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DEVELOPMENT OF GENETIC LINKAGE MAP OF LEAF RED COLOUR IN COTTON (*GOSSYPIUM HIRSUTUM*) USING DNA MARKERS

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Abstract

Red leaf colour in cotton (*Gossypium hirsutum* L.) variety SL 7-9 and green leaf colour variety FH-634 was crossed to raise F_2 and F_3 segregating populations for RAPD and SSR analysis. A total of 400 RAPD and 54 SSR primers were used to find out DNA markers linked with the red leaf colour. Two RAPD primers (OPL-09 and OPZ-11) and three SSR primer pairs CM-43, CM-162 and JESPR-204 amplified polymorphic DNA fragments for leaf colour trait. These findings provide basic information about the inheritance of red colour in cotton using RAPD and SSR for linkage analysis and the construction of genetic linkage map. The value of framework markers will be enhanced if locus information (fragment sizes, restriction enzymes, primers, etc.) is fully explored in each map.

Introduction

Insect pests represent one of the most serious biotic constraints to crop production. In recent years, yield of cotton has become static rather it is declining due to the infestation of insect pests and diseases (Anon., 2001). One of the major factors of low yield is the infestation of insect pests. The cotton crop is handicapped due to 96 insect and mite pests (Younus *et al.,* 1980). Among these, *Pectinophera gossypiella* (cotton bollworm) and sucking pests such as *Bemisia tabaci* Genn. (whitefly), *Amrasca devastans* Dist.(cotton jassid), *Thrips tabaci* Lind. (thrips) and *Aphis gossyii* Glov (aphid) are very serious in Pakistan. The sucking insect pests are reported to cause 4.6 % loss in yield of seed cotton (Raza, 2000). Beside jassid, thrips infestation has frequently caused serious injury to young cotton plants (David, 1958). Whitefly is ranked the most serious pest and has been proved a vector for cotton leaf curl virus disease (Malik *et al.,* 1995). In 1996, the crop failure in Pakistan was related to whitefly damage, which appeared in epidemic form all over the country.

Red leaf colour confers resistance to the cotton bollworm and whiteflies, *Trialeurodes spp.* or *Bemisia spp.*, (Frisbie *et al*., 1994). Jones *et al*., (1978) found that red leaf colour was the first phenotypic trait of a cotton plant that was found to confer a measurable amount of *Anthonomus andis* Boheman (boll weevil), resistance in cotton. In non-preference studies using near-isogenic lines, Jones *et al*., (1978) showed that red plants were significantly less preferred by the boll weevil compared to their green isolines.

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Red plant colour is a morphological trait that could potentially be useful in cotton as a host plant resistance mechanism. Contrasted with the normal, green plant phenotypes, red cotton phenotypes vary widely, ranging from an intense, whole plant colour e.g., R1, R1dar, and Rd genotypes to a marginal Rm genotype leaf colour (Frisbie *et al*., 1994). Red plant colour is, however, associated with poor agronomic performance in cotton. Nevertheless, a compromise balancing the positive attributes of host plant resistance against the demerits of insecticide control measures may be attractive to cotton production economics. Genome research promises to promote continued and enhanced plant genetic improvement. As a world's leading crop and a model system for studies of many biological processes, genomics research of cotton has advanced rapidly in the past few years (Zhang *et al.*, 2008).

The development of near isogenic lines is a costly, tedious and time-consuming process. A little later, bulked segregant analysis (BSA) was proposed to overcome the non-availability of near-isogenic lines (Michelmore *et al.,* 1991). BSA has been extensively used for the identification of RAPD markers or AFLP markers linked to different genes (Eujayl *et al.,* 1998; Kondo *et al.,* 2000; Mackay & Caligari, 2000; Ni *et al.,* 2001).

