

# Mapping and genomic targeting of the major leaf shape gene (*L*) in Upland cotton (*Gossypium hirsutum* L.)

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## Introduction

Cotton (*Gossypium* spp.) is the most important source of natural fiber in the world. Nearly all Upland cotton (*G. hirsutum*) cultivars possess the normal or broad leaf type. Along with normal (*l*), numerous mutant leaf types make up an allelic series at the leaf shape locus including okra (*L<sup>o</sup>*), sub-okra (*L<sup>u</sup>*), super-okra (*L<sup>s</sup>*), and sea-island (*L<sup>f</sup>*). The normal leaf shape in Upland cotton is broad and palmate with five readily observable, yet insipid lobes (Fig. 1). The okra leaf shape is characterized by reduced photosynthetic area per leaf, a more pronounced lobing pattern with deeper sinuses, and ectopic outgrowths leading to abnormal leaf margins (Fig. 1). Heterozygotes are intermediate between the two parental types and all three types can be easily categorized with brief visual observation (Fig. 1).

The advantages of okra leaf cultivars included reduced incidence of boll rot, accelerated flowering rates, early maturity, and increased resistance to whitefly and pink bollworm (1). Okra leaf cultivars also benefit from reduced lint trash, evapotranspiration, and chemical application rates due to their diminished leaf area. However, this reduced leaf area leads to sub-optimal light capture and reduced photosynthetic rates, causing higher rates of boll shedding and a lower yield potential under optimal conditions.

Despite its importance, the molecular and genetic control of leaf shape in cotton is poorly characterized. The factor underlying okra leaf shape is known to act early in leaf development, during the initiation of the primordia (2). Furthermore, the increase in leaf complexity seen in okra leaf cotton is phenotypically similar to the ectopic primordial expression of *KNOX1* genes in other species (3). Classical genetic analyses and mapping studies have placed the leaf shape locus on chromosome 15 (Chr15) of the D sub-genome of Upland cotton (4-5). The objective of the present study was to use an intraspecific *G. hirsutum* population to identify SSR and STS markers linked to the leaf shape locus. These markers will serve as the basis for expanded efforts currently underway to fine map and clone the leaf shape gene in cotton.

## Materials & Methods

The role of *KNOX1* genes in leaf shape variation in cotton was studied by semi-quantitative RT-PCR analysis. Primers were designed off of expressed sequence tags (ESTs) in the DFCI Cotton Gene Index with high similarity to *Arabidopsis KNOX1* genes. RNA samples were then collected from four different tissues types of the normal leaf genetic standard TM-1 and the okra leaf NC05AZ21. RNA was then converted to cDNA that was used as the template in PCR reactions in a semi-quantitative approach to measure mRNA copy number and, by extension, transcript levels of *KNOX1* genes.

For genetic analyses and molecular mapping of leaf shape, the okra leaf breeding line NC05AZ21 was crossed to the normal leaf landrace accession NC11-2100. A single F<sub>1</sub> plant was self-fertilized to obtain F<sub>2</sub> seeds. Leaf shape phenotype was scored on 236 F<sub>2</sub> plants and bulk leaf samples were collected from each individual as well as the parents. Genomic DNA was isolated from the leaf samples using a modified CTAB approach.

Forty SSR markers mapped to Chr15 in a high-density consensus (HDC) genetic map (6) were selected and evaluated for polymorphism between the two parents. Additionally 23 RFLP markers placed on Chr15 by the HDC were converted into 40 STS markers. Two candidate leaf shape genes present in the orthologous region of the sequenced *G. raimondii* genome delineated by the flanking SSR markers were also used to develop STS markers. All primers were labeled with the M13 tail sequence, amplified using a Touchdown PCR protocol and run on high-resolution 3% agarose gel as well as an ABI 3730XL capillary-based gel electrophoresis sequencer. The mapping software JoinMap 4.1 and a LOD score  $\geq 6.0$  were used to develop the linkage map for the leaf shape gene.

