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Full Length Research Paper

An environmentally induced adaptive (?) insertion event in flax

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Genomic changes in flax induced by the environment include the sequences encoding the ribosomal RNAs, many repetitive sequence families and a novel single copy insertion termed LIS-1, comprising a 5.7 kilobase (kb) DNA fragment. The variation in these sequences has been followed in the flax variety Stormont Cirrus under four different growth conditions. LIS-1 was observed to always become both homozygous and inherited in subsequent generations under two of the growth environments. In the third environment, LIS-1 sporadically appeared but was never transmitted to the progeny. Under non-inducing conditions, LIS-1 has been observed neither during growth nor in the next generation. Thus, LIS-1 reached a specific endpoint that depended only on the growth environment. The ribosomal RNA genes also altered during growth under inducing conditions but specific end points that correlated with specific nutrient treatments were not observed. None of these genomic changes were part of the normal developmental processes in flax. In a responsive flax variety that did not produce stable genotrophs, LIS-1 was subsequently lost unless the appropriate inducing conditions were maintained. The results show that the environment can act as both the inducer of targeted genetic variation and as the selective agent for advantageous mutations.

Key words: Flax, adaptive variation, insertion events, environmental induction.

INTRODUCTION

Mutation is the central player in the Darwinian theory of evolution – it is the ultimate source of heritable variation, providing the necessary raw material for natural selection. In general, mutation is assumed to create heritable variation that is random and undirected. Natural selection then discriminates among the initial variants by sorting them according to their adaptive values. Genome structure has been shown to be dynamic and responsive to conditions of stress resulting in mutations that can arise at widely varying frequencies (Cullis, 1987; McDonald, 1983; Walbot and Cullis, 1985). This genomic response does not assume that the mutations induced by any particular challenge are more likely to directly address that challenge simply that the sites at which these mutations occur is not randomly distributed through the genome.

Accumulating evidence for such non-randomness of change includes several documented instances where parts of the genome apparently alter in direct response to the environment, be it the external growth environment or the internal genomic environment (Walbot and Cullis, 1985; Evans et al., 1966; Cullis and Charlton, 1981; McClintock, 1984; Johnston et al., 1996; Wang et al., 2001; Rosenberg, 2001; Wright, 2004; Miller, 2005). In each case DNA variations arise in an apparent direct response to an environmental or genomic cue. However, the variations have generally not been shown to be directed to generate specific adaptive changes, with the possible exception of pathogen induced DNA rearrangements (Kovalchuk et al., 2003). Following this study it has also been shown that exposure to stress can alter the recombination rate in subsequent generations (Molinier et al., 2006) which has been termed a transgenerational memory. Transgenerational transmission of epigenetic information has also been reported in two other systems, namely the RNA-directed chromatin changes that mediate paramutation in maize (Alleman et al., 2006; Chandler and Alleman, 2008) and the role of RNA in paramutation in mice (Rassoulzadegan et al., 2006) and has recently been reviewed (Bond and Finnegan, 2007). These examples of an increased rate of mutation (rather than an epigenetic heritable change) in response to

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to stress still results in a low frequency of mutation. However, the most striking of all the current examples of genomic reorganization in response to stress is the induction of heritable changes in flax. Flax (*Linum usitatissimum*) undergoes heritable changes in phenoltype and genotype in response to the growth environment (Durrant, 1962, 1971; Evans et al., 1966; Cullis, 1973, 1977, 1983, 1986, 2005; Cullis and Charlton, 1981, Cullis and Cleary, 1986; Oh and Cullis, 2003; Cullis, 2005; Chen et al., 2005).

Several stable lines (termed genotrophs) have been derived from the inbred flax variety, Stormont Cirrus (Pl), following growth under specific environmental conditions, including nutrient regimes such as an imbalance of nitrogen or starvation. These regimes consistently lead to significant genomic DNA changes in the progeny of Pl individuals (Evans et al., 1966; Cullis, 1973; Cullis and Charlton, 1981; Cullis, 2005; Chen et al., 2005). The genotrophs have been referred to as stable since, unlike the original Pl line, they breed true when grown in a number of different environments, while in Pl changes associated with environmental factors in these growth environments continued to occur. Characteristics such as plant weight and height at maturity, capsule septa hair number and the mobility of various isozymes of peroxidase and acid phosphatase have been associated with the environmental induction of heritable changes and have been previously described in detail (Durrant, 1962, 1971; Tyson and Fieldes, 1982; Cullis, 1977, 1983, 1986). Most of the genomic differences characterized between the parent line and the genotrophs have involved the repetitive fraction of the genome, including the genes for the ribosomal RNAs (Cullis, 1973, 1983, Cullis and Cleary, 1986) although additional low copy sequence variation has also been identified (Oh and Cullis, 2003; Chen et al., 2005). The progression of the genomic variation during the growth of Pl under inducing conditions has been followed using the total nuclear DNA content (Evans et al., 1966) and the number of ribosomal RNA genes (Cullis and Charlton, 1981) and shown to change during vegetative growth under the inducing conditions. Another variable sequence, LIS-1 was identified as a single copy complex insertion present at a specific genomic site in some of the genotrophs but absent from Pl (Chen et al., 2005). The presence of LIS-1 is the result of a targeted, highly specific, complex insertion event that occurs during the formation of some the genotrophs, and occurs naturally in many flax and linseed varieties. Therefore the presence of LIS-1 can be used as an easily scorable marker that can be directly followed during the growth of plants in various environments to monitor the induction of nuclear DNA changes. The emergence of quantitative PCR has also facilitated the monitoring of the changes in the copy number of the known repetitive fragments during growth under inducing conditions.

