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ORIGINAL PAPER

Interaction of novel Dobzhansky–Muller type genes for the induction of hybrid lethality between *Gossypium hirsutum* and *G. barbadense* cv. Coastland R4-4

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Abstract Hybrid lethality was identified in interspecific hybrids between two cotton species, *Gossypium hirsutum* and *G. barbadense* cv. Coastland R4-4 (R4-4). Genetic analysis indicated that the lethal symptom was controlled by two dominant complementary genes, one from *G. hirsutum* and another from R4-4. Microsatellite mapping identified the location of the causal gene in *G. hirsutum* as chromosome D8, while the R4-4 gene was placed on chromosome D11. Our data indicate that these genes conform to the Dobzhansky–Muller model, and are novel for the induction of hybrid lethality in *Gossypium*. Following the genetic nomenclature, we propose that the two novel Dobzhansky–Muller genes from *G. hirsutum* and from R4-4 be named Le_3 and Le_4, respectively. Given what we know about their inheritance patterns, their genotypes should be $Le_3Le_3le_4le_4$ in *G. hirsutum*, and $le_3le_3Le_4Le_4$ in R4-4. Data from this study supported previous information in that expression of the lethal symptom might be affected by the dosage of causal alleles and the environment in which plants are growing.

Introduction

One of the classic definitions of a species is reproductive isolation, which is usually enforced by some physical, biochemical or behavioral mechanism. These restrictive mechanisms can be classified into two groups: prezygotic and postzygotic. Hybrid lethality occurs because of postzygotic mechanisms that maintain species integrity and reproductive isolation (Stebbins 1966). Phenotypic manifestations of hybrid lethality include cell death, tissue necrosis, wilting, yellowing, reddening, chlorosis, dwarfism and reduced growth rate. Often these manifestations lead to actual death, although in a small number of cases the individual hybrids do reach reproductive maturity. Few, however, are sufficiently vigorous to reproduce. This phenomenon has been observed in F_1 hybrids, as well as later generations. Hybrid lethality, and the implications for speciation, have been described in the interspecific and intraspecific hybrids of several animals (Provine 1991; Sasa et al. 1998; Presgraves 2002; Price and Bouvier 2002) and plants (Hollingshead 1930; Wiebe 1934; Caldwell and Compton 1943; Savant 1956; Saunders 1952; McNaughton and Harper 1960; Tsunewaki 1970; Chu and Oka 1972; Sato and Hayashi 1983; Christie and MacNair 1984; Singh and Gutiérrez 1984; Valkonen and Watanabe 1999; Yamada et al. 2001; Mino et al. 2002; Moyle and Graham 2005; Marubashi and Tezuka 2006; Bomblies et al. 2007).

Several cases of hybrid lethality in *Gossypium* (cotton) have been reported. Hutchinson (1932) studied crosses between certain strains of *G. arboreum* var. *soudanensis* and a range of other *arboreum* and *herbaceum* strains. Silow (1941) reported hybrid lethality in *G. herbaceum* and other species including *G. arboreum* and *G. anomalun*. Hybrid lethality has also been recognized between certain strains of *G. hirsutum* var. *marie-galante* and *G. barbadense* (Stephens 1946), Asiatic *G. arboreum* (sanguineum) and upland strains of *G. hirsutum* (Gerstel 1954), *G. hirsutum* and *G. gossypioides* (Philips and Merritt 1972), *G. klotzchianum* ($D_{3\text{-k}}$) and other species containing A (two species), E (four species), F (one species) and other D genomes (Phillips and

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Reid 1975). In these cases, the hybrid phenotype resulted from the interaction of alleles at one or two loci in the parental genomes.

In the instance of *G. davidsonii*, Lee (1981a) suggested that the hybrid lethality resulted from allelic and interlocus interactions of Le_2^{dav} from *G. davidsonii* with Le_1 and Le_2 from *G. hirsutum* and *G. barbadense*. Le_1 and Le_2 were not linked to each other, but Le_2^{daw} was identified as a Le_2 allele. Monotelodisomic tests indicated the Le_2 locus was located in the long arm of chromosome D12, or within 5 cM of the centromere in the short arm (Stelly 1990). The Le_1 locus was found in the long arm of chromosome A12; its distance from the centromere was about 13 cM (Samora et al. 1994).