Linked SSR and STS markers from the present genetic map and markers in the genomic region of the *L* locus from the HDC (6) were used to establish the orthologous genomic region in the sequenced *G. raimondii* genome (7). Marker sequences were obtained and BLAST searched against the draft sequence of the *G. raimondii* genome. Highest scoring matches on *G. raimondii* Chr02, the homeolog of *G. hirsutum* Chr15, were used to order relevant markers in relation to one another using the Strudel software. The region encompassed by the closest proximal and distal markers, NAU2343 and Gh565 respectively, was annotated using the transcript, protein homolog, and gene ancestry features of the Phytozome *G. raimondii* genome.

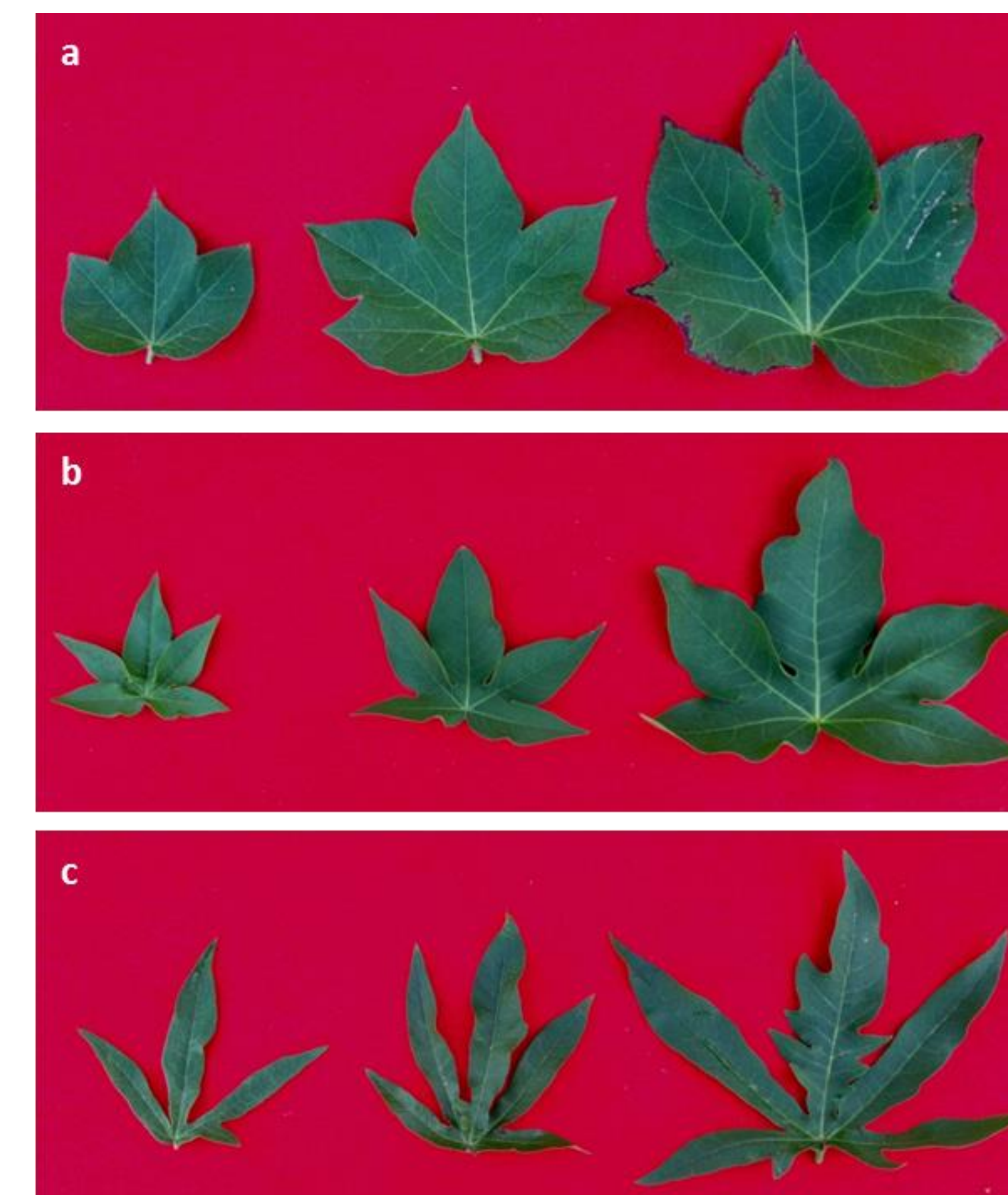
## Results and Discussion

The okra leaf NC05AZ21 showed expression of multiple putative *G. hirsutum KNOX1* genes later in leaf development than the normal leaf genetic standard TM-1 (Fig. 2). This differential expression was most noticeable during the leaf primordia stage of development (Fig. 2). The ectopic expression of *KNOX1* genes at this stage has been shown to result in increased leaf complexity in numerous species, including the increased