The data reported in this paper primarily follow the appearance of LIS-1 in one flax variety in response to four different growth conditions. Three of the four growth environments were ones in which environmentally induced heritable changes have previously been observed (inducing environments). The fourth growth environment was one identified for the maintenance of the ability to respond and so has been assumed to be non-inducing. These changes in genotype and phenotype in flax following growth under inducing conditions have not been subject to rigorous testing for adaptive value. Thus the large and small genotrophs have not been grown in competition under the equivalent inducing conditions to demonstrate that the changes are adaptive to those environments. Adaptive value cannot simply be inferred because all the individuals respond in a specific manner as observed for the appearance of LIS-1. However, the results reported here indicate that the environment can act as both the inducer of targeted genetic variation and subsequently as the selective agent for these mutations. The changes in LIS-1 are consistent with this being a reproducible insertion event that can occur in direct response to the growth environment and, based on the frequency of appearance of this element and its reversibility, which this event may also be under direct selection.

MATERIALS AND METHODS

Plant Materials

The plant material used included the inbred flax (*Linum usitatissimum*) variety Stormont Cirrus (referred to as the plastic line or Pl), the *L. usitatissimum* accession CI1303, the genotroph L1 (Cullis 1977, Oh et al., 2000) and the flax varieties Hollandia and Saskia. The Hollandia seeds were obtained from the North Central Regional Plant Introduction Station of the USDA-ARS, accession number PI 249245, with an origin from Hungary. The seeds of Saskia were obtained from Kweekbedrijf Ropta-Zpc, The Netherlands.

Plant growth

Seeds from the third generation of the selfed progeny of a single plant of the variety Stormont Cirrus were grown under four different conditions. All the plants were grown in 5" pots with compost comprising 7 parts soil, 3 parts peat and 2 parts of granite chippings in a greenhouse. N-treatment - plants were watered with 100 ml per pot of a 1% ammonium sulfate solution every seven days. NPK treatment - plants had 100 ml per pot of a commercial fertilizer solution (Peter's Professional) applied weekly. Water treatment (W) - plants only had tap water applied throughout their growth. The control (C) treatment – the plants were fed at 100 ml of a $1/10^{th}$ strength a commercial fertilizer application (Miracle Grow 15 - 30 - 15% nitrogen - phosphate - potassium) every two weeks to ensure moderate soil fertility (Durrant 1971). These conditions were based on those previously shown to result in heritable stable changes in Stormont Cirrus (Pl) (Durrant, 1958, 1962; Cullis, 1981), or not to result in any changes in nuclear DNA or phenotype (Durrant, 1958, 1962; Evans et al., 1968). One treatment (NPK) has previously been shown to result in a large genotroph, in which the insertion was not present (Chen et al., 2005). The other two growing conditions (N and water) have both been shown to result in small genotrophs with the appearance of LIS-1 in the progeny (Cullis and Charlton, 1981; Chen et al., 2005). The final conditions (C) were

Table 1. Primers used for PCR amplifications. All the primers are written in the 5' to 3' direction.

Primer name	Primer sequence	Tm (°C) of primer using Primer 3
2	GGGTTTCAGAACTGTAACGAA	56.87
3'	GAGGATGGAAGATGAAGAAGG	57.78
Pl9	GCTTGGATTTAGACTTGGCAAC	60.14
18	CATAAATTCAGTCCTATCGAC	59.50
19'	TGTAACAGCTCGGATCTAGGC	59.49
5S coding qPCR Left	GCGATCATACCAGCACTAATGCAC	64.96
5S coding qPCR Right	GCAACACGAGGACTTCCCAAG	64.28
pBG13 spacer qPCR Left	CGACGTTATGGGCACGCTTA	59.70
pBG13 spacer qPCR Right	TACATTTTGCCCCGTTCCTG	63.01
18SF	GGGGGCATTCGTATTTCATA	59.62
18SR	CCCCAACTTTCGTTCTTGAT	59.03
25SF	CGATGTCGGCTCTTCCTATC	59.80
25SR	AACCTGTCTCACGACGGTCT	59.76
FAD3L	TACCTACGTGGAGGGCTGAC	60.13
FAD3R	AGTGTGGCATTTGAGGGAAG	60.11

known to leave Pl unaltered in the next generation (or at least able to respond in subsequent generations with no evidence of the appearance of LIS-1).

