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DNA hypomethylation in 5-azacytidine-induced early-flowering lines of flax

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Abstract HPLC analysis was used to examine the cytosine methylation of total DNA extracted from four early-flowering lines that were induced by treating germinating seeds of flax (*Linum usitatissimum*) with the DNA demethylating agent 5-azacytidine. In the normal lines that gave rise to the induced early-flowering lines, flowering usually begins approximately 50 days after sowing. The early-flowering lines flower 7–13 days earlier than normal. The normal level of cytosine methylation was approximately 14% of the cytosines and 2.7% of the nucleosides. In the early-flowering lines, these levels were 6.2% lower than normal in DNA from the terminal leaf clusters of 14-day-old seedlings and 9.7% lower than normal in DNA from the cotyledons and immature shoot buds of 4-day-old seedlings. This hypomethylation was seen in lines that were five to nine generations beyond the treatment generation. The level of hypomethylation was similar in three of the four early-flowering lines, but was not as low in the fourth line, which flowers early but not quite as early as the other three lines. Unexpectedly, the degree of hypomethylation seen in segregant lines, derived by selecting for the early-flowering phenotype in the F_2 and F_3 generations of out-crosses, was similar to that seen in the early-flowering lines. Analysis of the methylation levels in segregating generations of out-crosses between early-flowering and normal lines demonstrated a decrease in methylation level during the selection of early-flowering segregants. The results suggest an association between hypomethylation and the early-flowering phenotype, and that the hypomethylated regions may not be randomly distributed throughout the genome of the early-flowering lines.

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Introduction

The early-flowering flax lines were induced by treating germinating seeds with the DNA demethylating agent 5-azacytidine [(5-azaC) Fieldes 1994], and have provided two indications that the induced heritable changes in the genome are epigenetic. First, the induction of six early-flowering lines reflected a rate of mutation that was considerably higher than expected using classical mutagens (Fieldes 1994). Furthermore, the possibility that classical mutation was involved became even less likely when subsequent genetic analysis demonstrated that the early-flowering phenotype of most of the induced lines is controlled by the combined effects of changes (epimutations) at three independent loci (Fieldes and Amyot 1999a). Second, although there have been no indications of reversion since the third generation after induction, low levels of reversion were observed in the generations immediately following induction in some lines, and one had completely reverted by the fourth generation after treatment (Fieldes and Amyot 1999a). This type of reversion is characteristic of epigenetic effects rather than classical mutations (Jablonka and Lamb 1989).

The early-flowering phenotype, which includes reduced height at maturity and a reduction in the number of leaves produced on the main stem (Fieldes and Amyot 1999a; Fieldes and Harvey 2004), was the most striking effect induced by the 5-azaC treatment. Albeit, in total, 27% of the progeny of the plants grown from treated seeds displayed altered phenotypes in terms of flowering time and/or height, and much of this induced phenotypic variability was inherited into subsequent generations (Fieldes 1994). Similar heritable effects of treating seeds

with 5-azaC have also been reported for triticale (Heslop-Harrison 1990; Amado et al. 1997), *Brassica oleracea* (King 1995), and rice (Sano et al. 1990). In rice, the treatment induced dwarfism and a concomitant reduction in the level of 5-methylcytosine (5mC) in the DNA (Sano et al. 1990), which were shown to be transmitted into the second generation after treatment. Vyskot et al. (1995) have also demonstrated the meiotic transmission of hypomethylation induced by 5-azaC. The heritable effects of 5-azaC are thought to result from the demethylation of sites associated with loci that control phenotypic or developmental characteristics. This demethylation is likely to be part of a generalised reduction in the level of genomic 5mC, which results from the incorporation of 5-azaC into the DNA and its inhibitory effects on DNA methyltransferases and the maintenance of methylation (Santi et al. 1983; Jones 1984).

DNA methylation plays a role in the regulation of gene expression and is involved in transcriptional gene silencing (Furner et al. 1998; Matzke and Matzke 1998; Mittelsten Scheid et al. 2002), the regulation of transposons (Miura et al. 2001; Cui and Fedoroff 2002), nucleolar dominance (Chen and Pikaard 1997; Houchins et al. 1997), and imprinting (Alleman and Doctor 2000). In each function, the methylated state is usually associated with inactivation of gene expression and, conversely, gene activation is associated with demethylation. DNA methylation has implications in terms of the control and organisation of chromatin and its role in controlling gene expression. The relationships between chromatin structure and gene expression, and between histone–protein complexes, DNA methylation, and chromatin organisation, which have been recognised for decades (e.g., Conklin and Groudine 1984), are being unravelled for plant species (for review, see Li et al. 2002; Tariq et al. 2003; Steimer et al. 2004).

It has also been recognised for some time that epigenetic changes, such as alterations in DNA methylation status, are likely to be involved in regulating ontogenetic changes in gene expression in plants (Finnegan et al. 1996; Richards 1997), and direct effects of 5-azaC treatments on gene expression and cell differentiation have provided part of the information supporting this contention (e.g., LoSchiavo et al. 1989; Burn et al. 1993; Galaud et al. 1993; Vyskot et al. 1993; Chen and Pikaard 1997; Tatra et al. 2000; Horváth et al. 2002; Santos and Fevereiro 2002). Nevertheless, it is only recently that definitive examples of epimutations (epi-alleles) resulting from changes in methylation status have been described for plant genes that are developmentally regulated (Hoekenga et al. 2000; Jacobsen et al. 2000; Soppe et al. 2000; Stokes et al. 2002). In *Arabidopsis*, loss-of-function mutations (*ddm1)* at the decreased DNA methylation 1 (*DDM1*) locus (Vongs et al. 1993), loss-of-function mutations (*met1)* at the DNA methyltransferase 1 gene (*MET1*) (Kankel et al. 2003), or antisense forms of *MET1* (Ronemus et al. 1996; Finnegan et al. 1998; Genger et al. 2003) have been used to produce lines with reduced levels of 5mC that display a range of heritable morphological defects and developmental changes. These methods of inducing hypomethylated genomes have indicated a role for DNA methylation in regulating genes that control flowering time, in particular, the *FWA* locus (Kakutani 1997; Soppe et al. 2000; Kankel et al. 2003). They also provide support for other evidence that suggests DNA methylation is involved in regulating the *FLC* locus, which is part of the vernalization-response pathway (Finnegan et al. 1998; Sheldon et al. 2000a, 2000b; Gendall et al. 2001; Genger et al. 2003; Bastow et al. 2004).

The objective of this study was to determine whether total DNA from the early flowering flax lines is hypomethylated, and whether there is an association between the level of DNA methylation and flowering age. HPLC analysis was used to determine the 5mC levels in DNA samples from seedlings. All four 5-azaC-induced early-flowering lines were shown to be hypomethylated. DNA samples from three early-flowering segregant lines, obtained by selection from early-flowering segregants in the F_2 and F_3 generations of out-crosses, were also found to hypomethylated; DNA from the progeny of plants grown in the first generation after treatment (A1) demonstrated that 5-azaC treatments induce heritable demethylation of flax DNA. Finally, the progeny of F_2 and F_3 plants were used to assess the methylation levels in segregating generations, and to examine the relationship between cytosine methylation level and flowering age.

Materials and methods

Ten plant lines were examined: three inbred "normal" lines, which flower at the usual time (L, S, and R); four inbred early-flowering lines (LE1, LE2, RE1, and RE2′) induced by 5-azaC; and three early-flowering segregant lines (LE1s, LE2s, and RE1s). L and S are Durrant's large and small genotrophs derived from the fibre cultivar Stormont Cirrus (Durrant 1971). The normal line, R (Royal), is an oilseed cultivar. LC and RC are lines from untreated (control) plants of L and R that were grown in the 5-azaC treatment experiments that gave rise to the early-flowering lines. The 5-azaC induction and derivation of LE1, LE2, and RE1, from L and R, are described elsewhere (Fieldes 1994; Fieldes and Amyot 1999a). RE2′ was recently re-established from the same source as the original RE2 line that reverted (Fieldes and Amyot 1999a). The seedlings of the early-flowering lines used for DNA extraction in the initial experiment were nine (LE1 and LE2), seven (RE1), or five (RE2′) generations beyond the 5-azaC treatment generation.