leaf lobing and ectopic outgrowths that are characteristic of okra leaf cotton (3). These findings are in agreement with previous reports that the mechanism underlying okra leaf shape acts early on in leaf development (2).

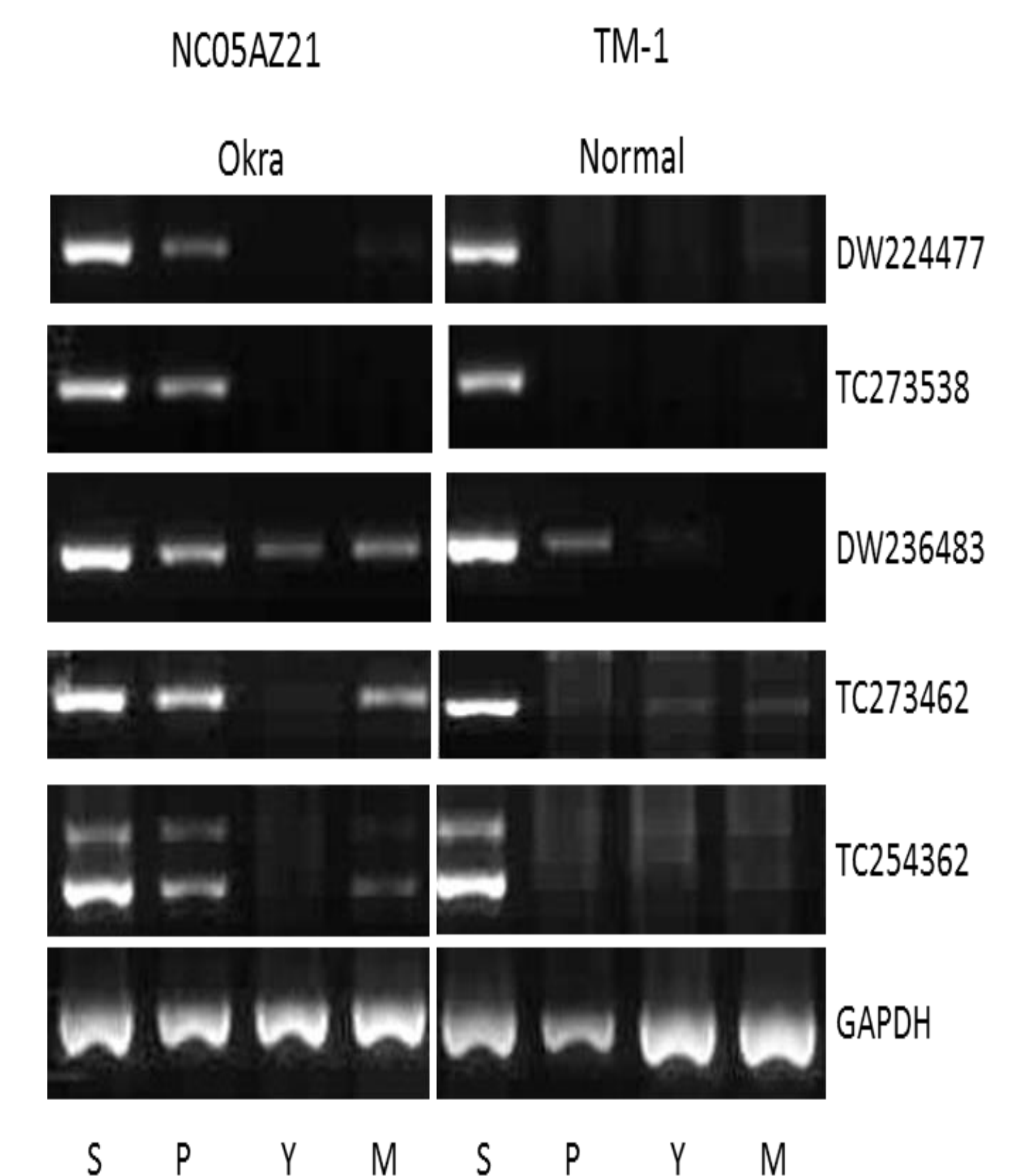
The phenotypic ratio of individuals within the population fit the expected 1:2:1 segregation ratio ( $\chi^2=0.31$ ,  $p=0.86$ ) confirming the single gene nature of okra leaf shape in cotton. Further, the alleles of the okra and normal leaf shape trait show an incompletely dominant phenotypic expression in the heterozygote. Of the 80 SSR and STS markers genetically mapped to Chr15, only five SSR markers were polymorphic, showing varying degrees of linkage to the leaf shape locus on Chr15 (Fig. 3). The two closest markers, Gh565 and NAU2343 mapped 2.6 cM distally and 2.8 cM proximally, respectively. Comparison of the *G. hirsutum* marker sequences to the *G. raimondii* draft genome indicates that the physical distance of the 5.4 cM region between Gh565 and NAU2343 is ~337kb and contains 34 putative genes (Fig. 3). Furthermore, the leaf shape locus (*L*) is mapped to a high-recombination, gene rich telomeric region of Chr15 in cotton (Fig. 3).

