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Evaluating the Potential of Using 5-Azacytidine as an Epimutagen

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Abstract: A number of early flowering lines were induced when 5-azacytidine was applied to germinating flax (*Linum usitatissimum* L.) seed. The genetics of these lines indicate that the induced changes are epigenetic and probably result from demethylation of the genomic DNA at loci that affect flowering age. Although the growth and development of three stable early flowering lines are altered and the percentage of filled seed was reduced in all three lines compared with controls, measures of seed productivity demonstrated that harvest index was unaffected in two of the lines. In the third, harvest index was lower than normal and both seed set per capsule and seed mass per 100 seed were reduced. Furthermore, six generations after induction this line began to display relatively high levels of polyembryony. The late appearance of this twinning and other aspects related to working with lines induced by 5-azacytidine and using 5-azacytidine as an epimutagen are discussed.

Key words: *Linum usitatissimum*, flax, flowering, epigenetic, polyembryony.

Résumé : On obtient un certain nombre de lignées à floraison hâtive, lorsqu'on applique de la 5-azacytidine sur des graines de lin (*Linum usitatissimum* L.) en germination. La génétiques de ces lignées indique que les changements induits sont épigénétiques et résultent probablement d'une déméthylation de l'ADN génomique à des lieux qui affectent l'âge de floraison. Bien que la croissance et le développement de trois lignées stables à floraison hâtive soient altérés et que le pourcentage de graines pleines soit réduit dans ces trois lignées comparativement aux témoins, les mesures de productivité en graines indiquent que l'indice de récolte n'est pas affecté dans deux lignées, Dans la troisième lignée, l'indice de récolte est plus faible que la normale et le nombre de graines par capsule ainsi que le poids des graines par 100 graines sont réduits. De plus, six générations après l'induction, cette lignée commence à montrer des niveaux relativement élevés de polyembryonie. Les auteurs discutent l'apparition tardive de cette polyembryonie et d'autres aspects reliés à l'utilisation de lignées induites par la 5-azacytidine, ainsi que l'utilisation de la 5-azacytidine comme agent épimutagène.

Mots clés : *Linum usitatissimum*, lin, floraison, épigénétique, polyembryonie.

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Introduction

When the DNA demethylating agent, 5-azacytidine (azaC), is applied to seeds, it will induce heritable changes in the growth and development of a number of diverse crop species, e.g., *Oryza sativa* L. (Sano et al. 1990), *Triticale* (Heslop-Harrison 1990), *Linum usitatissimum* L. (flax) (Fieldes 1994), and *Brassica oleracea* L. (King 1995). The most obvious changes affect height, branching or tillering, and in some cases flowering age. In flax, a number of early flowering lines were induced. Studies have focussed on three of these lines and have emphasized genetic aspects (Fieldes and Amyot 1999) and the quantification of morphological and growth characteristics associated with the early flowering phenotype (Amyot 1997).

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Although azaC may sometimes induce classical mutations, i.e., alterations in the primary sequence of DNA, it usually results in a marked reduction in the level of cytosine methylation in the genomic DNA. These induced changes in methylation level and any associated phenotypic alterations may be transient; however, in those cases where the azaC-induced phenotypic alterations are heritable, the genetics indicate that epimutations are involved (e.g., Fieldes 1994; Janousek 1996). Furthermore, in those studies where the effects of azaC treatments on DNA methylation have been examined, heritable changes in cytosine methylation status have been demonstrated (Sano et al. 1990; Heslop-Harrison 1990; Finnegan et al. 1996). There are a number of examples which illustrate that changes in methylation status in plants and associated changes in gene expression occur during development (Finnegan et al. 1993, 1998). Thus, the heritable phenotypic changes induced by azaC in plants almost certainly result from demethylation and altered gene expression. Furthermore, it is likely that the heritable demethylation of any locus that normally undergoes developmental changes in methylation status will alter development.