The segregant lines, LE1s and LE2s, were F_4 progeny from two out-crosses, LC × LE1 and LE2 × LC. In each case, the F_4 seedlings came from a single F_3 plant that was a member of an F_3 progeny group. The F_2 parent of the F_3 group had flowered earlier than normal, but not necessarily as early as the corresponding early-flowering

line. The F_3 progeny groups for LE1s and LE2s were phenotypically uniform and early flowering. In contrast, variability among the F_3 progeny of the corresponding RE1 × RC out-cross indicated that RE1s was still heterozygous. For RE1s, the F_4 seedlings used for DNA extraction were progeny of the earliest-flowering F_3 plant (flowering age, day 42; height, 68.2 cm). F_4 progeny, grown to maturity, indicated that this F_3 plant was also heterozygous.

Based on information obtained for the three segregant lines, more detailed analyses were done for the LE1, LE2, and RE1 lines. In the first approach, DNA samples from groups of A2 (second generation after treatment) seedlings were examined using seed from five A1 siblings for each of LE1 and RE1, and of the single viable sibling of LE2. Progeny groups from the actual A1 plant that had produced RE1 were also examined. (The corresponding seed stocks for the A1 plants that gave LE1 and LE2 had been depleted.) In the second approach, the DNA samples examined came from groups of F_3 and F_4 seedlings that were the progeny of F_2 and F_3 plants from reciprocal out-crosses between LC and LE1 (made in 1996) and between LC and LE2 (made in 1995). The genetic analyses of data from large populations of these out-crosses had substantiated the three locus genetic model described previously for LE1 (Fieldes and Amyot 1999a) and indicated that a similar, although not quite identical, model can also be applied to LE2 (unpublished data). For RE1, the DNA samples used came from groups of F_3 and F_5 seedlings, that were progeny of F_2 and F_4 plants from an RE1 × RC out-cross made in 1992 (Amyot 1997; Fieldes and Amyot 1999a).

All plants were grown in vermiculite supplied with constant volumes of inorganic nutrient solutions starting 7 days after sowing (Fieldes 1994). Phenotypic data were from the greenhouse-grown plants that provided the seeds used for the DNA studies and from the groups of greenhouse-grown plants that were their progeny. The plants used for DNA extraction were grown in a growth chamber with a dark/light cycle of 8/16 h, at 18/25°C, supplied by cool white fluorescent tubes with a light intensity of 225 μmol m^{-2} s^{-1}, at pot level. DNA was extracted from the terminal leaf clusters of 14-day-old seedlings, or from the cotyledons and immature shoot buds of 4- or 5-day-old seedlings. Usually, 10–12 seedlings were used per extract for 14-day-old seedlings, and 7–10 seedlings were used for the younger seedlings. The DNA extracts were prepared using DNeasy plant mini-kits (Qiagen) with the following modifications: (1) 100–180 mg, fresh weight, of tissue was used per extract; (2) in the final step of the protocol, the DNA was eluted from the DNeasy membrane using 100 μl of 10 mM Tris prepared in sterile water and filtered through a 0.2-μm sterile filter; and (3) the 5-min incubation in this buffer was done at 65°C. A second elution from the membrane gave a final volume of 200 μl per sample. Samples were stored at −20°C.

Each sample was acidified prior to hydrolysis by adding 1.75 μl of 1.0 N HCl. The DNA was degraded to nucleosides, as described elsewhere (Matassi et al. 1992). For hydrolysis to nucleotides: (1) each sample was boiled for 2 min and rapidly transferred to an ice bath, (2) 10 μl of filtered 10 mM $ZnSO_4$and 20 μl (2 U phosphodiesterase activity) nuclease P_1 [Sigma (St. Louis, Mo., USA) N-8630, or US Biological (Swampscott, Mass., USA) N7000] prepared in filtered 30 mM sodium acetate (pH 5.4) were added, and (3) the reaction mixture was incubated for 17 h at 37°C. For dephosphorylation to nucleosides: (1) 28 μl of filtered 0.67 M Tris buffer, pH 8.3, warmed to 37°C, and 12 μl (2 U) of bovine Type VII-S alkaline phosphatase (Sigma P-5521) in 2.5 M ammonium sulphate were added, and (2) the reaction mixture was incubated for an additional 2–3 h at 37°C. Immediately prior to HPLC analysis, the samples were centrifuged at 7,500 *g* for 30 min at room temperature, and the supernatants were collected. Reference samples of 4 μg/200 μl calf thymus DNA (Sigma D-1501) were hydrolysed, processed, and analysed at the same time as the plant DNA samples.

A Varian (Mississauga, Ont., Canada) ProStar Analytical HPLC system with a Timberline 101 column heater was used. The column (150×4.6-mm Supelcosil C-18S, with LC-18C Supelguard) was held at 30°C. The elution protocol, modified slightly from that described elsewhere (Matassi et al. 1992), used mixtures of methanol and 50 mM KH_2PO_4, pH 4.0. The system was programmed to: (1) hold at methanol/KH_2PO_4 [2.5/97.5 (v/v)] for 7 min, followed by (2) a 9-min, linear gradient to methanol/KH_2PO_4 [20/80 (v/v)], and then to (3) hold at methanol/KH_2PO_4 [20/80 (v/v)] for 5 min. The flow rate was 1 ml min^{-1}. Two 50-μl aliquots of each sample were analysed. The chromatography was monitored at 260 nm except for a 2-min period, spanning the retention time for 5mC deoxyriboside, when 280 nm was used. Purified nucleosides were used to validate the identities of the peaks. A_{260} values for the five deoxyribonucleosides (dC 6150, 5mC 4600, dG 11300, dT 8750, and dA 14100) and the four ribonucleosides (C 6400, U 9950, G 11750, and A 14300), as well as the A_{280} value of 9,300 for 5mC (Dawson et al. 1969), were used to standardise the peak areas as concentrations (micromolar). Data from the two aliquots were averaged prior to analysis.

Data were analysed by analysis of variance (ANOVA) using orthogonal comparisons to examine the differences among the plant lines (Sokal and Rohlf 1981) and arcsine transformations for percentages. Two complete sets of samples (one sample per plant line) were prepared from the plants grown in each experiment (the plantings for the two sets were offset by a day to facilitate sampling and DNA extraction). At each step of the procedure, the samples (plant lines) in each set (replicate) were processed together, and the two replicates were usually processed on consecutive days. In the two-way ANOVA for the initial experiment using 14-day-old plants the planned, orthogonal comparisons compared (1) the means for normal (LC, S, RC) and early-flowering (LE1, LE2, RE1) lines, and

(2) the mean for early-flowering segregant lines (LE1s, LE2s and RE1s) to the mid-point between normal and early-flowering lines. They also examined (3) the two orthogonal comparisons among the normal lines; (4) the three comparisons among the early-flowering lines, LE1, LE2, RE1 and RE2′; and (5) the two comparisons among the early-flowering segregant lines. The sets of comparisons in (3), (4), or (5) are pooled in Table 2. When comparison (2) was significant, it was interpreted using a non-orthogonal comparison, which compared (6) the means for early-flowering and early-flowering segregant lines.

The data for 4-day-old plants were from duplicate samples for each of four pairs of normal and early-flowering lines, grown in two experiments, one for L lines and another for R lines. In the one-way ANOVAs, four of the seven orthogonal comparisons, (1)–(4), compared the normal and early-flowering lines within each pair. The remaining three comparisons examined (5) the differences among the pairs of normal and early-flowering lines, that is, the difference between LC-LE2 and LC-LE1, the difference between RC-RE1 and RC-RE2, and the difference between the two LC–LE differences and the two RC–RE differences. These three comparisons are shown as pooled values in Table 3. When the comparisons in (5) were not significant, a non-orthogonal comparison (6) was used to summarise the difference between normal and early-flowering lines.