Microsatellites have become the target sequences of choice for a wide range of applications in genetic mapping and genome analysis (Siu *et al.,* 2000; Mba *et al.,* 2001; Saal *et al.,* 2001; Chethana *et al.,* 2003; Saha *et al.,* 2003). They produced new insights into genetic relationship among closely and distantly related populations (Matsuoka *et al.,* 2002). Microsatellites are exceptionally useful because of highly variable nature among taxa, mainly due to a variable number of tandem repeats (Fisher *et al*., 1998) present in large number, and more or less evenly distributed throughout the genome. Moreover, these are probably nonfunctional and therefore selectively neutral.

The red leaf colour is an important feature of insect resistance in cotton. The objective of the present study was to construct the genetic linkage map for inheritance of red leaf colour trait in cotton using DNA markers.

Materials and Methods

Plant growth, DNA analysis and generation of bulks: Seeds of red leaf colour variety SL 7-9 and green leaf colour variety FH-634, kindly provided by K.N. Shah, Ayub Agricultural Research Institute, Faisalabad, Pakistan, were crossed to raise F_2 and F_3 segregating populations. The genomic DNA was extracted (Iqbal *et al*., 1997) for RAPD analysis.

Concentration of the DNA was measured spectrophotometrically from the absorbance ratio of the extracted genomic DNA at 260 nm and 280 nm. Quality of DNA was observed by running 50 ng DNA in 0.8% agarose gel. DNA bulks contrasting for red and green colour trait were used for RAPD analysis by mixing the equal amount of DNA from each individual.

RAPD analysis and PCR amplification: A total of 400 primers belonging to Operon series were used in PCR reactions. PCR was performed in reaction mixture of 25 µl containing 2.5 µl 10X [(750 mM Tris-HCl (pH 8.8)], 200 mM $(NH_4)_2SO_4$, 3 µl $MgCl_2$, (25 mM), 2.5 µl 0.001% gelatin, 1µl each of dATP, dCTP, dGTP, dTTP (2.5 mM), 2 µl primer (15 ng/µl), 3 µl of genomic DNA (15 ng/µl), 0.2µl (1 unit) *Taq polymerase* and 7.8 µl ddH_2O.

Amplicons were analyzed by electrophoresis in 1.2% agarose gel run in 0.5% TBE buffer and detected by staining with ethidium bromide (10 ng/100 ml of agarose solution in TBE). The gel was loaded with DNA markers of known molecular weight to determine the size of polymorphic fragments. The amplified products were photographed using the

Stratagene Eagle Eye Still Video System. Amplified fragments were scored but bands of less than 250 bp, which in some cases difficult to score, were not considered. The primers that amplified polymorphic DNA were used to amplify genomic DNA of individual plants from each contrasting bulk.

Microsatellite markers/SSR analysis: A total of 54 primer pairs custom-synthesized from GIBCOBRL (Life Technologies, USA) were used in SSR analysis. The micro-satellite primers used were: CM-25, CM-27, CM-29, CM-42, CM-43, CM-56, CM-60, CM-66, CM-67, CM-68, CM-162, JESPR=7, JESPR=14, JESPR=21, JESPR=55, JESPR=56, JESPR=58, JESPR=62, JESPR=64, JESPR=67, JESPR=84, JESPR=87, JESPR=89, JESPR=92, JESPR=102, JESPR=148, JESPR=152, JESPR=153, JESPR=160, JESPR=171, JESPR=176, JESPR=180, JESPR=185, JESPR=195, JESPR=204, JESPR=205, JESPR=210, JESPR=214, JESPR=215, JESPR=218, JESPR=220, JESPR=221, JESPR=223, JESPR=224, JESPR=228, JESPR=229, JESPR=230, JESPR=231, JESPR=232, JESPR=235, JESPR=236, JESPR=286, JESPR=291, JESPR=296, JESPR=297, JESPR=298, JESPR=300, JESPR=310

The PCR reaction mixture of 20 µl final volume was used for SSR analysis. The reagents included DNA 2 µl, dNTPs 6.4 µl, buffer 2 µl, $MgCl_2$ 1.6 µl, primer-F and primere-R 1 µl, Taq polymerase 2 µl and ddH_2O 5.8 µl. After PCR amplification, the concentration of amplicons was determined on 1.2 % agarose gel stained with ethidium bromide. Then loading concentrations for polyacrylamide gel electrophoresis (PAGE) were made according to the brightness of bands on 2% agarose gel. Linkage analysis was conducted by Mapmaker 3.0 software (Lander *et al.*, 1987). Map units were computed by applying the Kosambi function (Kosambi, 1944).