A separate set of 20 seeds of Pl were grown with the water treatment, selfed and seeds from each of the 20 plants subsequently grown under the C treatment. The LIS-1 status of each of the progeny was tested on a bulked sample of leaves after 6 weeks of growth.

DNA isolation

Leaves were sampled starting three weeks after planting for up to nine weeks. Single leaves were ground with a pestle in a 1.5 ml microfuge tube containing 10 µl 0.5 N NaOH per mg of tissue. 5 ml of the homogenate was diluted with 495 µl of 100 mM Tris-HCl (pH 8.0). 1µl of this solution was used in the PCR amplifications. Additionally collections consisting of 5 leaves, from each of the lower (L), middle (M) and top (U) of the plants, were made. A sample of leaves from the later flower branches (including flowers in the sample) was also collected as a second top leaf sample. DNA was extracted from these larger leaf collections using the Qiagen DNeasy kit as per the supplier's instructions.

Polymerase chain reaction (PCR)

PCRs were performed in either a 25 µl or a 50 µl reaction mixture containing either 2.5 U AmpliTaq DNA polymerase (Perking Elmer or Amersham Life Science) or Takara rTaq polymerase (Takara), 4mM $MgCl_2$, 2.5 mM of each of the 4 dNTPs and 10 /mol of each of the primers in a Programmable Thermal controller (MJ Research Inc.). All the basic components for the PCR reactions were supplied with the Taq polymerase including the 10x buffer. The primers were supplied by either Operon or MWG. The PCR programs used were 96°C for 2 minutes, followed by 35 cycles of denaturing DNA at 94°C for 1 minute, annealing primers at 54°C to 65°C for 30 s, and extending DNA chain at 72°C for 1 min followed by a final extension at 72°C for 7 min. The annealing temperature, ranging from 54 - 65°C, had previously been determined for each pair of primers (Chen et al., 2005). The PCR products were analyzed on 2% agarose gels.

Primers (Table 1) used for amplifications included those across the ends of LIS-1 or the uninserted site (Chen et al. 2005), for the 5S gene family represented by pBG13 (Goldsbrough et al., 1981), within the 5S coding region for all 5S genes, within conserved regions of the 18S and 25S ribosomal RNA repeat unit and for FAD3 as a control gene (Clouthier et al., 2008).

Quantitative PCR

Quantitative PCR was performed using the ABI StepOne Real Time PCR System. Briefly, approximately 10 ng of template was mixed with 10 µl of 2× SYBR Green PCR master mix (ABI) and 5 pmol each of the forward and reverse primers in a final volume of 20 µl. The amplification program was one of the standard protocols of the thermocycler – 10 min at 95°C, 15 s at 95°C and 1 min at 60°C for 40 cycles. Following amplification a thermal denaturing step was run to generate the dissociation curves to verify amplification specificity. All reactions were performed in triplicate, and the sizes of the PCR products were validated by electrophoresis on a 2% agarose gel. Normalization of the reactions was to a low copy flax sequence, the fatty acid desaturase (FAD3) gene. The primers used would amplify from both the FAD3A and FAD3B genes in flax resulting in a 2 copies per haploid genome control. The plasmid pBG35 (Goldsbrough and Cullis 1981) was used at various dilutions representing 1, 5, 50, 500 and 5,000 copies per flax haploid genome as specific copy number standards to confirm this copy number value for the FAD probe as well as the copy numbers for the 25S and 18S ribosomal RNA genes and 5S ribosomal RNA genes. All QPCR experiments included duplicate samples of the FAD amplification as a control. The sequence abundance relative to the reference was calculated using the formula where ΔCT = CT sample - CT reference. CT represents the threshold cycle, and the absolute number of copies was calculated as twice this number since the FAD gene was used as the standard. Up to 15 replicates were done for some of the reactions. All data were considered reproducible if the spread of CT was less than 1 cycle within the triplicate samples, and that the replicate experiments using the standard primers with the same template was also lie within this range.

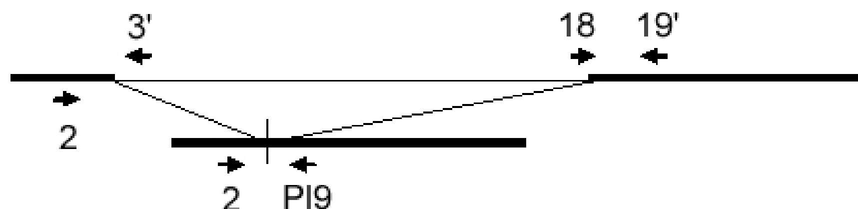

Figure 1. The structure of the locus at which LIS-1 is inserted. The position of the insertion of LIS-1 (thin line) with respect to the target sequence (thick line) is identical all the insertions events that have been sequenced. The position of the primers used for the amplifications are as shown with the arrow indicating the 5' to 3' direction of the primer sequence. The sequences of the primers are given in Table 1. The sequences of Pl9 and 19' overlap in a region where there are differences in the sequence between the inserted and the uninserted sites. The sizes of the fragments amplified are using primers 2 and Pl9 – 417 bp; primers 2 and 3' – 416 bp; primers 18 and19' – 398 bp.