The designs for the ten subsequent experiments were similar. In each, two complete sets of DNA samples were obtained from groups of 5-day-old seedlings. In total, 178 DNA extracts were prepared from 178 groups of seedlings that were the progeny of 89 individual plants. Ten extracts were lost or unreliable and did not provide data. Missing data were fitted, and the degrees of freedom in the ANOVAs were adjusted accordingly (Sokal and Rolf 1981). Four of the ten experiments examined DNA samples from the A1 plants following treatment. Half of the A1 plants came from untreated A0 plants (controls), the others were from 5-azaC-treated A0 plants and were siblings of the A1 plants that had produced the early-flowering lines; each ANOVA compared the mean for control plants to the mean for the siblings of the early-flowering line. Six experiments examined DNA from a normal line and an early-flowering line (parental lines), and from the progeny of seven to ten plants in an out-cross generation. In each ANOVA, orthogonal comparisons compared: (1) means for the two parents lines, (2) means for the reciprocal out-crosses (C × E vs E × C), and (3) the mean for the hybrids (C × E and E × C) to the mid-point between the parents. Scatter plots were used to compare the mean methylation levels for the samples, in an experiment, to the phenotypes of the plants that produced the progeny groups. In some cases, the methylation levels were also compared to the mean phenotypes for progeny groups, from the same plants, that had been grown to maturity.

Results

Comments on the protocol

The final extraction buffer supplied with the DNeasy mini-kits is not appropriate if the extracted DNA is to be used directly for nucleoside analysis, because it contains EDTA and has a high pH. EDTA interferes with the nuclease hydrolysis, and the high pH, which is needed to optimise the amount of DNA recovered, is outside the optimal range for this hydrolytic reaction. This problem can be circumvented by using 10 mM Tris in the final extraction step and acidifying the DNA extracts prior to hydrolysis. With this modification, an average of 4–6 μg of DNA was routinely recovered from 100 mg to 150 mg fresh weight of the tissues used, and the DNA concentration (4–6 μg DNA/200 μl sample) was suitable for HPLC analysis.

Traces of ribonucleosides were detected in all samples (including the calf thymus samples) and, except for the peaks for cytosine deoxyriboside (dC) and uridine, the chromatographic peaks for deoxyribonucleosides and ribonucleosides were well separated (Table 1). Others have also encountered this difficulty, and an elution protocol that overcomes it has been reported (Jaligot et al. 2000). In our work, at pH 4.0, the presence of uridine could be monitored as a shoulder on the trailing edge of the peak for dC. To avoid problems related to the presence of U, we used the data for dG to estimate the levels of cytosine methylation and for all other estimates that relied on the total concentration of dC, except for the G/C ratio. The G + C contents, which

Table 1 Summary of retention times and nucleoside concentrations in the flax DNA extracts, using the data from the 14-day-old plants. Means ($n = 20$) and standard errors of the means (± SE) for the retention times (minutes) and concentrations (micromolar) of the ribonucleosides and the deoxyribonucleosides are given. Comparable values are also given for the mean ($n = 4$) concentration of the nucleosides (*d-* prefix indicates deoxyriboside) in 4 μg/200-μl samples of calf thymus DNA

Nucleosides	Retention time (min)	Concentration (μM)	
		Flax samples	Calf thymus
C	3.72 ± 0.005	1.1 ± 0.09[b]	0.3
dC	4.89 ± 0.003	14.5 ± 1.24	17.9
U[a]	5.27 ± 0.006	0.8 ± 0.10	
5-Methylcytosine (5mC)	9.33 ± 0.004	2.1 ± 0.15	1.0
G	11.48 ± 0.004	3.5 ± 0.24	0.4
dG	12.81 ± 0.003	17.0 ± 1.33	19.4
dT	13.96 ± 0.003	25.3 ± 2.00	22.4
A	15.25 ± 0.002	1.6 ± 0.10	0.1
dA	15.80 ± 0.002	26.3 ± 2.16	23.2

[a]Values based on the 11 (of 20) flax samples for which the chromatographic peak for U was resolved. The peak for U was not resolved in any of the calf thymus samples

[b]The SEs for the deoxyribonucleoside concentrations reflect the variability, among the 20 samples, in the amount of DNA extracted

were consistently 40% and 46%, for flax and calf thymus DNA, respectively, were in good agreement with values quoted elsewhere (Vanyushin and Belozerskii 1959; Sober 1970). Under the chromatographic conditions used, the monophosphate forms of the deoxyribonucleosides would elute at 2.9, 5.4, 5.5, and 8.5 min (for dCMP, dTMP, dGMP, and dAMP, respectively). Absence of these peaks indicated that the dephosphorylation step was complete. Similarly, the absence of peaks with retention times higher than 16 min indicated that the initial hydrolysis was complete.

Phenotypic differences among the plant lines

The significant differences in phenotype that distinguish the early-flowering lines from the normal lines include early flowering (Fig. 1a), reduced main stem height at maturity (Fig. 1b), and the production of fewer leaves on the main stem prior to flowering (Fig. 1c). The phenotype of LE1 is usually slightly less extreme than the phenotype of LE2 (Fig. 1). The normal (C) lines (L, S, and R) have similar flowering ages and leaf numbers at maturity, but differ in height. There were no significant differences in flowering age between the early-flowering segregant (Es) lines and their corresponding early-flowering (E) lines (Fig. 1a), but, compared to the early flowering lines, the segregant lines tended to be slightly taller and have a small number of additional leaves (Fig. 1b, c). The plants in the F_3 progeny groups of LE1s and LE2s were phenotypically uniform but the F_3 progeny group of RE1s was not uniform; 4 of 19 plants in this group did not flower early.

Hypomethylation in 14-day-old seedlings

In DNA from 14-day-old seedlings of the normal lines (LC, S, and RC), 2.6% of the nucleosides and 12.8% of the cytosines were methylated (Table 2). The DNA from 14-day-old seedlings of the early-flowering lines (LE1, LE2, RE1) was significantly hypomethylated, relative to DNA from the normal lines [Table 2, comparison (1)], and the level of cytosine methylation in the recently established early-flowering line (RE2′) was comparable to that of the other early-flowering lines [Table 2, comparison (4)]. Unexpectedly, the level of cytosine methylation in the early-flowering segregant lines (LE1s, LE2s, and RE1s) was significantly lower than the mid-point between the normal and early-flowering lines [Table 2, comparison (2)], and resembled the level in the early-flowering lines [Table 2, comparison (6)]. In addition, although it was noted that LE1 was less hypomethylated than LE2, there were no significant differences in the level of hypomethylation among the early-flowering lines, among the segregant lines, or among the normal lines [Table 2, comparisons (4), (5) and (6), respectively]. Means for the A/T ratio, the G/C ratio, and the G + C content illustrated the uniformity of the data (Table 2). In this, and in the other experiments, there were no significant differences among the plant lines for any DNA characteristics, other than cytosine methylation, except in the data for the G/C ratio where there were occasional anomalies related to effects of the uridine peak (see explanation above).