The main advantage of using azaC to induce new plant variants may, therefore, lie in the fact that it induces a new type of genomic change, which is epigenetic and also likely

to be ontogenetic. Because epimutations are typically induced at high rates (Jablonka and Lamb 1989), they are easier to obtain than mutations induced by mutagens that alter the primary sequence of the DNA. However, epimutations are also characterized by high rates of reversion (Jablonka and Lamb 1989). Spontaneous remethylation at the loci responsible for the particular azaC-induced phenotype can lead to reversion and the stability of azaC-induced lines is an important consideration. Another potential disadvantage is that cytosine methylation levels in plant genomes can be as high as 30% (Finnegan et al. 1993), and therefore, the demethylation induced by azaC-treatments can be extensive. This means that much of the induced demethylation might occur without causing any phenotypic effect(s) and, alternatively, that many simultaneous, and possibly unrelated, epimutations might be induced within a given line.

Flax is a completely inbreeding annual. It has a cymose growth habit and stem growth effectively terminates at first anthesis. The three early flowering lines that were azaC-induced are all shorter than normal, mostly because they flower early (Amyot 1997). The only differences in main stem growth in the early flowering lines, compared with controls, were slight increases in height during the vegetative phase, which appeared to be associated with ectopic bolting. In contrast, the number of leaves on the main stem at flowering was reduced, in part because the main stems are shorter than normal, but also because the rate of leaf production during vegetative growth was lower than normal. Leaf production and other measures of early vegetative growth have all indicated that the small size of the early flowering lines, compared with controls, is generalized and affects most, if not all, tissues (Amyot 1997). An objective of this study was to determine the relationship between the reduced size of the early flowering lines and seed productivity.

Materials and methods

The plant lines came from azaC treatments of flax seeds conducted in 1990 (Fieldes 1994). In the treatment generation (A0), seeds were placed in water for 12 h, transferred to aqueous treatment solutions for 24 or 72 h, rinsed, and then transplanted and grown under normal conditions. Three levels of azaC treatment (0.5, 1.0, and 1.5 mM) were used in addition to controls (0 mM). Four lines, LE1, LE2, RE1, and RE2, came from two inbred types of flax, the large (L) genotroph (Durrant 1971) and the Royal (R) oilseed genotype. They were derived from four early flowering plants among the first generation (A1) progeny of plants of azaC-treated seeds. Lines of control plants, LC and RC, were derived from untreated seeds (0 mM azaC). The phenotypes seen in LC and RC are referred to as "normal." LE1, LE2, and RE1 continue to display the early flowering phenotype and are referred to collectively as "the early flowering lines." In contrast, the flowering age of RE2 had completely reverted to normal after three progeny generations. Seven progeny generations (A1 to A7) have now been grown for two of the lines, LE1 and LE2, six progeny generations have been examined for RE1, and five for RE2. Data presented are for A5 (LE1 and LE2) and A4 (RE1 and RE2) generation plants, grown in 1996, or from A7 (LE1 and LE2) and A5 (RE1) generation plants, grown in 1998. The populations were grown in late spring and summer in a greenhouse at Wilfrid Laurier University, Waterloo, Ont. The plants were potted in vermiculite and supplied with inorganic nutrient solution. In 1996, the populations were grown in four replicates with five plants of each line per replicate. In 1998, three replicates were grown with six plants per line per replicate.

Height refers to main stem height (cm) from cotyledons to (*i*) the tip of young plants, or to (*ii*) the base of the inflorescence. Flowering age is the number of days from sowing to first anthesis. Leaf number is the number of leaf nodes on the main stem, from the cotyledons to (*i*) the base of the terminal leaf cluster in young plants, or to (*ii*) the base of the inflorescence. Mature plant mass was recorded as dry mass (g) at ambient humidity, more than 2 months after the plants had senesced and dried out, and included the mass of the stem, inflorescence, and tap root; it did not include the mass of the secondary roots, leaves, seeds, and capsules. Estimates of a harvest index were seed mass (for filled seed) as a proportion of total mass, where total mass was the sum of seed mass and plant mass (vegetative biomass). Total seed set per plant was the number of filled seeds per plant plus the number of "unfilled" seeds per plant. Seeds were classified as unfilled when they lacked a mature seed coat and had little or no indication of seed filling. Capsules were classified as "good" when they were similar in size and appearance to the capsules on control plants. The number of good capsules needed to account for the total seed set per plant was estimated. This number was the total seed set per plant divided by seed set per good capsule. Replicate means were used in the data analyses; all data were analysed in standard two-way analyses of variance. Data for L and R were analysed in separate analyses. Percentages were analysed as arcsine transformations.

