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Simple Sequence Repeat (SSR) Markers Linked to the *Ligon Lintless* (Li_1) Mutant in Cotton

M. Karaca, S. Saha, J. N. Jenkins, A. Zipf, R. Kohel, and D. M. Stelly

Ligon lintless (Li_1) is a monogenic, dominant mutant in cotton, whose expression results in extreme reductions in fiber length on mature seed. The objectives of this research were to compare fiber initiation between the Li_1 mutant and TM-1 to reveal the fiber initiation differences between normal and mutant phenotypes, to develop a linkage map of simple sequence repeat (SSR) markers with the Li_1 locus, and to identify the chromosomal location of the Li_1 locus. Comparative scanning electron microscopy studies of fiber development in a normal TM-1 genotype and the near-isogenic Li_1 mutant at 1 and 3 days postanthesis revealed little differences between the two during early stages of development, suggesting that Li_1 gene expression occurs later, probably during the elongation phase. Thirty-eight SSR loci were found to be polymorphic between TM-1 and Li_1 and were used for mapping in an F_2 population. Twenty-two SSR loci, along with Li_1, were located on eight linkage groups, covering a total genetic distance of 218.3 cM. Analysis of individual monosomic and monotelodisomic plants indicated that two SSR loci (MP4030 and MP673) from the Li_1 linkage group were located on chromosome 22.

Cotton (*Gossypium* sp.) is the world's most important textile fiber and the second most important oil seed source. Competition from synthetic fibers and challenges to improve fiber quality are the two major economic forces driving the current global cotton market. However, records indicate that improvement in fiber yield and quality has recently reached a plateau (Meredith 1995). One of the major limitations in the genetic improvement of fiber is the paucity of information at the molecular level about genes controlling fiber development. This is especially surprising, considering that a large number of fiber-specific mutants are available.

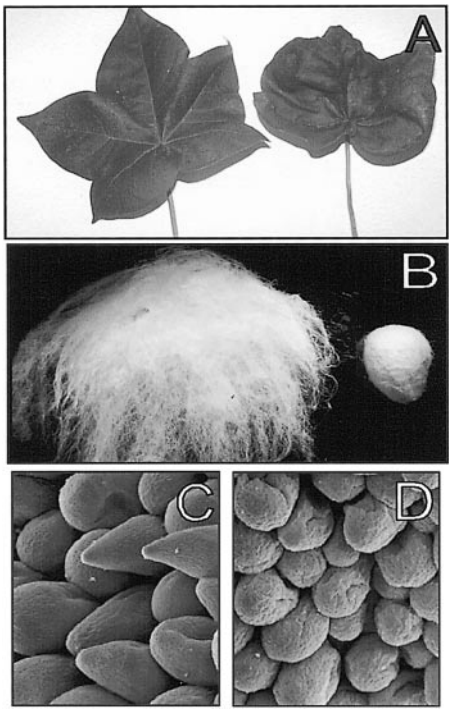


Figure 1. Comparison of fiber initiation and leaf morphology between mutant (Li_1) and normal (TM-1) cotton lines: **(A)** leaf (TM-1, left; Li_1, right); **(B)** mature cotton seeds with fibers (TM-1, left; *Li-1,* right); **(C,D)** 3-day postanthesis of fiber development on TM-1 and Li_1 ovules, respectively (magnification 839×).

Identification of a locus specific to fiber development is an important step toward manipulation and improvement of cotton fiber properties. Griffee and Ligon first discovered the *Ligon lintless* mutation in 1929 and Kohel (1972) documented its genetic characteristics. Li_1 is a monogenic, dominant mutant characterized by short fibers (~6 mm long; Figure 1) and distorted plant growth in the leaves, stems, and flowers (Kohel 1972). The pleiotropic effects of Li_1 suggest that it is a regulatory gene. This mutant has important ramifications because fiber price depends on the content of short versus long fibers in a cultivar (Cotton Incorporated 1994).

