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IDENTIFICATION OF DNA MARKER FOR NECTARILESS TRAIT IN COTTON USING RANDOM AMPLIFIED POLYMORPHIC DNA TECHNIQUE

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Abstract

Nectaries are nectar producing epidermal glands located on lower side of cotton leaves. Nectar is sugar rich food source for insect pests. The leaf nectaries provide nectar source long before the plants begin to flower and attract insects. Double recessive genotype produces nectariless phenotype which confers resistance to pink bollworm. Incorporation of double recessive trait would be easier if DNA marker for the trait is developed and used in marker assisted selection/backcrossing. Random Amplified polymorphic DNA technique was used to identify DNA markers linked with this trait. More than 300 RAPD primers were used to find linkage with the trait. Only one primer GLE-11 was found linked to the nectariless trait, this primer amplified a polymorphic DNA fragment of 1145bp between the parents and bulks. Three recombinants were observed out of 30 individual plants showing the presence of this marker at 10cM distance from the nectariless loci. This DNA marker was named as GLE-11_{1145}. This marker can be converted into SCAR and may be used in breeding.

Introduction

Insect/pests infestation is the main reason for lower cotton production. Pesticides are being used since long to save cotton crop from insect pest infestation. The application of pesticides not only deteriorates environment but also increases the cost of crop production. In Pakistan the import of pesticides has reached to 35299 metric tons with a cost of 12900 million rupees (Anonymous, 2005). The use of pesticides is expected to increase. These chemicals account for more than 50% of the total cost of cotton production. Pesticides used on cotton may enter human food chain through cottonseed oil used in processed foods and results in contamination of meat and milk of livestock fed on cotton seedcake. In addition to introducing insect resistant genes from other species there is still considerable value in conventional host plant resistant traits like hairiness, leaf shape, nectariless, gossypals, bract type etc. for many insect pests. Cotton plant possess floral, extra floral and leaf nectaries. *Gossypium tomentosum* is unique among tetraploid cotton, due to the reason that it is naturally devoid of leaf and extra floral nectaries. The trait was introduced into upland cotton from *Gossypium tomentosum* (Meyer &Meyer, 1961). Absence of nectaries deprived bollworm moths of the source of adult food resulting in lower egg deposition and in turn lower insect population in cotton field.

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Incorporation of nectariless trait is important in tailoring insect resistant cotton.

Flint *et al*. (1991) observed resistance to pink boll worm in nectariless and okra leaf cultivar (WC-12NL) compared to the nectaried and normal leaf one (DPL-61). Soares *et al*. (1998) reviewed the importance of nectariless trait in cotton and reported that 57.14% papers verified reduction in Lepidoptera on nectariless cotton. Platt & Stewart (1999) in a study of near isogenic nectaried and nectariless cotton lines observed significantly lower tarnished bugs in nectariless variety than in Bt and conventional varieties with nectaries. The nectariless trait has been reported to confer partial resistance to pink bollworm in cotton (Percy, 2001). Results of the study of Khan *et al.* (2002) also indicated that nectariless cotton was less affected by pink bollworm compared to nectaried cotton.

Nectariless trait is controlled by two recessive genes (Amir *et al*., 2002b). Manipulation and incorporation of traits especially, the recessive ones in a breeding programme would be easier if DNA markers for such traits could be identified and used in marker assisted introgression. The window for selection of having all the useful traits in one plant becomes limited when we select a plant with recessive trait having homozygous recessive genotype. The selection option would be wider and inbreeding can be avoided if DNA markers linked to the recessive genes could be identified. It would significantly help in breeding of insect pest resistant plants with combination of good yield and fibre quality. DNA markers facilitate incorporation of a recessive gene to a useful line through backcrossing in lesser time. In ordinary backcrossing the backcrossed population is selfed in each generation which results inbreeding depression in open pollinated species like cotton and hence reduce the chance of tailoring a vigorous cultivar. If we use DNA markers, we can identify plants with recessive alleles and there would be no need of selfing after each backcross.

With the invention of Polymerase Chain Reaction (PCR), the PCR based techniques like RAPD (Random Amplified Polymorphic DNA), SSR (Simple Sequence Repeats), AFLP (Amplified Fragment Length Polymorphism) etc. are preferred in DNA marker identification due to their ease in use (Malik *et al*., 2007). Among these RAPD is relatively less expensive and easier. Although RAPDs are dominant markers and are comparatively less reproducible but once it is identified, can be converted into more reliable, reproducible co-dominant markers like SCAR (Sequence characterized amplified regions). The objective of the present study was to develop DNA markers for nectariless trait in cotton through RAPD.

