse	64.00
2	CGCGTTGCACTGTCTGGTC	Forward	64.00
2	GGGAAGGTACATAAACAAGCC	Reverse	58.00
3	GARYTBGATGABATGATGA	Forward	49.00
4	GGDGAAAGKTATGGHATYAARGC	Forward	62.00
3,4	TTNCCHCCHGCMACYTCHCC	Reverse	69.00
3,4	CTTDGTDGGRYAYTTYTCHCC	Reverse	59.00
5	GGDGARGTKGCDCGDGCNAA	Forward	70.00
6	GGDGARGRTRYCCHACHAAG	Forward	61.00
5,6	GCYTGTCTCCAWGMNCCMAC	Reverse	64.00
7	CCTACGTTCTCCCCTCTTTCC	Forward	64.83
8	CGTTCTCCCCTCTTTCCTTG	Forward	64.82
7,8	CGTGCATTGCTACGCGT	Reverse	64.87
9	CTCCTACGTTCTCCCCTCTTTCCTTGA	Forward	68.52
9	AGGCACTACGTGCATTGCTACGCG	Reverse	71.95
10	CTCCTACGTTCTCCCCTCTTTCCTTGA	Forward	68.52
10	AGGCACTACGTGCATTGCTACGCG	Reverse	71.09

Note: Numbers correspond to the combinations in which the primers were tried. For example, the reverse primer notated "3,4" was used with both of the forward primers, numbers 3, and 4. Alternatively, forward primer 9 was only tried with reverse primer 9. These primers were found by: a) design from the published sequences for the gene in question, and b) from the OligoPerfect function from Invitrogen. All primers were ordered from Invitrogen, Canada (Hamilton, ON) and follow the degenerate code of Invitrogen (see Appendix G).

Appendix Hb: *TFL1* primers

Primer Combinations	Sequence	Direction	Melting Temperature (°C)
1	AATGGCCATGAGCTCTTTCCTTC	Forward	68.28
1	AACGYCTKCKRGCGGCRGTTTC	Reverse	71.00
2	GGATTTGATCAAGTATGAATGATGAA	Forward	60.00
2	TGGCTCCCGATATGTCATT	Reverse	59.00
3	GAGTSRTWGGWGAWGT	Forward	47.00
4	CAAGTYTNCAAYGGACA	Forward	49.00
5	AGATCMTTCTTYACWYTGGT	Forward	52.00
3,4,5	ACCARWGTRAAGAAKGATCT	Reverse	52.00
3,4,5	GGAYATYWGYRAHRAT	Reverse	43.00
6,7	AATGGCCATGAGCTCTTTCCTTC	Forward	64.96
6	TGKATCCCTATGYTYGGCCTTGG	Reverse	67.87
7	CTYCTGGCAGCRGTYTCKCKCTG	Reverse	69.64
8	AARCADGTDWVDAAYGGDCAYGA	Forward	63.91
9	ATGAYDGAYCCDGAYGTDCC	Forward	61.06
8	TTRAARAADACDGCDGCDAC	Reverse	58.89
9	AADACRWADCKRTGDATDCC	Reverse	55.87
10	GGTGGTGATCTCAGGTCCTT	Forward	58.96
10	CGGCATCTCATAGCTCAACC	Reverse	60.77

Note: Numbers correspond to the combinations in which the primers were tried. For example, the reverse primers notated "3,4,5" were used with all three of the forward primers, numbers 3, 4, and 5. Alternatively, forward primer 1 was only tried with reverse primer 1. These primers were found by: a) design from the published sequences for the gene in question, b) from the OligoPerfect function from Invitrogen, and c) from published papers (set 1 was from Esumi, Tao, and Yonermori 2005, sets 6 and 7 were from Amaya, Ratcliffe, and Bradley 1999, and sets 8 and 9 were from Boss, Sreekantan, and Thomas 2006). All primers were ordered from Invitrogen, Canada (Hamilton, ON) and follow the degenerate code of Invitrogen (see Appendix G).

APPENDIX I: Band Extraction Procedure

(Derived from the QIAquick® Spin Handbook from Qiagen)

1. The DNA band of interest was excised from the gel with a clean, sharp razor blade.
2. Gel slices were weighed in a 1.5 mL eppendorf tube and 3 times the volume of the gel weight of buffer QG was added (where 100 µL buffer = 100 mg gel).
3. The gel and buffer were incubated at 50 ºC until the gel had dissolved, while samples were vortexed every three min.
4. Isopropanol was added to gel in the same volume of the gel (i.e. 100 µL isopropanol = 100 mg gel).
5. The solution was pipetted into a QIAquick spin column and centrifuged at 15 690 g for 1 min. to bind DNA to the filter.
6. Liquid waste was discarded from the collection tube.
7. 0.5 mL Buffer QG was added to the column and centrifuged at 15 690 g for 1 min. to remove all agarose traces. Flow-through was discarded.
8. DNA was washed by addition of 0.75 µL of Buffer PE (with ethanol) to column, followed by centrifugation at 15 690 g. The liquid waste and collection tube were discarded.
9. The QIAquick column was put into a new collection tube.
10. 30 µL molecular grade water was added to centre of the QIAquick filter and allowed to stand for 1 min.
11. The column was centrifuged for 1 min. at 15 690 g.
12. Samples were stored in 1.5 mL eppendorf tubes at –20 ºC.