Clarification of reproductive isolating mechanisms will provide important information about biological speciation and genes that may be employed for targeted crop breeding. To this end, representative examples are identified to study the underlying genetic basis and, subsequently, to clone the genes involved in the process. In this study, we identified hybrid lethality between *G. hirsutum* and *G. barbadense* cv. Coastland R4-4 (hereafter referred to as R4-4). We conducted genetic analyses and mapped the causative genes using a detailed linkage map (Guo et al. 2007).

Materials and methods

Plant materials

The *G. barbadense* cv. Coastland R4-4 (R4-4) line and three inbred lines of *G. hirsutum* were used, including a dominant fuzzless seed mutant (N_1FLM), a recessive fuzzless seed mutant (n_2FLM) and the genetic standard upland stock, TM-1. The phenotypes and origins of the parents are presented in Table 1.

The three inbred lines of *G. hirsutum*, N_1FLM, n_2FLM and TM-1, were used as female parents in crosses with R4-4 at the Jiangpu Breeding Station of Nanjing Agricultural University (JBS-NAU), Jiangsu province of China. The F_1 plants were self-pollinated to produce the F_2s and backcrossed to develop the BC_1 on Hainan Island in China. In addition, TM-1 and N_1FLM, were used as male parents in crosses with R4-4 at JBS-NAU.

All the resulting F_1, F_2, and BC_1 plants as well as the parents were grown for field evaluations at JBS-NAU, and the numbers of plants are listed in Table 2. All these plants were grown in rows spaced 80 cm apart by row and 30 cm by plant. Field conditions consistent with those for normal cotton production were employed. Field evaluations for (N_1FLM × Coastland R4-4)F_2, (N_1FLM × Coastland R4-4)N_1FLM, (n_2FLM × Coastland R4-4)F_2, (n_2FLM × Coastland R4-4)n_2FLM, (N_1FLM × Coastland R4-4)F_1 and (n_2FLM × Coastland R4-4)F_1 along with their parents were conducted in 2006; other crosses as well as the parents were evaluated in 2007.

Phenotypic analysis

The phenotype was scored every 2 weeks beginning at the first true leaf stage and continuing to maturity of individual plants. Phenotypes were designated as lethal or normal, depending upon leaf appearance. Leaves of unusual appearance characterized by reddening and lethal necrosis on the surface and back, were assigned to the lethal phenotype. Normal green leaves were marked as the normal phenotype.

For all of the field tests, F_1 and the two parents were used as controls. The numbers of lethal and normal plants were counted in the F_2 and BC_1 populations. Segregation of lethal and normal plants was tested using a chi-square test to determine if the observed ratios fit Mendelian genetic models at the 5% level. Heterogeneity was assessed with chi-square to test if the segregation ratios are the same across different populations within F_2 and BC_1 progenies. The heterogeneity χ^2 value was obtained by subtracting the χ^2 value for the total number of plants from the sum of all the individual χ^2 values within F_2 or BC_1 populations, respectively. The heterogeneity degree of freedom was the number of crosses minus one within F_2 or BC_1 populations.

Table 1 Phenotypes and origins of the parents

Materials	Phenotypes	Genotypes	Species	Origins
Texas Marker 1, TM-1	Normal	$Le_3Le_3le_4le_4$	*G. hirsutum*	USDA-ARS, College Station, Texas, USA
Dominant fuzzless mutant, N_1FLM	Normal	$Le_3Le_3le_4le_4$	*G. hirsutum*	USDA-ARS, College Station, Texas, USA
Recessive fuzzless mutant, n_2FLM	Normal	$Le_3Le_3le_4le_4$	*G. hirsutum*	Cotton Research Institute, Nanjing Agricultural University (CRI/NAU)
Coastland R4-4, R4-4	Normal	$le_3le_3Le_4Le_4$	*G. barbadense*	Coastal Plain Experiment Station, Tifton, Georgia, USA