DNA sequencing

DNA sequencing on gel-purified bands was carried out either by Cleveland Genomics (Cleveland, OH, USA) or by MWG, Huntsville, Alabama.

RESULTS

Induction of LIS-1 in Pl

The appearance of the LIS-1 insertion element in the flax variety Stormont Cirrus was followed while the plants were growing under four different growth conditions. Leaves were sampled during growth at weekly intervals and the site-specific primers (Figure 1) were used to determine the presence or absence of the junctions of LIS-1. The complete data sets for all six plants from each of the inducing treatments, as well as a series from a control plant are shown in Table 2. The gel profile of the amplified products for all the individual leaves from one plant from each of the N and water treatments, and two plants from the NPK treatments are shown in Figure 2. Only 3 samples for the N-treatment were obtained due to the stunting of growth under these conditions.

The appearance of LIS-1 was most rapid in the N treated plants (Table 2). In all except one case LIS-1 was homozygous at each tested time. Even though the plants grew more slowly under these conditions it is not apparent that the developmental stage of the first leaf samples is different between the three treatments. Thus under the N treatment, LIS-1 is rapidly induced and becomes homozygous almost immediately.

In contrast, the plants treated with water (Table 2, Figure 2, lanes 17 - 22) demonstrated a slightly different pattern of appearance of LIS-1.

Five of the six lower leaves still had the uninserted site present, although one of these also had evidence for the insertion of LIS-1. In the central portion of the plant all the leaves had evidence of LIS, but two still also had the uninserted site also present. Finally, at flowering, all the plants were homozygous for the presence of LIS-1. In two separate independent experiments with this growth environment where only the progeny have been tested, all the progeny of ten (Cullis and Charlton, 1981) and twenty individual plants were also homozygous for LIS-1.

The results for leaves from plants treated with NPK had the most variable pattern of appearance of LIS-1 (data for two plants are shown in Figure 2, lanes 5 - 16). Plant 1 (lanes 5 - 10) only had the bands characteristic of the insertion in the second and sixth leaves sampled (Figure 2, lanes 6, 10), while the other 4 leaves were homozygous for the original site. The sixth leaf sampled (Figure 2, lane 10) was homozygous for the insertion although none of the progeny from this plant had the insertion present. Plant 2 (lanes 11 - 16) was homozygous for the original site for all the leaves except the second and third leaves sampled (lane 12, 13). Only in four samples was there no evidence for the uninserted site, although in nine cases there was also evidence for some of the presence of LIS-1. Of particular interest was that in a number of cases amplification occurred with the primers for both the uninserted site and both ends of the inserted site.

Additionally, in lanes 12 and 13, only one end of the inserted site was amplified. In no cases was LIS-1 identified in the progeny of these plants. The NPK treatment applied in this experiment has also previously been used in two independent experiments that yielded stable genotrophs (Durrant, 1958, 1962; Cullis and Charlton, 1981). In every one of these cases (a total of 25 independent treated plants), no insertion of LIS-1 was observed in the genotrophs resulting from these experiments.

Collecting the data for all the experiments using the water treatment (36 plants) and the NPK treatment (25 plants) gives 36 progeny inheriting LIS-1 as a homozygous insertion and 25 without LIS-1, with the presence of LIS-1 being completely associated with the particular treatments.

The possibility that LIS-1 always arises in Pl, in contrast to stable varieties, irrespective of the growth conditions was tested by growing Pl under control conditions which

Table 2. The amplification of target and the junction fragments of LIS-1 in the leaves of Pl growing under different conditions. The inducing treatments are denoted by N, P or W.

Low leaves

Primers	Growth conditions Plant number																	
	N 1	N 2	N 3	N 4	N 5	N 6	NP K 1	NP K 2	NP K 3	NP K 4	NP K 5	NP K 6	W 1	W 2	W 3	W 4	W 5	W 6
2 and Pl9	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	-	+	+
2 and 3'	+	+	+	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-
18 and19'	+	+	+	-	+	+	-	-	-	-	-	-	-	+	-	+	-	-

Middle leaves

Primers	Growth conditions																	
	N1	N2	N3	N4	N5	N6	NPK 1	NPK 2	NPK 3	NPK 4	NPK 5	NPK 6	W1	W2	W3	W4	W5	W6
2 and Pl9	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	-	+	-
2 and 3'	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	+	+
18 and 19'	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+

Upper leaves

Primers	Growth conditions																	
	N 1	N 2	N 3	N 4	N 5	N 6	NP K 1	NP K 2	NP K 3	NP K 4	NP K 5	NP K 6	W 1	W 2	W 3	W 4	W 5	W 6
2 and Pl9	-	-	-	-	-	-	+	-	-	+	+	+	-	-	-	-	-	-
2 and 3'	+	+	+	+	+	+	-	-	+	-	+	-	+	+	+	+	+	+
18 and 19'	+	+	+	+	+	+	-	+	+	-	+	-	+	+	+	+	+	+