APPENDIX J: Bacterial Cloning Procedure and Plasmid Mini Prep

Appendix Ja: Bacterial Cloning Procedure

(Derived from the Invitrogen TOPO TA Cloning Kit Procedure)

1. 4 µL fresh PCR product, 1 µL salt solution and 1 µL TOPO® vector were combined into a 1.5 mL eppendorf, mixed and incubated for 30 min. at room temperature.
2. 2 µL of mixture from step 1 was added to a vial of OneShot® Chemically Competent TOP10 *E. coli* and gently mixed.
3. The tube was incubated on ice for 30 min.
4. Cells were heat-shocked for 20 s. at 42 °C to stop the reaction and they were immediately transferred to ice.
5. 250 µL of room temperature S.O.C. medium was added.
6. Samples were placed on a horizontal shaker at 200 rpm at 37 °C for 1 h.
7. 25 µL, 100 µL and 150 µL (or remaining) cells were spread onto 50 µg/mL kanamycin plates and incubated overnight at 37 °C.
8. Six colonies were cultured overnight in 3 mL of LB containing 50 µg/mL kanamycin.

Appendix Jb: Plasmid Mini Prep

(Derived from the Invitrogen PureLink Quick Plasmid MiniPrep Kit Procedure)

1. Samples of overnight culture from Appendix Ja were centrifuged (all 3 mL) and all medium was removed from the cell pellet.
2. Pellets were resuspended in 250 µL of solution R3 with RNase A.
3. 250 µL solution L7 were added and mixed by inverting the tube.
4. Samples were incubated at room temperature for 5 min.
5. 350 µL of buffer N4 were added and solutions were mixed by inversion until homogeneous.
6. Samples were centrifuge at 12 000 x g for 10 min. at room temperature and supernatant was loaded into a spin column.
7. Columns were centrifuge at 12 000 g for 1 min. and flow through was discarded.
8. 500 µL W10 (ethanol added) was added and samples were incubated for 1 min. at room temperature.
9. Samples were centrifuged at 12 000 g for 1 min. and flow through was discarded.
10. 700 µL W9 buffer (with ethanol) were added and tubes were centrifuged at 12 000 g for 1 min.
11. Flow through was discarded and samples were centrifuged for an additional minute at 12 000 g.
12. Spin columns were placed into a clean 1.5 mL Recovery Tube and 75 µL of preheated (65-70 °C) TE buffer was added.
13. Samples were incubated at room temperature for 1 min. and centrifuged at 12 000 g for 2 min.
14. The purified DNA was stored at -80 °C for future use.

APPENDIX K: Procedure for Extraction of RNA

(Derived from the RNeasy® Mini Handbook from Qiagen)

1. Approximately 100 mg 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 to avoid thawing of the plant material.
2. 450 µL of the Buffer RLT/B-meracaptoethanol (10:1) solution were added.
3. Sample material was pipet into the QIAshredder spin column in a collection tube and centrifuged for 2 min. at 8000 g.
4. The supernatant was put into a new eppendorf tube and 225 µL 100 % ethanol were added and mixed by pipetting.
5. The sample was moved into an RNeasy mini column in a collection tube and centrifuged for 15 s. at 8000 g.
6. Flow-through was discarded.
7. 700 µL of Buffer RW1 was put onto the RNeasy column, centrifuged for 15 s. at 8000 g, and then discarded.
8. The RNeasy column was put into a new collection tube and 500 µL of Buffer RPE was pipette onto it.
9. The column was centrifuged for 15 s. at 8000 g to wash the column and the flow-through was discarded.
10. 500 µL of Buffer RPE were again put onto the column and centrifuged at 8000 g, but for two minutes.
11. The column was once again transferred to a new collection tube and centrifuged for 1 min. at 8000 g to thoroughly remove all remaining Buffer RPE and completely dry the membrane.
12. The column was moved to a new collection tube and 30-50 µL of molecular grade water were pipette onto the membrane.
13. The material was centrifuged for 1 min. at 8000 g and the flow-through was transferred to a 1.5 mL eppendorf tube for storage at –80 ºC until use.

APPENDIX L: RT-PCR Procedure

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

1. All required solutions (*i.e.* oligo-dT, 10 X buffer, RT, dNTP mix, and water) were thawed at room temperature, briefly vortexed and stored on ice. 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 was 2 µL 10 X buffer, 2 µL dNTP mix, 2 µL oligo-dT (Invitrogen), 1 µL (RNase inhibitor), and 1 µL Omniscript Reverse Transcriptase. Also added were 2 ng RNA (volume determined by spectrophotometer reading). The volume was topped to 20 µL with DNA/RNA-free water.
4. The tube was briefly centrifuged to collect residual liquids.
5. The tube was incubated at 37 °C for 1 h.
6. cDNA was stored at -80 °C until further required.
7. Samples were run using HotStartTaq® provided by Qiagen.

APPENDIX M: Determining the Optimal Number of PCR cycles for *LFY* and Actin

These graphs represent three PCR runs using different tissue (leaf or shoot tip) at different ages (7, 56, and one age between) to find the optimal cycle number for each gene. Graph a) is the result of the actin linear range (optimal cycle no.: 25), and b) is the result of the *LFY* linear range (optimal cycle no.: 31).