Table 2 Segregation rations for sensitivity to hybrid lethality

Population/cross	No. lethal plants	No. normal plants	χ^2	Probability %
(N_1FLM × R4-4)F_1	105	0	–	–
(n_2FLM × R4-4)F_1	38	0	–	–
(TM-1 × R4-4)F_1	32	0	–	–
(R4-4 × N_1FLM)F_1	42	0	–	–
(R4-4 × TM-1)F_1	29	0	–	–
(N_1FLM × R4-4)F_2	485	404	1.0370(9:7)	0.250–0.500
(n_2FLM × R4-4)F_2	229	165	0.5610(9:7)	0.250–0.500
(TM-1 × R4-4)F_2	133	91	0.8889(9:7)	0.250–0.500
Total	847	660	0.0013(9:7)	>0.900
Heterogeneity ($df = 2$)			2.4856	0.250–0.500
(N_1FLM × R4-4)N_1FLM	160	183	1.5423(1:1)	0.100–0.250
(N_1FLM × R4-4)R4-4	170	179	0.2321(1:1)	0.500–0.750
(n_2FLM × R4-4)n_2FLM	132	107	2.6151(1:1)	0.100–0.250
(TM-1 × R4-4)R4-4	181	224	4.5654(1:1)	0.025–0.050
(TM-1 × R4-4)TM-1	207	248	3.6945(1:1)	0.050–0.100
Total	850	941	4.6237(1:1)	0.025–0.050
Heterogeneity ($df = 4$)			8.0257	0.050–0.100

DNA extraction, PCR amplification, and electrophoresis

Cotton genomic DNA extraction followed the procedure of Paterson et al. (1993). Simple sequence repeat polymerase chain reaction (SSR-PCR) amplifications were performed using a Peltier Thermal Cycler-225 (MJ Research, USA). Electrophoresis of the products was conducted as described by Zhang et al. (2000, 2002).

Molecular mapping of the hybrid lethality genes

In comparison with the F_2 population, BC_1 populations are able to generate more genotypic information for linkage analysis since only one of the two necrosis genes actually segregates, making it easier to identify genotype. The two backcross populations (N_1FLM × R4-4)R4-4 and (N_1FLM × R4-4)N_1FLM, consisting of 349 and 343 individuals, respectively, were used to map the causal genes.

For the linkage maps, normal and lethal plants were scored as 1 and 3, respectively, and the parental genotype and the heterozygous genotype were scored as 1 and 3, respectively, in the two BC_1 populations. Missing data were designated as "–". The chi-square (χ^2) test was used to determine if normal segregation had occurred at the 5% level.

JoinMap 3.0 (Van Ooijen and Voorrips 2001) was employed to construct the genetic linkage map. Recombination frequency was converted to genetic map distance (centiMorgen, cM) using the Kosambi mapping function (Kosambi 1944). The linkage groups were isolated with log-of-odds (LOD) scores ≥10.

Results

Hybrid lethality in interspecific hybrids between *G. hirsutum* and R4-4

To map the fuzzless genes N_1 and n_2 in cotton, the two fuzzless seed mutants N_1FLM and n_2FLM, containing N_1 and n_2, respectively, were crossed with R4-4 with fuzzy seed. However, the lethal phenotype was detected in these F_1 hybrids. To further study the lethal symptom, a genetic standard line of Upland cotton, TM-1, was used for an additional cross with R4-4.

In the interspecific crosses between *G. hirsutum* acc. N_1FLM, n_2FLM and TM-1 with R4-4 (Table 2), all F_1 seeds initially germinated and developed normally. However, the lethal symptoms appeared in the F_1 plants during the pre-budding stage, beginning with the first true leaf. While the young leaves initially appeared normal, they quickly developed a reddish color between veins on both the top and back surfaces. Leaf color gradually changed to purple and the leaves began drooping. Finally, the mature leaves dropped, leaving naked stems behind (Fig. 1b).

The F_1 hybrids grown in Nanjing developed more severe lethal symptoms, and produced no flowers. However, the F_1 hybrid plants from Hainan Island (in southern China) were normal, and produced viable seed, allowing for additional research. The observed variability in the degree of lethal symptoms may be attributable to environmental conditions, as has been previously reported (Gerstel 1954; Philips 1977; Shii et al. 1980; Manabe et al. 1989; Yamada et al. 2000; Yamada and Marubashi 2003; Bomblies et al. 2007).