Plants grown under control conditions

Primers	Leaf number								
	30	40	50	55	60	65	80	165	Flower
2 and Pl9	+	+	+	+	+	+	+	+	+
2 and 3'	-	-	-	-	-	-	-	-	-
18 and 19'	-	-	-	-	-	-	-	-	-

+ indicates the amplification of a band of the expected size (using 2 and Pl9 – 417 bp; 2 and 3' – 416 bp; 18 and19' – 398 bp) _ indicates the absence of a band of the expected size.

have previously been shown not to induce any heritable changes. Under the control environment three plants were sampled and 10 pairs of leaves at various points up the plant were sampled. In addition DNA was isolated from bulked leaves from the top of the plants at flowering. The data from some the leaves from one of the plants grown under the control (C) conditions are shown in Table 2 and Figure 3. No evidence for the insertion of LIS-1 or the loss of the inserted site was observed under these conditions. Therefore, as predicted, LIS-1 insertion appears to be in response to specific growth conditions and more specifically in response to growth conditions that are generally sub-optimal.

The three bands from all the six samples in which bands were amplified with all three primer pairs (such as those shown in lanes 6 and 18, Figure 2) were isolated from the gel, cloned and sequenced. The sequences were identical to those expected for these fragments based on the sequences of LIS-1 (GenBank sequence AJ131994.1) and the target region previously documentted (Genbank AJ131991.1) (Chen et al. 2005). Therefore, all of the nucleotide polymorphisms associated with the insertion of LIS-1 into the target locus was present as soon as LIS-1 itself could be detected. The presence of only one end of LIS-1 in some of the samples (lanes 12, 13, 17 in Figure 2) lends support to the suggestion that LIS-1 is itself the result of a series of events, that most probably occur at the site of insertion, rather than being assembled and then inserted as a complete fragment (Chen, Schneeberger and Cullis, 2005). Since either end could be present as a sole fragment, it would appear that LIS-1 could be assembled in either direction.

Appearance of LIS-1 is not part of normal flax development

The appearance of LIS-1 could be a part of the normal developmental program of the flax genome under particu-

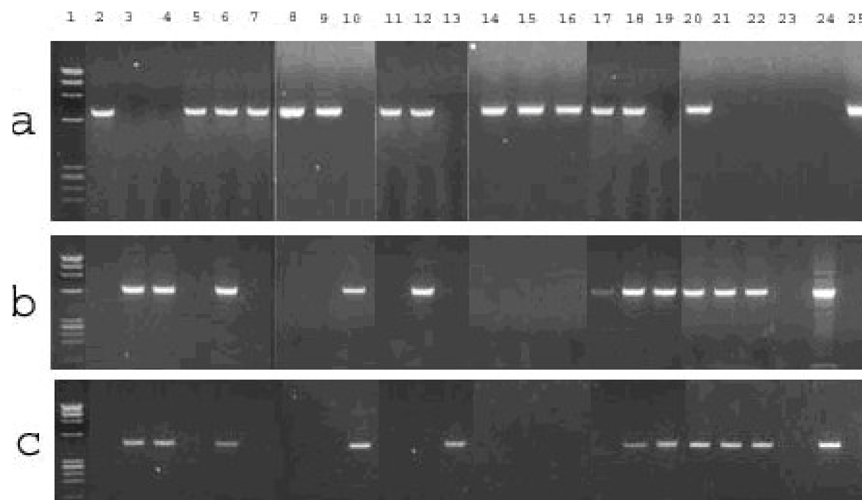

Figure 2. Amplifications of DNAs extracted from individual leaves of plants of Stormont Cirrus grown under three different conditions. The products were separated on a 2% agarose-TBE gel. The uninserted site is shown in Panel a, the two ends of the inserted site are shown in Panels b and c. Panel a. Primers 2 and Pl9; Panel b. Primers 2 and 3'; Panel c. Primers 18 and 19'
1 Boehringer-Manheim Molecular Weight Marker VI
2 – 4 N1-treated plant leaf samples 1 - 3
5 – 10 P1-treated plant leaf samples 1 - 6
11 – 16 P2-treated plant leaf samples 1 - 6
17 – 22 Water-treated plant leaf samples 1 - 6
23 – no DNA only control
24 LH genotroph (containing LIS-1) DNA
25 Pl DNA