Of the 34 putative genes proposed to lie between the most closely linked SSR markers, only two (*Gorai.002G244000* and *Gorai.002G244200*) showed substantial homology to genes implicated in leaf shape. Both of these candidate genes show high sequence similarity to the known leaf shape modification gene *LMI1/ATHB51*. *In silico* mapping of candidate genes of the model plant leaf shape traits also showed that none of the *KNOX1* genes mapped in the genomic region of the *L* locus, indicating that *LMI1* genes were the likely candidates. Loss-of-function mutations to *LMI1* in *Arabidopsis* have been shown to lead to elevated expression levels of a *KNOX1* gene and leaf morphogenesis defects characteristic of ectopic *KNOX1* expression (8). Hence, *LMI1* likely serves as an upstream negative regulator of *KNOX1* gene expression (8). Furthermore, the STS marker 13-LS-195 developed from the candidate gene *Gorai.002G244000* showed co-segregation with the leaf shape phenotype in the F<sub>2</sub> population (Fig. 3). This indicates that 13-LS-195 is tightly linked to the leaf shape locus and that both *Gorai.002G244000* and *Gorai.002G244200* are strong candidates for the leaf shape gene in cotton.

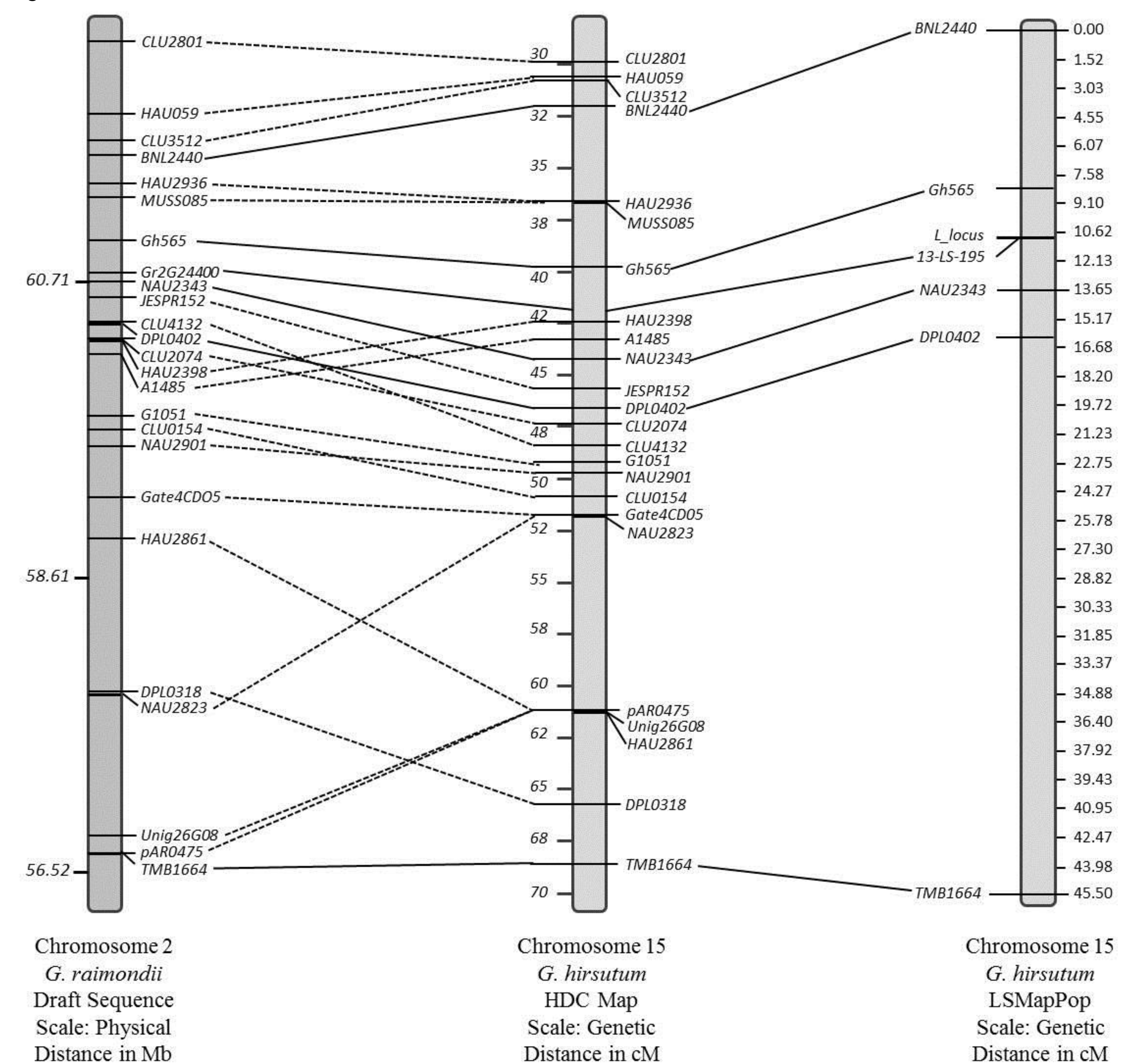
Taken together, the above findings and inferences indicate that modifications to a *LMI1*-like ortholog could possibly be the candidates for the leaf shape locus *L* in cotton. Identification and molecular characterization of the genes involved in leaf shape are essential prerequisites to elucidate the molecular mechanism controlling leaf shape and permit its manipulation for cotton cultivar improvement. Efforts are currently underway to fine map the leaf shape locus and definitively determine the underlying gene.



