Specific DNA Alterations Associated With the Environmental Induction of Heritable Changes in Flax

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ABSTRACT

Several flax varieties have been shown to undergo environmentally induced heritable changes resulting in stable lines termed genotrophs. The most notable of these is the variety Stormont Cirrus, also termed "plastic" or Pl. A number of morphological, biochemical and genetic differences are associated with environmental induction of heritable changes in flax. We have studied 5S rDNA alterations as a model system for understanding environmental induction of heritable changes in flax. This paper reports the isolation of a flax 5S rRNA gene variant which identifies genotroph specific restriction fragment length polymorphisms (RFLPs) in flax. Restriction fragment patterns for several enzymes were observed in both large and small genotrophs which consistently differed from the progenitor, Stormont Cirrus. Identical RFLP profiles for all restriction endonucleases tested were observed in four small genotrophs produced from separate environmental induction experiments. Comparison between Stormont Cirrus and these small genotrophs showed at least six differing bands in addition to several high molecular weight polymorphisms. Genetic data indicate that the polymorphisms were all produced from a repetitive 5S rRNA gene cluster at a single chromosomal locus. Similar, but not identical, polymorphisms are also detected in other flax varieties and *Linum* species suggesting that the induced variation is related to that which occurs naturally. The results are evidence that a specific set of DNA alterations occur in association with the induction of heritable changes in flax. This is the first genetic marker which is altered to an identical state in one type of genotroph. The results are discussed with respect to mechanisms for environmentally induced heritable change in plants.

THE effect of the environment on the phenotype of an organism is largely thought to be physiological, with the genome remaining unchanged. However, several instances have been documented in which the genome does alter in response to the environment (McCLINTOCK 1984; WALBOT and CULLIS 1985). Examples include adaptive evolution in *Escherichia coli*, sporulation in *Bacillus*, mating type switching in yeast, activation of transposable elements, tissue culture induced variation, and gene amplification as well as others (HALL 1988; STRAGIER *et al.* 1989; SPRAGUE, BLAIR, and THORNER 1983; McCLINTOCK 1984; ROTH *et al.* 1989; STARK *et al.* 1989). Each of these systems shares the common feature of DNA rearrangements or mutation in response to an environmental cue. The environmental induction of heritable changes in the inbred flax variety Stormont Cirrus is one well described plant system in which genome alterations occur in response to specific, defined environments. Growth of the progenitor, termed Plastic (Pl), in different fertilizer combinations results in phenotypic and genotypic differences in the first generation which are inherited by the self fertilized progeny in subsequent generations (DURRANT 1962; CULLIS 1977, 1981). While much remains to be learned about the induction process in flax, there are four established aspects. First, Stormont Cirrus is a predominantly self fertilizing plant since anther dehiscence and pollination occur during flower opening. Second, nearly all of the seeds planted grow under the inducing conditions and can contribute to the next generation (DURRANT 1962). Thus, simple selection is not the causative agent for the observed change. Third, all of the self fertilized progeny from all individuals growing in a specific environment were identical, but different from all the progeny of the individuals grown in a different environment. Fourth, the induction has been repeated with Pl, resulting in the appearance of similar phenotypic, biochemical and molecular changes (DURRANT 1962; CULLIS 1977, 1981). Similar induced changes have also been described for other flax varieties as well as inbred lines of the tobacco species *Nicotiana rustica* (EVANS, DURRANT and REES 1966; FIELDS, GAUDREAULT and TYSON 1989; HILL 1965). The phenotypically altered plants, called genotrophs, were first defined by differences in total dry weight and classed as either large or

We dedicate this work to the kind memory of NORMAN ALLDRIDGE whose inspiration, guidance and friendship will be sorely missed by all who knew him.

small types (DURRANT 1962). The genotrophs differ from Pl in genetic, biochemical and morphological characters. Among these are total nuclear DNA content as determined by Feulgen microdensitometry, the number of hairs on false capsule septa, the isozyme band patterns for peroxidase activity, and the copy numbers of rRNA genes, 5S rRNA genes and all highly repetitive DNA fractions except one (EVANS, DURRANT and REES 1966; EVANS 1968; DURRANT and NICHOLS 1970; CULLIS and KOLODYNSKA 1975; FIELDS and TYSON 1972; CULLIS 1976; GOLDSBROUGH, ELLIS, and CULLIS 1981; CULLIS and CLEARY 1986). The 5S rRNA gene copy number can be particularly labile resulting in a 50% reduction in the genotroph L^H compared with Pl (GOLDSBROUGH, ELLIS and CULLIS 1981) (see Table 1).

In an effort to further understand the nature of rapid DNA sequence alterations and their involvement in heritable genomic change, we have conducted a detailed molecular analysis of the 5S rRNA gene family (5S rDNA) in flax. The 5S rDNA accounts for 3% of the flax genome in the variety Stormont Cirrus with 117,000 copies per diploid genome and is dispersed over most of the chromosomal complement unlike all other angiosperms studied to date (GOLDSBROUGH, ELLIS and CULLIS 1981; SCHNEEBERGER, CREISSEN and CULLIS 1989). Five groups of 5S rRNA genes were identified based on homology to a previously described flax 5S rRNA gene clone, pBG13. A large amount of heterogeneity was observed between groups with up to 30% divergence in the sequence of the repeat unit. Group-1 and group-2 5S rRNA genes represent the majority of the 5S rDNA in flax. These two groups differ from each other in the amount of homology they share in the intergenic spacer region. The derived 5S rRNA sequence for group-1 and group-2 is very homologous to that reported for other plant 5S rRNAs (GOLDSBROUGH, ELLIS and LOMONOSSOFF 1982; R. G. SCHNEEBERGER and C. A. CULLIS, unpublished). Three other groups of 5S rRNA genes were characterized which have a low representation in the genome. These groups also showed low homology to the group-1 clone pBG13 (SCHNEEBERGER, CREISSEN and CULLIS 1989; GOLDSBROUGH, ELLIS and LOMONOSSOFF 1982).

This paper describes one group-4 clone, pRS20.7, which identifies a well defined set of restriction fragment length polymorphisms (RFLPs) in flax, between Pl (Stormont Cirrus) and environmentally induced genotrophs. Identical patterns are detected in four, independently induced small genotrophs, suggesting that specific DNA alterations have occurred in association with the induction of the small phenotype. In addition, similar but nonidentical polymorphisms are also detected in other flax varieties and Linum species, although the relationship of these "naturally" occurring polymorphisms and the induced RFLPs is not yet known. The data are consistent with the RFLPs having resulted from DNA rearrangements, including gene amplification, deletion and possibly gene conversion. The results demonstrate that specific DNA alterations are associated with the environmental induction of heritable changes in flax.

