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Molecular Characterization of BSRI Stevia Using RAPD Markers

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ABSTRACT

For molecular characterization, an easy and efficient method was developed for isolation of quality DNA from shoot tip, stem segment and mature leaf of *Stevia rebaudiana*. The quality and quantity of DNA were determined by visual estimation of agarose gel electrophoreses and by UV spectrophotometer. The yield of DNA was 1200 to 5600 ng/µl. Sufficient amount of quality DNA isolation was possible without use of liquid nitrogen, tissue homogenizer and using a simple micro-centrifuge. The amount of recovered DNA was enough for Polymerase Chain Reaction (PCR) amplification and marker studies such as random amplified polymorphic DNA (RAPD). Molecular characterization of Stevia was performed using RAPD primers as markers. Among the 10 primers the primer OPB-09 was reproducible for PCR amplification for BSRI Stevia DNA. The results could be used for protection of the variety using OPB-09 as molecular marker. The technique could be efficiently used for identification of the variety and molecular characterization for development. The results give the positive indications of the further possibilities of linkage map analysis and Marker Assisted Selection for Stevia.

Key words: Stevia, molecular characterization, RAPD marker.

INTRODUCTION

Stevia (*Stevia rebaudiana* Bert.) is a small, herbaceous, semi-bushy, perennial shrub of Compositae family originated from Paraguay. Centuries ago, natives of Paraguay used the leaves of this 'honey plant' to sweeten their bitter drinks. It is one of 154 members of the genus Stevia, which produces sweet stevioside, a diterpenoid glycoside isolated from this plant leaves (Robinson 1930; Soejarto *et al.,* 1982). The dry leaves of this plant are 30 times sweeter than sugar, with zero calories. Where as pure extract stevioside is non-caloric and 300 times sweeter than sugar (Bhosle, 2004). Stevioside is of special interest to diabetics, persons with hyperglycemia and the diet conscious. The product can be added to tea and coffee, cooked or baked goods, processed foods and beverages. It is also used as a table top sweetener, in soft drinks, pickles, fruit juices, tobacco products, confectionery uses, jams and jellies, candies, yogurts, pastries, chewing gum, sherbets, etc.

In Bangladesh propagation is being practiced through seed germination along with the tissue culture technique to multiply the plants but any step towards the variety development is still to be undertaken. Some selection characteristics of Stevia are based on agronomic and morphological traits. Such traits are highly affected by the environment. Isolation, purification and quantification of DNA are the prerequisites of DNA fingerprinting, molecular characterization and marker assisted selection. The DNA

isolation methods involve the initial removal of the cell wall and nuclear membranes, separation of the DNA from all other cell components, and the protection of the DNA during the procedure from nucleases and mechanical shearing. A variety of problems may be encountered during the isolation and purification of high molecular weight DNA from plant species. It is also argued that DNA markers with significant linkage to the phenotypic characters could be useful in the breeding programmes since they would facilitate accurate, rapid and early screening of progeny independent of environmental or ontogenic factors. A number of markers linked to dominant genes in important crops have been characterized (Martin *et al.*, 1991; Haley *et al.*, 1993; Adam-Blondin *et al.*, 1994). However, the techniques like RAPD markers are usually dominant and may be carried out with no prior knowledge of the genome.

The RAPD analysis has been used extensively for various purposes which include identification and classification of accessions (Fukuoka *et al.*, 1992; Virk *et al.*, 1995), identification of hybrids (Qian *et al.*, 1996), genetic diversity analysis (Yu and Nguyen, 1994; Mackill, 1995; Cao and Oard, 1997), for map construction and linkage analysis (Reiter et al., 1992; Staub et al., 1996) and predicting quantitative variation within germplasm (Virk *et al.*, 1995). Therefore, the investigation was undertaken for molecular characterization of BSRI Stevia (developed through the tissue culture from the collected stevia plants) using RAPD markers.

