Theor Appl Genet (2013) 126:963–970
DOI 10.1007/s00122-012-2029-x

ORIGINAL PAPER

Discovery and identification of a novel Ligon lintless-like mutant (Lix) similar to the Ligon lintless (Li_1) in allotetraploid cotton

Caiping Cai · Xiangchao Tong · Fengju Liu · Fenni Lv · Haihai Wang · Tianzhen Zhang · Wangzhen Guo

Received: 21 October 2011 / Accepted: 6 December 2012 / Published online: 9 February 2013

Abstract Mutants are a powerful resource for studying gene structure, function, and evolution. In this present study, a novel Ligon lintless-like mutant (Lix), that has short fibers and deformed leaves and stems, was isolated from the progeny of transgenic cottons. The Lix mutant is similar in morphology to the Ligon lintless (Li_1) mutant. Genetic analysis and molecular mapping were performed for the Lix and Li_1 mutants. These two mutants are monogenic dominant mutants with distorted growth of vegetative and reproductive structures. Seedlings of the dominant homozygote Li_1Li_1 genotype are lethal, while *LixLix* plants are viable but show no reproductive growth. Molecular tagging showed that the *Lix* gene is located on Chr. 04 in a 30.9-cM region spanned by NAU8376 and NAU3469. In a previous study, the Li_1 gene was mapped to Chr. 22, and Chr. 04 and Chr. 22 are homoelogous chromosomes in tetraploid cotton. So, we propose that Lix and Li_1 mutants have similar mutated morphology, and *Lix* is mapped to a homoelogous chromosome carrying Li_1. The identification and genetic mapping of Lix/Li_1 genes using mutants provides a foundation for isolating these genes. In turn, this will permit studies to elucidate the functional and evolutionary roles for these genes in cotton growth and development.

Communicated by R. Snowdon.

C. Cai · X. Tong · F. Liu · F. Lv · H. Wang · T. Zhang · W. Guo (✉)
National Key Laboratory of Crop Genetics and Germplasm Enhancement, Cotton Research Institute, Nanjing Agricultural University, Nanjing 210095, Jiangsu Province, People's Republic of China
e-mail: moelab@njau.edu.cn

Introduction

Cotton (*Gossypium* spp.) is the world's most important natural textile fiber and a significant oilseed crop. Tens of thousands of genes are expressed during cotton development. Mutants can provide insight for elucidating the genetic factors that are critical for controlling a given phenotype. Thus, mutants are a powerful resource for studying gene structure, function, and evolution. To date, there have been many mutants reported in cotton including open-bud (ob_1, ob_2), virescent (v_1), sterile-dwarf (sd^a), restores fertility (Rf), and nitrate nonutilizing gene (nit) (Endrizzi and Nelson 1989; Endrizzi and Ray 1991; Qian et al. 2009; Killough and Horlacher 1933; Wu et al. 2009; Yin et al. 2006; Korolev et al. 2008). Moreover, some important fiber mutants have been discovered and studied for their biology and genetic mechanisms of control, including Ligon lintless-1 (Li_1), Ligon lintless-2 (Li_2), dominant naked seed (N_1), recessive naked seed (n_2), Xuzhou-142 lintless-fuzzless (XZ142WX), Xuzhou-142 linted-fuzzless (XZ142FLM), Xinxiangxiaojilintless-fuzzless (XinWX), Xinxiangxiaojilinted-fuzzless (XinFLM) and immature fiber (im) (Griffee and Ligon 1929; Narbuth and Kohel 1990; Kearney and Harrison 1927; Ware et al. 1947; Zhang and Pan 1991; Zhang et al. 2007; Kohel and McMichael 1990). Some of these cotton mutants have been used to identify and characterize the function of the genes (Lee et al. 2006; Wu et al. 2006).

Genetic mapping of mutation genes provides an invaluable step towards the isolation of a gene and the elucidation of its function. In combination with high-density molecular maps, mutation genes can be located on corresponding chromosomes quickly and accurately. Furthermore, using chromosome location analysis, we can identify the possible homologous or homoeologous

relationships of mutation genes expressing similar morphologies in allopolyploids. Cultivated *Gossypium hirsutum* and *Gossypium barbadense* are allotetraploid cotton species. To date, several genetic maps of cotton genomes have been constructed using diverse DNA molecular markers and mapping populations (Ulloa and Meredith 2000; Ulloa and Meredith 2002; Zhang et al. 2002; Lacape et al. 2003; Rong et al. 2004; Mei et al. 2004; Zhang et al. 2005; Frelichowski et al. 2006; Guo et al. 2008; Yu et al. 2011; Yu et al. 2012). Rong et al. (2004) constructed a tetraploid cotton map of sequence-tagged sites that was composed of 2,584 loci at 1.72 cM intervals in 26 linkage groups covering 4447.9 cM. Yu et al. (2011) reported a genome-wide microsatellite-based tetraploid cotton genetic map that included 2,316 loci on the 26 cotton chromosomes, covering a total length of 4418.9 cM with an average distance of 1.91 cM. In our laboratory, a tetraploid cotton genetic linkage map using a [(TM-1 × Hai7124) × TM-1] inter-specific BC_1 mapping population has been constructed and enhanced during recent years (Song et al. 2005; Han et al., 2004, 2006; Guo et al. 2007a, 2008). Until now, this was mostly microsatellite-based, gene-rich, saturated cotton linkage map composed of 3,147 loci in 26 linkage groups that covered 3615.5 cM with an average inter-marker distance of 1.15 cM (Zhao et al. 2012). Based on molecular mapping analysis, the novel Dobzhansky–Muller type interaction genes Le_3 and Le_4, that cause interspecific hybrid lethality between two cotton species (*G. hirsutum* and *G. barbadense*), were identified on Chr. 8 and Chr. 11 (Song et al. 2009). Similarly, fine mapping of open-bud duplicate genes (ob_1 and ob_2) in homoelogous chromosomes Chr. 13 and Chr. 18 in tetraploid cotton has also been reported (Qian et al. 2009). In addition, several fiber mutation genes, including four dominant (Li_1, Li_2, N_1, and *Fbl*) and three recessive (n_2, *sma-4(ha)*, and *sma-4(fz)*), were genetically mapped using seven mapping populations (Rong et al. 2005). The location of these mutation genes lays a foundation for map-based cloning of genes, which will allow for functional and evolutionary analysis studies.