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Once a gene, such as *Li*$_1$, has been identified by its phenotypic effects, mode of expression, and manner of inheritance, it is then important to determine the precise genomic position of the gene, since this information typically facilitates isolation and manipulation of the gene at the organismal and molecular levels. One approach relies on identifying linked marker loci, based on recombination in a segregating population. This strategy has several benefits, including identification of molecular markers useful for marker-assisted selection (MAS), map-based cloning, and physical mapping of the gene(s) of interest.

Microsatellites, or simple sequence repeats (SSRs), are useful molecular markers because they are based on a simple polymerase chain reaction (PCR)-based technique; highly polymorphic and multiallelic; normally codominant, abundant, and randomly distributed throughout the cotton genome (Karaca 2001; Saha et al. 1999); amenable to high-throughput usage; and easily shared among research and breeding laboratories. Many SSR loci have already been assigned to specific chromosomes or chromosome arms (Liu et al. 2000b).

The objectives of this research were to compare fiber initiation between *Li*$_1$ mutant and TM-1 to reveal the fiber initiation differences between normal and mutant phenotypes, develop a linkage map of SSR markers with the *Li*$_1$ locus, and identify the chromosomal location of the *Li*$_1$ locus based on marker-assisted identification.

Materials and Methods

Comparative Analysis of Fiber Initiation

Scanning electron microscopy (SEM) was used to compare fiber initiation between TM-1, which produces a normal phenotype consisting of both short and long fibers, and *Li*$_1$, which only produces short fibers. Ovules [1 and 3 days postanthesis (dpa)] were collected from TM-1 and *Li*$_1$ flowers and fixed in half-strength Karnovsky's fixative, pH 7.2, overnight at 4ºC. Specimens were rinsed, postfixed with 2% osmium tetroxide (OsO_4), dehydrated, and critical point dried. Samples were mounted on aluminum stubs, coated, and imaged in a LEO S360 scanning electron microscope.

Genetic Inheritance of *Li*$_1$

A total of 147 individuals from a segregating F_2 population of the cross TM-1 × *Li*$_1$ were scored to confirm the monogenic dominant inheritance of the *Li*$_1$ phenotype. The *Li*$_1$ parent line used in the crosses was developed from six successive backcrosses to the recurrent TM-1 parent, accompanied by selection for the *Li*$_1$ phenotype. When plants were 3 weeks old they were scored (normal versus mutant) to confirm the inheritance pattern of the *Li*$_1$ locus using the chi-square test.

Chromosomal Assignment of the *Li*$_1$ Locus

Cytologically identified monotelodisomic (25 II + Ii) and monosomic (25 II + I) chromosome substitution lines (BC_0F_1), along with euploid F_1, were used to identify the chromosomal location of the *Li*$_1$ locus following Lui et al. (2000b). Monotelodisomes included chromosomes 1Lo, 2Lo, 2sh, 3Lo, 3sh, 4sh, 5Lo, 6Lo, 7Lo, 7sh, 9Lo, 11Lo, 14Lo, 15Lo, 15sh, 16sh, 16Lo, 18Lo, 18sh, 20Lo, 22sh, 25Lo, and 26sh. Note that Lo is long arm and sh is short arm; that is, monotelodisomic 1Lo contains a normal chromosome 1 and a telosome for the long arm of chromosome 1; it is disomic for the long arm but hemizygous for the short arm. Monosomes included chromosomes 1, 2, 3, 4, 6, 7, 9, 10, 12, 16, 17, 18, 20, 23, and 25.

Genomic DNA Extraction

Ninety-six F_2 plants segregating for *Li*$_1$ from the cross of TM-1 and *Li-1* were used for molecular mapping. Genomic DNAs from leaves of monosomic and monotelodisomic chromosome substitution stocks, bulked samples of 3-79, TM-1, and 3-79 × TM-1 (a euploid F_1 as control), *Li*$_1$, TM-1 × *Li*$_1$, along with the individual F_2 progenies of TM-1 × *Li*$_1$ were extracted using the DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) with minor modifications (Karaca 2001).