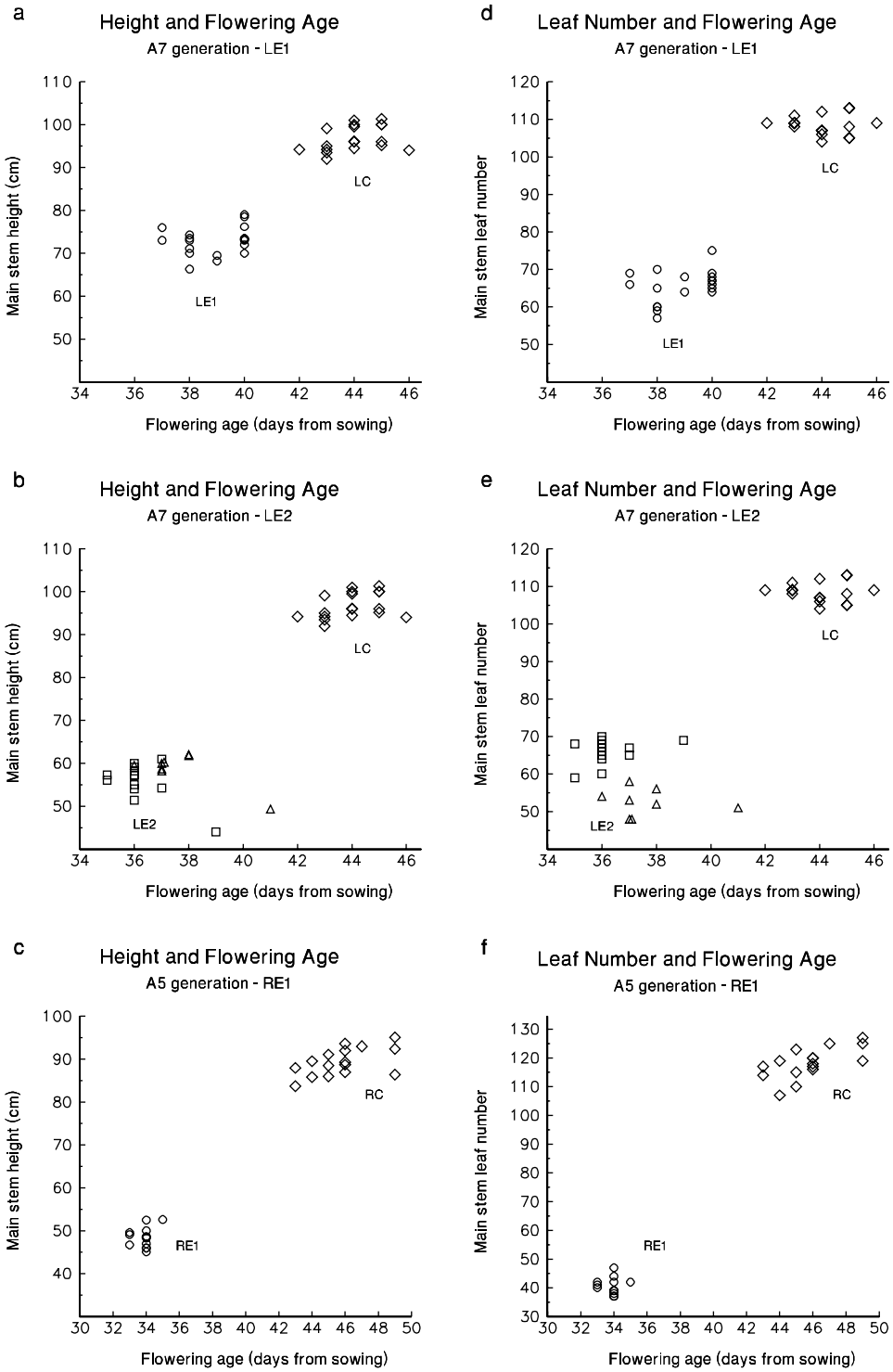
Results

Plant size and seed production

The reduced size of the early flowering lines, compared with their controls, was apparent for a number of vegetative parameters. Main stem height (Figs. 1*a*, 1*b*, and 1*c*), leaf number (Figs. 1*d*, 1*e*, and 1*f*), and plant masses (Table 1) at maturity were reduced in all three lines. One of the interesting observations was that the tissue masses, including plant mass (Table 1), tended to be reduced to a similar extent in both LE1 and LE2, even though LE2 had a more extreme phenotype in terms of height and flowering age (Fig. 1). The reverted line, RE2, did not differ from controls for any of the size or productivity parameters. Seed yields per plant, based on the number of seeds produced or on the mass of fully formed seeds, was significantly reduced in the three early flowering lines, but only one, LE2, had a significantly reduced harvest index (Table 1). In LE2, the drop in seed yield was proportionally greater than the decrease in plant mass because of reductions in both seed number per plant and seed mass per 100 seed. However, the decrease in harvest index in LE2, compared with LE1, appeared to be caused by a slight difference in plant mass and not by seed number or seed mass (Table 1). This difference in plant mass was not significant, but probably reflected the reduced inflorescence in LE1, compared with LE2 (Table 2). Despite the reduction in plant size, inflorescence growth was normal in LE2 and RE1.

The early flowering lines produced a higher than normal percentage of unfilled seeds, and total seed set was lower than normal in LE1 and LE2, with a similar trend in RE1 (Table 2). In LE1, seed number per capsule was normal and the reduced seed set per plant reflected the lower than normal number of capsules per plant (Table 2). In contrast, in LE2, the number of capsules per plant was normal, but the average seed set per capsule (based on all capsules per plant)

