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Molecular characterization of transgenic castor and identification of polymorphism between sunflower ray and non-ray florets

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SUMMARY

The present investigation was carried out with an objective of the conformation of the transgenic in T_1 progeny of the transgenic plants of castor and sunflower by using PCR technique. A total of 74 plants in the T_1 progeny were tested for transgenic conformation by using PCR technique for the hpt primers. In them 59 showed PCR positive and 15 PCR negative. The presence of polymorphism between these two rays and non-ray florets are of interesting task in the development of hybride varieties. Considering the importance of these issues the present work was conducted using molecular markers (RAPD) for assessment of diversity among the various collections of *Helianthus annuus*.

Key words : Sunflower, Castor, PCR technique and RAPD markers

Castor (*Ricinus communis*) is an important non-edible oilseed crop widely cultivated in tropical, sub-tropical and temperate countries for its high utilitarian value (Atsmon, 1989). Castor seed oil and its derivatives have become important commodities and an increasing number of uses are being found for them in the industrial world. The derivatives are used in a range of sectors including agriculture, the textile industry, plastics engineering, rubber and pharmaceuticals (Vignolo and Naughton, 1991).

Sunflower (*Helianthus annuus*) is an important oilseed crop worldwide. Successful cultivation of the crop is limited by the vulnerability of the crop to several biotic stresses (Morris *et al*., 1983; Sujatha *et al*., 1997). Genetic improvement of any species through genetic engineering techniques requires an efficient *in vitro* regeneration system, which is rapid, reproducible and applicable to a broad range of genotypes (Sujatha and Reddy, 1998). This study reveals that wild *Helianthus* species not only have valuable genes to the improvement of cultivated sunflower but also preserve large genetic variability in them that can be exploited for further improvement of this crop.

MATERIALS AND METHODS

Materials were collected from DOR field, Rajendar Nagar, Hyderabad. The plant material are been young leaves of *Helianthus annuus* and *Ricinus communis* were collected from in and around Thanjavur, Tamilnadu.

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Total genomic DNA was isolated from young leaves of 60 plants of T_1 progeny derived from PCR positive. To plant and untransformed (control) plants using the CTAB method (Doyle and Doyle, 1987). 100-150mg of leaf sample was taken in an eppendorf and was kept in liquid nitrogen for 30 minutes. The samples were ground to fine powder-using micro pestles followed by addition of 400 µl of extraction buffer and kept at 65°C in water bath. Then equal volumes of chloroform: iso-amyl alcohol (24:1) was added and kept for centrifugation at 8000 rpm for 8 minutes. The supernatant was collected and CI treatment was repeated. To the resultant supernatant 2/3 volume of isopropanol was added and kept at -20°C for 30 minutes. DNA was pelleted at 10,000 rpm for 15 minutes and washed with 70% alcohol, air dried and dissolved in 40-50 µl of $T_{10}E_1$. Quality and size of DNA were checked through agarose gel electrophoresis.

PCR analysis for Ricinus communis:

Genomic DNA extracted from the samples was subjected to PCR analysis for confirmation of hpt gene. The PCR reaction mixture (20 µl) contained 0.48 U Taq DNA polymerase, 10 mM Tris-Hcl (pH 9.0), 50mM KCL, 1.5 mM $MgCl_2$, 0.01% gelatin, 150 µM of each dNTP, 1 µl of each forward and reverse primer at a final concentration of 0.25 µl and 100 ng template DNA. For the positive control, 50 Pg of the Pcambia 1304 DNA was used. DNA from untransformed control and reaction profile included 30 cycle of strand separation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. The program was extended for 5 min at 72°C. The amplification products were analyzed on 1.4% agarose ethidium bromide gels. Further, to overrule the possibility of nonspecific amplification in PCR-amplified

fragment generated using primers specific to the hpt gene were sequence characterized (Bioserve, India). The forward and reverse primers (Bangalore Gebeum, India) used for the hpt gene were used to amplify a 520bp fragment.

RAPD analysis for Helianthus annuus:

A total of 200 RAPD primers from Operon kits (Operon technologies, Alameda, USA) were used for DNA amplification according to the method of Williams *et al*. (1990). PCR reaction mix except DNA for all the samples was prepared and aliquoted in to individual tubes for each reaction, DNA was added last. This minimizes pipetting error and increases uniformity across the reactions within a batch.

When preparing the cocktail all PCR components should be placed on ice and the components are to be added according to the numerical order specified in the cocktail recipe. It is advisable to prepare the cocktail in excess (around 10%) so as to make up for any pipetting error. Assemble RAPD reaction mix as described in the following table using the Taq polymerase holoenzyme.