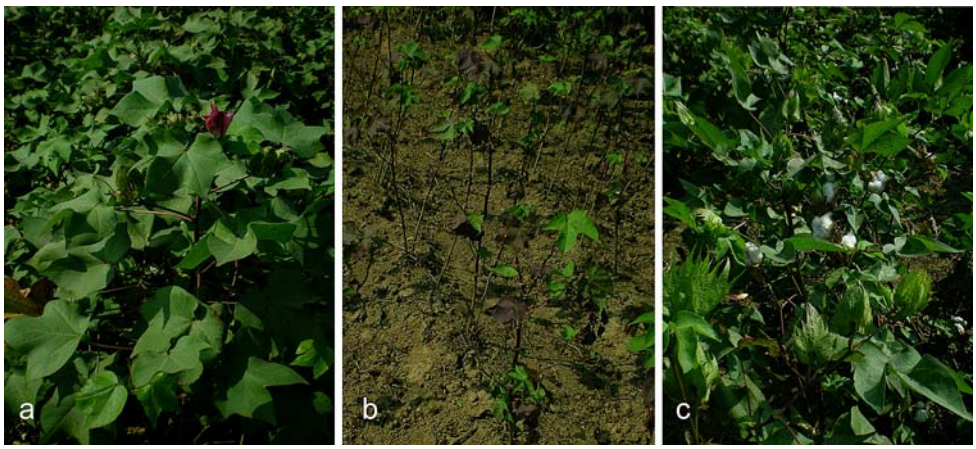

Fig. 1 Phenotype of N_1FLM, N_1FLM/R4-4 F_1 hybrid, and R4-4 at the open boll stage; **a–c** denote N_1FLM, N_1FLM/F_1 hybrids and R4-4, respectively

Genetic analysis of the lethal phenotype in the interspecific hybrids

To investigate the genetic mechanism(s) producing the lethal phenotype, three inbred lines, N_1FLM, n_2FLM and TM-1 in *G. hirsutum* and R4-4, along with their resulting F_1, F_2 and BC_1 progenies, were grown at JBS-NAU. The parents were normal, but in the interspecific crosses of N_1FLM × R4-4, n_2FLM × R4-4 and TM-1 × R4-4, all F_1 hybrids showed similar symptoms, indicating that the lethal characteristic was caused by an interaction of genes from *G. hirsutum* and R4-4 (Fig. 1). Their reciprocal F_1 hybrids, R4-4 × N_1FLM and R4-4 × TM-1, exhibited the same lethal phenotype as their respective original cross, indicating that the cytoplasmic genome had no impact on the symptoms.

The segregations of (N_1FLM × R4-4)F_2, (n_2FLM × R4-4)F_2 and (TM-1 × R4-4)F_2 fit a two dominant complementary genes model, with a 9:7 (lethal:normal) ratio. Likewise, the BC_1 population of (N_1FLM × R4-4)N_1FLM, (n_2FLM × R4-4)n_2FLM, (TM-1 × R4-4)TM-1 and (N_1FLM × R4-4)R4-4 segregated in a 1:1 (lethal:normal) ratio, as expected under the two dominant complementary genes model. There was a slight deviation ($\chi^2 = 4.5654$) in (TM-1 × R4-4)R4-4. Homogeneity tests indicated that populations within F_2 and BC_1 progenies were homogeneous (Table 2).

In these F_2 and backcross populations, some plants had more severe symptoms than the F_1 hybrids, and died at an early developmental stage, including the seedling stage (Fig. 2). The earlier the symptoms of lethality were manifested in the plants, the more severe they were. Symptoms occurred earlier in F_2 than in backcross populations. The variation of lethal symptoms within a population (Fig. 2) was probably caused by the dosage of causal alleles, as previously reported (Shii et al. 1980; Rooney and Stelly 1990; Chu et al. 2006; Bomblies et al. 2007; Ichitani et al. 2007).