Results

RAPD markers for red leaf colour: In the present study, 400 RAPD primers were tested only two primers (OPL-09 & OPZ-11) produced fragments that clearly and consistently differentiated the red and green leaf coloured bulks. These primers produced markers $OPZ\text{-}11_{950}$ and $OPL\text{-}09_{960,}$ which were subsequently tested on all the individuals of the red leaf and green leaf colour populations. Figures 1 and 2 show the banding pattern of all red and green populations of these fragments. The polymorphic fragment amplified by the primer (OPL-09) in red population was 5 cM from the locus, while polymorphic band amplified by the primer (OPZ-11) in the green population showed a distance of 6.8 cM from OPL-09.

SSR Markers for red leaf colour: The primer pairs CM-43, CM-162 and JESPR-204 amplified polymorphic DNA fragments for leaf colour trait. The primer pair CM-43 amplified an allele a_1 approximately of 150 bp from the genomic DNA of plant leaf red colour while a_2 allele amplified around 145 bp (Fig. 3). The primer pair CM-162 amplified an allele of around 125 bp. However, a_2 allele of approximate size 105 bp was detected in green plants (Fig. 4). The primer pair JESPR-204 amplified a DNA fragment of 220 bp (a_1) linked with red leaf colour and of 200 bp (a_2) linked with the green leaf colour (Fig. 5). The SSR loci CM-43, CM-162 and JESPR-204 amplified polymorphic products among the parents were selected to amplify the genomic DNA of 40 plants each of F_2 red leaf colour and the green leaf colour.

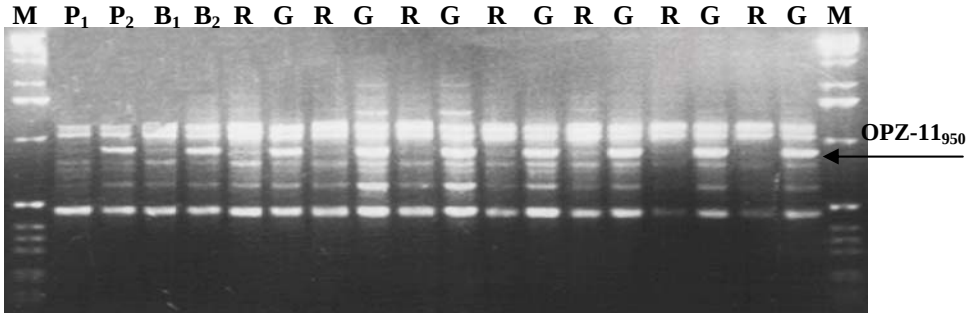

Fig. 1. RAPD marker amplified by the primer OPZ-11 linked with the red leaf colour locus: M=1 kb ladder, P_1=Red parents, P_2=Green parents, B_1=Red bulk, B_2=Green bulk, R=Red, G=Green.

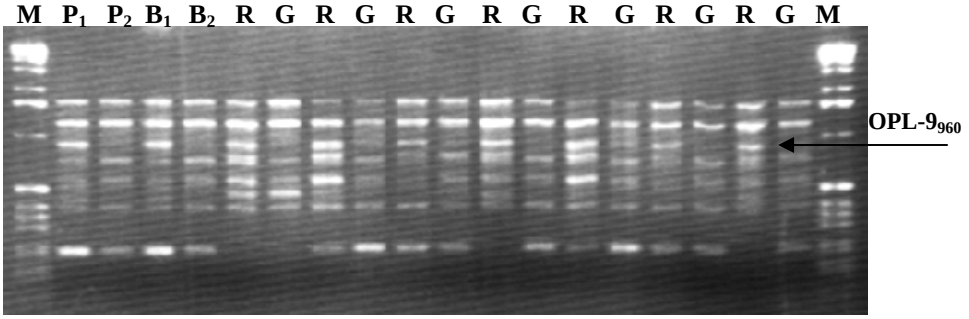

Fig. 2. RAPD marker amplified by the primer OPL-09 linked with the red leaf colour locus: M=1 kb ladder, P_1=Red parents, P_2=Green parents, B_1=Red bulk, B_2=Green bulk, R=Red, G=Green.