Fig. 1. Comparisons of characteristics using data for individual plants of the early flowering lines and their controls. Comparing main stem height at flowering and flowering age in (*a*) the A7 generation of LE1 (circles) and LC (diamonds), (*b*) the A7 generation of LE2 (squares and triangles) and LC (diamonds), and (*c*) the A5 generation of RE1 (circles) and RC (diamonds). Comparing main stem leaf number at flowering and flowering age in (*d*) the A7 generation of LE1 and LC, (*e*) the A7 generation of LE2 and LC, and (*f*) the A5 generation of RE1 and RC. In Figs 1*b* and 1*e*, the LE2 plants came from 13 seeds which each produced a single plant (squares) and four seeds each of which produced two plants (triangles). Some data points are overlapping in some plots, the number of data points plotted were for LC, n = 18; LE1, n = 18; LE2 single plants, n = 13; LE2 twins, n = 8; RC, n = 17; RE1, n = 13.



was lower than normal. Eight normal-looking capsules of each plant were examined. In these (good) capsules, seed set was normal, but the number of unfilled seeds per capsule was higher than normal for both LE1 and LE2 (Table 2). The number of good capsules needed to account for the total seed set per plant (Table 2) illustrated that most capsules of

Table 1. Measures of productivity for control plants of plant types L and R, for early flowering lines LE1, LE2, and RE1, and for the reverted line RE2.

		Line			$F_{1,9}$		
Parameter	Plant type	Control	E1	E2	C–E1	C–E2	E1–E2
Plant mass (g)	L	1.86	0.85	1.1	54.1 **	31.5**	2.3ns
	R	1.23	0.76	0.96	8.6*	2.8ns	1.2ns
Seed no./plant	L	146.9	59.6	63.2	63.8**	58.6**	<1.0
	R	174.9	103.3	139.7	8.2*	2.0ns	1.6ns
Seed mass/plant (g)	L	0.61	0.26	0.24	67.6**	73.0**	<1.0
	R	0.9	0.55	0.7	6.1*	2.0ns	<1.0
Seed mass/100 seed	L	0.42	0.43	0.38	3.1ns	27.8**	37.0**
	R	0.53	0.53	0.5	<1.0	<1.0	<1.0
Estimate of harvest index	L	0.34	0.3	0.22	<1.0	16.0**	7.0*
	R	0.74	0.71	0.73	<1.0	<1.0	<1.0