Ligon lintless mutant (Li_1) is a monogenic, dominant mutant characterized by short fibers (no more than 6 mm long) and distorted leaf, stem, and flower growth; however, the dominant homozygote (Li_1Li_1) genotype is lethal (Narbuth and Kohel 1990; Rong et al. 2005). Genetic mapping showed that Li_1 is located on Chr. 22 (Karaca et al. 2002; Rong et al. 2005). Zhu et al. (2003) reported that the Li_1 mutant's deformed leaf and stem trait is similar to the polar auxin transport mutant of *Arabidopsis*; thus, impaired polar auxin transport in the Li_1 mutant may inhibit its vegetative and reproductive development. Recently, microarray analysis using the Li_1 mutant has been carried on to identify and characterize the function of the mutated gene (Bolton et al. 2009). In this present study, a novel Ligon lintless-like mutant that exhibited the similar morphology to the Li_1 mutant was found in transgenic cotton plants knocked down by antisense against *GhPEL* that encodes pectate lysase. This newly-discovered mutant had deformed leaves and stems and short fibers. However, in contrast to Li_1, the dominant homozygote in the new mutant can survive (though reproductive growth is inhibited), while the dominant heterozygote shows late maturation in the boll opening stage. We speculate that this novel mutant, which is similar to Li_1 in morphology, is controlled by an incomplete dominance gene that has been temporarily termed *Lix*. To clarify the relationship between the two mutants exhibiting similar phenotypes, we present the results of a genetic analysis of the Lix and Li_1 mutants that includes the molecular mapping of *Lix*. The results provide a foundation for isolating and cloning Lix/Li_1, and understanding their distribution and functional roles in allotetraploid cotton.

Materials and methods

Plant materials

Two mutated materials and two allotetraploid cultivated species were chosen for this present study. The two mutants were Li_1 and Lix. The Li_1 mutant was a gift from Dr. Kohel (USDA-ARS, College Station, TX, USA), while the novel Lix mutant was found in our laboratory in transgenic cotton knocked down by antisense for *GhPEL*; the antisense construct of *GhPEL* was driven by the E6 promoter (E6ASP) that was developed using pBI121 and was introduced into *G. hirsutum* acc. W0 (Wang et al. 2010). TM-1 is a genetic standard line of Upland cotton and Hai7124 is a commercial Sea island *Verticillium*-resistant cultivar and both are allotetraploid.

For genetic analysis, we combined different crosses of the Lix mutant with TM-1, Hai7124 and Li_1, respectively, from 2008 to 2011. In the F_1 populations, plants with deformed leaves were further self-pollinated or crossed with TM-1 or Hai7124 to produce segregating populations. In addition, the Lix mutant was also self-pollinated each year to confirm its genetic characteristics. In different segregating populations, the normal and deformed leaf traits were investigated during the plant seedling and maturation stages. The χ^2 test for goodness of fit was used to assess the Mendelian 1:1 (F_1 or BC_1 populations) or 1:2:1 (self-pollinated or allelic analysis populations) inheritance of the deformed leaf traits. As contrast, Li_1 mutant were also self-pollinated or crossed with Hai7124 to produce segregating populations for inheritance confirmation.

For the molecular mapping of the *Lix* gene, we constructed [(Lix × Hai7124)$_{(\text{deformed leaves})}$ × Hai7124] backcross populations. The mapping population comprised of 469 BC_1 plants, and the deformed leaf and stem traits of the BC_1 segregation population were investigated during the plant seedling stage. The fiber phenotypes of the BC_1 segregation population were investigated during the boll opening stage.

Two sets of genetic materials, one for newly enlarged (Lix × Li_1)F_1 population, and another for 50 $F_{2:3}$ plant lines with the phenotypes of deformed leaves and 10 $F_{2:3}$ plant lines with the normal leaf and stem phenotype by selfing corresponding individuals in (Lix × Li_1) F_1, were obtained, respectively, in the Jiangpu experimental field of Nanjing Agricultural University, Nanjing, Jiangsu Province, China in 2011 summer, and planted in Cotton Plantation of Nanjing Agricultural University, Hainan Island, China, in 2011 winter for further allelic analysis of *Lix*/*Li*$_1$ genes. Other materials involved in the study were all planted in the Jiangpu experimental field of Nanjing Agricultural University, Nanjing, Jiangsu Province, China.

DNA extraction and construction of the genetic linkage map

Cotton genomic DNA was isolated from two parents (Lix and Hai7124), F_1 and 469 BC_1 individuals, as described by Paterson et al. (1993). Simple-sequence repeat polymerase chain reaction (SSR–PCR) amplifications were performed using a Peltier Thermal Cycler-225 (MJ Research), and electrophoresis of the products was performed as described by Zhang et al. (2000, 2002).

All SSR primer pairs were developed in our laboratory. The primer information can be downloaded from http://www.cottonmarker.org/. The normal leaf genotype (*lixlix*) and the heterozygous genotype (*Lixlix*) were scored as 2 and 3 in the BC_1 population, respectively. Missing data were noted as "–". The χ^2 test for goodness of fit was used to assess the Mendelian 1:1 inheritance in the BC_1 segregating population.