All of these data indicate that the lethal phenotype in interspecific hybrids is controlled by two dominant complementary genes: one derived from the *G. hirsutum* parent and the other from R4-4 in *G. barbadense*. We did not know then if the genes identified in the present research are the same as Le_1 and Le_2 that were reported by Lee (1981a, b). Therefore, they were tentatively designated as Le_h from *G. hirsutum* and Le_b from *G. barbadense* cv. Coastland R4-4.

Molecular mapping of two dominant complementary genes responsible for the hybrid lethality

Because the lethal phenotype in interspecific hybrids is controlled by two dominant complementary genes, it is possible to segregate each one separately in backcross (BC_1) populations. To map Le_h from *G. hirsutum*, R4-4 was used as the recurrent parent to produce the backcross population, whereas, to map Le_b from *G. barbadense*, *G. hirsutum* cultivars were used as the recurrent parents.

Molecular mapping of Le_h derived from G. hirsutum

The (N_1FLM × R4-4) R4-4 backcross population, comprised of 349 individuals, was used to map the hybrid lethality gene Le_h from *G. hirsutum*. Earlier studies determined that the two genes, Le_1 and Le_2, interacted with an allele at the Le_2 locus–Le_2^{daw} to induce hybrid lethality; Le_1 and Le_2 were assigned to A12 (Chr. 12) and D12 (Chr. 26), respectively (Lee 1981b; Stelly 1990; Samora et al. 1994). Given this location information, SSR markers anchored on Chr. A12 and Chr. D12 (Guo et al. 2007) at about 10 cM intervals were used to screen 65 randomly selected plants from the (N_1FLM × R4-4) R4-4 population. Linkage analysis by JoinMap 3.0, however, failed to detect any marker on Chr. A12 and Chr. D12 that was linked to the hybrid lethality gene Le_h.

In order to quickly determine on which chromosome the Le_h gene was found, the bulked segregant analysis (BSA) method (Michelmore et al. 1991) was employed. Seven plants with lethal characteristics and seven normal plants

Fig. 2 Observed phenotypes in the F_2 population of the cross N_1FLM × R4-4 at the budding stage. **a** Wild type (wt); **b–f** lethal types

from the mapping population were selected to build the lethal and normal pools, respectively. Four hundred and thirty (430) SSR markers, positioned at about 10 cM intervals, were selected from all chromosomes (24) except A12 and D12 (Guo et al. 2007) to detect the polymorphism between the parents and DNA pools. The analysis identified three SSR markers—NAU2306, NAU738 and NAU2439—on Chr. D8 that were polymorphic between the two parents and two pools, suggesting that these three SSR markers may be linked to the hybrid lethality gene Le_h. The three SSR markers were further used to screen their association with the Le_h gene using the same 65 randomly selected individuals from the (N_1FLM × R4-4)R4-4 population. Linkage analysis indicated that Le_h was linked to these same three SSR markers on Chr. D8. Eleven of the SSR markers around Le_h from the linkage group in Chr. D8 (Guo et al. 2007) were then selected to analyze the whole population for fine mapping of the gene Le_h. The resulting map illustrated the position of Le_h relative to 11 neighboring markers on Chr. D8 (Fig. 3). The closest marker to Le_h was NAU2306, at a distance of 3.3 cM.

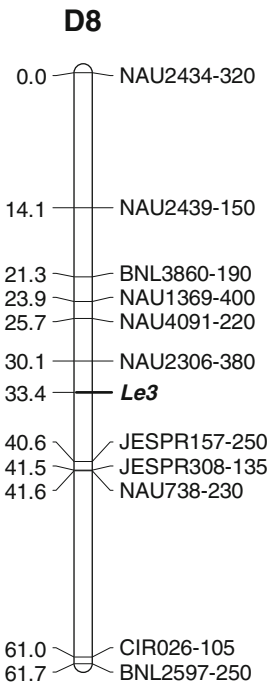

Fig. 3 Linkage map of the region of D8 surrounding Le_3 derived from *G. hirsutum* from analysis of the (N_1FLM × R4-4) R4-4 population

Molecular mapping of Le_b from R4-4

The (N_1FLM × R4-4)N_1FLM population, comprised of 343 individuals, was used to map Le_b, the complementary hybrid lethality gene from *G. barbadense*. Because Le_h was mapped on Chr. D8, another hybrid lethality gene, Le_y, was expected to be on Chr. A8, a homoeologous chromosome of Chr. D8. SSR markers anchored on Chr. A8 (Guo et al.