Note: Total plant mass recorded at ambient humidity after senescence was used as the measure of vegetative biomass in the calculation of estimated harvest index (seed mass/vegetative biomass). Seed number/plant refers to the number of filled seed per plant. Means are $n = 39$ for LC, $n = 40$ for RC, $n = 20$ for LE1, LE2, and RE2, and $n = 19$ for RE1. F values from the analyses of replicate means compare each E1 line with its control (C–E1), each E2 line with its control (C–E2), and the E1 and E2 lines (E1–E2). **, significant at $P < 0.01$; *, significant at $P < 0.05$; ns, not significant at $P = 0.05$.

Table 2. Detailed analysis of seed production on a per plant basis, for control plants of plant types L and R, for plants of early flowering lines LE1, LE2, and RE1, and for the reverted line RE2 and on a per capsule basis for control plants of LC and for plants of LE1 and LE2 using six to eight good capsules per plant.

		Line			$F_{1,9}$		
Parameter	Plant type	Control	E1	E2	C–E1	C–E2	E1–E2
Mean production per plant							
Inflorescence height (cm)	L	38.8	25.6	35.1	53.9**	4.2ns	20.9**
	R	26.1	26.9	24.2	<1.0	<1.0	<1.0
% height inflorescence	L	27.6	23.2	37.5	16.4**	57.8**	102*
	R	26	37.8	26	7.6*	<1.0	6.2*
Total seed set/plant	L	171.8	84	106.1	43.8**	24.5**	2.1ns
	R	182.5	120.8	142	4.9ns	2.1ns	<1.0
% unfilled seed/total seed	L	14.5	29.2	41.5	18.3**	53.1**	6.8*
	R	4	14.3	1.7	18.7**	1.3ns	22.2**
Mean production per capsule							
Number of capsules/plant	L	27	14.2	25.6	23.2**	<1.0	13.7**
Seed set/capsule per plant	L	6.4	5.9	4.3	<1.0	15.7**	16.6**
Seed set/good capsule	L	6.8	6.4	6.1	<1.0	2.1ns	<1.0
Filled seed/good capsule	L	6.3	4.7	4.1	16.3**	27.9**	1.2ns
% unfilled/good capsule	L	7.5	27.5	32.6	470**	684**	15.1**
Capsules for total seed set	L	25	13.1	17.4			

Note: Means are $n = 39$ for LC, $n = 40$ for RC, $n = 20$ for LE1, LE2 and RE2, and $n = 19$ for RE1. F values from the analyses compare each E1 line with its control (C–E1), each E2 line with its control (C–E2), and the E1 and E2 lines (E1–E2). L and R lines were populations of A5 and A4 generation progeny, respectively. **, significant at $P < 0.01$; *, significant at $P < 0.05$; ns, not significant at $P = 0.05$.

LE1 plants had normal levels of seed set, whereas in the smaller capsules of LE2, the seed set was lower than normal.

Twinning and other abnormal development in LE2

The reduced seed set in LE2 was of interest because in the A6 generation some LE2 sublines displayed an increased level of polyembryony (Table 3), as evidenced by the germination of two seedlings from a single seed ("twinning"). Twinning was seen in a small percentage of plants in the early generations of LE2. It was more prevalent in the later generations of some sublines, and even though the percentage of twinning was no higher among the progeny of twinned plants than among those of their nontwinned siblings (Table 4), it appeared to be heritable. The two plants usually developed slightly slower than other LE2 plants, and sometimes, one would develop slower than the other or die. On average, twin plants also flowered slightly later than other LE2 plants but had lower leaf numbers (Fig. 1*e*). In the A7 LE2 group (Figs. 1*b* and 1*e*), 4 of the 18 seeds produced twins. One of the eight twinned plants was slow to develop and flowered after the other LE2 plants on day 41 (Fig. 1*b*). An apparently nontwinned plant also developed slowly and flowered on day 39 (Fig. 1*b*); this plant may have had a twin that did not emerge.