Materials and Methods

Contrasting genotypes, HRVO1= (Nectaried) and 3722LA-566= (Nectariless) were used in the study. The genotypes were grown for crossing during the normal crop season (May-November) of 2004 in the area of the Department of Plant Breeding and Genetics. The F_1 population was raised in the glasshouse in December, 2004. Sufficient number of flowers were selfed to produce pure seed for the development of F_2 population. The F_2 population was raised during the normal crop season of 2005. From the seed of individual F_2 plants, separate progeny rows of F_3 generation were raised in glasshouse. The objective to raise F_3 population was to select homozygous F_2 plants to be used in bulked segregant analysis.

Young leaves from the F_2 plants (150) and parents were collected early in the morning for DNA isolation. The leaves were transported to the laboratory in ice and

stored at -20 ^{o}C. The standard CTAB method was used for DNA extraction (Doyle & Doyle, 1990). DNA concentration in the samples was measured using spectrophotometer. Quality of DNA was observed by running 50 ng DNA on 0.8% agarose gel. The quantity of DNA was also confirmed by comparing with Quantification Standards Phage λ DNA (Fermentas) on 0.8% agarose gel. The DNA samples giving smear in the gel were rejected. Dilutions of 15ng/µl were prepared from the stock solutions. The DNA of homozygous F_2 plants was used in bulks. Heterozygous plants can be identified from homozygous ones in case of an incomplete dominant trait whereas, heterozygotes of the recessive traits cannot be identified from dominant homozygotes. Such homozygous and heterozygous F_2 plants were distinguished by growing progeny rows of individual F_2 plant seeds. The F_3 families showing no segregation for the trait were tagged and corresponding F_2 plant's DNA samples were used in PCR.

DNA bulk pairs were formed (Michelmore *et. al*., 1991) by mixing equal amount of DNA of nectaried and nectariless F_2 plants. The DNA of only homozygous F_2 plants (nectaried and nectailess) were used to construct bulks. Fifteen DNA samples of homozygous nectaried plants were used to form nectaried and fifteen DNA samples of homozygous nectariless plants were used to form nectariless bulk. These DNA bulks were then used as template in a PCR to find polymorphism linked with the trait.

Random amplified polymorphic DNA (RAPD) technique as described by Williams *et al*. (1990) was used to screen the parents, bulks and F_2 individuals from which bulks were constructed. First the PCRs were started for the parents (HRVO1 and 3722LA-566) to find the polymorphic primers. A total of 320 primers (160 from Gene link, USA, 120 from Genosys Biotechnologies UK and 40 from Operon Technologies, USA) were used in PCR. The PCR was performed in a volume of 25µl containing 2.5 µl 10X [(750 mM Tris-HCL (pH 8.8), 200 mM $(NH_4)_2SO_4$], 3µl $MgCl_2$ (25 mM), 2.5µl 0.001% Gelatin, 1µl each of dATP, dCTP, dTTP (2.5 mM), 2µl primer (15ng/µl), 2.5µl of genomic DNA (15 ng/µl), 0.2µl (1 unit) *Taq* polymerase and 8.3µl dH_2o.

Taq polymerase, together with buffer, $MgCl_2$ and dNTPs were purchased from MBI, Fermentas. Amplification was performed in Eppendorf DNA thermal cycler 9600 programmed for a first denaturation step of 5 min at 94^{o}C followed by 40 cycles of 1 minute at 94^{o}C, 1 minute at 36^{o}C and 2 min at 72^{o}C. The reactions were kept at 72^{o}C for 10 min for final extension step. To confirm that the observed bands were amplified from genomic DNA, and not primer artifact, genomic DNA was omitted from control reaction. Amplification products were analyzed by electrophoresis in 1.2% agarose gel in 0.5X TBE (Tris Borate EDTA) buffer and detected by staining with ethidium bromide. Before loading PCR products in the gel, 5 µl of bromophenol blue dye mixed with 10% glycerol, 0.1 M EDTA, and 2% SDS was added. Only 10µl of the reaction mixture was loaded on gel. A 100 bp DNA ladder from MBI Fermentas, USA with known molecular weight was loaded on both or either side of the gel to calculate the size/molecular weight of the polymorphic DNA fragments. Samples were electrophoresed for approximately two hours at 50 volts. After electrophoresis, the amplified products were viewed under ultraviolet transilluminator and photographed using the Syngene Gel Documentation System.