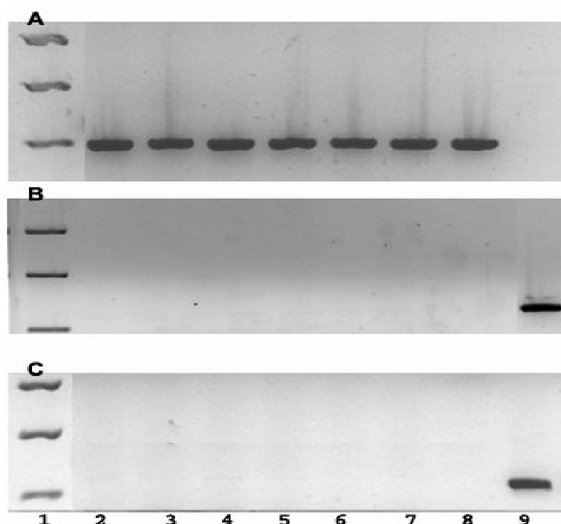

Figure 3. DNAs isolated from leaves of Pl grown under non-inducing conditions and then amplified with the primers (2 and Pl9 - panel A, 2 and 3' – panel B, 2 and 19' - panel c) to detect the presence or absence of LIS-1.
Lane 1 – Bioline molecular weight marker I;
Lanes 2 – 8 leaves from Pl grown in non-inducing environment. Lane 2 leaf 34; Lane 3 leaf 40; Lane 4 leaf 50; lane 5 leaf 55; Lane 6 leaf 60; Lane 7 leaf 80; Lane 9 – DNA from leaf 80 from the S genotroph (which contains LIS-1) grown at the same time under the same non-inducing conditions.

lar growth conditions, rather than just occurring in responsive varieties under specific growth conditions. To test this possibility, three other flax lines, none of which respond to the growth conditions by undergoing heritable genomic variation, were grown under the same three conditions (N, NPK and water). These lines were the variety Saskia, the flax accession CI1303 and the stable genotroph L1. None of these lines have LIS-1 present, nor did it arise in any of the leaves sampled under any of the growth conditions, nor was it present in any of the progeny. The same sampling strategy and number of plants used for these lines was the same as that as used for Pl. Therefore the appearance of LIS-1 is specifically limited to those individuals that respond to the growth environment by modifying their genome in the specific growth environments.

Reversion of the induction of LIS-1

The experiments performed using the variety Stormont Cirrus, which loses the ability to respond in subsequent generations when grown under these specific environment in which the heritable changes are induced, precluded the testing of the reversibility of the induced changes, hence the appearance of stable genotrophs. A second flax variety, Hollandia, was also found to respond when the plants are grown under these inducing conditions and does not appear as readily to lose the ability to subse-

Table 3. Presence of LIS-1 in the lower and upper leaves of Hollandia grown in NPK. The parent plant from which the seeds were taken was homozygous for LIS-1.

DNA from leaves	Primer pair (2 and Pl9)	2 and 3'	18 and 19'
Plant 1 upper	+	-	-
Plant 1 lower	+	+	+
Plant 2 upper	+	-	-
Plant 2 lower	-	+	+
Plant 3 upper	+	+	+
Plant 3 lower	-	+	-
Plant 4 upper	+	+	+
Plant 4 lower	+	+	+

+ Appropriate size fragment amplified (using 2 and Pl9 – 417 bp; 2 and 3' – 416 bp; 18 and19' – 398 bp).
- Appropriate size fragment not amplified.

quently respond. Therefore generations can be grown in alternating environments and the genomic changes monitored. When Hollandia individuals that contain LIS-1 were grown in NPK (which does not induce LIS-1 insertion in subsequent generations) many of the plants (but not all) lose LIS-1 and either became homozygous or heterozygous for LIS-1 (Table 3). Therefore, in unstabilized plants, the inducing environment is necessary to maintain the presence of LIS-1.

Induction of changes in the 5S ribosomal RNA genes

The 5S ribosomal RNA genes form a very highly repeated, diverse set of sequences in flax (Schneeberger and Cullis 1991). One of the families, represented by the clone pBG13 (Goldsbrough and Cullis 1981), contains two length variants. A set of primers from within the spacer region of pBG13 that spanned the variable region within this spacer was designed as well as a pair within the 5S RNA coding region (Table 1). These primers were used in qPCR to determine the relative contributions of the two classes to the overall number of 5S rRNA genes in Pl grown under the various growth conditions. It is clear (Table 4) that the 5S gene copy number is very variable within most of the Pl plants grown under inducing environments, but is not associated with any particular growth environment. It is also clear that the relative numbers of the overall 5S gene number and that for the set identified by the spacer region in pBG13 vary independently. Thus these data again confirm the earlier observations that subsets of the 5S gene family are differenttially affected during growth under inducing conditions (Cullis 1983).

Changes in the number of copies of the repeat unit for the large (25S and 18S) ribosomal RNAs

Again qPCR experiments using primers in conserved regions of the 18S and 25S RNAs (Table 1) were used with the DNA samples extracted from leaves of plants grown under all the environments. Most of the data were derived from using the 18S primer pair, but the same gene copy number was also obtained with the 25S primer pair. The data are shown in Table 4. Variation occurred in the number of large ribosomal RNA genes within Pl plants under all the inducing environments, with a 5-fold variation between the highest and lowest values. As observed with the 5S gene copy number, the changes in the rDNA copy number were not unidirectional and did not occur in all the individuals in a given growth environment. Therefore, although it is clear that this fraction of the genome is modulated in response to the growth environment, it does not result in a defined end point correlated with the growth environment.