MATERIALS AND METHODS

Derivation of flax genotrophs: All genotrophs were induced from the flax variety Stormont Cirrus, termed Pl (plastic). L refers to the large genotroph phenotype. S refers to the small genotroph phenotype. However, L_6 is also a small genotroph which was derived from a large genotroph, L_1 (CULLIS 1977). Genotroph characteristics and induction conditions have been previously described in detail (DURRANT 1962, 1971; CULLIS 1977). All genotrophs have been subsequently maintained by growth under standard greenhouse conditions. L^H and S^h genotrophs were derived from Pl in a separate induction experiment from those described above. The conditions and genotrophs have been described (DURRANT 1971; CULLIS 1977). C3 is a small genotroph obtained from an induction experiment carried out in 1980. The characteristics of C3 have been described (CULLIS 1981). Flax varieties were obtained through the USDA-SEA, North Dakota State University. *Linum grandiflorum caeruleum* was obtained from the Hortis Botanicus, Romania. Genetic crosses were performed according to standard procedures.

DNA isolation, restriction digests, electrophoresis and blotting: Genomic DNA was isolated as described by CULLIS 1976. The DNA used from C3 was from leaves grown from self fertilized seed from the original induced plants. Restriction enzyme digests contained 2–4 μg of DNA and 10 units of enzyme per μg of DNA. Additional enzyme (10 units) was added after 2 hr and digested for a further 2 hr. Enzymes and reaction buffers were purchased from Boehringer Mannheim Biochemical. Digested DNAs were electrophoresed on 0.8% agarose gels run in Tris-acetate buffer (0.04 M Tris-HCl, 0.001 M Na_4EDTA, 0.005 M Na_3acetate, pH 7.9) at 1.5 V/cm for 20 hr. Denaturation of the DNA in the gels was carried out for 30 min in 1.5 M NaCl, 0.5 M NaOH, and then neutralized for 30 min in 3.0 M NaCl, 0.5 M Tris-HCl, pH 6.5. The DNA was then transferred to Zetaprobe filters (Bio-Rad) in 20 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M Na_3citrate) for 16 hr. Post transfer membranes were rinsed in 2 × SSPE buffer (1 × SSPE = 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M Na_2EDTA, pH 7.4).

Copy number reconstructions: Copy number reconstructions were carried out by diluting pRS20.7 plasmid DNA to 10, 50, 100, 200, 500, 1000 and 2000 copies. The samples were digested with *Bam*HI. Digestion was terminated by addition of 1 μl of 0.5 M EDTA and loading buffer after 1 hr and 1 μg of sonicated herring sperm DNA was added as carrier. These samples were electrophoresed on a vertical 1% agarose gel along with 1 μg *Sca*I digested samples of Pl, L^H, S^h and *L. grandiflorum caeruleum*. *Sca*I digests 99% of the pRS20.7 genomic sequence into 353- and 706-bp bands. The gel was subsequently blotted to a nylon membrane and hydridized as above to random labeled 706-bp insert of plasmid pRS20.7. Autoradiography was performed using preflashed Fuji RX X-ray film. The resultant autoradiograms were scanned on a Shimadzu densitometer and the peak areas of standards and genomic samples quantitated. In order to make more accurate determinations of copy

number changes between the genomic samples, the filters were also hybridized with a cloned sequence shown to be a single copy sequence in Pl, L^H, S^h and *L. grandiflorum caeruleum*. (R. G. SCHNEEBERGER and C. A. CULLIS, unpublished data). The autoradiograms were scanned for this sequence hybridization and the copy numbers normalized to the Pl value to correct for any error in genomic DNA loading. Reconstructions of RFLP band copy numbers were carried out essentially as above except the genomic DNA was digested with *Eco*RI.

Probe preparation, labeling and hybridization: The spacer probes of pRS20.7 shown in Figure 2 were isolated from a preparative restriction enzyme digest by electroelution of the appropriate bands from low melting point agarose (SAMBROOK, FRITSCH and MANIATIS 1989). The 209-bp A probe was subsequently blunt end cloned into pBluescript vectors according to conventional procedures (STRATAGENE La Jola, California). Radioactive DNA probes were generated using the random primer method with [α^{32}P]dCTP (Amersham; 3000 Ci/mmol) (FEINBERG and VOGELSTEIN 1984). Filters were prehybridized for 4 hr to overnight at 68° in 2 × SSPE, 0.5% dried milk powder, 1.0% $NaDodSO_4$, 0.05 mg/ml denatured herring sperm DNA (Sigma). Hybridization was carried out with 10^6 cpm/ml at 68°.

Filters were washed 1 hr each in 0.1 × SSC, 0.1% $NaDodSO_4$, at 50° and exposed to Fuji RX film with intensifying screen at −70° unless stated otherwise.

Field inversion gel electrophoresis (FIGE): FIGE was performed using DNASTAR computer software and switching modules (DNASTAR Madison, Wisconsin). Gels were made in 0.5 × TBE buffer (1 × = 90 mM borate, 100 mM Tris-HCl, 1 mM EDTA). Gel temperature was constant at 14°. Beginning forward pulse was 0.3 sec, ending forward pulse was 0.8 sec. Beginning reverse pulse was 0.15 sec, ending reverse pulse was 0.4 sec. Gels were run for 11 hr at 10 V/cm. After electrophoresis the gels were transferred to Zetaprobe membranes as described above. High molecular weight DNA standards were supplied by Bethesda Research Laboratories.

DNA sequencing: Several 706-bp repeats from the λ5S-20 EMBL 4 genomic clone were subcloned into pBluescript vectors (Stratagene) at the *Bam*HI restriction site according to standard proceedures (SAMBROOK, FRITSCH and MANIATIS 1989). Single-strand DNA was prepared for two clones according to the manufacturer's instructions (Stratagene). Positives were sequenced using T7 DNA polymerase and chain terminating dideoxy nucleotides (United States Biochemical). Sequence for the opposite strands was obtained by double strand sequencing.

RESULTS

5S rRNA gene isolation and description: A 706-bp 5S rRNA gene clone, pRS20.7, was isolated from a flax EMBL4 genomic clone, λ5S-20 (SCHNEEBERGER, CREISSEN and CULLIS 1989). This sequence, shown in Figure 1A, was found by hybrid duplex stability to be weakly homologous to the previously described flax 5S rRNA gene clone pBG13 (SCHNEEBERGER, CREISSEN and CULLIS 1989; GOLDSBROUGH, ELLIS and LOMONOSSOFF 1982). Comparison of pRS20.7 and pBG13 DNA sequences shows a high degree of divergence in both the spacer region and the 5S rRNA transcription unit with an overall homology of approximately 75% (R. G. SCHNEEBERGER and C. A. CULLIS, unpublished). Restriction mapping and sequence analysis identified this clone as a tandemly arranged 5S rRNA gene dimer (Figures 1A and 2). Several features of this clone are noteworthy. First, the 706-bp repeat sequence contains two 5S rRNA gene transcription units designated 5S1 and 5S2. The 5S1 and 5S2 transcription units and spacer regions separating the genes are significantly diverged from one another as shown by their restriction maps in Figure 2. Sequence comparisons of both 5S1 and 5S2 transcription units and spacers, show 76% sequence homology (Figure 1B) This type of heterodimer repeat has not been identified in any of the other five groups of flax 5S rRNA genes studied to date. Normally 5S rDNA sequences found in tandem arrays are nearly homogenous with the exception of micro-sequence heterogeneity (LONG and DAWID 1980). This was previously shown in flax for pBG13 (group 1) 5S rRNA gene sequences (GOLDSBROUGH, ELLIS and LOMONOSSOFF 1982). Different copies of the 5S rDNA repeats present in λ5S-20 are very homogeneous with respect to restriction enzyme sites. Two different copies of the λ5S-20 706-bp repeat array (λ5S-20 contains a total of seven repeats) have been sequenced and found to differ in eight positions (Figure 1A). Thus, the dimer repeat would appear to be a unit which is being maintained in a homogeneous tandem array in a concerted fashion. This situation may be structurally similar to that described for the *Xenopus laevis* oocyte type 5S rDNA repeat which contains a pseudogene alternated with an active gene in a tandem array (MILLER *et al.* 1978).