The rate of emergence, which is largely a measure of germination, was also lower than normal in some LE2 progeny groups and a small percentage of the LE2 plants displayed abnormal development during early seedling growth (Ta-

Table 3. Pedigrees for three sublines (*a*, *b*, and *c*) of LE2 showing the percentage of polyembryony (twinning), and other abnormalities, in the A3 to A7 generations.

Generation	Twinning (%)	Details*	
A3	0.0	30 planted: 1 ne	
A4	9.1	(*i*) 12 planted: 1 tw	(*ii*) 10 planted: 1 tw
A5	5.0	(*a*) 10 planted: 1 tw, 3 ne, 1 d	(c) 5 planted: all normal
		(*b*) 5 planted: all normal	
A6	26	(*a*) 20 planted: 10 tw, 1 ab	(*c*) 10 planted: all normal
		(*b*) 20 planted: 3 tw, 1 ab, 1 ne	
A7	23	(*b*) 18 planted: 5 tw, 1 ab	(*c*) 18 planted: 1 tw, 2 ne, 1 ab

Note: In each generation, each subline was derived from a single plant in the previous generation. Sublines *a* and *b* came from two different plants of A3 (*i*) and subline *c* came from a single plant of A3 (*ii*).
*ab, abnormal; d, died; ne, not emerged; tw, twinned.

Table 4. Twinning, and other abnormalities, in the A7 generation of five progeny groups from subline *a*.

	A7 progeny group					
	1	2**	3	4	5**	Totals
No. planted	10	10	10	18	10	58
No. twinned	3	3	2	4	4	16
Not emerged	1	3	0	1	1	6
Abnormal	1	0	0	0	0	1

Note: The progeny groups came from five different A6 plants.
*Groups 2 and 5 each came from an A6 plant that had a twin.

bles 3 and 4). Abnormalities included reduced elongation of the hypocotyl, lack of main stem growth, and sometimes failure of both main stem growth and basal branching.

Discussion

Early flowering can be a desirable characteristic in crop plants, and given the reduced size of the early flowering lines, one of the questions was whether flowering age affected seed productivity or, more specifically, harvest index. The number of seeds set per plant was lower than normal in all three of the stable early flowering lines, and in each line the percentage of unfilled seeds was higher than normal. Any of the morphological effects associated with the early flowering phenotype could have resulted in resource limitations that would explain these effects. Whatever the cause, the low seed number and the increased number of poor-quality seeds did not affect harvest index for LE1 and RE1. Only LE2 had a lower than normal harvest index, and in this line, seed productivity was affected by low seed set and decreased seed mass per 100 seed as well as the reduction in seed filling. The reduced seed set, the abnormal seedling development, and the twinning seen in LE2 may all be caused by azaC-induced genomic changes that affect embryonic development. For example, twinning is likely to reflect epimutations that alter proembryonic development, while reduced seed set may result from epimutations that are lethal during any of the early stages of embryo development. By extrapolation, some of the unfilled seeds may result from epimutations that are lethal in the later stages of seed development.

The stability of the azaC-induced effects has been a consideration throughout the seven generations of work on the early flowering lines. In the early generations it was apparent that, because the effects of azaC on the genome are extensive, the timing of the treatments, treatment concentration, and treatment duration were all critical for induction of stable, or relatively stable, heritable changes. AzaC-induced demethylation requires mitotically active cells (Jones 1984), and treatment needs to coincide with the correct stage of germination. In flax, treatments applied for 24 h, but without the initial 12 h imbibition period, failed to elicit a response, presumably because they were applied too early for cell division in the apical meristem (M.A. Fieldes, unpublished data). Prolonged treatment at high concentrations was deleterious in terms of seed set, whereas lower treatment concentrations induced fewer abnormal A1 progeny. Furthermore, subsequent generations indicated that the heritable effects induced by low treatment concentrations may be less stable (Fieldes 1994). The compromise seems to be a short-duration treatment at a higher concentration; the three early flowering lines were all induced by the 24-h treatment with 1.5 mM azaC, whereas the line that reverted, RE2, came from the 24-h treatment with 0.5 mM azaC.