JoinMap v3.0 software was employed to construct the genetic linkage map, and all linkage groups were determined at LOD scores ≥6. Mapping was completed using Mapchart software (Van Ooijen and Voorrips 2001; Voorrips 2002).

Results

Discovery of a novel Ligon lintless-like mutant (Lix) similar to the Ligon lintless (Li_1)

To confirm the functional role of *GhPEL* encoding pectate lysase in fiber elongation, we regenerated *GhPEL*-knockdown transgenic plants by *Agrobacterium*-mediated transformation (Wang et al. 2010). In *GhPEL*-knockdown transgenic plants, a novel mutant (Lix) similar to the Li_1 mutant was identified. PCR analysis by promoter-gene specific primers (Wang et al. 2010) showed that the mutant trait had no relationship with T-DNA insertion, suggesting it should be caused by point mutation in the process of tissue culture. The Lix mutant had deformed leaves and stems and short fibers compared with those observed for its parent, *G. hirsutum* acc. W0. In Lix self-pollinated segregating populations, we found that there were three types of leaf trait, specifically normal, deformed and super-deformed (Fig. 1a). Following Li_1 inheritance in Ligon lintless plants, we suppose that the recessive homozygote (*lixlix*) exhibited the normal leaf characteristic and fibers, the heterozygote (*Lixlix*) displayed the short fibers and deformed leaf and stem phenotype, while the dominant homozygote (*LixLix*), that showed only vegetative growth and no reproductive growth, exhibited the super-deformed leaves. For the Li_1 genetic model (Fig. 1b), the heterozygote (Li_1li_1) genotype is characterized by short fibers and distorted leaf, stem, and flower growth, while the dominant homozygote (Li_1Li_1) genotype is lethal (Narbuth and Kohel 1990; Rong et al. 2005). Thus, we speculated that the Lix mutant was controlled by an incomplete dominance gene with different genetic and functional roles from those in the Li_1 mutant.

Genetic analysis of Lix/Li_1 mutants

Genetic analysis of the Lix mutant

Deformed leaves and stems and short fibers were two phenotypes observed for the Lix mutant. In the genetic analysis, we focused on the deformed leaf phenotype because it was simpler to distinguish during vegetative growth.

From 2009 to 2011, using TM-1 and Hai7124 as female parents, we made several combinations by crossing the Lix mutant with TM-1 or Hai7124. Then, individuals with deformed leaves in F_1 were self- or backcrossed with TM-1 or Hai7124 to create the F_2 or BC_1 progenies. In addition, the Lix mutant plants with deformed leaves were self-pollinated to produce a segregating population for each year, and the Lix mutant was crossed with the Li_1 mutant to produce an F_1 population with 81 individuals in 2011 summer. For accurately identifying the genetic relationship between *Lix* and Li_1, newly enlarged (Lix × Li_1)F_1 population, and 50 $F_{2:3}$ plant lines with the phenotypes of deformed leaves and 10 $F_{2:3}$ plant lines with the normal leaf and stem phenotype by selfing corresponding individuals in (Lix × Li_1) F_1, were further analyzed in 2011 winter. In each segregating population, the deformed leaf

Fig. 1 Morphology in self-pollinated population of Lix and Li_1 mutants. a Phenotype of Lix mutant self-pollinated population. a–c recessive homozygote (lixlix), heterozygote (Lixlix) and dominant homozygote (LixLix), respectively. d leaf type (from *left to right*) of the recessive homozygote (lixlix), heterozygote (Lixlix) and dominant homozygote (LixLix). e fiber morphology of the recessive homozygote (lixlix, *up lane*) and heterozygote (Lixlix, *down lane*). b Phenotype of Li_1 mutant self-pollinated population. a, b the recessive homozygote (li_1li_1) and heterozygote (Li_1li_1), respectively. c the leaf type (from *left to right*) of the recessive homozygote (li_1li_1) and heterozygote line (Li_1li_1). d fiber morphology of the recessive homozygote (li_1li_1, *up lane*) and heterozygote line (Li_1li_1, *down lane*)

plants were investigated in the plant seedling and maturing stages.

During three consecutive years of analysis, 1193 self-pollinated segregating populations in total from Lix mutants were surveyed, which consisted of 284 plants with the super-deformed leave phenotype, 588 plants with the deformed leaf and stem phenotype and 321 plants with the normal leaf phenotype. These data fitted Mendelian 1:2:1 inheritance ($\chi^2 = 2.537 < \chi^2_{0.05,2} = 5.99$). Further, the segregations of 167 (TM-1 × Lix) F_1 individuals and 585 (Hai7124 × Lix) F_1 individuals were determined to fit Mendelian 1:1 inheritance ($\chi^2 = 0.000 < \chi^2_{0.05,1} = 3.84$, $\chi^2 = 1.156 < \chi^2_{0.05,1} = 3.84$) (deformed leaf plants:normal leaf plants, 1:1). Moreover, the self-pollinated segregating populations of F_1 deformed leaf plants of (TM-1 × Lix) and (Hai7124 × Lix) showed Mendelian 1:2:1 ratio ($\chi^2 = 2.336 < \chi^2_{0.05,2} = 5.99$, $\chi^2 = 1.525 < \chi^2_{0.05,2} = 5.99$) (super-deformed leaf plants:deformed leaf plants: normal leaf plants, 1:2:1). In the cross [(Lix × Hai7124)$_{(\text{deformed leaves})}$ × Hai7124] that gave 469 BC_1 plants, we found 220 plants that had a deformed leaf and stem phenotype while 249 plants had the normal leaf phenotype. These data also fitted Mendelian 1:1 inheritance ($\chi^2 = 1.672 < \chi^2_{0.05,1} = 3.84$). Genetic analysis from different combinations indicated that the Lix mutant is a monogenic, dominant mutant, and the deformed leaf trait in the Lix mutant was controlled by an incomplete dominance gene, temporarily named *Lix* (Table 1).