To determine whether or not the variations in ribosomal gene copy number were a part of the normal flax development, the copy number was determined in both Pl grown in the non inducing environment and the L1 genotroph in various inducing environments. The data are shown in Table 5. For the Pl grown under non-inducing conditions the values are not significantly different, except for the highest and lowest values for pBG13 family spacer region (2970 and 1750, $P < 0.05$). This compares to a variation in copy number for this family under the various inducing treatments of 4100 - 24000. Although L1 is denoted as a stable genotroph there was a single observation of significant variation in the ribosomal RNA gene copy numbers when it was grown in the various inducing environments, (NPK2 upper and lower values of 6600 and 2000 for the 18S rDNA). However, these differences were rarer and much smaller than those observed with Pl under the same conditions.

DISCUSSION

The role of the environment in generating adaptive mutations is still a contentious subject (Cairns et al. 1988; Cairns and Foster 1991; Foster and Cairns 1992; Foster and Trimarchi 1994; Hall 1990; Lenski et al. 1989; Rosenberg 2001; Layton and Foster 2003; Hastings et al. 2004; Miller 2005; Wright 2004; Henikoff, 2005). Some of the instances of adaptive mutation involve either an increase in mutation rate or gene amplification (Cairns et al. 1988; Cairns and Foster 1991; Foster and Cairns 1992; Foster and Trimarchi 1994; Hall 1990; Lenski et al. 1989; Rosenberg 2001; Layton and Foster 2003. Hastings et al. 2004; Miller 2005; Wright 2004). The high frequency programmed rearrangements in the macronuclei of ciliates and the immune system that do occur in a reproducible fashion are excluded from the germline so do not directly affect the genotype of the next generation (Prescott, 1999; Honjo et al. 2004). The results reported here show that in flax a reproducible insertion event can occur in direct response to the growth environment and this event may be directly selectable. The insertion arises

Table 4. Haploid genome copy numbers of the large ribosomal RNA, 5S RNA coding sequence and pBG13 family spacer region in Pl grown in various inducing environments, based on the FAD3 gene copy number of 2 per haploid complement. Inducing treatments denoted by N, P or W. For example N1L refers to the DNA isolated from the lower leaves of plant 1 grown in the N environment. The coding plant number sampled is indicated by the numeral and either the lower (L) or Upper (U) leaves as the origin of the DNA.

DNA source	Copy number		
	18S RNA gene number	5S coding region number	pBG13 family spacer region copy number
N1L	6500	19200	20000
N1U	6500	36400	11600
N2L	9100	9400	6500
N2U	3300	23000	11600
N3L	9100	36000	20000
N3U	9100	19200	24000
NPK2L	2000	13000	10000
NPK2U	4400	9100	4100
NPK3L	6200	13000	18600
NPK3U	4100	14000	18600
NPK4L	6800	23000	13200
NPK4U	11200	5640	10000
NPK5L	7000	39000	5200
NPK5U	4300	9100	10000
W1L	13000	116000	16000
W1U	5800	19000	5800
W2L	10000	19000	10000
W2U	4000	14000	12000
W4L	10000	13200	11600
W4U	9500	9700	18400
W5L	5200	13000	9400
W5U	5400	32000	13200

during the development of the plant and appears in the leaves. In this respect the results here confirm earlier observations concerning changes in both the nuclear DNA amount and the number of ribosomal RNA genes during growth of Stormont cirrus under inducing conditions (Cullis and Charlton, 1981; Evans, Durrant and Rees 1966; Cullis 2005). The timing of the appearance of LIS-1 is dependent on the particular conditions under which the plant is growing. The results for both the N and water treatments indicate that as the plant grows under these conditions the insertion becomes homozygous in all the leaves and inherited by all the progeny. Previous experiments have also shown that the insertion is present in all the progeny following these treatments (Chen et al. 2005). The presence of the insertion in subsequent generations confirms that it had occurred in the meristematic tissue and so had been incorporated into the germline at flowering. This occurrence within the meristem is also consistent with the nuclear DNA content determinations of Pl growing under inducing conditions (Evans et al., 1966). Therefore a possible explanation of the results with the water and N treatments is that all the apical meristematic cells giving rise to leaves and gametes became altered and homozygous for LIS-1, and therefore all leaves and gametes contained LIS-1.

Since whole leaf extracts were used in these experiments it is impossible to determine the reason for the appearance of amplified bands with all three of the primer pairs (Figure 2, lanes 6, 12, 18, 20). Two possibilities are that either some of the cells were heterozygous for the insertion or that the leaf itself was a mosaic, with some cells being homozygous for the insertion and the remainder being homozygous for the uninserted site. If the latter case represented the actual situation, then, once one insertion occurred in a cell, that cell must have become homozygous immediately or very soon thereafter. LIS-1 has always been observed to be homozygous in the generation following its appearance which is consistent with all the observations concerning the induction of the heritable changes in flax where every change has been observed to be homozygous after the first generation (Durrant, 1962; Cullis and Charlton, 1981; Cullis and Cleary, 1986; Cullis et al., 1999; Schneeberger and Cullis, 1991).