2007) at about 10 cM intervals were used to initially screen 93 randomly selected plants from the (N_1FLM × R4-4) N_1FLM population. Linkage analysis, however, failed to associate any marker on Chr. A8 with Le_b.

Using the BSA method (Michelmore et al. 1991), seven lethal plants and seven normal plants from the mapping populations were selected to build the lethal and normal pools, respectively. 455 SSR markers, drawn from all 25 chromosomes other than A8 (Guo et al. 2007), were selected at about 10 cM intervals to screen for polymorphism. Four markers, NAU3740, BNL3279, NAU2602 and NAU3889 anchored on Chr. D11, were found to be polymorphic between the two parents and between the two pools, suggesting that the Le_b gene might lie on Chr. D11. The four polymorphic markers were further used to screen their association with the Le_b gene with the same 93 randomly selected individuals from the backcross population (N_1FLM × R4-4) N_1FLM. As expected, the gene Le_b was linked to the four SSR markers on Chr. D11. Nine of the SSR markers located around Le_b from the linkage group in Chr. D11 (Guo et al. 2007) were selected to screen the 343 individuals for fine mapping of the gene Le_b. The resulting map illustrated the position of Le_b relative to nine neighboring markers on D11 (Fig. 4). The nearest marker to Le_b was BNL1154, at a distance of 4.1 cM.

The mapping of Le_h in *G. hirsutum* on D8 and Le_b in R4-4 on D11 indicates that these genes differ from Le_1 and Le_2, which are located on A12 and D12, respectively. Therefore, they are novel loci responsible for hybrid lethality in *Gossypium*. Based on standard genetic nomenclature (Kohel 1973), Le_h from *G. hirsutum* and Le_b from R-4 were tentatively designated as Le_3 and Le_4, respectively. The corresponding genotypes are $Le_3Le_3le_4le_4$ in *G. hirsutum*, and $le_3le_3Le_4Le_4$ in *G. barbadense* cv. R4-4.

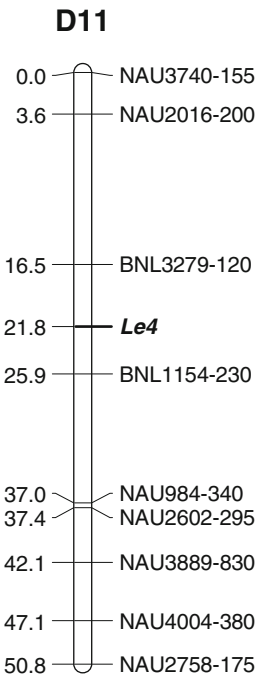

Fig. 4 Linkage map of the region of D11 surrounding Le_4 derived from *G. barbadense* from analysis of the (N_1FLM × R4-4) N_1FLM population

Discussion

Hybrid lethality in *Gossypium*

Hybrid lethality plays an important role in reproductive isolation and speciation (Orr and Presgraves 2000). To date, several cases of interspecific hybrids exhibiting lethality have been reported in cotton (Stephens 1946, Phillips 1976, Lee 1981a). Stephens (1946) reported the presence of lethal hybrids between certain strains of *G. hirsutum* var. *marie-galante* and *G. barbadense*. The hybrids have a characteristic bushy habit owing to their shortened internodes and excess production of lateral branches; leaves are inrolled and exhibit a yellowish mottling. The symptoms depend on the interaction of two dominant complementary alleles located on the same chromosome in the D-genome, carried by the *marie-galante* and the *G. barbadense* parents. Phillips (1976) characterized the lethal symptom as tumorigenesis. In addition, Lee (1981a) identified the lethal symptom in interspecific hybrids of *G. davidsonii* Kell. (D_3) and cultivated tetraploid cottons, *G. hirsutum* and *G. barbadense*. Lethality resulted from the intralocus and interlocus interaction of Le_2^{dav} (the D_3 complementary hybrid lethality system factor) with alleles at the Le_1 and/or Le_2 loci (Lee 1981a, 1981b). Linkage and monotelodisomic tests indicated the Le_2 locus was located in chromosome D12, and Le_1 in chromosome A12 (Stelly 1990; Samora et al. 1994; Wang et al. 2006).