Genetic analysis of the Li_1 mutant

In a previous study, the Li_1 mutant was shown to be a monogenic dominant mutant. The dominant homozygote (Li_1Li_1) genotype is lethal, while the heterozygote (Li_1li_1) displays short fibers (no more than 6 mm long) and distorted leaf, stem, and flower growth (Narbuth and Kohel 1990; Rong et al. 2005). In our (Hai7124 × Li_1) F_1 combination, the segregation of 56 F_1 individuals fitted Mendelian 1:1 inheritance ($\chi^2 = 0.018 < \chi^2_{0.05,1} = 3.84$) (deformed leaf plants:
normal leaf plants, 1:1). The self-pollinated segregating populations comprised of 242 individuals from Li_1 mutants, and this consisted of 158 plants with the deformed leaf and stem phenotype and 84 plants with the normal leaf phenotype. These data fitted Mendelian 2:1 inheritance ($\chi^2 = 0.149 < \chi^2_{0.05,1} = 3.84$) and deviated Mendelian 3:1 inheritance ($\chi^2 = 11.658 < \chi^2_{0.05,1} = 3.84$). In the cross of [($Li_1$ × Hai7124)$_{(\text{deformed leaves})}$ × Hai7124] that generated 609 BC_1 plants, 308 plants had the deformed leaf and stem phenotype while 301 plants showed the normal leaf phenotype. These data fitted Mendelian 1:1 inheritance ($\chi^2 = 0.059 < \chi^2_{0.05,1} = 3.84$). In the boll opening stage, all individuals with the deformed leaf and stem phenotype displayed very short fibers, indicating the pleiotropic effects of Li_1 on vegetative and reproductive structures in cotton. The genetic analyses of the different combinations led us to draw the same conclusions as in a previous study (Narbuth and Kohel 1990), in that the Li_1 mutant is a monogenic dominant mutant and that the dominant homozygote (Li_1Li_1) genotype is lethal.

Allelic analysis of Lix/Li_1 genes

Because Lix and Li_1 mutants are all controlled by an incomplete dominance gene *Lix*/Li_1, we crossed Li_1 and Lix mutants to produce an F_1 combination in 2011 (Table 1). Of the 81 F_1 individuals, 50 plants had deformed leaves and stems while 31 plants showed the normal leaf phenotype. Of the 50 plants with deformed leaves and stems, 23 had similar morphology to the Lix mutant while 27 showed similar morphology to the Li_1 mutant. To

Table 1 Chi-squared test for the segregation of cotton deformed leaf phenotype caused by the *Lix/Li₁* genes

Cross/generation	Total	No. super-deformed leaves plants	No. deformed leaves plants	No. normal leaves plants	Expected ratio	χ^2	Pr
Lix⊗	1,193	284	588	321	1:2:1	2.537	0.25–0.75
(TM-1 × Lix)F_1	167	–	84	83	1:1	0.000	0.90–0.99
(Hai7124 × Lix)F_1	585	–	279	306	1:1	1.156	0.25–0.75
(TM-1 × Lix)$_{(deformed\ leaves)}$⊗	262	71	136	55	1:2:1	2.336	0.25–0.75
(Hai7124 × Lix)$_{(deformed\ leaves)}$ ⊗	263	58	133	72	1:2:1	1.525	0.25–0.75
[(Lix × Hai7124)$_{(deformed\ leaves)}$ × Hai7124]	469	–	220	249	1:1	1.672	0.10–0.25
Li_1⊗	242	–	158	84	2:1	0.149	0.25–0.75
(Hai7124 × Li_1)F_1	56	–	29	27	1:1	0.018	0.75–0.90
[(Li_1 × Hai7124)$_{(deformed\ leaves)}$ × Hai7124]	609	–	308	301	1:1	0.059	0.75–0.90
(Lix × Li_1)F_1	543	–	348	195	2:1	1.624	0.20–0.30

further identify the allelic relationship *Lix/Li₁* genes, we enlarged (Lix × Li_1) F_1 population and selfed 50 plants with the deformed leaf and stem phenotype and 10 plants with the normal leaf and stem phenotype in (Lix × Li_1) F_1 to produce $F_{2:3}$ plant lines. 462 F_1 individuals in new (Lix × Li_1) F_1 combination were obtained, with 298 plants showing deformed leaves and stems and 164 plants showing the normal leaf phenotype. Integrating the phenotypes of the 81 F_1 individuals in (Lix × Li_1) F_1 in 2011 summer, statistics analysis showed that the data fitted Mendelian 2:1 inheritance ($\chi^2 = 1.624 < \chi^2_{0.05,1} = 3.84$) and deviated Mendelian 3:1 inheritance ($\chi^2 = 16.782 < \chi^2_{0.05,1} = 3.84$) (Table 1). In 50 self-pollinated plants with the deformed leaf and stem phenotype in (Lix × Li_1) F_1, 23 $F_{2:3}$ plant lines, which F_1 had similar morphology to the Lix mutant, showed three types of leaf traits: normal, deformed, and super-deformed, and 27 $F_{2:3}$ plant lines, which F_1 had similar morphology to the Li_1 mutant, showed two types of leaf traits: normal and deformed. However, 10 $F_{2:3}$ plant lines, which F_1 had normal leaf phenotype, all showed the normal leaf trait. This result shows that the F_1 progeny of Lix crossed with Li_1 contained three genotypes (*lixlixli₁li₁*, *Lixlixli₁li₁*, *lixlixLi₁li₁*) indicating that *Lix* and *Li₁* were non-allelic. The pyramiding of *Lix/Li₁* in the same genotype (*LixlixLi₁li₁*) is impossible and should be lethal just like *lixlixLi₁Li₁*.