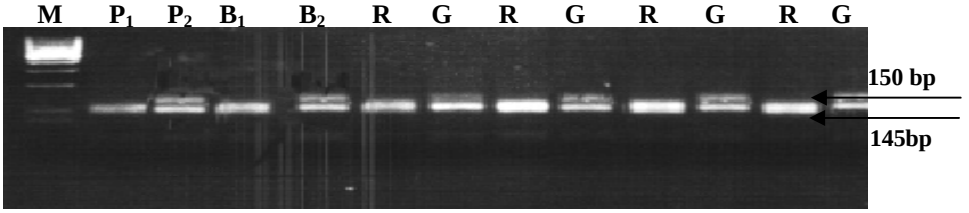

Fig. 3. SSR marker amplified by the primer pair CM-43: M=1 kb ladder, P_1=Red parents, P_2=Green parents, B_1=Red bulk, B_2=Green bulk, R=Red, G=Green.

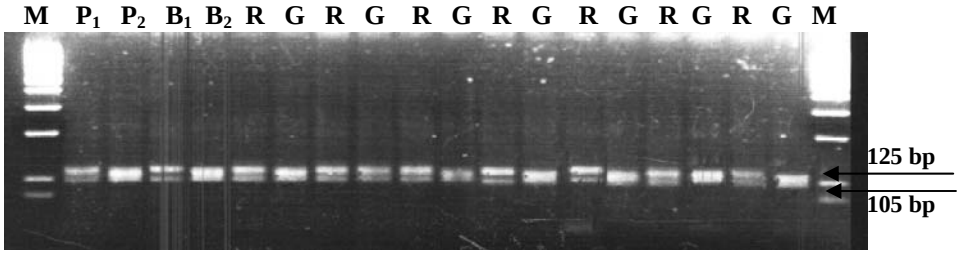

Fig. 4. SSR marker amplified by the primer pair CM-162: M=1 kb ladder, P_1=Red parents, P_2=Green parents, B_1=Red bulk, B_2=Green bulk, R=Red, G=Green.

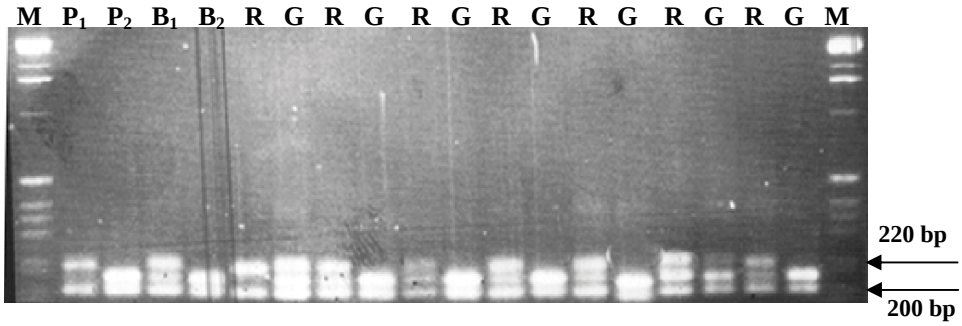

Fig. 5. SSR marker amplified by the primer pair JESRR-204: M=1 kb ladder, P_1=Red parents, P_2=Green parents, B_1=Red bulk, B_2=Green bulk, R=Red, G=Green.

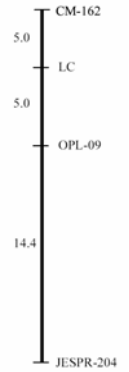

Fig. 6. Preliminary genetic linkage map of red leaf colour.