In this study, hybrid lethality between *G. hirsutum* and R4-4 was characterized by leaf reddening and necrosis, leaving bare stems and leading to death in many cases. Through genetic analysis we were able to determine that the deleterious characteristics were controlled by two dominant complementary genes: one on D8 in *G. hirsutum* and the other on D11 in R4-4. Based upon a review of similar research in cotton, the interactions of these two genes appear to be a novel mechanism for the induction of hybrid lethality in these two taxa.

Dobzhansky–Muller genes act as an interspecific isolating mechanism between *G. hirsutum* and *G. barbadense* cv. Coastland R4-4

In our study, Le_3 and Le_4, the two dominant complementary genes from *G. hirsutum* and R4-4, interact to cause lethality in interspecific hybrids, a situation which supports the Dobzhansky–Muller model, where hybrid incompatibilities,

such as death or sterility, are caused by genes that have evolved from a common ancestor but diverged in each of the species. In the common ancestor, these genes may have worked in a complementary manner, but evolutionary changes have resulted in their current antagonism (Dobzhansky 1937; Muller 1942; Brideau et al. 2006; Bomblies and Weigel 2007).

Based on the Dobzhansky–Muller model, we suggest that *G. hirsutum* and R4-4 alleles evolved from a common ancestor with a genotype of $le_3le_3le_4le_4$. Through evolution, le_3 diverged in *G. hirsutum* to Le_3, and le_4 diverged in R4-4 to Le_4. While functioning well within the genome of each species, the Le_3–Le_4 hybrid interaction is detrimental. This hypothesis is consistent with previous propositions wherein *G. hirsutum* and *G. barbadense* diverged from the original AD tetraploid entity, and the AD tetraploid species ($2n = 4\chi = 52$) arose from interspecific hybridization between A- and D-genome diploid *Gossypium* species (Seelanan et al. 1997; Small et al. 1998).

Although *G. barbadense* and *G. hirsutum* are identified as two distinct species, most of the interspecific hybrids between these species are vigorous, and some hybrids are used as commercial varieties. A species can be defined as a group of actually, or potentially, interbreeding natural populations that are reproductively isolated from other such groups (Mayr 1942). In this study, we discovered one case of interspecific hybrid lethality between *G. hirsutum* and *G. barbadense* cv. Coastland R4-4, thus demonstrating the existence of mechanisms to discourage hybridization between them. Our data indicate the Dobzhansky–Muller genes, Le_3 and Le_4, act as an interspecific isolating mechanism between *G. hirsutum* and *G. barbadense* cv. Coastland R4-4.

Gossypium barbadense originated in South America (Hutchinson 1959). Until the 16th century, it was the primary fiber crop on the continent (Kerr 1960). The ancestral cotton plants were photoperiodic, and the fiber was medium staple (23.8–27.0 mm in length) and coarse, as typified by current Tanguis cottons of Peru. *Gossypium barbadense* first appeared in the United States ca. 1785, where it became known as Sea Island (Hutchinson 1959). Sea Island is characterized as long staple fiber. The exact origin of Sea Island is unknown, but Kerr (1960) and Stephens (1975, 1976) suggested that the most logical development path was by transgressive inheritance through the introgression of length genes from outside the species, possibly from *G. hirsutum*. Coastland, on the other hand, was developed from intraspecies crossing of three *G. barbadense* types: Sea Island, Egyptian and Tanguis (Jenkins 1953). We presume, therefore, that the hybrid lethality allele in the other cultivars of *G. barbadense* was eliminated or modified by selection, whereas in R4-4 it was maintained by crossing of the three *G. barbadense* types. If this is the case, the lethal combination evaluated in our study might be novel and provide additional insight into the divergence of *G. hirsutum* and *G. barbadense*.