Table 5. Haploid genome copy numbers of rDNA and 5S gene families from individual leaves from a PI plant grown under non-inducing conditions, and from the genotroph L1 grown in various inducing environments based on the FAD3 gene copy number of 2 per haploid complement. The inducing treatments are denoted by N, P or W. For example N1L refers to the DNA isolated from the lower leaves of plant 1 grown in the N environment. The coding plant number sampled is indicated by the numeral and either the lower (L) or Upper (U) leaves as the origin of the DNA.

Leaf number of sample	18S RNA Gene Number	5S coding region number	pBG13 family spacer region copy number
34	3200	23200	2480
40	2900	23000	2600
50	3200	19200	2480
55	3560	22000	1900
60	2600	19200	1870
65	3220	28200	2970
80	3660	19000	1750
165	3320	21400	1980
L1 N1 L	5000	14000	
L1 N1 U	4200	14000	
L1 N2 L	7600	7000	
L1 N2 U	6300	11200	
L1 NPK1 L	2500	14600	
L1 NPK1U	2500	14600	
L1NPK2L	2000	8100	
L1 NPK2U	6600	8000	
L1 W1 L	5400	7900	
L1 W1U	2900	8200	
L1 W2 L	5600	15800	
L1 W2 U	5500	13000	

The difference in the timing of the appearance of the insertion in the N and water treatments would then be due to the differences in how LIS-1 is "activated". The results indicate that the activation is earlier and more frequent under growth with high levels of nitrogen than with no added nutrients. A consistent explanation of the results with added NPK is that although the insertion can arise in these plants as part of the overall genomic destabilization in response to this environment, it does so less frequently and is not incorporated into all the meristematic cells. Since the progeny do not inherit LIS-1, it cannot be inserted in those cells giving rise to the germline. These data demonstrate that LIS-1 can arise under all three inducing growth conditions tested here. What is the mechanism by which it appears in all the meristematic cells in two of the treatments and none of the meristematic cells in the third? One possibility is that the presence of the insertion has a phenotypic effect within the meristem that is beneficial (adaptive) under the N and water treatments, but is either deleterious or neutral in the NPK environment. Therefore, the cells in the meristem containing LIS-1 would be selected for in N and water treatments but the reverse selection (or no selecion) would have occurred in the NPK treatments. If the selection occurred in leaf cells rather than in meristematic cells, then there should be no, or at least a variable, transmission to the next generation. Since this is not observed, it is likely that the origin of LIS-1 is in the meristem, and this is also the site of selection. The loss LIS-1 in Hollandia grown under NPK treatment again supports the notion that this region is under some direct selection in various growth environments, positive selection in two of the growth environments and negative in the third, but it is unlikely to be neutral. Whether or not the actual presence of LIS-1 and/or some adjacent region is the adaptive event cannot be determined on the basis of this data.

The use of stable flax varieties grown under inducing conditions demonstrated that the appearance of LIS-1 was not part of the normal developmental program of flax since the appearance of LIS-1 was specifically limited to those individuals that respond to the growth environment by modifying their genome. Additionally, the possibility that LIS-1 always appeared during the growth of plants capable of responding to the growth environment was shown not to be the case since when PI was grown in non-inducing environment, LIS-1 was never observed.

LIS-1 is only one of the labile sites in the flax genome that are affected during the induction of the genotrophs (Cullis and Charlton 1981; Cullis et al. 1999). Therefore the apparent high frequency of appearance of LIS-1 could simply be as a result of this being one of many

events that occurred at very high frequency. However, the data for the 5S and the large ribosomal RNA genes indicates that these labile sites do not act in a consistent fashion. Therefore it is unlikely that the appearance and inheritance of LIS-I is just because it is a high frequency event. The three parameters, nuclear DNA content (Evans et al., 1966), ribosomal RNA gene number (Cullis and Charlton, 1981) and LIS-1 which have been characterized during growth under inducing environments all change during vegetative development and the value transmitted to the next generation is associated with the value at the end of the vegetative growth period (Cullis, 2005). Quantitative PCR using the DNAs isolated during growth will indicate whether or not the appearance of LIS-1 and any of the other quantitative changes in the repetitive fraction of the genome are coordinated, or all occur independently. The preliminary evidence from the ribosomal RNA genes indicates that the changes occur in these sequences independently with changes in the ribosomal RNA gene number occurring throughout the growth under inducing conditions. More information is needed to determine how many of the sites are possibly the subject of positive or negative selection during vegetative growth.

The results reported here demonstrate that the environment can act both as an inducer of variation within a limited subset of the genome and then as the subsequent selective agent among the variants generated to genetically alter the majority, or all, of a population as has been previously proposed (Cullis, 1977). The results have clear evolutionary implications for any organism in which the germline is not set aside very early in development as the this mechanism can give rise to a selectable, coordinated set of mutations under particular environmental stresses that can result sizeable, rapid, adaptive evolutionary responses.

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