The genomic organization of this 5S rRNA gene family was examined further in Pl (Stormont Cirrus), genotrophs, and other flax varieties and Linum species. The DNA probes used in this study are shown in Figure 2. The 209-bp A spacer probe is specific to the spacer sequence preceding the 5S2 transcription unit. Probe A: starts 16 bp downstream of 5S1 transcription unit and ends 2 bp upstream of the 5S2 transcription unit. Probe B is an 201 bp fragment specific to the spacer sequence preceding 5S1. Probe B: starts at the first bp past 5S2 transcription unit and ends 16 bp upstream of the 5S1 transcription unit. Probe C contains all of probe B in addition to 40 bp of 5S2 transcription unit sequence. Probe C: starts 40 bp upstream of probe B and ends in the same position as probe B. The 706-bp *Bam*HI fragment represents a single repeat from λ5S-20.

Genomic reconstructions hybridized at high stringency (2° below Tm) indicate that the pRS20.7 5S rDNA sequence is present at approximately 500 copies per Pl haploid genome (Table 1; no hybridization to any other group of flax 5S rRNA gene is seen at this stringency; SCHNEEBERGER, CREISSEN and CULLIS

A.

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                    20                  40                  60                  80                  100
      t                         a                                                       g
  1 gttcgataatcctgtggaac cgagaccaagatgtagtaaa aagtaatcgaaacgagtcta aaataaggtaggaagtagag aaagtttattcatgtacata
                              C                                                         G
100 tgtgcatgagaagcagacGA GTGAGGTCATACCAGTAGTA ATTCATCGGATCCCATCATC ACTCTGAAGTTAAACGTACT TGGCCAAGACTAGTACTACG
                           C
200 ATGGGTGACCTTTTGATCCT TCCCCGAGTACACCCCTTtt attttctattttagcagcta tttttaccccaatgtgcaat ggaaacgtttgactatccaa
                           ^                                    *
300 catccgctagtagatgttta gtaatgactacggggaacca tgagttcgggtttgttcgat gctccagcagaaatgagaac aagatgttgtaagacgtaag

400 agaaatgagtgaaaaagaag ggaggaagtagtgaaagaca atttatgtagttatgagcac gagaagctgatGAATGAGAT CATACCAACACTAATTCATT

500 GGATCTTGTCATCACTCTTA AATTAAACGTACTTGGTTGA GTGTAGTACTACGATGGGTG ACCTTTTGATCCATCCTCCG ATTGCACACCTtctttttttc
                              g                         a
600 ttctttttttctatttttac cctgacgtgtaatagaaatg tttaactaaacaacatcctc tactagaacataggtaatga ctctggggaaccgtgagtgc

700 gatata
```

B.

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    1                   20                  40                  60                  80                  100

5S1 gttcgataatcctgtggaac cgagaccaagatgtagtaaa aagtaatcgaaacgagtcta aaataaggtaggaagtagag aaagtttattcatgtacata
5S2       gc   a ca   a t   a       t   g c   ga   t   ga    g   g        t     aca   t    gt

5S1 tgtgcatgagaagcagacga GTGAGGTCATACCAGTAGTA ATTCATCGGATCCCATCATC ACTCTGAAGTTAAACGTACT TGGCCAAGACTAGTACTACG
5S2  a   c       t  t   A   A       AC C        T    TTG          T  A              TTG  TG

5S1 ATGGGTGACCTTTTGATCCT TCC-CCGAGTACACCCCTTT tattttctattttagcagct atttttaccccaatgtgcaa tggaaacgtttgactatcca
5S2                  A   T   T G   A   C t      tc   tttt-          tg c   t   a   t   a   aa

5S1 acatccgctagtagatgtt tagtaatgactacggggaac catgagttcgggttt
5S2       t   c    aca  ag        ct        g    g  ata a
```

FIGURE 1.—A, Nucleotide sequence of the 706-bp insert of pRS20.7. Differences between this sequence and a second 706-bp repeat isolated from λ5S-20 are shown above the pRS20.7 sequence. Spacer sequence is shown in lower case letters and 5S rRNA coding sequence (based on comparison to the plant consensus sequence in ERDMANN and WOLTERS 1986) is shown in upper case letters. The (^) symbol indicates the insertion of the base appearing above the pRS20.7 sequence. The asterisk at base 353 defines the position where 5S1 and 5S2 repeats were defined for comparison in panel B. B, Sequence comparison of the two 5S rRNA gene repeats present in pRS20.7. Differences between 5S2 and 5S1 are shown below the 5S1 sequence. (-) indicates gaps introduced to optimize the overlap of homologous bases.

1989). The sequence is organized largely in tandem arrays of 706-bp *Bam*HI fragments containing the repeat unit shown in Figure 1 (SCHNEEBERGER, CREISSEN and CULLIS 1989). Thus, this 5S rRNA gene group shares characteristics of other tandemly arrayed genes despite the marked divergence of the 5S1 and 5S2 repeats from each other and from other flax 5S rRNA gene groups. However, a considerable amount of sequence variation is observed with many restriction enzymes and is particularly apparent with enzymes which do not have sites within the 706-bp repeat isolated from λ5S-20. The hybridization pattern of the 706-bp probe to genomic Southern blots of flax genotroph and P1 DNAs digested with three different enzymes is shown in Figure 3 (SOUTHERN 1975). Figure 3, A, B and C, shows the hybridization pattern detected in *Eco*RI, *Dra*I and *Eco*RV digests, respectively. A large number of bands are generated with these enzymes, indicating a high degree of sequence variation in the 706-bp repeat family. However, many of the bands do not correspond to multimers of 706 or 353 bp (one-half of the 706-bp dimer repeat). These results are interpreted as evidence of large amounts of length and sequence heterogeneity. Group-4 clone λ5S-12 also displays length variation and is approximately 85% homologous to pRS20.7 (SCHNEEBERGER, CREISSEN and CULLIS 1989). This suggests that flax group-4 5S rDNA sequences are exceptionally heterogeneous in comparison to group-1 and group-2 flax 5S rDNA and are not as homoge-