Amplification of the SSR Loci

SSRs were amplified in 25 μl reactions containing 1× GeneAmp PCR Gold Buffer, 2.5–3.0 mM $MgCl_2$, 0.15 μM SSR primer pairs (Map-Pairs, Research Genetics, Huntsville, AL), 0.2 mM each dNTP, 0.5 unit Ampli*Taq* Gold DNA polymerase (Perkin-Elmer, Norwalk, CT), and 80–100 ng of template DNA following the overall methods of Karaca (2001).

Capillary Electrophoresis (CE)

DNA fragment separations were performed utilizing the ABI Prism 310 Genetic Analyzer or ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA) using the method of Karaca (2001). To eliminate instrument noise and the effect of comigration, a DNA marker was considered to be valid if it had a peak height of at least 100 fluorescent units and ±1 base size difference with the nearest DNA fragment peak, respectively. To adjust fluorescent intensities, PCR products, amplified with fluorescent-labeled primers, were loaded (2 FAM : 3 HEX : 5 NED) in a 13 μl run reaction mixture containing 2.5 μl 1/30-diluted (in sterile H_2O) PCR product and deionized formamide.

Linkage Analysis

Linkage analysis between DNA markers, and *Li*$_1$ was performed using MapMaker (Lander et al. 1987). Linkage was considered significant if the LOD score was greater than or equal to 3.0.

Results and Discussion

Comparative Analysis of Fiber Initiation

Primordial fiber cells that initiate elongation between the day of anthesis and 4–6 dpa are destined to become long or lint fiber. Short or fuzz fibers are initiated 4–10 dpa, but never obtain lengths greater than 10 mm (Basra and Saha 1999). SEM studies indicated that morphological development of almost the same number of fiber begins in the ovule epidermal cells beginning at 1 dpa, first evident by bulging and spherical expansion, followed by cell elongation in a similar fashion in both TM-1 and *Li*$_1$ (Figure 1C,D). This similarity in development indicated that the fiber initiation factor(s) acted at the same time in TM-1 and *Li*$_1$. Differences in fiber lengths at the time of harvest further suggest that elongation factors might account for the difference in length between TM-1 and *Li*$_1$. Wilkins and Jernstedt (1999) reported that two membrane-bound electrogenic proton pumps, the vacuolar H^+-ATPase and the plasma membrane H^+-ATPase (PM-ATPase), play an important role in regulating cell expansion by controlling the cell turgor in fiber cells. They reported, based on molecular and biochemical analysis of excised ovules spanning −3 to 3 dpa, that the level of these two proteins was higher during fiber differentiation. However, these increased protein levels were not accompanied by increases in enzyme activity, and regulation of these proton pumps was subject to posttranscriptional regula-

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tion during the early stages of fiber development.

Li$_1$ fibers appeared to be more contorted than TM-1 at 3 dpa (Figure 1C,D). The *Li*$_1$ locus caused several pleiotropic effects, including contorted leaves, cotyledons, stems, and flowers. Given its profound pleiotropic effect, *Li*$_1$ might be a regulatory gene involved in all plant tissues and also has an effect on fiber elongation.

Genetic Inheritance of *Li*$_1$

Kohel (1972) reported a monogenic dominance pattern of inheritance for the *Ligon lintless* trait. Segregation among 147 F_2 progeny of the cross TM-1 × *Li*$_1$ produced 32 normal phenotype individuals and 115 mutant individuals. The chi-square value (0.036) was not significant, suggesting that the F_2 plants segregated as per the expected Mendelian ratio of 3:1, and according to the single dominant gene model reported by Kohel (1972).