Hypomethylation in 4-day-old seedlings

In the 14-day-old seedlings, the cytosine methylation level was 6.2% lower than normal in the early-flowering lines, and, contrary to expectation, the early-flowering segregant lines displayed a similar (6.3%) reduction (Table 2). One possibility was that this unexpected result reflected a developmental effect and that the early-flowering lines and segregant lines display the same level of hypomethylation, because they all have accelerated developmental programmes. DNA from 3- to 7-day-old seedlings was used to examine the hypothesis that the difference in cytosine methylation levels between the early-flowering and normal lines would be smaller, or absent, in younger, less-mature plants. As illustrated by data from 4-day-old seedlings (Table 3), the results did not support the hypothesis. At all ages, the reduction in methylation level in the early-flowering lines was as great, or greater, than the reduction seen in the 14-day-old seedlings. Nevertheless, cytosine methylation increased in all lines during this period of seedling growth. The possibility that the difference in methylation level in the 4-day-old seedlings reflects delayed development in the early-flowering lines was therefore considered. Although germination is slightly delayed in LE2 and various weight and size differences have demonstrated that the early-flowering lines are generally smaller-than-normal, shoot elongation begins at the same time (day 4) in all lines (Fieldes and Amyot 1999b; Fieldes and Harvey 2004). That is, lower-than-normal tissue weights are characteristic of most of the early-flowering lines and do not necessarily reflect delayed development. Nevertheless, the weights of the tissues sampled for DNA analysis were used to normalise the methylation data so that any potential effects of differences in seedling development were removed. As the average weight of the tissues sampled (milligrams per 10 seedlings) increased in the 3- to 7-day-old seedlings, there was a linear increase in the methylation level, over the range from 100 mg to 220 mg for L lines, and from 100 mg to 240 mg for R lines. The rate of increase, 0.02% mg^{-1}, was the same in all lines, but slightly (not significantly) higher in the normal lines. Normalising the methylation data for the 4-day-old seedlings to adjust for differences in the weight per 10 seedlings among the groups of seedlings did not alter the interpretation of the results (Table 3). In the early-flowering lines, the level of hypomethylation in the 4-day-old seedlings was as great or greater (9.7%, raw data; 9.2%, adjusted data) than the level in 14-day-old seedlings (6.2%).

Table 2 Composition of DNA from 14-day-old plants of the normal (*N*), early-flowering (*E*) and early-flowering segregant (*Es*) lines. Means ($n=2$) for the average nucleoside concentration, the A/T and G/C ratios, the G+C content (percentage), and the 5-methylcytosine content (*5mC content*) relative to the total deoxyribonucleoside content (*%Total*) and relative to the G content (*%C*) are given

Line	Type	Concentration (μM)	A/T ratio	G/C ratio	G+C content	5mC content	
						%Total	%C
LC	N	17.4	1.02	1.06	40.1	2.62	13.09
LE1	E	18.7	1.02	1.06	40.1	2.47	12.35
LE1s	Es	12.4	1.03	1.08	39.9	2.46	12.33
LE2	E	20.0	1.03	1.04	40.0	2.41	12.02
LE2s	Es	26.2	1.06	1.03	39.6	2.43	12.29
S	N	29.9	1.07	1.00	39.3	2.53	12.86
RC	N	26.3	1.04	1.03	39.8	2.50	12.58
RE1	E	20.8	1.04	1.02	39.9	2.39	11.98
RE1s	Es	21.0	1.04	1.02	39.8	2.34	11.77
RE2'	E	20.3	1.03	1.01	39.9	2.35	11.81
Mean ($n=20$)		21.3	1.038	1.035	39.83	2.451	12.31
SE mean[a]		0.98	0.0052	0.0040	0.071	0.0130	0.065
F-values from the analyses of variance[b]							
Comparison							
(1) N vs E	$F_{1/9}$		1.24^{NS}	1.13^{NS}	2.65^{NS}	14.42**	19.48**
(2) Es vs (N and E)	$F_{1/9}$		< 1.0	< 1.0	< 1.0	6.50*	5.84*
(3) Among N lines	$F_{2/9}$		2.27^{NS}	6.05*	3.12^{NS}	2.28^{NS}	1.50^{NS}
(4) Among E lines	$F_{3/9}$		< 1.0	3.25^{NS}	1.62^{NS}	1.55^{NS}	1.27^{NS}
(5) Among Es lines	$F_{2/9}$		< 1.0	7.63*	< 1.0	2.47^{NS}	2.44^{NS}
Non-orthogonal comparison							
(6) E vs Es	$F_{1/9}$		1.05^{NS}	1.24^{NS}	1.54^{NS}	< 1.0	< 1.0

**Significant at $P=0.01$, *significant at $P=0.05$, *NS* not significant at $P=0.05$

[a]SEs (for means of $n=20$) were computed using the error terms from the analyses of the non-transformed data

[b]*F*-values examined differences among the three types of lines and among the lines within each type (see "Materials and methods")

Table 3 Composition of DNA from 4-day-old seedlings of the normal and early-flowering lines. Means ($n=2$) for average nucleoside concentration, A/T and G/C ratios, G+C content (percentage), tissue weight (milligrams) per 10 seedlings, and the percentage of 5mC relative to %Total and %C

Experiment	Line	Concentration (μM)	A/T ratio	G/C ratio	G+C content	Weight (mg/10 seedlings)	5mC content		
							%Total	%C	Adjusted[a]
(a)	LC	20.8	1.03	0.97	39.4	119	2.78	14.08	14.60
	LE1	19.7	1.04	0.97	39.6	117	2.57	12.89	13.45
	LC	22.2	1.05	0.97	39.3	139	2.77	13.99	14.12
	LE2	13.6	1.03	0.96	40.0	120	2.40	12.02	12.53
(b)	RC	15.9	1.03	0.99	39.3	162	2.84	14.45	14.21
	RE1	21.5	1.03	0.97	39.6	191	2.65	13.32	12.50
	RC	19.6	1.03	0.99	38.3	173	2.87	14.50	14.04
	RE2′	18.3	1.03	1.03	39.0	151	2.65	13.21	13.18
Mean ($n=16$)		19.0	1.034	0.983	39.3	146	2.69	13.56	13.58
SE mean[b]		1.02	0.0029	0.0067	0.15	2.3	0.019	0.080	0.11
F-values from the analyses of variance									
Comparison									
(1) LC-LE1	$F_{1/8}$		< 1.0	< 1.0	< 1.0		8.12*	14.34**	6.30*
(2) LC-LE2	$F_{1/8}$		4.90^{NS}	< 1.0	1.38^{NS}		24.69**	41.34**	12.90**
(3) RC-RE1	$F_{1/8}$		< 1.0	< 1.0	< 1.0		6.35*	12.68**	14.74**
(4) RC-RE2′	$F_{1/8}$		< 1.0	1.74^{NS}	1.31^{NS}		8.54**	16.70**	3.60^{NS}
(5) Differences[c]	$F_{3/8}$		1.81^{NS}	< 1.0	< 1.0		1.07^{NS}	1.76^{NS}	< 1.0
Non-orthogonal comparisons									
(6) N vs E	$F_{1/8}$		< 1.0	< 1.0	< 1.0		43.97**	79.77**	35.03**

**Significant at $P=0.01$; *significant at $P=0.05$; *NS* not significant at $P=0.05$

[a]Normalised to 125 mg/10 seedlings for L lines and 170 mg/10 seedlings for R lines

[b]SEs (for means of $n=16$) were computed using the error terms from the analyses of the non-transformed data

[c]See "Materials and methods"

Normalising the data from the other experiments also had little effect

The experimental conditions for the other ten experiments were kept as constant as possible, but some differences in the level of methylation were observed among these experiments (Tables 4, 5). Normalisation of the data from these experiments reduced the variability among experiments but did not eliminate it. At this time, the differences in methylation level between some experiments cannot be entirely explained. Albeit, normalising the data did not alter the interpretation of the results obtained from the raw data for any of the experiments. In all ten experiments, each methylation level was obtained for DNA from a group of progeny and each is, therefore, representative of the average methylation level in the progeny group and of the methylation status in the plant that produced the group.