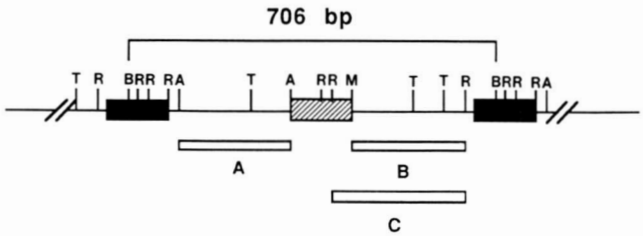

FIGURE 2.—Restriction map of pRS20.7. Filled and hatched boxes represent 5S RNA transcription units 1 and 2, respectively. The 706 bp bracket designates one repeat unit as defined by *Bam*HI which was cloned from λ5S-20 (SCHNEEBERGER, CREISSEN and CULLIS 1989). The open boxes below show the sub-probes used in hybridization experiments (probes described in text under *5S gene isolation and description*). The interrupted lines on either side of the 706-bp repeat indicate that this sequence is organized in tandem arrays in the original genomic clone. Restriction enzyme sites; T, *Taq*I; R, *Rsa*I; B, *Bam*HI; A, *Alu*I; M, *Mbo*II.

neous as would be suggested from the analysis of just the sequences present in λ5S-20 alone (Figure 1).

The 706-bp probe identifies RFLPs in flax genotrophs: Polymorphisms have been detected in comparison of Pl, L and S genotrophs by Southern analysis with nine different restriction enzymes (*Eco*RI, *Dra*I, *Eco*RV, *Sca*I, *Spe*I, *Bcl*I, *Bgl*I, *Hin*dIII and *Bst*NI). Comparison of the *Eco*RI, *Dra*I and *Eco*RV restriction fragment patterns detected by the 706-bp probe between Pl (Figure 3, lane 6, panels A, B, and C) and both large (lanes 7 and 8, panels A, B and C) and small genotrophs (lanes 1–5, panels A, B and C) shows distinct RFLPs. A large number of bands are detected in each lane, a subset of which are polymorphic. Large and small genotroph patterns differ from one another as well as from Pl. The RFLP patterns are stable and have been reproduced with several independent DNA samples. The stability of the RFLP patterns in stable genotrophs is shown in families such as S_1 and S_6 which are separated by many generations but have identical patterns (Figure 3, lanes 2 and 3, panels A, B and C; CULLIS 1977).

TABLE 1

Copy numbers of 706-bp repeat in comparison to total 5S rDNA

Plant line/species	Copy No. 5S rDNA[a]	Copy No. pRS20.7[b]
Pl	117,000	1,000
L^H	49,600	960
S1	52,800	1,060
L. grandiflorum caeruleum	ND[c]	220

[a] Determined using radiolabeled cytoplasmic 5S rRNA (GOLDSBROUGH, ELLIS and CULLIS (1981).

[b] Mean of two determinations. The standard error for these experiments is less then 5% (RIVIN, CULLIS and WALBOT 1986).

[c] Not determined.

Small genotroph specific RFLPs: An identical hybridization pattern is detected for each restriction enzyme in four small genotrophs, produced from separate, independent environmental induction experiments (Figure 3, compare lanes 1–5, panels A, B, and C). This RFLP pattern will be referred to as the S pattern. The S pattern has been identified in all small genotrophs studied to date. This observation suggests that common sequence alterations have occurred in each of the small genotrophs. An identical S pattern was found in DNA isolated from four separate C3 plants, one of which is shown in Figure 3. Seed for each of these C3 plants was individually collected from four separate, self fertilized Pl plants after environmental induction (CULLIS 1981). The largest number of polymorphic bands are detected in the S pattern for each enzyme. The *Eco*RI, *Dra*I and *Eco*RV blots show seven, ten and nine polymorphic bands (below 15 kb), respectively, between small genotrophs and Pl (compare Figure 3 lanes 1–5 with 6).

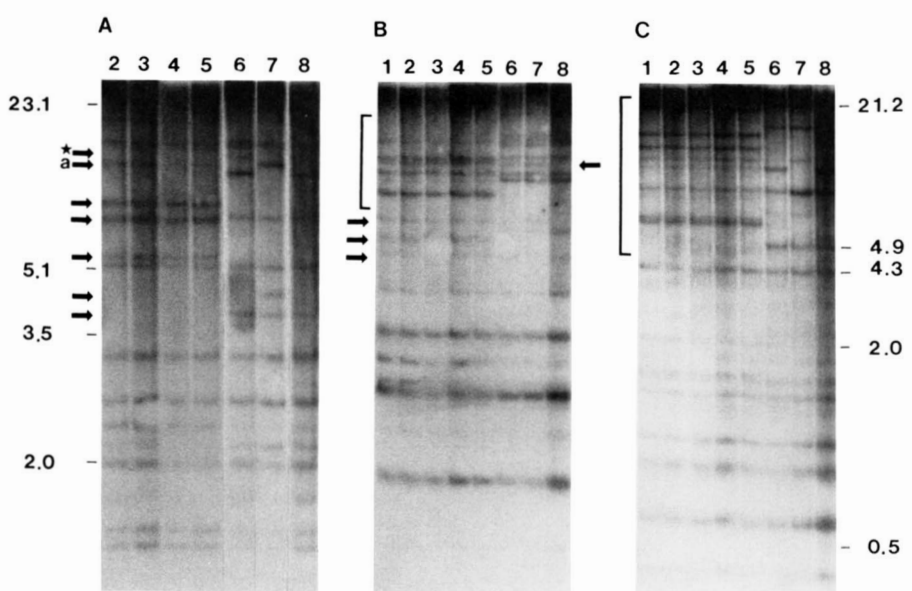

FIGURE 3.—Southern blot hybridization patterns detected by pRS20.7 in *Eco*RI (A), *Dra*I (B) and *Eco*RV (C) digests of flax Pl and genotroph DNAs. Lane 1, S^h; lane 2, S_1; lane 3, S_6; lane 4, C3; lane 5, L_6; lane 6, Pl; lane 7, L^H; lane 8, L_1. Panel A does not show S^h (see Figure 6, lane 5). Arrows indicate RFLPs. Bracketed areas designate multiple RFLPs. DNA standard sizes are in kilobases. DNA standards for panel A are on the left side. DNA standards for panels B and C are on the right side. Band "a" is 3–5-fold more intense in L^H (lane 7) than in small genotrophs (lanes 1–5). Asterisk denotes a potential single copy band in Pl; see RESULTS, *The 706-bp probe identifies RFLPs in flax genotrophs.*

In contrast to the situation described above for the S pattern, the RFLP patterns detected in two independently induced large genotrophs are nonidentical. Comparison of L^H and Pl shows four, two and three polymorphisms, respectively, in panels A, B and C. Only one polymorphic band is detected for L_1 in the *Dra*I digest, (arrowed band on the right side of panel B). The *Eco*RI and *Eco*RV hybridization patterns are the same for L_1 and Pl.