Molecular Linkage Mapping

Two hundred twenty SSR primer pairs were initially used to screen polymorphisms between TM-1 and *Li*$_1$. A total of 45 of the 249 (some primer pairs yielded two loci) SSR loci were identified to be polymorphic. It was surprising that such a high percentage (18%) of polymorphism existed at the molecular level between the two parents, given that they were near-isogenic lines developed from six backcrosses. Similar results were obtained using SSR markers on the backcross conversion program to day-neutral stocks in cotton (Liu et al. 2000a). They suggested that this could be due to linkage drag adjacent to genes controlling day neutrality coming from the donor parent. Our results indicate that selection based on phenotypic traits, such as *Li*$_1$, may not be a suitable method, as there may be many large linkage blocks within the Upland cotton genome that are not easily amenable to recombination in backcross breeding.

Linkage analysis was performed using the *Li*$_1$ morphological locus and easily scorable 38 SSR loci, which consisted of two dominant (MP3502-204 and MP3594-196) and 36 codominant markers. A total of 23 loci were linked on eight different linkage groups, covering a total distance of 218.3 cM (Figure 2). Linkage group 1 (LNK-1) consisted of 4 SSR loci and *Li*$_1$ covering 116.1 cM, while 16 polymorphic SSR loci were not linked to any linkage groups, including several that were located on different chromosomes (Liu et al. 2000b). Only SSR locus MP4030-113 was linked to the flanking *Li*$_1$ gene, with a distance of 12.5 (cM) and an LOD score of 10.31.

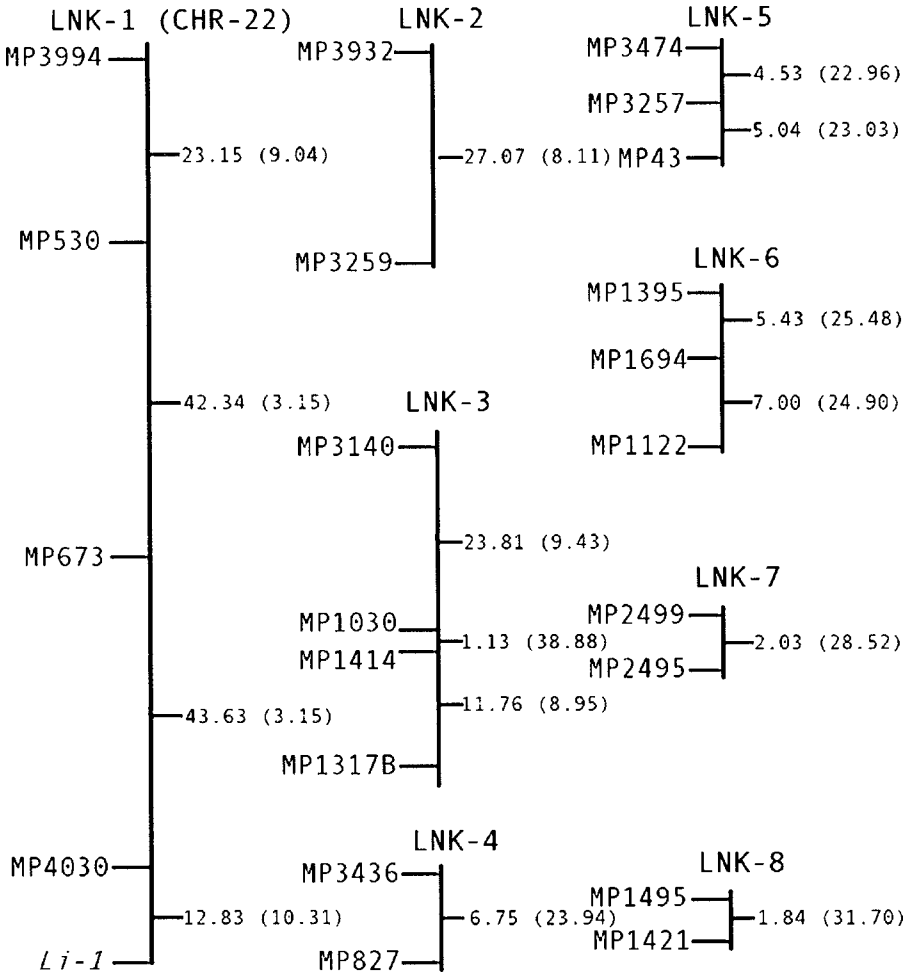


Figure 2. Linkage map of 22 SSR LP loci and the *Li*$_1$ locus. Loci are listed on the left and the distances (cM) and LOD scores (within parentheses), respectively, are on the right.