Methylation levels in the first (A1) generation after the 5-azaC treatments were applied

Average methylation levels for the A2 groups from the A1 siblings of the plants that produced the three early-flowering lines, LE1, LE2 and RE1, were significantly lower than normal (Table 4) and variable (Fig. 2). One of the LE1 siblings that flowered earlier than normal (Fig. 2a) produced an A2 group ($n = 15$) that contained two plants with intermediate flowering ages and had a mean flowering age that was 4 days earlier than normal (Fig. 2b). This sibling also had a low methylation level (Fig. 2a, b). None of the other siblings of LE1 displayed any indication of early flowering in the A2, but 5-azaC-induced height differences were seen in all five of the A1 siblings of LE1 (Fig. 2c) and also in the five A2 progeny groups (not shown). In contrast, all RE1 siblings had normal phenotypes (not shown) and produced A2 groups that were uniform and phenotypically normal (Fig. 2d). The actual A2 group for RE1 was uniformly early flowering (Fieldes and Amyot 1999a) with low methylation level (Fig. 2d). The LE2 sibling had a low level of methylation but displayed no indication of the early flowering in the A2 generation (Table 4).

Methylation levels in segregating generations of out-crosses

The resemblance between the methylation level in each early-flowering segregant line and its corresponding early-flowering line (Table 2) suggested an association between methylation level and flowering age, and that the 5-azaC-induced hypomethylation may cosegregate with loci that control early flowering. Methylation levels in segregating generations of out-crosses were used to examine this possibility. The hypothesis was that methylation level and flowering age assort independently and was based on the assumption that the F_1 hybrids are heterozygous for methylation status, at all of the sites that are hypomethylated in the early-flowering line, and that the hypomethylated sites are randomly distributed. This hypothesis predicts that the methylation levels in F_2 plants will be variable, but that the F_2 population will have an average methylation level mid-way between

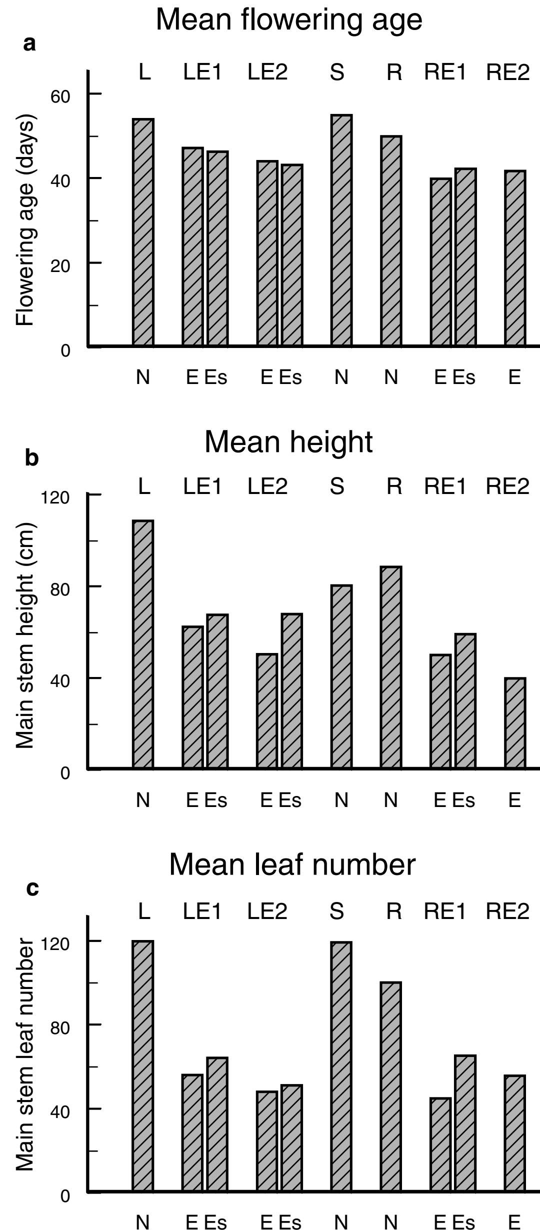

Fig. 1 Phenotypic characteristics. Mean **a** flowering age (days), **b** main stem height (centimetres), and **c** main stem leaf number for the three normal (*N*) lines, LC, S and RC, the four early-flowering (*E*) lines, LE1, LE2, RE1, and RE2′ and the three early-flowering segregant (*Es*) lines, LE1s, LE2s, and RE1s. For pairs of bars, the *left* and *right bars* are for the E lines and the corresponding Es lines, respectively. Means are for $n = 10$ plants, except for LE2s ($n = 18$), RE1s ($n = 19$), and RE2′ ($n = 15$), and the average standard errors (SEs) of the means are 0.83 days for flowering age, 1.6 cm for height, and 1.7 for leaf number

Table 4 Cytosine methylation levels in DNA from groups of 5-day-old seedlings that were progeny of offspring of plants grown in the first generation after treatment (A1) plants. The A1 control plants (LC and RC) came from untreated plants. The five LE1 and RE1 siblings (*LE1sibs* and *RE1sibs*) and the single LE2 sibling (*LE2sib*) were A1 plants from the three azaC-treated plants that gave the LE1, RE1, and LE2 lines. Data are also shown for the A1 plant that produced RE1. Means ($n=10$ or 2) for the percentage of 5mC based on guanine content (*5mC content*), and for the flowering age (days from sowing) and main stem height (centimetres) of the A1 plants that provided the seed. Means for flowering age and stem-height data in the second generation after treatment (*A2*) are for the A2 progeny groups ($n=18$–20) of the A1 plants used

Experiment	Line	A1[a] plants	5mC content	*F*-value[b]	Mean A1 phenotype		Means for A2 groups	
					Flowering	Height	Flowering	Height
(a)	LC	5	13.70	$F_{1/7}=16.0$**	57.8	80.8	49.5	100.8
	LE1sibs	5	12.54		51.0	50.1	49.1	91.2
(a′)	LC	1	14.17	$F_{1/2}=15.4^{NS}$	46	81.7	56.5	82.9
	LE2sib	1	12.87		43	68.6	63.5	82.1
(b)	RC	5[c]	13.31	$F_{1/5}=8.2$*	50.6	54.5	54.2	77.3
	RE1sibs	5	12.57		54.2	67.8	55.4	74.1
(b′)	RC	1	13.85	$F_{1/2}=19.8$*	48	74.5	50.7	56.1
	RE1	1	11.77		33	32.4	38.2	36.2

*Significant at $P=0.01$; *significant at $P=0.05$; *NS* not significant at $P=0.05$

[a]Number of A1 plants used

[b]*F*-values compare the mean 5mC content for siblings of the early-flowering lines and their corresponding controls

[c]The A2 phenotypic data for RC are based on only two groups

Table 5 Cytosine methylation levels in DNA from groups of 5-day-old seedlings that were progeny of F_2 and F_3 (or F_4) plants from out-crosses between early-flowering and normal lines. Means ($n=2$ or 10) for the percentage of 5mC are based on guanine content (*5mC content*), and *F*-values from the analyses provide a general summary of the results

	Line	F_2 plants	5mC content	*F*-values[a]		F_3 plants[b]	5mC content	*F*-values
(a)	LC	1	14.63		(d)	1	14.60	
	LC × LE1	5	14.10	(1) $F_{1/11}=7.95$*		2	13.68	(1) $F_{1/8}=14.03$**
	LE1 × LC	5	13.84	(2) $F_{1/11}=1.01^{NS}$		6	14.53	(2) $F_{1/8}=12.71$**
	LE1	1	13.04	(3) $F_{1/11}<1.0$		1	13.10	(3) $F_{1/8}=4.26^{NS}$
(b)	LC	1	13.76		(e)	1	14.34	
	LC × LE2	6	12.95	(1) $F_{1/10}=39.78$**		6	12.96	(1) $F_{1/8}=84.38$**
	LE2 × LC	4	13.27	(2) $F_{1/10}=4.29^{NS}$		4	13.63	(2) $F_{1/8}=60.51$**
	LE2	1	11.62	(3) $F_{1/10}=4.72^{NS}$		1	12.61	(3) $F_{1/8}=4.40^{NS}$
(c)	RC	1	14.67		(f)	1	15.52	
	RC × RE1	3	14.17	(1) $F_{1/8}=12.43$**		0[b]	–	(1) $F_{1/9}=6.59$*
	RE1 × RC	4	13.54	(2) $F_{1/8}=6.39$*		8	14.76	(2) N/A
	RE1	1	13.04	(3) $F_{1/8}<1.0$		1	14.35	(3) $F_{1/9}<1.0$