Molecular mapping of *Lix* and chromosome confirmation of *Li₁/Lix* genes

A [(Lix × Hai7124)$_{(deformed\ leaves)}$ × Hai7124] backcross population was constructed for the molecular mapping of *Lix*. Based on our laboratory's saturated genetic linkage map information from *G. hirsutum* × *G. barbadense*, SSR markers at ca. 10 cM intervals in each chromosome were used to screen for polymorphisms between Hai7124 and Lix parent plants. Of these markers, 120 primer pairs with polymorphisms were selected to screen 94 randomly selected members of the BC_1 segregating population from the [(Lix × Hai7124)$_{(deformed\ leaves)}$ × Hai7124] cross. Further, the marker genotypes were recorded and linkage analysis was performed. *Lix* was tentatively located on Chr. 4 by Join map analysis. Further molecular mapping was done using all of the polymorphic SSR markers anchored on Chr. 04 (Guo et al. 2008; Zhao et al. 2012). Ultimately, *Lix* was located between NAU7998 and NAU3469.

Based on the preliminary tagging results, 469 individuals from the BC_1 segregating population were further screened to obtain more accurately linked markers with *Lix*. Along with NAU7998 and NAU3469, 14 additional markers (linked with *Lix* on both sides of the interval from 25.0 to 86.7 cM of the preliminary map) were selected to screen the enlarged BC_1 population. Chi-square tests showed that thirteen markers fitted the Mendelian segregation model except for NAU3469, which deviated from Mendelian inheritance. Finally, *Lix* was located on Chr. 04 between NAU8376 and NAU3469 by genetic distances of 6.4 and 24.5 cM, respectively (Fig. 2). No closer markers flanking *Lix* were tagged using the enlarged BC_1 population.

In a previous report, *Li₁* was located on Chr. 22 by SSR marker analysis (Karaca et al. 2002). Subsequently, a more detailed mapping of *Li₁* was carried out using the closest markers flanking *Li₁*, Gate4CA09 and Coau1J04 which are located 2.7 and 1.3 cM away, respectively (Rong et al. 2005). Both Chr. 04(A4) and Chr. 22 (D4) are homoelogous chromosomes in tetraploid cotton. Integrating the morphological, genetic and allelic analysis of Lix/Li_1 mutants, we propose that Lix and Li_1 mutants have similar mutated morphology, and *Lix* is mapped to a homoelogous chromosome carrying *Li₁*. The study for further cloning *Lix/Li₁* and understanding their functional roles in allotetraploid cotton is being processed.

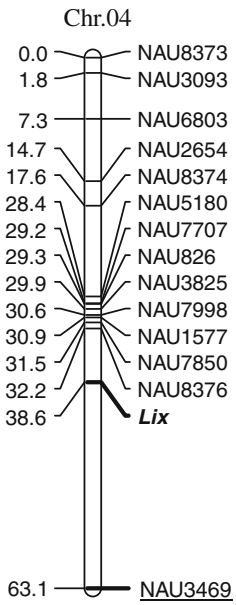

Fig. 2 Molecular mapping of *Lix* gene. The *underlined marker* indicates deviated loci

Discussion

The cotton genus comprises of ca. 50 species. Of these, there are five allopolyploid species, including two domesticated allopolyploids, *G. hirsutum* and *G. barbadense*. Polyploidy can result in gene duplications and this can be an origin of evolutionary novelty. Allopolyploid cotton species are believed to have formed about 1–2 million years ago by hybridization between a maternal Old World diploid A-genome (*Gossypium herbaceum*) and paternal New World diploid D-genome (*Gossypium raimondii*) (Wendel 1989). So, there are two homologs in tetraploid cotton species representing gene descendants from the A- and D-genome donors at the time of polyploidy formation. In general, the evolutionary fate of duplicate genes leads to functional diversity following mutation in the coding and/or regulatory regions (Moore and Purugganan 2005). In detail, when duplicate genes play their functional roles, they can follow one of three evolutionary paths. First, one copy may evolve into a nonfunctional pseudogene (Kondrashov and Kondrashov 2006). Second, the multiple copies can contribute to an increase in gene expression level (Lynch and Force 2000) or both copies can suffer mutations, but the combined action of both gene copies is necessary to maintain original function and expression levels (subfunctionalization) (Clark 1994). Third, one copy may gain a novel beneficial function (neofunctionalization) that is selectively maintained within the genome (Adams et al. 2003; Blanc and Wolfe 2004). To elucidate the function and expression mechanism of duplicate genes in polyploids, mutants with special phenotypes can be useful resources to trace back to gene structure and function. In the present study, we found a novel mutant (Lix) from the progeny of transgenic cotton, which exhibited similar morphology to the Li_1 mutant. Genetic analysis and molecular mapping showed that the two mutants are monogenic dominant mutants that are characterized by short fibers and distorted leaf, stem, and flower growth; however, the dominant homozygote Li_1Li_1 genotype is lethal, but *LixLix* plants are viable though reproductive growth is inhibited. So, the Li_1 gene located on the D-subgenome that controls the distorted vegetative and reproductive traits has a more important function than the *Lix* gene located on A-subgenome of tetraploid cotton. Further cloning and functional analysis of Li_1/*Lix* using Li_1/Lix mutants could reveal the relationship between these two genes in terms of structure and function.