RAPD primers which showed polymorphism between the parents were used to screen the bulks constructed for the trait. The DNA fragments that were repeatedly present in one bulk and absent in the other were scored as polymorphic fragments. The primers that amplified polymorphic DNA were used to amplify genomic DNA of

individual plants from which the bulks were constructed. The polymorphic DNA fragment repeatedly present in one bulk and absent in the others was regarded as DNA marker linked to the trait for which the bulks were constructed. The DNA marker was named as the primer name with the size of the polymorphic fragment as subscript. The recombination frequency of the polymorphic fragment was considered as the distance of the marker in cM from the trait locus and was calculated as by Boora *et al.* (1998).

Results and Discussion

Parental (HRVO1 and 3722LA-566) survey was conducted using more than three hundred RAPD primers of Gene Link (GLA to GLS and Operon series). These RAPD primers generated thousands of DNA fragments/loci with an average of 5.40 loci per primer. Minimum number of bands/loci produced by a primer was one whereas maximum numbers of bands/loci amplified by a single primer were 10. Thirty six primers differentiated both the parents on the basis of their banding patterns. Amplification profiles of parents with some of the primers are shown in Fig-1. Assay reproducibility was improved by eliminating variation in DNA concentration and taking care to ensure consistent reaction conditions and thermal profile during amplification. All the reactions showing polymorphism among the parents were performed twice and only those primers giving repeatable amplification profiles were used in further studies on bulks and individual plant analysis.

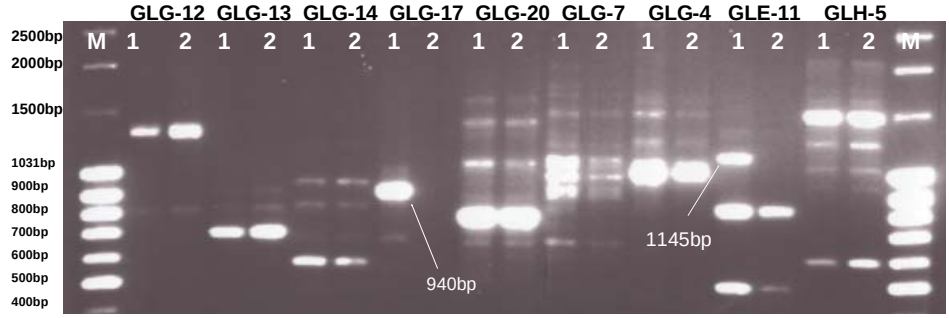

Fig. 1. PCR profile of parents, HRVO1 (1) and 3722LA-566 (2) with various RAPD primers. M is DNA ladder.

Some primers did not amplify the genomic DNA, indicating that they could not find homology with cotton DNA. Most of the primers amplified genomic DNA with similar profiles in parents showing the amplification of conserved sequences, present in both the genotypes. Iqbal *et al*. (1997) found that 98% of the primers amplified cotton genomic DNA, but the level of genetic divergence was quite low. Whereas, in the present study almost 11% of the RAPD primers distinguished two parents.

Lu & Myers (2002) studied relationships and discrimination of ten cotton varieties using RAPD and observed that 26% primers did not amplify, whereas, in the present study only 4.1% primers failed to amplify genomic DNA. Lu & Myers (2002) observed 5 DNA fragments per amplified primer. In the present study, almost similar number of fragments per primers (5.4) were observed. An average of 8.7 RAPD bands per primer

have been reported in sugarcane (Harvey *et al*., 1995) and 9 RAPD bands per primer in lettuce (Michalmore *et al.*, 1991). The range of fragments in the present study (1-10) was also similar to that of Lu & Myers (2002). Demeke *et al.* (1992) reported 8.3% of the RAPD bands as polymorphic in *Brassica.* In the present studies, few faint RAPD bands were also observed, which may be the result of amplification when primer template homology was not perfect. The chance of binding a primer with the target sequence not matching 100% would be less so amplification would be quantitatively less from those loci. The minimum size polymorphic DNA fragment (575 bp), was generated by primer GLH-16, whereas, polymorphic DNA band with maximum molecular weight (1850 bp) was amplified by the primer GLF-20.