In the present paper, the two novel Dobzhansky–Muller genes, Le_3 and Le_4, were both mapped on D-genome chromosomes. In the Dobzhansky–Muller model, the hybrid lethality genes are differentiated between the parent lineages. Evidence from several studies of *Drosophila* and *A. thaliana* suggest that hybrid lethality may arise as a by-product of adaptive evolution, since the few known Dobzhansky–Muller genes are all rapidly evolving (Coyne and Orr 2004; Orr 2005; Mallet 2006; Bomblies et al. 2007). The evolutionary rates and levels of nucleotide diversity are positively correlated (Hudson et al. 1987). We suppose that the differentiated hybrid lethality genes between *G. hirsutum* and R4-4 might be a result of differential evolutionary pressure acting on the two subgenomes. This hypothesis is strongly supported by previous reports that indicate the D-subgenome harbors greater nucleotide and allelic diversity than the A-subgenome in both *G. hirsutum* and *G. barbadense*, based on a comparison of duplicated paralogous *Adh* loci (Small and Wendel 2002; Small et al. 1999). However, an understanding of the evolution of Le_3 and Le_4 must await their cloning and identification of their biochemical function. Their fine mapping will help to clone them.

Molecular mechanisms of hybrid lethality

By clarifying the mechanism(s) associated with hybrid lethality, important insights into biological speciation and the general properties of postzygotic reproductive isolation are obtained. As an initial step to clone the causal genes following the Dobzhansky–Muller model, several genes have been positively located on linkage maps for many plant taxa, including wheat, rice and *Arabidopsis* (Chu et al. 2006; Ichitani et al. 2001, 2007; Bomblies et al. 2007). Some of the Dobzhansky–Muller genes in animals that have been cloned are the *Drosophila* Hybrid male rescue (*Hmr*) and its interacting partner Lethal hybrid rescue (*Lhr*) (Barbash et al. 2003; Brideau et al. 2006), and *Nucleoporin96* (*Nup96*) (Presgraves et al. 2003; Presgraves and Stephan 2007). Diverse functions are indicated for these genes. It has been speculated that the MYB-related gene *Hmr*, which is expressed at all stages during the life cycle, plays a role in normal gene regulation (Barbash et al. 2003). *Lhr* is an interacting partner of Heterochromatin Protein 1 (HP1) with, as yet, an unknown function (Brideau et al. 2006). *Nup96* encodes a structural protein of the nuclear pore complex (Presgraves et al. 2003). Common features of these genes are their rapid evolution (Coyne and Orr 2004; Orr 2005; Mallet 2006) and positive Darwinian selection (Barbash et al. 2003; Presgraves et al. 2003; Orr 2005;

Presgraves and Stephan 2007). In *Arabidopsis*, one allele of an *NB-LRR* gene interacts with another allele at the same locus to cause hybrid lethality (Bomblies et al. 2007). The *NB-LRR* genes are the most common type of plant disease-resistance genes. The known diversity of these genes has been interpreted as the result of positive selection (Bakker et al. 2006). If this is the case, however, it suggests the possibility that selective pressure exerted by pathogens can promote rapid evolution of gene variants that may prove beneficial to the parents, but deleterious to hybrid progeny. This link between hybrid lethality and disease-resistance pathways has also been suggested in tobacco (Masuda et al. 2007). Bomblies et al. (2007) and Bomblies and Weigel (2007) hypothesized that hybrid lethality could result from autoimmunity, perhaps as a pleiotropic effect of the evolution of genes that are involved in pathogen response.

Based upon the extant literature, it is apparent that studies of the molecular mechanisms underlying hybrid lethality have primarily focused on the model organisms, *Drosophila* sp. and *Arabidopsis thaliana*. Because the hybrid lethality mechanisms are potentially diverse, additional examples can only help to strengthen our understanding of the evolution of postzygotic reproductive isolation and speciation. In cotton, however, no genes causing hybrid lethality have been tagged with molecular markers, or cloned. The current study is the first occasion where two Dobzhansky–Muller genes have been located on the genetic linkage maps with SSR markers. The next step toward a more complete understanding of the key molecular mechanisms must be cloning, which will further uncover the structure, function, products and actions of the two causal genes.

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