RAPD reaction mix

Reagent	Stock	Amount used
DNA	2.5 ng/µl	1 µl
PCR buffer	10X	1 µl
dNTP mix	2 µM	2 µl
Taq polymerase	3U/ µl	0.1 µl
Milli Q water		Complete to 10 µl

Then the tube were capped and placed in the thermocycler and cycling was started.

Thermocycler programme:

Step 1: Initial denaturation = 94^{0}C - 4 min
Step 2: Denaturation = 94^{0}C - 30 sec
Step 3: Primer annealing = 36^{0}C - 45 sec
Step 4: Primer extension = 72^{0}C - 2 min
Step 5: Go to step 2 repeat for 45 cycles
Step 6: Final extension = 72^{0}C - 7 min
Step 7: Hold at 4^{0}C

Electrophoresis of RAPD-PCR product:

After completion of the thermal cycling, mix 1 x Bromophenol blue (1ml of 50% glycerol, 100 µl 1M Tris HCL, 50 µl of 0.5M EDTA and appropriate volume of Bromophenol blue) gel loading dye with appropriate volume of PCR amplified sample. Prepared 1.5% agrose gel and added 6 µl of 10 mg/ml EtBr. Loaded total PCR product along with dye on 1.5% agrose dye. Electrophorese gel examined on a UV transilliminator and documented the resulting Polaroid film on gel documentation system under UV-rays after completion of electrophoresis.

RESULTS AND DISCUSSION

A total of 74 T_1 progeny plants derived from PCR positive to putative transforments were used to screen the presence of hpt gene. In this study the T_1 generation plants genomic DNA was PCR amplified using hpt genes specific primer.

Information of primers used for confirmation of Transgenics

Primers	Sequence	Tm(^{o}C)	Product size (bp)
Hpt For	5'CACAATCCCACTATCCTTCGC3'	59.8	520
Hpt Rev	5'GCAGTTCGGTTTCAGGCAGGT3'		

The PCR amplification of T1 progeny genomic DNA using hpt gene specific primers resulted in a band size of 520 bp. Out of 74 plants screened 59 are PCR positive and the remaining 15 negative. By taking this in to consideration, the PCR analysis is shown to be that, the ratio of hpt gene expression was in ratio 3.9:1 (positive: negative).

Sr. No.	Total no. of T_1 plants screened	No. of plants PCR positive	No. of plants PCR negative
1.	74	59	15

Interpretation for Helianthus:

Among the molecular markers, PCR based markers offer promise over hybridization based markers, as they are simple, rapid and cost effective. Hence, the two sunflower germ plasm were characterized using RAPD markers, as this method does not demand high purity DNA. The study proved the effectiveness of this marker system in estimating the genetic relatedness of sunflower accessions.

The RAPD marker systems being employed to assess the genetic diversity in germ plasm were quite informative and were able to generate adequate polymorphism between the two sunflower lines. A total of 200 primers screened 168 were amplified and remaining 32 primers were not amplified. Out of 168 primers tested 14 were

polymorphic and 154 were monomorphic. The size of the amplification products of these primers ranged between 0.2 to 2.5 Kb.

Marker	Tot. No. of primers screened	No. of primers amplified	No. of primer polymorphic	No. of primers monomorphic
RAPD	200	168	14	154

The present research work aimed to characterize the T_1 progeny for the confirmation of transgenic formed. The transgenicity of the plant was confirmed through PCR analysis by using hygromycin gene specific (hpt) primers. DNA was isolated following CTAB method, as polyphenolic compounds were more in the isolated samples. PVP treatment was given for purification of the DNA. Then the samples were quantified on 0.8% gel, diluted and standardized. Due to the presence of more phenols even after purification standardization has taken long time. Then by using hygromycin specific primers PCR was conducted. Out of 74 plants taken 59 PCR were positive and 15 were negative this conformed that the transgenic formed in the T_1 progeny are in the ratio of 39:1.

DNA was isolated following CTAB method, as polyphenolic compounds were more in the isolated samples. PVP treatment was given for purification of the DNA. The RAPD marker systems being employed to assess the genetic diversity in germplasm were quite informative and were able to generate adequate polymorphism between the two sunflower lines. A total of 200 primers screened 168 were amplified and remaining 32 primers were not amplified. Out of 168 primers tested 14 were polymorphic and 154 were monomorphic. The size of the amplification products of these primers ranged between 0.2 to 2.5 Kb.

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