*Significant at $P=0.01$; *significant at $P=0.05$; *NS* not significant at $P=0.05$

[a]For *F*-values, from the analyses: comparison (1) examines the difference between the control line and the early flowering line. Comparison (2) examines the reciprocal difference. Comparison (3) compares the mean for the hybrids to the mid-point between the two parents. *N/A* Not applicable

[b]Eight F_4 plants were examined for the out-cross between RC and RE1; all came from a single F_3 plant

the levels in the two parents. Because the relationship between methylation level and flowering age was of interest, the F_2 plants used were chosen to represent a range of phenotypes. In contrast, more than 90% of the plants in the F_2 populations for all three out-crosses had normal flowering ages. Albeit, if methylation level and flowering age assort independently, the under-representation of normal F_2 plants in the samples examined does not change the prediction; variable methylation levels, with a mid-point between the parents, would be expected in the normal plants and in the plants that flowered earlier than normal. The flowering age data for groups of progeny generated means and phenotypic ratios, which classify flowering age as “early” (in or close to the range for the early-flowering parent), “intermediate,” or “normal” (in or close to normal).

Methylation levels in F_2 and F_4 generations of the RE1 out-crosses

A single, slightly shorter-than-normal plant had been found among the F_2 plants of the RE1 × RC cross (Fieldes and Amyot 1999a). The segregant line for RE1 (RE1s, Table 2) had come from the earliest flowering plant in the F_3 progeny of this plant, and a group of F_4 progeny ($n=20$) demonstrated that this F_3 plant was heterozygous. Most of the F_4 plants were intermediate, but five had normal flowering ages. Groups of F_5 progeny ($n=20$) from eight of the intermediate plants were variable; most contained only intermediate and normal plants, but one contained 13 early and 7 intermediate plants. Methylation levels were examined for progeny groups from seven F_2 and eight F_4 plants. As

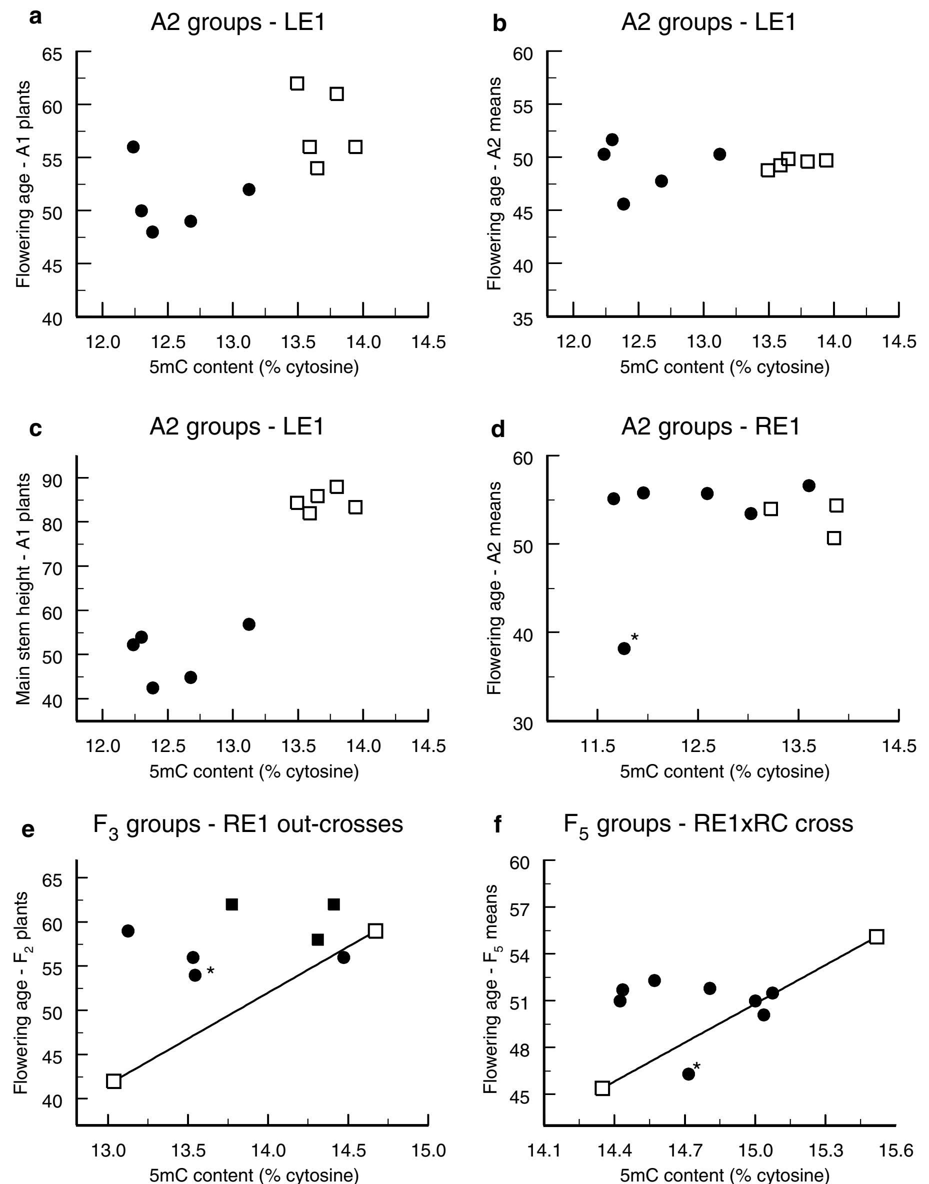

Fig. 2 Flowering age and methylation level in progeny of plants grown in the first generation after treatment (*A1*) generations and in RE1 out-crosses. Comparisons of flowering ages (days from sowing) for individual plants, or mean flowering ages for their progeny groups (generally, $n = 18$–20), and mean ($n = 2$) methylation levels (*5mC content*) obtained from groups of progeny. In **a**–**c**, 5mC levels were for second-generation-after-treatment (*A2*) groups, from siblings of the plant that produced LE1 (*circles*) and LC plants (*open squares*); in **c**, 5mC levels and heights are compared. In **d**, 5mC levels were for A2 groups, from the plant that produced RE1 (*asterisks*) and its siblings (*circles*) and from RC plants (*open squares*). In **e** and **f**, data points for RE1 (*open square, left*) and RC (*open square, right*), connected by a line, show the relationship among the parameters in the parental lines. In **e**, 5mC levels were for F_3 progeny groups from the RC × RE1 (*closed squares*) and RE1 × RC (*closed circles*) crosses, and the *asterisk* indicates the progenitor of the F_5 groups (and the source of RE1s). In **f**, 5mC levels were for F_5 groups of RE1 × RC, and the *asterisk* indicates the group with 13 early:7 intermediate plants. SEs for 5mC content (for means of $n = 2$), computed using the error terms from the analyses of the non-transformed data, averaged 0.34 and ranged from 0.29 to 0.40

expected, the F_2 levels were variable (Fig. 2e), with an average at the mid-point between the two parents [Table 5, (c)]. The level for the progenitor of the eight F_4 plants was lower than the mid-point (Fig. 2e). The average level for F_4 plants was below, but not significantly lower than, the mid-point between the parents [Table 5, (f); Fig. 2f]. The levels were less variable in the F_4 generation than in the F_2, and the level for the F_4 plant that gave a 13:7 ratio was low, but higher than that for RE1 (Fig. 2f).