MATERIALS AND METHODS

The present investigation was carried out at the Biotechnology Laboratory, Bangladesh Sugarcane Research Institute (BSRI), Ishurdi, Pabna, Bangladesh from April to June, 2007. In the present investigation, a method modified from Aljanabi *et al.* 1999. and mini prep-method adopted by BAU, Mymensingh, 2005 has been used to isolate total genomic DNA from Stevia.

Plant materials and sample collection: Three plant materials such as shoot tip, stem segment, and mature leaf of *Stevia rebaudiana* (Bert.) plant were used as the plant materials for DNA isolation. Collection of sample is the most important part of the DNA isolation procedure. Due to the handling error while sampling, the quality of the isolated DNA varies considerably. In this investigation, collection of samples was carried out with special care. Shoot tips, stem segments and mature leaves were used as sample. The samples were collected from 4-6 month old yard grown plants at Biotechnology Laboratory, BSRI. The samples were cut into small pieces (about 1cm long) with sterile scissors and weighted required amount (0.2g) using a fine balance which was then washed with ddH_2O. Care was taken during sampling to avoid bare hand touch to the sample. For this hand gloves were worn.

DNA isolation: A modified method of Aljanbi *et al.* (1999) was used to isolate total genomic DNA from BSRI Stevia. Cut pieces shoot tips, stem segments and mature leaves (about 5 mm long with 3 mm dia) 0.2g was taken in small mortar (dia 6.5cm and 3.5cm depth) and homogenized with pestle in 800 µl of extraction buffer (200mM Tris HCl, pH 8.0; 50mM EDTA.H_2O, pH 8.0; 2.2M NaCl; 2% CTAB; 0.06% Sodium Sulfite) until finely shredded within 40-50 second. Total grinded sample was taken into 2 ml eppendorf tube then added 150µl of each 5% SDS, 10% PVP, 20% CTAB mixed well quickly and incubated at 65 °C at water bath during incubation 3-4 times inversion were

done. After incubation the samples were cooled to room temperature and equal volume of Phenol : Chloroform: Isoamyl Alcohol (25 : 24 : 1) was added mixed well by inversion and centrifuged at 10,000 rpm for 30 minutes at room temperature. Then aqueous phase (about 800 µl) was recovered and transferred to a fresh ice-cold 2ml eppendorf tube and an equal volume of ice-cold isopropanol was added followed by 120µl of 6M NaCl. The samples were incubated at -20 °C for at least 1hr and centrifuged at 10,000 rpm for 20 min at room temperature. The upper layer of solution was discarded carefully by using adjustable micropipette and 70% ice-cold ethanol about 2.5 times of the solution was added. Again centrifuged at 10,000 rpm for 10 min at room temperature. After pellet formation the solution was discarded from the tube carefully so that the DNA pellet remains constant and undisturbed. Then 70% ethanol was added up to the slant of the tube. The ethanol was discarded from the tube carefully so that the DNA pellet remains constant and undisturbed. The DNA pellet was dried for at least 30 min by putting the tubes upside down on a filter paper. Then the DNA pellet was re-suspended in 50µl of TE buffer (10mM Tris HCl, pH 8.0; 1mM EDTA.H_2O pH 8.0) and stored at -20°C for lateral use.

Primers used: Selected ten decamer primers (Operon Technologies, Inc., USA) of random sequence (Table 1) were used for the whole sample set of Stevia. Primers were evaluated on the basis of intensity or resolution of bands, repeatability of markers and consistency within individual and potential to differentiate varieties/accessions (polymorohism).