The primer pair CM-43 amplified DNA marker of 150 bp was present at locus in 37 out of 40 green plants and of 145 bp in 38 of 40 red plants with the recombination frequency of around 6 %. The primer pair CM-162 amplified a DNA fragment of size 125 bp linked with the red leaf colour was present in 37 out of 40 plants. The other DNA marker of allele size105 bp linked to the green leaf colour trait was present in 37 out of 40 individual sample plants showing the recombinant frequency of 6%.

The primer pair JESPR-204 amplified DNA band of size 220 bp ($a_{1)}$) in 37 of 40 homozygous red plants, while 200 bp ($a_{2)}$) in 31 out of 40 homozygous green plants .The recombination frequency with this primer pair was 15%.

Genetic linkage map for red leaf colour: Three SSR markers CM-43, CM-162 and JESPR-204 and two RAPD markers OPL-09_{960} & OPZ-11_{950} were initially identified in red and green population. Of these two markers, OPZ-11 and CM-43 were linked with each other at a map distance of 6.8 cM but seemed not linked with the trait of red colour. Two SSR markers CM-162 & JESPR-204 and one RAPD marker OPL-09_{960} linked with the red leaf colour were used to construct the preliminary genetic map for the red colour in cotton. SSR marker CM-162 was present at 5 cM and JESPR-204 at 19.4 cM from the locus responsible for red leaf colour. SSR marker CM-162 is linked to the SSR marker JESPR-204 at a distance of 24.4 cM on the linkage map. RAPD marker OPL-09_{960} is linked to the locus at a distance of 5.0 cM (Fig. 6).

Discussion

In the present study two DNA markers OPL-09_{960} and OPZ-11_{950} for leaf colour were identified using RAPD methodology in conjunction with BSA, after screening of 400 primers. This study is the first report of the identification of RAPD markers linked to the leaf colour. Benet *et al*., (1995), using a BSA approach found only 3 RAPD markers linked to black spot resistance in Chinese elm after testing 220 primers. Koller *et al*., (1994) identified only two primers out of 400 that showed PCR products using bulks for cultivar resistance to apple scab. Pomper *et al*., (1998) identified two primers after the screening of 250 that yielded the polymormphic pattern for the identification of self compatibility alleles in *Corylus avellana* L.

Diploidization and self-fertilization in many polyploids result in homozygosity for loci within each genome, a process known as an evolutionarily dead end or genetic "bottleneck" (Stebbins, 1971). As a result, genetic variation is extremely limited in polyploid species such as *G. hirsutum*, especially among elite cultivated types. Thus in the present study, the level of RAPD detected within *Gossypium* species is relatively low and only two polymorphic RAPD markers were found after the screening of 400 RAPD primers.

Bulked segregant analysis with RAPD has been exploited to discover DNA markers linked with different traits (Gardiner *et al*., 1996; Mackay & Caligari, 2000) and allele-specific markers (Bentolila *et al*., 1998). In the present studies, two primers OPO-07 and OPP-17 amplified polymorphic DNA bands in red leaf bulk, but could not be linked with the red colour trait. Warburton & Blisss (1996) reported "false positives" which occurred at a frequency of about 30% and could be identified as non-linkages by running RAPD assays on several of the individuals with each phenotype of a trait. In a false positive situation the band of interest was often amplified from individuals with both phenotypes, but is only expected to amplify from the individuals with either one of the two phenotypes, but not the other. OPL-09_{960} was closely linked to the leaf colour and was present at 5.0 cM from the R locus. This marker can be utilized in marker assisted screening for the leaf colour in cotton even in the cultivars with genetic background very different from that of the bulking population.

Microsatellite markers possess several important positive qualities for cotton genomic research. SSRs have been widely employed in genetic diversity analyses of cotton (Lacape *et al.*, 2007; Liu *et al.*, 2006; Rungis *et al.*, 2005; Zhang *et al.*, 2005) and several genetic linkage maps based mostly on SSRs have now been developed (Song *et al.*, 2005; Han *et al.*, 2004, 2006; Guo *et al.*, 2007).