Identifying and cloning novel genes using mutants has been widely applied in functional genomics research, especially to understand the biological significance and evolution of polyploidy. Auxin is a signal factor that activates a series of downstream signal pathways that play important roles in many aspects of plant growth and development, such as apical dominance, tropism, and lateral root and flower formation (Friml 2003). In cotton, auxin plays an important role in fiber development (Lee et al. 2006; Zhang et al. 2011); however, the exact role auxin plays in cotton vegetative and reproductive development needs to be clarified. In a previous study, the Li_1 mutant was found to exhibit the deformed leaf and stem trait, and this is similar to the polar auxin transport mutant of *Arabidopsis* where impaired polar auxin transport in the Li_1 mutant was shown to inhibit its vegetative and reproductive development (Zhu et al. 2003). Using this knowledge, we speculate that the function of Li_1/*Lix* might be related with polar auxin transport. Using Li_1/Lix mutants, we hope in future to isolate these two genes, so to elucidate their molecular mechanisms by analyzing the DNA variation in their coding and/or regulatory regions. This should allow us to further understand the relationship between polar auxin transport and the deformed leaf/stem and short fiber phenotype, and to clarify the mechanism behind the lethality of the dominant homozygote.

Genetic analysis and molecular mapping of mutants is a first step toward the isolation and cloning of the genes. Fine mapping of objective genes is a foundation for map-based cloning. In a previous study, the genetic mapping of Li_1 gene located it to Chr. 22 (Rong et al. 2005). The region containing Li_1 had an unusually high marker density, with the closest markers flanking Li_1, Gate4CA09 and Coau1J04, located just 2.7 and 1.3 cM away, respectively (Rong et al. 2005). In this present study, we elucidated that the two genes, *Lix* and Li_1, were incomplete dominance genes and were located on the A4/D4 homoelogous chromosomes in tetraploid cotton. However, molecular mapping of *Lix* with the closest markers, NAU8376 and

NAU3469, delimited it to a region of 30.9 cM in length on the Chr. 04, even after using all the polymorphic markers on Chr. 04 from our latest detailed genetic linkage map of *G. hirsutum* × *G. barbadense*. Further, we continuously checked other three high-density genetic maps composed of more than two thousand loci in cotton reported previously (Rong et al. 2004; Yu et al. 2011; Yu et al. 2012), and screened polymorphic SSR loci neighboring NAU8376 and NAU3469 on Chr. 04, however, no closer new markers flanking *Lix* were tagged. This suggests that there is a higher level of polymorphism in Chr. 22 than in Chr. 4 between *G. hirsutum* and *G. barbadense*. This also indicates that the A-subgenome may have experienced lower divergence rates than the D-subgenome during the evolution of different cotton species, which is similar to findings reported elsewhere (Guo et al. 2007b; Zhu et al. 2009). To elucidate the subfunctionalization of Li_1/*Lix* based on a map-based cloning method, we suggest focusing initially on isolating Li_1 and its regulatory regions by further fine mapping and using the flanking Li_1 genetic markers as probes to screen, and then, after functional confirmation, *Lix* and its regulatory regions can be cloned using a homologous method.

Recently, cotton whole-genome sequencing has entered into a calendar by international coalition of cotton genome scientists. A pilot study for the whole-genome scaffold sequence of a diploid cotton *G. raimondii* by the U.S. Department of Energy Joint Genome Institutes (http://www.jgi.doe.gov/) has been released at the end of 2011, and the draft genome of *G. raimondii* has been published recently (Wang et al. 2012). Based on whole-genome reference sequences, genomic resequencing will make it possible to directly map mutations responsible for phenotypes of interest. Using MutMap method, several agronomically important traits has been successfully harbored in rice (Abe et al. 2012). In the future, using Lix/Li_1 mutant and their corresponding recessive homozygote plant, it is hopeful to directly tag *Lix*/Li_1 by genomic resequencing and bioinformatics analysis. This will accelerate the process of cloning *Lix*/Li_1, and ultimately reveal the molecular relationship and functional roles of Lix and Li_1 in tetraploid cotton.

Acknowledgments This program was financially supported in part by National Science Foundation in China (30871558, 31101183), Natural Science Foundation in Jiangsu Province (BK2010438) and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

Abe A, Kosugi S, Yoshida K, Natsume S, Takagi H, Kanzaki H, Matsumura H, Yoshida K, Mitsuoka C, Tamiru M, Innan H, Cano L, Kamoun S, Terauchi R (2012) Genome sequencing reveals agronomically important loci in rice using MutMap. Nat Biotechnol 30(2):174–178

Adams KL, Cronn R, Percifield R, Wendel JF (2003) Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. Proc Natl Acad Sci USA 100(8):4649–4654

Blanc G, Wolfe KH (2004) Functional divergence of duplicated genes formed by polyploidy during *Arabidopsis* evolution. Plant Cell 16:1679–1691

Bolton JJ, Soliman KM, Wilkins TA, Jenkins JN (2009) Aberrant expression of critical genes during secondary cell wall biogenesis in a cotton mutant, Ligon lintless-1 (Li-1). Comp Funct Genomics. 659301. (Epub 2010 Jan 28)

Clark AG (1994) Invasion and maintenance of a gene duplication. Proc Natl Acad Sci USA 91(8):2950–2954

Endrizzi JE, Nelson R (1989) Linkage analysis and arm location of the open bud (ob_1) and Yellow Petal (Y_2) loci in chromosome 18 of Cotton. Genome 32:1041–1043

Endrizzi JE, Ray DT (1991) Linkage analysis of open bud (ob_2) and yellow petal (Y_1) in cotton. Genome 34:461–463