In each panel several polymorphisms appear to be identical in the S and L^H RFLP patterns. However, close inspection indicates that these bands are not identical. For example, band "a" in Figure 3, panel A, is consistently 3–5-fold more intense in L^H than in the S pattern. The difference in intensity could indicate a higher copy number of the same fragment in L^H, or a different fragment with more homology to the probe. The difference in band intensity is clearly seen when using a sub-probe of the 706-bp repeat as shown in Figure 5 (compare A with 706 patterns; also discussed below). In each case the polymorphisms in the genotrophs represent both novel bands and deleted bands with respect to Pl. The band intensities of many of the RFLPs differ from one another within and between lanes and do not reflect a linear increase in fragment size. RFLP generation as a result of loss or gain of restriction enzyme sites would predict the loss and appearance of the same number of bands between Pl and the genotrophs. However this type of predictable band shift is not observed from the calculated band sizes and numbers. This indicates that the RFLPs are not due to point mutation or DNA modification (*i.e.* methylation). In addition, the intensities of some similarly sized bands between lanes are different, indicating a difference in 706-bp sequence homology or copy number representation (Figure 3B, bracketed area). Copy number reconstructions were performed to determine the copy number of the 706-bp sequence in polymorphic bands detected by the 706-bp probe in an *Eco*RI digest of genomic DNA. The results indicate that the majority of the polymorphisms are not single copy in the genome, with each band representing several copies (data not shown). However, due to the heterogeneity of group-4 sequences it is difficult to determine the exact number of copies represented by each band. Several of the high molecular weight bands may correspond to single copy bands (Figure 3A, starred arrow).

High molecular weight RFLPs: The large degree of hybridization to DNA fragments above 11 kb obscures identification of polymorphisms in this region using conventional gel electrophoresis. The hybridization pattern observed with *Eco*RI-digested genomic DNA fractionated by FIGE is shown in Figure 4. A number of additional polymorphisms are detected in the high molecular weight DNA between Pl and the genotrophs (Figure 4, compare lane 3 with lanes 1 and 2). Several intense bands appear in L^H which have no obvious counterparts in Pl. These bands may represent DNA amplification events. Chromosomal amplification events in other systems (*e.g.* dihydrofolate reductase gene amplification in response to methotrexate selection in animal cells) are thought to result from DNA overreplication and integration into the chromosome (SCHIMKE *et al.* 1986). Such a process here would result in the production of novel bands and/or bands with increased copy number relative to unamplified bands.

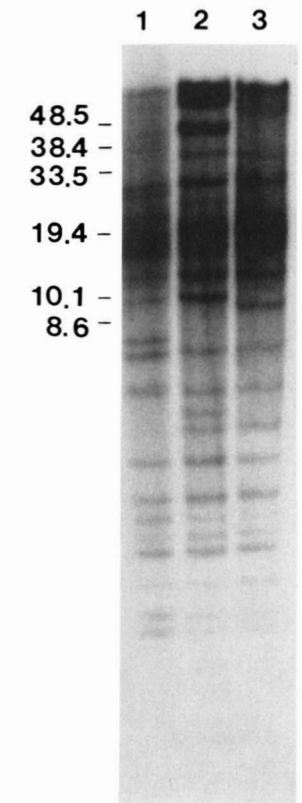

FIGURE 4.—Hybridization pattern detected by pRS20.7 in *Eco*RI digested S^h (lane 1), L^H (lane 2), and Pl (lane 3) DNAs separated by FIGE. DNA standard sizes are in kilobases.

Spacer probes: The spacer probes were found to detect a subset of the 706-bp probe RFLPs as well as additional sequence polymorphisms. The dimer organization of the 706-bp repeat shown in Figures 1 and 2 is unusual in that most 5S rDNA tandem arrays contain nearly homologous repeats (LONG and DAWID 1980). An exception to this generalization is the *Xenopus laevis* oocyte type 5S rDNA which contains a pseudogene alternated with a functional gene in each repeat (KORN 1982). In order to determine if the 706-bp repeat is representative of all members of the flax group-4 5S rDNA family including those which show polymorphism, spacer probes were isolated and used to hybridize *Eco*RI genomic blots of Pl, L^H and S^h DNAs (Figure 5). Clearly the patterns obtained with the different spacer probes are non-identical to that

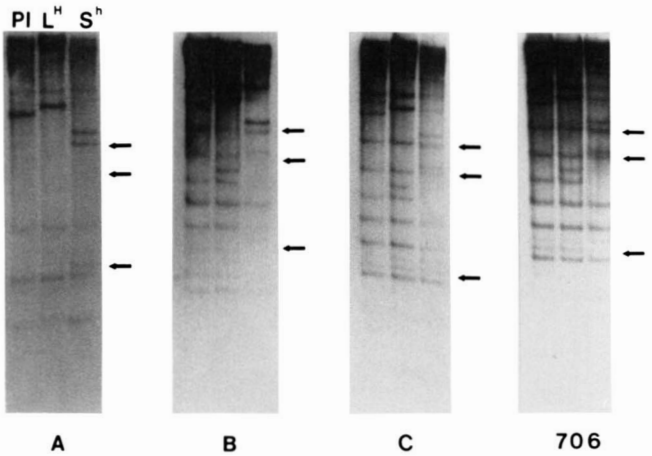

FIGURE 5.—Spacer analysis of pRS20.7. Autoradiograms of *Eco*RI-digested Pl, L^H and S^h total genomic DNA blots hybridized with the indicated pRS20.7-derived spacer probes. See Figure 1 for probe location on the 706-bp repeat restriction map and text for description. Each set of three lanes represents Pl, L^H and S^h DNAs, respectively, as shown for probe A. The position of bands which are differentially detected by the A and B spacer probes with respect to the 706-bp probe are indicated by arrows.

of the entire 706-bp probe. Both the A and B spacer probes identify a subset of the bands shown by the 706-bp repeat. Although the specific activities of the probes were similar in all of the experiments shown in Figure 5, the A spacer appears to be represented in only one-half of the bands detected by the entire 706-bp probe (Figure 5, compare A and 706). In addition the representation of the B spacer in S^h is reduced in comparison to Pl and L^H (Figure 5B). These results suggest that changes in the sequence copy number of group-4 5S rDNA family members has occurred during the induction process.

The A and B spacer probes distinguish different polymorphic bands as well as common, invariant bands. However, the polymorphisms all share the 5S2 transcription unit sequence since probe C (which contains 40 bp of 5S2 in addition to the A spacer) identifies all of the polymorphic bands (Figure 5C). The conclusion drawn from these data is that the 706-bp repeat does not always occur with the A and B spacers in the same organization as shown in Figure 2. Comparison of the A and B spacer probe blots indicates the spacers are separate from each other in some cases as they each identify bands exclusive of the other spacer (Figure 5, A and B). This result could be due to the high degree of sequence divergence in group-4 flax 5S rRNA genes resulting in a population of sequences which can be differentiated by their spacer sequence. Other members of this family are presently being cloned and analyzed to test this possibility. The spacer probes also specifically differentiate between bands which appear to be identical in the 706-bp *Eco*RI band pattern (Figure 5, compare arrowed bands in 706 with those in A and B). This latter result suggests two important features of the observed polymporphisms. First, the 706-bp repeat family is highly diverged, as the polymorphic bands identified by the 706-bp probe are not homogenous with respect to the sequence presented in Figure 1. Second, the differences in band intensities of several of the RFLPs suggest complex changes in either the sequence or organization of these bands (discussed below).