**Fig. 1** Leaf shape phenotypes of cotton from upper, middle, and lower nodes (left to right). **a** Normal-shaped leaves of the landrace accession NC11-2100. **b** Leaf shape of the F<sub>1</sub> heterozygote from NC11-2100 x NC05AZ21 that is intermediate between that of its parents. **c** Okra leaves of the breeding line NC05AZ21. Note the increase in leaf lobing and abundance of ectopic outgrowths.



**Fig. 2** Comparison of *KNOX1* gene expression between okra and normal leaf cotton. Okra leaf cotton shows later and stronger expression of multiple *KNOX1* genes during the development of leaves when compared with normal leaf cotton. S - shoot apical meristem (SAM)-enriched stem, P - leaf primordia, Y - young leaf, M - mature leaf.



**Fig. 3** Orthologous relationship between Chr02 of the *G. raimondii* draft sequence (7), Chr15 of the *G. hirsutum* HDC map (6), and Chr15 of the *G. hirsutum* map of the present study (LSMapPop) at the region encompassing the leaf shape locus in Upland cotton. The leaf shape gene (*L*) maps between SSR markers NAU2343 and Gh565 in the LSMapPop. The *LMI1* candidate gene (*Gorai.002G244000*) based STS marker 13-LS-195 co-segregates with the leaf shape gene in the LSMapPop. Markers that showed conserved order and relative distances across all three maps are indicated with solid lines

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