Frelichowski JE Jr, Palmer MB, Main D, Tomkins JP, Cantrell RG, Stelly DM, Yu J, Kohel RJ, Ulloa M (2006) Cotton genome mapping with new microsatellites from Acala 'Maxxa' BAC-ends. Mol Genet Genomics 275(5):479–491

Friml J (2003) Auxin transport—shaping the plant. Curr Opin Plant Biol 6(1):7–12

Griffee F, Ligon L (1929) Occurrence of lintless cotton plants and the inheritance of the character 'lintless'. Amer Soc Agron 21: 711–717

Guo W, Cai C, Wang C, Han Z, Song X, Wang K, Niu X, Wang C, Lu K, Shi B, Zhang T (2007a) A microsatellite-based, gene-rich linkage map reveals genome structure, function and evolution in *Gossypium*. Genetics 176(1):527–541

Guo WZ, Sang ZQ, Zhou BL, Zhang TZ (2007b) Genetic relationships of D-genome species based on two types of EST-SSR markers derived from *G. arboreum* and *G. raimondii* in *Gossypium*. Plant Sci 172(4):808–814

Guo W, Cai C, Wang C, Zhao L, Wang L, Zhang T (2008) A preliminary analysis of genome structure and composition in *Gossypium hirsutum*. BMC Genomics 9:314

Han ZG, Guo WZ, Song XL, Zhang TZ (2004) Genetic mapping of EST-derived microsatellites from the diploid *Gossypium arboreum* in allotetraploid cotton. Mol Genet Genomics 272(3):308–327

Han Z, Wang C, Song X, Guo W, Gou J, Li C, Chen X, Zhang T (2006) Characteristics, development and mapping of *Gossypium hirsutum* derived EST-SSRs in allotetraploid cotton. Theor Appl Genet 112(3):430–439

Karaca M, Saha S, Jenkins JN, Zipf A, Kohel R, Stelly DM (2002) Simple sequence repeat (SSR) markers linked to the Ligon lintless (Li_1) mutant in cotton. J Hered 93:221–224

Kearney TH, Harrison GJ (1927) The inheritance of smoothness seeds in cotton. J Agric Res 35:193–217

Killough DT, Horlacher WR (1933) The inheritance of virescent yellow and red plant colors in cotton. Genetics 18(4):329–334

Kohel RJ, McMichael SC (1990) Immature fiber mutant of upland cotton. Crop Sci 30:419–421

Kondrashov FA, Kondrashov AS (2006) Role of selection in fixation of gene duplications. J Theor Biol 239(2):141–151

Korolev N, Pérez-Artés E, Mercado-Blanco J, Bejarano-Alcázar J, Rodríguez-Jurado D, Jiménez-Díaz RM, Katan T, Katan J (2008) Vegetative compatibility of cotton-defoliating *Verticillium dahliae* in Israel and its pathogenicity to various crop plants. Eur J Plant Pathol 122:603–617

Lacape JM, Nguyen TB, Thibivilliers S, Bojinov B, Courtois B, Cantrell RG, Burr B, Hau B (2003) A combined RFLP-SSR-AFLP

map of tetraploid cotton based on a *Gossypium hirsutum* × *Gossypium barbadense* backcross population. Genome 46(4):612–626

Lee JJ, Hassan OS, Gao W, Wei NE, Kohel RJ, Chen XY, Payton P, Sze SH, Stelly DM, Chen ZJ (2006) Developmental and gene expression analyses of a cotton naked seed mutant. Planta 223(3):418–432

Lynch M, Force A (2000) The probability of duplicate gene preservation by subfunctionalization. Genetics 154(1):459–473

Mei M, Syed NH, Gao W, Thaxton PM, Smith CW, Stelly DM, Chen ZJ (2004) Genetic mapping and QTL analysis of fiber-related traits in cotton (Gossypium). Theor Appl Genet 108(2):280–291

Moore RC, Purugganan MD (2005) The evolutionary dynamics of plant duplicate genes. Curr Opin in Pliant Biol 8:122–128

Narbuth E, Kohel R (1990) Inheritance and linkage analysis of a new fiber mutant in cotton. J Hered 81(2):131–133

Paterson AH, Brubaker C, Wendel JF (1993) A rapid method for extraction of cotton (*Gossypium* spp.) genomic DNA suitable for RFLP or PCR analysis. Plant Mol Biol Rep 11:122–127

Qian N, Zhang XW, Guo WZ, Zhang TZ (2009) Fine mapping of open bud duplicate genes in homoeologous chromosomes of tetraploid cotton. Euphytica 165:325–331

Rong J, Abbey C, Bowers JE, Brubaker CL, Chang C, Chee PW, Delmonte TA, Ding X, Garza JJ, Marler BS, Park CH, Pierce GJ, Rainey KM, Rastogi VK, Schulze SR, Trolinder NL, Wendel JF, Wilkins TA, Williams-Coplin TD, Wing RA, Wright RJ, Zhao X, Zhu L, Paterson AH (2004) A 3347-locus genetic recombination map of sequence-tagged sites reveals features of genome organization, transmission and evolution of cotton (*Gossypium*). Genetics 166(1):389–417

Rong J, Pierce GJ, Waghmare VN, Rogers CJ, Desai A, Chee PW, May OL, Gannaway JR, Wendel JF, Wilkins TA, Paterson AH (2005) Genetic mapping and comparative analysis of seven mutants related to seed fiber development in cotton. Theor Appl Genet 111(6):1137–1146

Song X, Wang K, Guo W, Zhang J, Zhang T (2005) A comparison of genetic maps constructed from haploid and BC_1 mapping populations from the same crossing between *Gossypium hirsutum* L. and *Gossypium barbadense* L. Genome 48(3):378–390