Methylation levels in the F_2 and F_3 generations of the LE1 and LE2 out-crosses

Information from the out-crosses for LE1 and LE2 supported the hypothesis of independent assortment. Average methylation levels for progeny groups from F_2 and F_3 plants did not depart significantly from the mid-points between the corresponding parents (Table 5; Fig. 3), and reciprocal differences in both F_3 generations ([Table 5, (d), (e)] resulted from biases generated by the relatively small sample size. Nevertheless, in the LE1 out-crosses, the methylation levels for F_2 plants with normal phenotypes were distributed across the expected range, but the levels for the F_2 plants that flowered earlier than normal were less evenly dispersed and mid-range (Fig. 3a). The levels for the plants that flowered earlier than normal in the F_2 of the LE2 out-crosses were also not very dispersed and mid-range (Fig. 3c). There were two exceptions to this pattern. Plants X and Y (Fig. 3a, c) had been classified as early flowering, but both had high methylation levels. In fact, plant X was not particularly early. For both, the F_3 progeny groups ranged from early flowering to normal with approximately 60% in the intermediate range. These distribu-

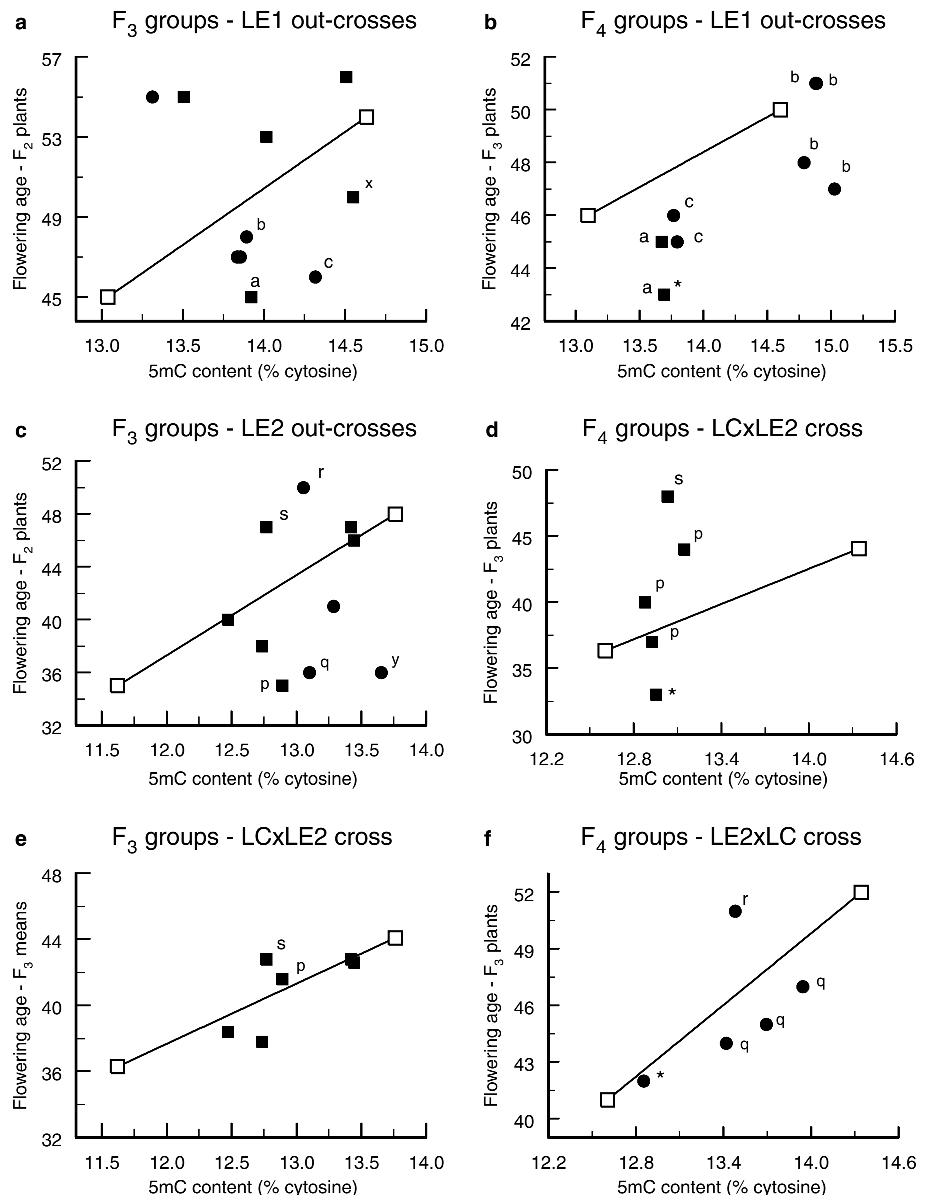

Fig. 3 Flowering age and methylation level in LE1 and LE2 out-crosses. The flowering ages (days from sowing) for individual plants, or mean flowering ages for their progeny groups (usually, $n = 18$–20), and mean ($n = 2$) methylation levels (5mC content) obtained from groups of progeny are compared for: **a** and **b**, the F_2 and F_3 generations of the LE1 out-crosses, and, **c**–**f**, the F_2 and F_3 generations of the LE2 out-crosses. In each plot, the data points for LE1, or LE2, (*open square, left*) and LC (*open square, right*), connected by a *line*, show the relationship between the two parameters in the parental lines. Data points from the C × E (*closed squares*) and E × C (*closed circles*) crosses that are designated by the same *lower case letter* are from the same source. In **b**, the *asterisk* indicates that plant that was the source of LE1s. In **d** and **f**, the *asterisk* indicates the "extra" plants; the extra plant from the LE2 × LC cross was a sibling of LE2s. SEs for 5mC content (for means, $n = 2$), computed using the error terms from the analyses of the non-transformed data, averaged 0.24 and ranged from 0.13 to 0.40

tions were unusual; plants in segregating F_3 groups usually fall into phenotypic clusters. For example, although the F_3 progeny group from F_2 plant A (Fig. 3a) was uniformly early flowering, the group from F_2 plant B (Fig. 3a) segregated, 6 intermediate:12 normal, and the group from F_2 plant C (Fig. 3a) segregated, 14 early:6 normal. In the next generation, methylation levels for progeny groups from four early and four normal F_3 plants clearly delineated the normal plants with high methylation levels, from the early flowering plants with low levels (Fig. 3b).

The LE2 outcrosses displayed similar effects. The group from F_2 plant P (Fig. 3c) segregated two early:eight intermediate:eight normal and that from F_2 plant Q (Fig. 3c) segregated 9 early:11 intermediate. In the next generation, methylation levels were obtained for progeny groups from two normal F_3 plants, from six plants in the F_3 groups produced by plants P and Q, and from two extra F_3 plants that flowered early (one of which was a sibling of the plant that produced LE2s). Two F_3 populations had been grown for the LE2 outcrosses, and, because the mean flowering ages of the two populations differed by 5 days, the information for plants from these populations had to be plotted separately (Fig. 3d,f). The two extra F_3 plants (Fig. 3d, f) had low methylation levels; F_4 groups for both of these plants flowered early, but also contained a few intermediate plants (1/11 for the LC × LE2 plant; 3/16 for the LE2 × LC plant).

The F_2 plants that flowered early generally had methylation levels in the mid-range even if the plant flowered as early as its early-flowering line. Nevertheless, the F_3 progeny groups for these plants often segregated and, as a result, plots using the mean flowering ages for

the F_3 groups sometimes revealed a more-apparent association between flowering age and methylation level than the equivalent plots using the flowering ages of the F_2 plants (e.g., Fig. 3e compared to data for LC × LE2 data in Fig. 3c). The explanation for this lies in the complexity of the genetic system that controls of the early-flowering phenotype (Fieldes and Amyot 1999a) and in a fundamental difference in the dominance relationships of the two parameters. A genomic region that is heterozygous for methylation status should have an intermediate methylation level but, if it is associated with a 5-azaC-induced epi-allele that is dominant, it could produce early flowering.