Genetic linkage of genotroph RFLPs: In order to determine whether the observed polymorphisms are in multiple genomic locations or at a single locus the RFLP segregation pattern was followed in $L_1 \times L_6$ genetic cross. DNA from 23 F_2 individuals was isolated and digested separately with *Eco*RI, *Dra*I and *Eco*RV. The resulting Southern blots with all 23 individuals represented for each enzyme were then probed with the 706-bp fragment (Figure 6A) and the A spacer probe (Figure 6B) independently to determine the linkage relations of the RFLPs (SOUTHERN 1975). Representative blots using conventional gel electrophoresis are shown in Figure 6, A and B (panels A and B represent all of the 23 F_2 individuals). Only parental and heterozygote type patterns (containing all of the polymorphic bands) for all probes are observed indicating that the RFLP patterns detected by both probes segregate as a single unit. This classification was independent of the enzyme used to digest the F_2 DNAs. The segregation pattern for both probes is identical indicating that the polymorphisms detected by each are linked to each other. A highly significant fit to a 1:2:1 (5-13-5) segregation ratio for each probe was obtained indicating that the RFLP patterns identified by the 706-bp probe and the A spacer probe follow a Mendelian single locus segregation ($\chi^2 = 0.37$, d.f. = 2). Therefore, the rearrangements must all be occurring at a single chromosomal locus. The lack of recombination between loci represented by any of the polymorphic bands indicates that either the RFLPs are physically close together or that recombination in this region was suppressed.

Linkage analysis was extended to the high molecular weight polymorphisms. *Eco*RI-digested genomic DNA of the 23 F_2 individuals represented in Figure 6, A and B, was fractionated by FIGE and hybridized with the A spacer probe (Figure 6C). Linkage is clearly observed for both the spacer probe polymorphisms and the high molecular weight polymorphisms which is identical to that obtained for the entire 706-bp probe (arrowed bands in Figure 6C; $\chi^2 = 0.37$, d.f. = 2). The segregation patterns for the high molecular weight polymorphisms are identical to those identified for the other probes indicating that these RFLPs are also linked to the same chromosomal region. Thus, all RFLPs detected by the pRS20.7 DNA sequence are linked to a specific chromosomal region. We pro-

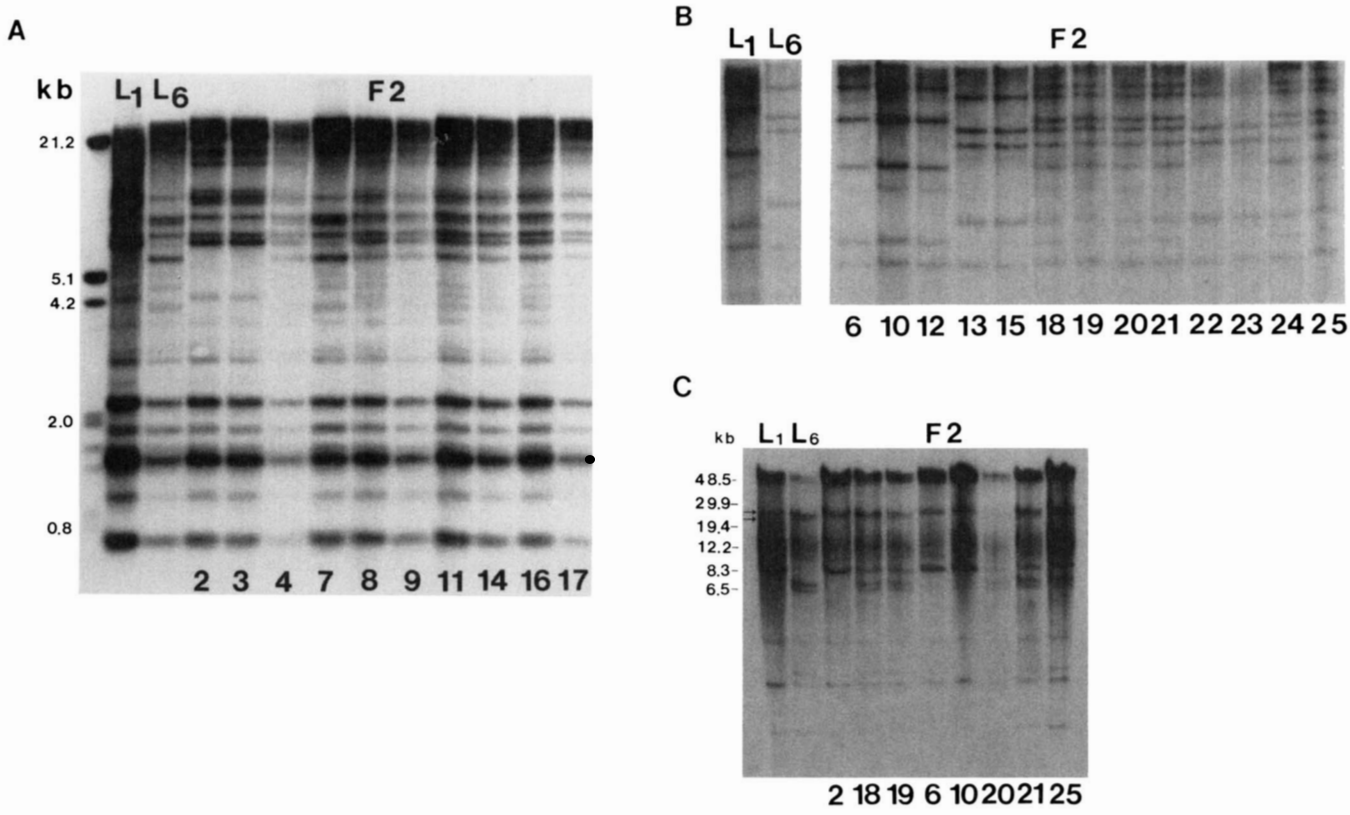

FIGURE 6.—Linkage analysis of pRS20.7 RFLP patterns. A, Autoradiogram of *Dra*I-digested total leaf genomic DNA from L_1, L_6 and F_2 progeny from an $L_1 \times L_6$ cross hybridized with pRS20.7. B, Autoradiogram of *Eco*RV-digested L_1, L_6 and $L_1 \times L_6$ F_2 individual total leaf genomic DNA hybridized with the A spacer probe. C, Autoradiogram of *Eco*RI digested total leaf genomic DNA from L_1, L_6 and $L_1 \times L_6$ F_2 individual total leaf genomic DNA separated by FIGE and hybridized with the A spacer probe (see Figure 2). F_2 individual numbers appear below the lanes for identification. The arrows identify two similarly sized restriction fragments, one of which is a segregating RFLP (lower band).

pose naming this locus Flp-1 for Flax polymorphic locus 1.