In present study, a total of 54 SSR primer pairs were used, out of which three SSR markers CM-43, CM-162 and JESPR-204 were found to be linked with the trait of red leaf colour. The SSR primer CM-43, CM-162 and JESPR-204 yielded two loci. The multiple products are the result of multiple priming sites along the genome (Fisher *et al*., 1998; Rallo *et al*., 2000; Saal *et al*., 2001; Rahman *et al*., 2002). The phenomenon is quite common since microsatellite sequences may be associated with highly repetitive DNA (Smith & Devey, 1994). Multiple-locus amplification is particularly common in species with an allopolyploid origin, or may be related to genome fusion and chromosome duplication events during evolution (Buteler *et al*., 1999). If products from different loci could be distinguished, they would be very interesting for multilocus genotyping. Yet, most of the time the patterns obtained are complex due to competition and the overlapping of bands (Rallo *et al*., 2000).

The progress on cotton genome mapping is impeded by relatively large genomes, inadequate DNA markers, and the polyploidy of widely cultivated tetraploid cottons. In spite of these disadvantages, several research groups have developed linkage maps of allotetraploid and diploid cottons (Brubaker *et al*., 1999; Lacape *et al*., 2003; Reinisch *et al.*, 1994; Shappley *et al*., 1998; Ulloa & Meredith Jr. 2000; Zhang *et al*., 2002). However, except for an integrated map developed recently (Lacape *et al*., 2003), many maps are developed independently and provide approximately 10–85% coverage of the approximate 5,500-cM cotton genome (Lacape *et al*., 2003; Reinisch *et al*., 1994; Stelly 1993).

In this study, segregation data between the marker and the red colour gene were used to construct the linkage map. The marker $OPL09_{960}$ linked in the coupling phase was located 5.0 cM from the R gene. Two SSR markers CM-162 and JESPR-204 also in the coupling phase were located 5.0 cM and 14.4 cM, respectively, from the locus but are located at the distance of 24.4 cM from each other. The total map distance for leaf colour was 24.4 cM.

Gossypium hirsutum x G. barbadense backcross population consists of 888 loci, including 465 AFLPs, 229 SSRs, 192 RFLPs, and 2 morphological markers, ordered in 37 linkage groups that represent most, if not all of the 26 chromosomes, altogether spanning 4400 cM (Lacape *et al*., 2003). Genetic linkage maps have been previously developed from four different populations of *G. hirsutum* (Delta, Acala & Texas plain), two previously reported by Ulloa & Meredith Jr. (2000) and Shappley *et al*., (1998). The map from the four populations comprises 283 loci, from 201 cDNA probe/enzyme combinations and the locus for P1, mapped to 47 linkage groups with the average distance between markers of 5.3 cM, covering 1502.6 cM. Genetic linkage maps of tetraploid cotton with SSRs, and RAPDs contain 566 loci amalgamated into 41 linkage groups with at least 3 markers in each group. The map covered 5141.8 cM with a mean interlocus space of 9.08 cM (Zhang *et al*., 2004).

There is a need to develop additional markers and integrate linkage maps. AFLP in combination with RFLP and SSR analyses is considered to be highly effective in comparative genomic studies (Saliba-Colombani *et al*., 2000). Several cotton genetic maps have been developed using RFLPs, SSRs and RAPDs in the analysis of $F_{2.3}$ populations of *G. hirsutum* (Ulloa *et al*., 2002) and a doubled haploid (Zhang *et al*., 2002) or F_2 populations (Reinisch *et al*., 1994; Ulloa & Meredith Jr., 2000) derived from interspecific hybrids between *G. hirsutum* and *G. barbadense*. There is a need to integrate genetic maps independently developed using different mapping populations. Ulloa *et al*., (2002) successfully integrated genetic maps developed from four mapping populations of upland cotton using a set of framework RFLP loci.

Framework markers have provided an effective means for integrating several genetic maps in cotton (Lacape *et al*., 2003). RAPD and SSR for linkage analysis and the construction of genetic linkage maps, and the value of framework markers will be enhanced if locus information (fragment sizes, restriction enzymes, primers, etc.) is fully disclosed in each map.

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