Discussion

Cytosine methylation levels demonstrated that the total DNA from the 5-azaC-induced early-flowering flax lines was hypomethylated and supported the contention that the early-flowering phenotype is controlled by epigenetic changes resulting from demethylation of the genome (Fieldes and Amyot 1999a). Furthermore, the reduced levels of cytosine methylation in early-flowering lines that were nine generations beyond the original treatment generation demonstrates the persistence of 5-azaC-induced hypomethylation and its stable transmission through both mitosis and meiosis, and parallels the observed transmission of hypomethylation that was induced by 5-azaC in the HRS60 repetitive DNA of tobacco (Vyskot et al. 1995). Three aspects of the initial studies of the methylation levels in flax were unexpected: (1) the overall level of cytosine methylation in the three normal plant lines was low relative to most other angiosperms (Sober 1970); (2) there was very little variation in the level of hypomethylation among the four early-flowering lines; (3) and the level of hypomethylation in the early-flowering segregant lines was similar to that seen in all three early-flowering lines.

Methylation level, genome size, and the impact of chloroplast DNA

The haploid nuclear genome of flax has been estimated as $7{\times}10^8$ nucleotide pairs, based on a value of the 1.52 pg/2C nucleus (Timmis and Ingle 1973), and as being in the range from $6{\times}10^8$ to $8{\times}10^8$ nucleotide pairs, based on estimates of total complexity (Cullis 1981), the proportions of the single-copy and middle-repetitive fractions in flax DNA (Cullis 1981; Cullis et al. 1999), and the assumptions described by Leutwiler et al. (1984). Thus, the flax genome is only approximately five times the size of the *Arabidopsis* genome. As might be expected, the highly repetitive fraction of the flax genome is 30–40% , compared to 10% for *Arabidopsis* and, at 14%, the level of cytosine methylation in the normal flax lines is higher than the 4.6% seen in total DNA from 5-week-old *Arabidopsis* plants (Leutwiler et al. 1984). That is, the level of methylation, in the normal flax lines, was consistent with the general observation that plant DNA methylation levels tend to be proportional to the percentage of highly-repetitive sequences. Nevertheless, 14% was slightly lower than expected, in comparison to *Arabidopsis*, and substantially lower than the 19% reported previously for flax seed (Vanyushin and Belozerskii 1959). It is possible that the cytosine methylation level is high in flax seeds and decreases during germination. In *Silene latifolia*, the DNA methylation levels in various seed and seedling tissues decrease rapidly during germination and early post-germination (Zluvova et al. 2001). Developmental differences in methylation levels have also been reported in tomato (Messeguer et al. 1991) and wheat (Follmann et al. 1990).

Uniform levels of hypomethylation suggest a non-random induction process

The four early-flowering lines were induced when germinating seeds were placed in solutions of 5-azaC for 24 h (Fieldes 1994). The treatments began just before the radicle emerged and ended before any marked elongation of the hypocotyl had occurred. Thus, the demethylation of the apical meristem is likely to have occurred during the mitotic cell divisions at the earliest stages of shoot growth. Until recently, we had assumed that these demethylation events were random and that, in each line, the early-flowering phenotype resulted from the chance demethylation of specific sites that regulate flowering age. We also predicted that the cell lines that gave rise to the early-flowering lines were extensively demethylated because, otherwise, it is difficult to explain the high rate of induction of an early-flowering genotype that involves two or three independent loci (Fieldes and Amyot 1999a). Furthermore, because the two A1 plants that gave LE2 and RE1 were homozygous for these loci (Fieldes 1994; Fieldes and Amyot 1999a), we anticipated more extensive hypomethylation in LE2 and RE1, than in LE1 and RE2′. Contrary to expectation, the level of hypomethylation seen in the early-flowering lines was relatively low and uniform, and this suggests that the initial demethylation events may preferentially affect loci that control flowering time in flax, or that these loci are preferentially protected from remethylation. A similar situation has been reported in the oil palm, where the "mantled" phenotype, which occurs as a somoclonal variant during clonal propagation by somatic embryogenesis, is associated with hypomethylation (Jaligot et al. 2000, 2004). Other examples have been seen in *ddm1*-induced, hypomethylated lines of *Arabidopsis*.The hypermethylated epi-alleles of the *SUP* gene occur frequently in these lines (Jacobsen and Meyerowitz 1997), and it has been suggested that the induction of a relatively high number of late-flowering mutations indicates preferential demethylation of the *FWA* locus, and, possibly, other flowering-time genes (Kakutani et al. 1996; Soppe et al. 2000).

The hypomethylation may not be uniformly distributed throughout the genome

The level of hypomethylation in the early-flowering segregant lines of flax was also unexpected. In *Arabidopsis*, DNA from F_1 plants, obtained by out-crossing *ddm1/ddm1* plants to wild-type plants, displayed intermediate levels of cytosine methylation, and on repeated back-crossing to the wild type, the intermediate level of methylation shifted towards the wild-type level (Vongs et al. 1993; Kakutani et al. 1999). Corresponding genetics were expected when the early-flowering flax lines were out-crossed. The F_1 plants were expected to have intermediate levels of cytosine methylation. Thus, with self-pollination, a random distribution of demethylated sites throughout the genome, recombination, and independent assortment, the progeny groups in subsequent generations were expected to have variable methylation levels; however, the average level of methylation was expected to remain intermediate between the levels in the normal and early-flowering lines. The low hypomethylation seen in all three early-flowering segregant lines initially suggested an association between the early-flowering phenotype and hypomethylation, and the subsequent studies on the methylation levels in the A1 generation and segregating generations of out-crosses provided support for this association.

The methylation levels observed in the A2 progeny groups, which reflect the levels in the corresponding A1 plants, were consistent with the idea that the 5-azaC treatments induced hypomethylation that was transmitted to subsequent generations. Variable methylation levels in the A1 generations indicate that the A0 plants are likely to be heterozygous and/or mosaic for their methylation status, and that the precise sites that contribute to the hypomethylation in the A1 generation differs from plant to plant. Nevertheless, in both of the cases where there was evidence of early flowering in the A1, the corresponding methylation levels were lower than normal. In the data from the segregating generations of out-crosses, there were three trends. First, as in the A1 and A2 generations, it was clear that plants could be hypomethylated without displaying the early-flowering phenotype. Second, plants with early or intermediate flowering ages generally had intermediate methylation levels in the F_2 generation, and produced segregating progeny groups. Finally, associations between flowering age and methylation began to appear in subsequent generations, where the methylation levels of early-flowering plants were lower than in the previous generation. Thus, selection for early-flowering, applied over two generation, seems to lead to lower levels of methylation, and, furthermore, the slow progression in the shift in methylation level and in the re-establishment of the pure breeding early-flowering segregant lines indicates that similar processes of reassortment are required to re-establish the early-flowering phenotype and the hypomethylation. The implication is that the epi-alleles that control the early-flowering phenotype may be adjacent to, or encompassed by, regions of the genome that has been substantially demethylated.

In contrast to site-specific changes in methylation, which can directly affect gene expression, changes in methylation over large regions of the genome are thought to have indirect effects on gene expression through the relationship between DNA methylation and chromatin structure (Li et al. 2002). For example, in maize, the altered pattern of pigmentation in *Pl-Blotched*, compared to *Pl-Rhoades*, which apparently results from lower expression of the *PL* gene and increased methylation of the gene, has been attributed to a difference in the structure of the chromatin domain associated with the gene (Hoekenga et al. 2000). In another example, the hypomethylation associated with *fwa* mutants (epi-alleles), which results in up-regulation of the *FWA* locus in *Arabidopsis*, has been detected in a 5-Mb region that spans the locus (Soppe et al. 2000). In addition, 5-azaC treatments are known to induce concomitant changes in cytosine methylation and chromatin condensation, at the chromosome level, and, although in some instances the chromatin becomes more condensed, the induced demethylation usually results in decondensation (e.g., Glyn et al. 1997; Kovarik et al. 2000). It is interesting, therefore, to speculate that the stability and transmission of the 5-azaC-induced early-flowering phenotype in flax may have as much to do with chromatin remodelling as with methylation status.

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