In the intermediate generations, the possibility of reversion was a major question. At the outset, it was assumed that the azaC-induced phenotypes would be single gene effects (Fieldes 1994). It was only after it had been shown that the early flowering phenotype is controlled by at least two loci (Fieldes and Amyot 1999) that it became apparent that most of the variability in the early generations of LE1 was genetic and not the result of reversion. Because the A1 plant of LE1 was heterozygous for two loci, it produced a range of phenotypic variability that reflected genetic variability. The genetic basis of this variability is illustrated by the fact that it has been possible to select for homozygosity within LE1, e.g., the phenotypically uniform A7 subline (Figs. 1*a* and 1*d*) was derived from an A5 plant that was predicted to be homozygous.

In contrast to LE1, the A1 plant of RE1 was homozygous and RE1 was phenotypically uniform for three generations. Therefore, when variability was seen in the A4 generation of RE1 it clearly demonstrated that spontaneous reversion of the induced effects had occurred during the A3 generation (Fieldes and Amyot 1999). By going back to the A2 generation, new sublines of RE1 have been established that have been uniformly early flowering and stable up to the A5 generation (Figs. 1*c* and 1*f*). However, the potential stability of these sublines does not rule out the possibility that reversion will reoccur.

Thus, in the flax lines, reversion has been less of a problem than anticipated. The variability seen in LE1 has mostly been due to segregation, and in the initially homozygous

line, RE1, the reversion seen in the A4 generation provided a useful source of heterozygous plants for genetic analysis (Fieldes and Amyot 1999). Nevertheless, the full reversion of RE2 and occasional reversion events seen in two of the other lines mean that the possibility of reversion should be an ongoing consideration. There are two practical implications: (*i*) because azaC-induced (epigenetic) effects are more likely to involve more than one locus, the number of plants examined in the A2 generation, or in an F_2 of crosses, should allow for the possibility of complex segregation ratios; and (*ii*) the possibility of reversion means that single-plant seed collection should always be used, even for a line that appears to be homozygous.

The late appearance of twinning in LE2 indicated another potential source of phenotypic instability that is genetic in origin. The inheritance pattern for the twinning phenotype suggests that it is only expressed when a specific genetic background, involving a number of loci, is produced by meiotic recombination. The genetic background required must involve some degree of heterozygosity; otherwise, twinning would be inherited by all progeny of plants that are twins themselves. In addition, it must also involve more than two loci, because the prevalence of twinning can be as low as 5%. The increased prevalence of twinning in later generations was almost certainly the result of inadvertently using progeny from plants with background genotypes that were closer to those needed for the expression of twinning. A similar situation has been seen in *Arabidopsis* where vegetative and floral phenotypes became progressively more abnormal in successive generations of a family (line) that was demethylated; this family did not inherit the antisense construct of a putative methyltransferase domain that was used to induce the genomic demethylation (Finnegan et al. 1996). Both of these situations demonstrate that lines induced by demethylating the DNA can have background genetic variability that is reshuffled each generation by recombination, so that new phenotypes, more extreme phenotypes, or a greater prevalence of an abnormal phenotype can occur in each subsequent progeny generation. In this regard, the epigenetic variability and recombination events seen in these lines are, in effect, no different from the genetic variability and meiotic recombination that are usually seen, for polygenic characteristics, in the inbred progeny generations of outcrosses between genotypes.

The twinning phenotype and other abnormalities in LE2 illustrate that the generalized demethylation induced by azaC may give rise to numerous genomic changes, some of which may only be expressed under specific conditions. They also illustrate that there may be a connection between the stability of the induced changes and the extent of the background genetic variability. With low levels of demethylation there should be less genetic variability but the phenotype of interest might tend to revert. Thus, background variability may be an unavoidable cost associated with line stability. Nevertheless, 5-azaC treatments provide a valuable source of epigenetic variability for studying the plant genome, and of new plant variants for studying plant growth and development.

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