Chromosomal Assignment of the *Li*$_1$ Locus Using Marker-Assisted Identification

Chromosomal location of several SSR loci (Liu et al. 2000b) and many morphological traits have already been reported in cotton (Endrizzi et al. 1984; Kohel et al. 2000) using aneuploid chromosomal substitution materials similar to this study.

SSR loci specific to *Li*$_1$ were assigned to chromosomes and chromosome arms according to Liu et al. (2000b). Two (MP4030-113 and MP673-189) of the four SSR loci of LNK-1, where *Li*$_1$ was located, were polymorphic between TM-1 and 3-79 and were screened against all of the aneuploid cytogenetic stocks to identify chromosomal location. Loci MP4030-113 and MP673-189 were also polymorphic at the interspecific level between the 3-79 and *Li*$_1$ lines.

Using the cytogenetic stocks, the TM-1 alleles from the two different loci, MP4030-113 and MP673-189, were located on the short arm of chromosome 22 (D subgenome), based on deletion analysis. However, considering *Li*$_1$ as the flanking marker of the linkage group and the possibility of the existence of the centromere between *Li*$_1$ and its closest SSR locus, we can still indirectly confirm the location of *Li*$_1$ on chromosome 22. This is the first morphological marker that has been assigned to chromosome 22. Identification of two loci linked to *Li*$_1$ on the short arm of chromosome 22 substantiated the initial association with linkage group 1 and also suggested the location of the *Li*$_1$ locus on chromosome 22 (subgenome D). Kohel et al. (1993) suggested that *Li*$_1$ was located on the D subgenome of Upland cotton, which has not been assigned to any chromosome.

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Fortunately more than 40 DNA markers (SSR and restriction fragment length polymorphism [RFLP] markers) have been assigned on chromosome 22 (Jiang et al. 1998; Reinisch et al. 1994), and it will be useful to identify the linkage relationship of these other DNA markers with the Li_1 locus. This might also provide a way to find DNA markers more closely associated with the Li_1 locus and help in map-based cloning of the Li_1 gene.

Conclusion

SEM results demonstrated that there were no differences between TM-1 and Li_1 during fiber initiation, suggesting that the genetic control of fiber initiation was very similar between both lines. A total of 22 SSR loci and the Li_1 locus were grouped onto eight separate linkage groups (LNKs), covering a total distance of 218.3 cM. LNK-1 consisted of Li_1, and the MP4030, MP673, MP530, and MP3994 loci. MP4030, MP673, and Li_1 were found to be located on chromosome 22. Thus we have identified the first morphological marker assigned to chromosome 22 in cotton.

From the Department of Field Crops, Faculty of Agriculture, Akdeniz University, Antalya 07759, Turkey (Karaca), USDA-ARS, Mississippi State University, Mississippi State, MS 39762 (Saha and Jenkins), Department of Plant and Soil Science, Alabama A&M University, Normal, AL 35762 (Zipf), USDA-ARS, College Station, TX 77845 (Kohel), and Department of Soil and Crop Sciences, Texas A&M University, College Station, TX 77843-2474 (Stelly). Mention of trademark or proprietary products does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, Akdeniz University, or Alabama A&M University and does not imply its approval to the exclusion of other products that may also be suitable. We thank L. D. Hendrix, M. Duke, D. Dollar, and W. Monroe for their technical assistance. The authors thank Drs. R. Percy, J. C. McCarty, K. M. Soliman, and M. Ullola for their helpful suggestions on the manuscript. Address correspondence to Sukumar Saha at the address above.

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