The 36 RAPD primers, which showed 14 polymorphisms between the parents were further used to identify polymorphisms among the bulks. Out of the polymorphic primers, four produced polymorphic bands among the bulks, but in segregation analysis, only the DNA fragment of 1145 bp amplified by GLE-11 primer was found to be reproducible and tightly linked with the trait. The primer GLE-11 consistently differentiated the bulks by DNA fragment of 1145bp (Fig. 2). The primer GLE-11 was used for amplification of DNA of 30 individual F_2 plants which were used in the construction of bulks, distinguished all the individual plant samples (except 3 recombinants) with nectaried and nectariless phenotypes (Fig.2). The plants marked with * are recombinants. In total, 3 recombinants were observed out of 30 (10% recombination). This shows that distance of this marker from trait locus is 10cM. This DNA marker for the trait was named as GLE-$11_{1145.}$

The RAPD markers can be converted into SCAR to improve their reliability and may be used in breeding, especially the introduction of trait by backcrossing. In every backcross the individuals with recessive alleles can be identified by the marker instead of selfing. In an often cross pollinated species like cotton selfing reduces the hetrozygossity of various loci, which is required for maintenance of productivity. Amir *et al.* (2002a) identified two polymorphic DNA bands of 840 and 970bp amplified by the primer OPI-18, linked with leaf nectaries trait using bulked segregant analysis and suggested its use in MAB (Marker assisted breeding). They constructed the DNA bulks by mixing equal amount of DNA from 10 nectariless and 10 nectaried plants respectively. In the present study the primer GLI-18 (Similar in sequence to OPI-18) did not differentiate the genotypes contrasting for the nectariless. This may be due to differences in the germplasm used in the two studies.

Nectariless trait is conditioned by duplicate recessive genes (Meyer & Meyer, 1961; Amir *et al.*, 2002b). In present study, one locus was identified by GLE-11_{1145}, whereas, the other locus could not be tagged. The marker identified by Amir *et al*. (2002a), amplified by the primer OPI-18 may be the other locus located on other chromosome. The location of the DNA marker identified in the present study can be confirmed by using the substitution lines of cotton for chromosomes 12 and 26, as the two loci responsible for nectariless trait have been reported to be located on homeologous chromosomes 12 and 26 (Endrizzi *et al.,* 1984). In present study DNA marker was at a distance of 10 cM from the trait locus. Zhang & Stewart (2004) identified two RAPD markers linked to the fertility restorer genes in cotton with an average genetic distance of 2.9 cM. Jiang *et al*., (2000) failed to identify DNA markers for nectariless trait in cotton.