Song L, Guo W, Zhang T (2009) Interaction of novel Dobzhansky-Muller type genes for the induction of hybrid lethality between *Gossypium hirsutum* and *G. barbadense* cv. Coastland R4–4. Theor Appl Genet 119(1):33–41

Ulloa M, Meredith WR Jr (2000) Genetic linkage map and QTL analysis of agronomic and fiber quality traits in an intraspecific population. J Cotton Sci 4:161–170

Ulloa M, Meredith WR Jr (2002) Shappley ZW, Kahler AL. RFLP genetic linkage maps from four $F_{2.3}$ populations and a join map of *Gossypium hirsutum* L. Theor Appl Genet 104(2–3):200–208

Van Ooijen JW, Voorrips RE (2001) JoinMapR Version 3.0: software for the calculation of genetic linkage maps. CPRO-DLO, Wageningen

Voorrips RE (2002) MapChart: software for the graphical presentation of linkage maps and QTLs. J Heredity 93(1):77–78

Wang H, Guo Y, Lv F, Zhu H, Wu S, Jiang Y, Li F, Zhou B, Guo W, Zhang T (2010) The essential role of GhPEL gene, encoding a pectate lyase, in cell wall loosening by depolymerization of the de-esterified pectin during fiber elongation in cotton. Plant Mol Biol 72(4–5):397–406

Wang K, Wang Z, Li F, Ye W, Wang J, Song G, Yue Z, Cong L, Shang H, Zhu S, Zou C, Li Q, Yuan Y, Lu C, Wei H, Gou C, Zheng Z, Yin Y, Zhang X, Liu K, Wang B, Song C, Shi N, Kohel RJ, Percy RG, Yu JZ, Zhu YX, Wang J, Yu S (2012) The draft genome of a diploid cotton *Gossypium raimondii*. Nat Genet 44:1098–1103

Ware JO, Benedict LI, Rolfe WH (1947) A recessive naked-seed character in upland cotton. J Hered 38:313–320

Wendel JF (1989) New World tetraploid cottons contain Old World cytoplasm. Proc Natl Acad Sci USA 86(11):4132–4136

Wu Y, Machado AC, White RG, Llewellyn DJ, Dennis ES (2006) Expression profiling identifies genes expressed early during lint fibre initiation in cotton. Plant Cell Physiol 47(1):107–127

Wu C, Zhou B, Zhang T (2009) Isolation and characterization of a sterile-dwarf mutant in Asian cotton (*Gossypium arboreum* L.). J Genet Genomics 36(6):343–553

Yu JZ, Kohel RJ, Fang DD, Cho J, Van Deynze A, Ulloa M, Hoffman SM, Pepper AE, Stelly DM, Jenkins JN, Saha S, Kumpatla SP, Shah MR, Hugie WV, Percy RG (2012) A high-density simple sequence repeat and single nucleotide polymorphism genetic map of the tetraploid cotton genome. G3: Genes Genomes Genetics 2(1):43–58

Yin J, Guo W, Yang L, Liu L, Zhang T (2006) Physical mapping of the Rf1 fertility-restoring gene to a 100 kb region in cotton. Theor Appl Genet 112(7):1318–1325

Yu Y, Yuan D, Liang S, Li X, Wang X, Lin Z, Zhang X (2011) Genome structure of cotton revealed by a genome-wide SSR genetic map constructed from a BC1 population between *gossypium hirsutum* and *G. barbadense*. BMC Genomics 12:15

Zhang TZ, Pan JJ (1991) Genetic analysis of a Fuzzless-lintless mutant in *Gossypium hirsutum* L. Jiangsu J Agric Sci 7:13–16

Zhang J, Wu YT, Guo WZ, Zhang TZ (2000) Fast screening of microsatellite markers in cotton with PAGE/silver staining. Cotton Sci 12:267–269

Zhang J, Guo WZ, Zhang TZ (2002) Molecular linkage map of allotetraploid cotton (*Gossypium hirsutum* L. × *Gossypium barbadense* L.) with a haploid population. Theor Appl Genet 105:1166–1174

Zhang ZS, Xiao YH, Luo M, Li XB, Luo XY, Hou L, Li DM, Pei Y (2005) Construction of a genetic linkage map and QTL analysis of fiber-related traits in upland cotton (*Gossypium hirsutum* L.). Euphytica 144:91–99

Zhang DY, Zhang TZ, Sang ZQ, Guo WZ (2007) Comparative development of lint and fuzz using different cotton fiber-specific developmental mutants in *Gossypium hirsutum*. J Integ Plant Biol 49:1038–1046

Zhang M, Zheng X, Song S, Zeng Q, Hou L, Li D, Zhao J, Wei Y, Li X, Luo M, Xiao Y, Luo X, Zhang J, Xiang C, Pei Y (2011) Spatiotemporal manipulation of auxin biosynthesis in cotton ovule epidermal cells enhances fiber yield and quality. Nat Biotechnol 29(5):453–548

Zhao L, Lv YD, Cai CP, Tong XC, Chen XD, Zhang W, Du H, Guo XH, Guo WZ (2012) Toward allotetraploid cotton genome assembly: integration of a high-density molecular genetic linkage map with DNA sequence information. BMC Genomics 13:539

Zhu YQ, Xu KX, Chen XY (2003) Impaired polar auxin transport in cotton li mutant. J Plant Physiol and Mol Biol 29(1):15–20

Zhu HY, Zhang TZ, Yang LM, Guo WZ (2009) EST-SSR sequences revealed the relationship of D-genome in diploid and tetraploid species in *Gossypium*. Plant Sci 176(3):397–405