Low plant height and weight measurements of induced plants in comparison to the progenitor Pl are used as discriminating values for classifying genotrophs as large or small (DURRANT 1962). Analysis of the 23 individuals described above indicates that plant height is not significantly correlated with segregation of an L or S RFLP pattern (correlation coefficient = 0.36). However, a larger sample size may be required to determine if height and other genotroph traits are linked to Flp-1.

Flp-1 locus variation in other flax genotypes: The 706-bp repeat was used to probe genomic blots of flax linseed varieties and another *Linum* species, *Linum grandiflorum caeruleum* (Figure 7). In no case is the hybridization pattern observed in these lines identical to that in the genotrophs (Figure 7, compare lanes 1–4 with lanes 5–7). The RFLP pattern detected in *L. grandiflorum caeruleum* lacks several of the invariable bands detected in the genotrophs as well as showing non-genotroph polymorphisms. In addition the overall copy number of the pRS20.7 sequence is reduced to approximately 110 copies/1C as determined by copy number reconstructions (compare lanes 1 and 7, Figure 7; Table 1). RFLPs are also detected in nine other flax varieties. Of these only two, Dakota and Abyssian Brown appear identical for *Eco*RI (Figure 7, lanes 2 and 3). The others (Royal, Williston Brown, Kenya, Victory, Koto, Leona and Barnes) show RFLPs in comparison to Pl (Barnes is shown in Figure 7, lane 2). Several of the polymorphisms detected in Barnes resemble those present in S^h. These results suggest that restriction fragment length polymorphism at this 5S rDNA locus is high in comparison to other flax 5S rRNA gene families and the induced polymorphism is similar to that which exists naturally.

DISCUSSION

DNA polymorphisms identified by pRS20.7: We describe the identification of a 5S rRNA gene probe, pRS20.7, which identifies RFLPs in environmentally induced flax genotrophs and the progenitor line Stormont Cirrus (Pl) in addition to other flax varieties and *Linum* species. Polymorphic patterns have been identified with nine restriction enzymes thus far, three of which are shown in Figure 3. The 706-bp repeat found in pRS20.7 displays some interesting character-

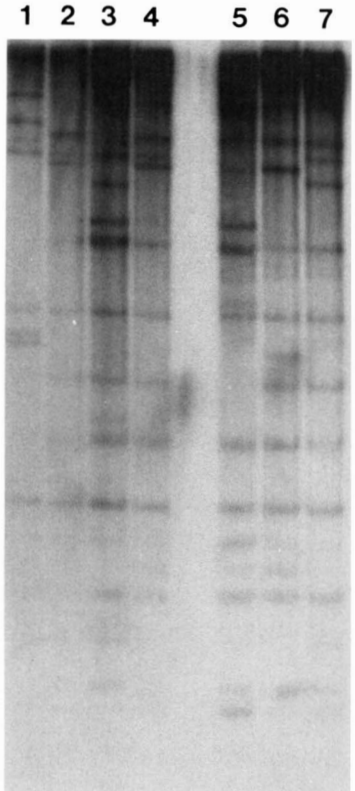

FIGURE 7.—Hybridization pattern detected by pRS20.7 in *Eco*RI-digested DNA of flax varieties and *L. grandiflorum caeruleum*. Lanes 5, 6 and 7 show the pattern detected in S^h, L^H and Pl, respectively, for comparison. Lane 1 is *L. gandiflorum caeruleum*. Lanes 2, 3 and 4 are Dakota, Barnes and Abyssian Brown, respectively.

istics including a heterodimer repeat and a high degree of divergence from the majority of flax 5S rRNA gene sequences (Figures 1 and 2). The amount of sequence divergence in the transcription unit is greater than that yet observed within any group of plant 5S rRNA sequences described to date (ERDMANN and WOLTERS 1986). The degree of heterogeneity displayed by this group of 5S rRNA genes is clearly seen in the DNA gel blots shown in Figure 3. Typically tandemly arrayed, repetitive genes such as rDNA and 5S rRNA genes are maintained in a concerted fashion and do not show large amounts of restriction enzyme site polymorphism (LONG and DAWID 1980). A similar situation is observed for pRS20.7 homologous sequences with enzymes such as *Bam*HI and *Sca*I which are highly conserved in plant 5S rRNA genes (SCHNEEBERGER, CREISSEN and CULLIS 1989). However, other enzymes such as *Eco*RI, *Dra*I and *Eco*RV produce fragments which do not fall into a multimer repeat size (Figure 3, A, B and C). This indicates the pRS20.7 gene family is heterogeneous with respect to sequence and repeat sizes and may not be subject to sequence homogenization characteristic of other tandemly arrayed genes, an interpretation which is strengthened by the analysis of pRS20.7 spacer sequences shown in Figure 5 (DOVER 1982). Comparison of the hybridization patterns of the A and B spacer probes illustrates differences in representation and organization, with each spacer detecting bands exclusive of the other spacer sequence. The results taken together show that group-4 flax 5S rRNA genes are highly variable. Analysis of the predicted 5S rRNA secondary structure for both 5S1 and 5S2 of pRS20.7 shows poor conservation of stem loop domains thought to be important for 5S rRNA structure (data not shown; ERDMANN and WOLTERS 1986). This result in addition to pRS20.7's high degree of divergence from the majority of flax 5S rRNA genes represented by pBG13 strongly suggests that this group may represent a pseudogene class and may have implications for the lack of conservation of this group of 5S rDNA sequences with respect to others in the genome (SCHNEEBERGER, CREISSEN and CULLIS 1989; DOVER 1982).

Induction of small genotrophs is associated with specific RFLPs: A defined set of RFLPs is observed in the comparison of the 706-bp probe hybridization pattern in Pl with that in both large and small genotrophs. The pattern identified in four small genotrophs is identical for all restriction enzymes tested (Figure 3). This result is of particular significance for several reasons. As described in MATERIALS AND METHODS, the four small genotrophs analyzed were induced in different environmental induction experiments. In addition all four share common characteristics including reduced plant weight, plant height, DNA content, copy number of rDNA genes, and an altered peroxidase isozyme band pattern (CULLIS 1977, 1981). Thus, a specific DNA RFLP pattern is associated with the induction of small genotrophs. That an induction event is required to generate these polymorphisms is evidenced by the derivation of the small genotroph L_6 from a large genotroph L_1. It has been observed that maintenance of stability for some genotroph lines requires growth under defined stabilizing conditions (Durrant 1962). L_6, a small genotroph, was derived from the large genotroph L_1 after growth outdoors for six generations (CULLIS 1977). DNA extracted from L_3 plants contains the same pRS20.7 polymorphisms detected in L_1 (data not shown). These plants had retained the large genotroph phenotype. However, there was a reduction in the total nuclear DNA content of L_3 plants (DURRANT and JONES 1971; JORDER *et al.* 1975). After three more generations of growth outdoors the resulting L_6 plants changed to a small phenotype as defined by lower DNA content, lower rDNA amount, reduced plant weight and height, concomitant with the acquisition of the small genotroph pRS20.7 RFLP pattern (CULLIS 1977; Figure 3, lane 5). An example of the stability of the small genotroph pattern is shown in a plant series derived from S_1. S_3 and S_6 are sublines separated from S_1 by