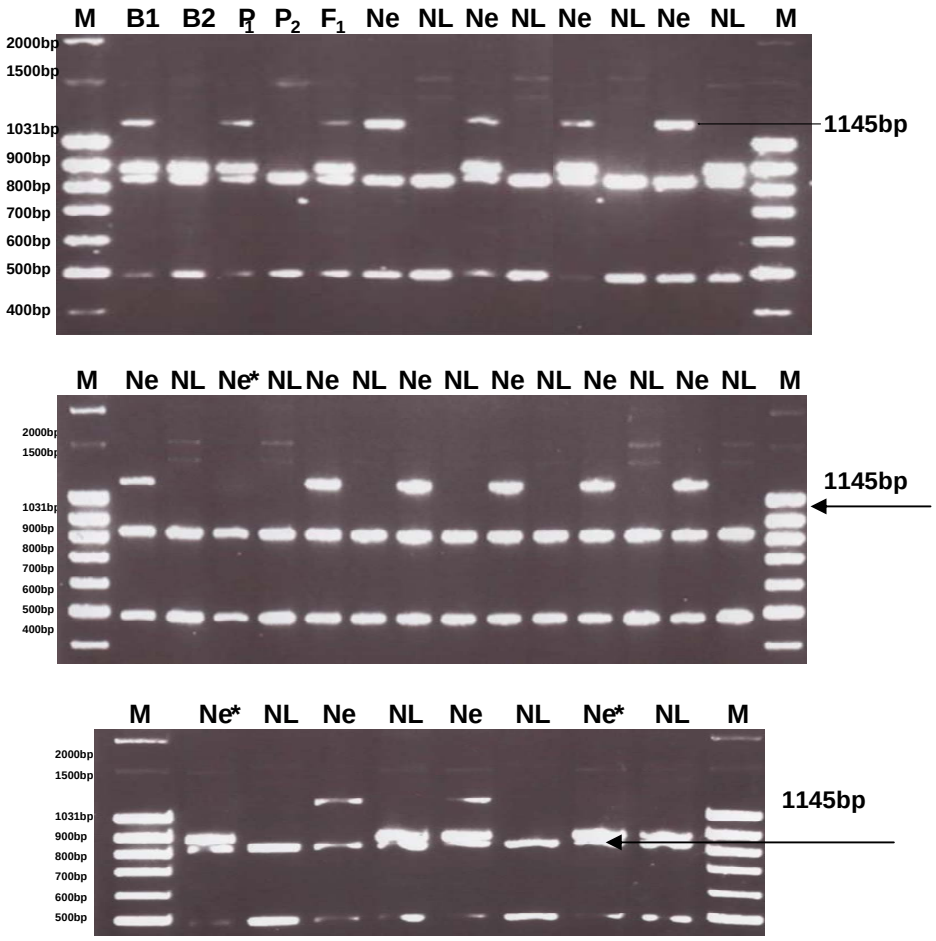

Fig. 2. PCR profile of bulks (B1, nectaried & B2, nectariless), Parents (P_1, HRVO1 & P_2, 3722LA-566), F_1 and F_2 plants (Ne, nectaried and NL, nectariless) with RAPD Primer GLE-11. The polymorphic DNA fragment of 1145bp (GLE-11_{1145}) is present in nectaried bulk (B1) parent (P1), F_1 and nectaried F_2 plants (Ne) but absent in parent (P2), nectariless bulk (B2), F_1 and nectariless F_2 plants (NL). M is DNA ladder. Recombinants are marked with*.

The size of the bulk is determined by the frequency with which unlinked loci can be detected as polymorphic between the bulked samples. This depends on the type of marker being screened (Dominant or Co-Dominant) and the type of population used to generate the bulks (F_2, back cross, fullsib etc.). A larger number of individuals in each pool increase the probability of a marker revealing polymorphism between the bulks, to be linked to the target interval. Too many individuals per bulk may also present a problem since the chance of adding a recombinant individual to the bulk increases with increasing number of individuals and it may become more difficult to identify the linkages if recombinants are present in both bulks (Warburton *et al.*, 1996). In present study 15 individuals were used in constituting the bulks. Different number of individuals and

varieties/genotypes were used to construct the bulks in different studies like, QTL for aluminum tolerance in wheat (Ma *et al.,* 2005 used 5 RILs for each bulk), mapping of maize stalk rot resistant gene (Yang *et al.,* 2004 used 15 individual F_2 plants for each bulk), blue mold resistance in tobacco (Milla *et al.,* 2005 used 7 DH lines for each bulk), thermo-sensitive genic male sterile gene in wheat (Xing *et al.,* 2003 used 15 individual DNAs for each bulk), root knot nematode in cotton (Wang *et al*., 2006 used 7 individuals in each bulk).

In present study, DNA of the individual F_2 plants was used to generate the bulks like many other studies (Wang *et al*., 2006). However, in some studies researchers used leaf samples to make bulks and the bulked leaves were used to isolate DNA. Zhang & Stewart (2004) collected leaves from 10 fertile and 10 sterile plants. The DNA was isolated after bulking those leaves to identify markers linked to fertility restorer genes in cotton. Literature survey shows that generally researchers prefer to use F_2 population (Wang *et al*., 2006), however, some prefer backcross populations to generate bulks (Feng *et al.,* 2005 used 10 plants from BC population for each bulk). Hu *et al.* (1997) used 6 F_6 plants to generate each contrasting bulk to identify RAPD markers linked to powdery mildew resistance gene in wheat.

In bulked segregant analysis care should be taken while selecting the individual plants for bulk constitution. Homozygous can be distinguished from the heterozygous, in dominant bulk by raising progeny of F_2 plants and only homozygous may be selected for bulk construction (Haley *et al.,* 1994). In present study, progeny test of F_2 plants was conducted to test their homozygosity to include in the bulks. The underlying principle of BSA is the grouping of informative individuals, so that a particular genomic region can be studied against a randomized genetic background of unlinked loci. RAPD technology has shortcoming of relatively low reproducibility and reliability (Weeden *et al*., 1992). Inspite of lower reproducibility of RAPD markers, these are still being used in identification of DNA polymorphism (Li *et al.* 2006, Rahman *et al.*, 2004)).

The RAPD marker identified in the present study was reproducible. There are reports that similar results can be achieved in different laboratories using different PCR machines, if the temperature profiles especially, the annealing temperature are kept uniform in different PCR machines (Malik & Price, 2003). Development of SCAR markers from these RAPDs would further increase their reliability (Huang *et al.,* 2000). It is reasonable to convert any dominant marker detected during the pre-selection stage into co-dominant markers, which will assure high efficiency of marker assisted selection. RAPD marker identified in this study is linked with dominant allele. The polymorphic DNA fragment amplified in the nectaried parent. The marker may be more useful if converted into SCARs or some other co-dominant maker.

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