three and six generations, respectively, of growth outdoors. All three show the same small RFLP pattern (compare S_1 and S_6, Figure 3, lanes 2 and 3, S_3 not shown). This indicates that pRS20.7 polymorphisms are rapidly generated in Stormont Cirrus only in response to an induction event in which plant stature is altered. The instability of the large pattern and the stability of the small pattern also suggests that different states of genomic plasticity/stability exist which may be differentially affected by environmental conditions. The detection of RFLPs which are identical in the small genotrophs suggests that this DNA alteration is specific to the induction of small genotrophs. In addition, RFLPs are detected in all small and large genotrophs indicating that the 706-bp probe may represent a valuable diagnostic marker for stress induced heritable changes in flax.

Although the exact nature of the RFLPs is unknown the results indicate that a DNA rearrangement, such as sequence rearrangement, deletion or amplification may be responsible for the altered band patterns. It is unlikely that the RFLP pattern is due to single site mutations since the patterns are observed with several enzymes. The polymorphic pattern is not lost when genomic DNAs are digested with methylation insensitive enzymes such as *Dra*I (Figure 3C). In addition, due to the enzymes used in this study, both specific adenine and cytosine methylation would be required to explain the polymorphisms as the result of DNA modification. If DNA modification is responsible then the modification is regulated and heritable. This situation would be similar to an imprinting mechanism (KERMICLE 1978). The results of FIGE analysis of high molecular weight polymorphisms indicate that DNA amplification and/or recombination may be responsible for the new bands in L^H.

Linkage data from L × S F_2s show that all of the RFLPs are tightly linked (Figure 6). Analysis of 23 F_2 individuals did not show any recombination of polymorphic bands. Therefore the polymorphisms constitute a single chromosome locus. Due to the localization of the polymorphisms to this region the locus has been termed Flax polymorphic locus 1 (Flp-1). Since segregation of the RFLPs is not observed, the rearrangements must be confined to this chromosomal region. This observation places restrictions on the type of mechanisms which can be envisioned for production of the RFLPs.

The mechanism(s) that are responsible for the described DNA alterations are not clear. Large changes in the DNA content and copy number of repetitive sequences have been documented in flax genotrophs, suggesting deletion and amplification of large amounts of DNA (CULLIS and CLEARY 1986). Specific deletions of 5S rDNA involving the loss of a cluster 5S rDNA repeats resistant to cleavage by *Taq*I has also been associated with a reduction in 5S rDNA copy number (CULLIS and CLEARY 1986). Thus, based on previous studies and the nature of the RFLPs described above it seems likely that a DNA rearrangement is responsible for the observed polymorphisms. The spacer regions of the 706-bp repeat differentiate between bands which appear to be identical when the entire repeat is used as a probe (Figure 5). This result may indicate that these bands represent a heterogeneous population of fragments of a specific size which contain different spacer regions. Differential modulation of these fragments as a result of deletion, amplification or copy correction (gene conversion) may result in different representations in the genotrophs. Copy number determinations for the entire 706 bp of Pl, L^H and S^h did not show a conclusive change (Table 1). However, the results presented in Figure 4 suggest that copy number alterations have occurred in this gene family. Further experimentation is required to determine the nature of these complex DNA alterations.

The results from hybridizations to other *Linum* species and flax varieties show that similar types of DNA polymorphisms exist in these lines and that the RFLPs are not the result of an artifact in the induction experiments. The mechanism(s) by which these polymorphisms arose is unknown, but may be due to the occurrence of "induction events" similar to those in Stormont Cirrus. Identification of specific "fingerprint" like polymorphisms in flax species and varieties suggests that this probe may also be useful in breeding or mapping experiments.

The possibility of selection of genetic variants at the whole plant level is unlikely since all plants undergoing induction survive, reproduce and their progeny are alike (DURRANT 1962; CULLIS 1977). Furthermore the S pattern is detected in four individual C3 plants which are each first generation progeny of different, simultaneously induced parent Pl plants. Thus, the changes are occurring in all plants undergoing induction and are not due to selection of preexisting variation in Pl. Since Stormont Cirrus is self pollinating and the RFLPs are homozygous, the changes may be occurring in all of the cells of a meristem before the development of gametes such that both micro- and megagametophytes contain the altered DNA. Alternatively there may be a selection for cells which contain specific alterations during the course of growth under inducing conditions. This somatic selection again results in a homogeneous meristem before gametogenesis. Since the cells undergoing induction are diploid and there is no evidence for heterozygosity in Pl, the alterations must be occurring on both homologues before gametogenesis. This can occur either by independent changes in both homologous chromosomes, chromosome loss, or gene conversion. Al-

ternatively gametophytic selection may result in preferential transmission of the altered genotypes. Previous data indicates that both nuclear DNA content and rDNA gene copy numbers change during growth under inducing conditions (EVANS, DURRANT and REES 1966; CULLIS and CHARLTON 1981). Experiments directed at determination of the RFLP pattern during growth under inducing conditions will clarify these possibilities. The appearance of homozygous RFLPs is consistent with the changes in peroxidase band patterns. In this case the changes in relative mobilities of anionic peroxidase isozymes is controlled by dominant and recessive alleles in L and S, respectively, which show simple 3:1 Mendelian inheritance (TYSON, TAYLOR and FIELDES 1978). The specificity and reproducibility of the S-RFLP pattern possibly indicates a limited repertoire of potential rearrangements. However, the patterns for the large genotrophs show that more than one type of rearrangement can occur.

How the environmental conditions are responsible for this very specific set of polymorphisms is not yet known. One possibility is that the new arrangement is advantageous in the new environment but the basis for this "adaptation" is not known. Generation of variation in this manner would clearly be advantageous to inbreeding plants with limited genetic variability. Alternatively, the genotypes of the plastic varieties may be unstable under certain environmental conditions. Instabilities such as this are common to traits resulting from insertion sequences (PETERSON 1988). Recent evidence indicates that such instabilities may result in "adaptive" variation in prokaryotes (HALL 1988). However, to date there is no evidence to suggest that any of the environmentally induced characters is directly advantageous to the genotrophs. Physical characterization of the pRS20.7 RFLPs and surrounding sequences will aid in determining the type of alteration as well as where and when in the development of the plant the changes are occurring during induction. In addition studies directed at the expression of genes in the vicinity of the rearrangements will help clarify if any adaptive advantage accrues through these alterations. These experiments will be important in gaining an understanding of environmentally induced heritable change in plants.

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