PCR amplification and electrophoresis: PCR amplification was done in an oil-free thermal cycler (Master Cycler Gradient, Eppendorf) following the PCR profile of 94 °C for 4 minutes followed by 35 cycles of 1 minute denaturation at 94 °C, 1 minute annealing at 36 °C and elongation or extension at 72 °C for 2 minutes. After the last cycle, a final step of 7 minutes at 72 °C was added to allow complete extension of all amplified fragments. After completion of cycling programme, reactions were held at 4 °C. The PCR reactions were performed on each DNA sample in a 10 µl reaction mixture containing 1µl of 10X Ampli Taq polymerase buffer, 1µl of 10µM Primer, 1µl of 2.5mM DNTPs, 0.2 µl of 3U/µl Ampli Taq DNA polymerase (Bangalore Genei Pvt. Ltd., India), 4µl of 25ng/µl genomic DNA and a suitable amount (2.8µl) of sterile deionized water. After amplification 2 µl loading dye was added to the amplification product and separated on 1.4% agarose gel (SRL, India) containing ethidium bromide (0.8µg/ml from 10mg/ml in 1xTBE buffer). Electrophoresis was performed at 100V for 2 hours. The DNA ladder 100 bp (Bangalore Genei Pvt. Ltd., India) was run alongside the RAPD reactions. DNA bands were observed on UV-transilluminator in Gel Documentation System (uvitec, DBT-2000LS), printed and saved on floopy disket (3.5") for lateral use.

Data analysis: All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their position on the gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The scores obtained using all primers in the RAPD analysis were then combined to create a single data matrix. This was used for estimating linkage distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) Dendogram among the populations using computer programme Statistica.

RESULTS AND DISCUSSION

The RAPD technology has provided a quick and efficient method for DNA sequence-based polymorphisms at a very large number of loci. The major advantage is that no prior DNA sequence information is required. The vast range of potential primers that can be used gives the technique great diagnostic power. Reproducible RAPD bands can be found by a careful selection of primers, optimization of PCR conditions for the target species, and replication to ensure that only the reproducible bands are scored.

Among the ten primers (OPA-01, OPA-03, OPA-08, OPA-12, OPB-05, OPB-09, OPC-02, OPC-04,OPB-18,OPB-20), primers OPB-9 (Figure-1) produced the high intensity bands with minimal smearing. The reproducible band amplified by the primers OPB-09 might be used for identification of BSRI Stevia. The results could be used for protection of the variety according to plant variety protection law of Bangladesh. Using the results the BSRI Stevia might be protected from bio-piracy. The technique could be efficiently used for identification of the variety and molecular characterization for further development. The results give the positive indications of the further possibilities of linkage map analysis and Marker Assisted Selection of Stevia varieties.

The size of the band produced by the primer OPB-09 lies in between 500bp-600bp. (Table 2). However, the other primers failed to amplify any band. This might be perhaps due to recessive allele were corresponding to these primers.

Results of this investigation revealed that sufficient amount of quality DNA isolation was possible without use of liquid nitrogen, tissue homogenizer, using a simple micro-centrifuge and using the minimum amount of chemicals. Isolated DNA well performed for PCR amplification and DNA Fingerprinting using RAPD markers.

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Table 1. Parameters of the operon random primers used in the present study for screening.

Primer Code	Sequence (5'-3')	G+C Content (%)
OPA-01	CAGGCCCTTC	70%
OPA-03	AGTCAGCCAC	60%
OPA-08	GTGACGTAGG	60%
OPA-12	TCGGCGATAG	60%
OPB-05	TGCGCCCTTC	70%
OPB-09	TGGGGGACTC	70%
OPC-02	GTGAGGCGTC	70%
OPC-04	CCGCATCTAC	60%
OPB-18	CCACAGCAGT	60%
OPB-20	GGACCCTTAC	70%

Table 2. RAPD primers with corresponding bands scored and their size ranges in Stevia plant DNA.

Primer codes	Sequences (5'-3')	Total number of bands scored	Size ranges (bp)
OPA-01	CAGGCCCTTC	-	-
OPA-03	AGTCAGCCAC	-	-
OPA-08	GTGACGTAGG	-	-
OPA-12	TCGGCGATAG	-	-
OPB-05	TGCGCCCTTC	-	-
OPB-09	TGGGGGACTC	1	500bp-600bp
OPC-02	GTGAGGCGTC	-	-
OPC-04	CCGCATCTAC	1	-
OPB-18	CCACAGCAGT	-	-
OPB-20	GGACCCTTAC	-	-

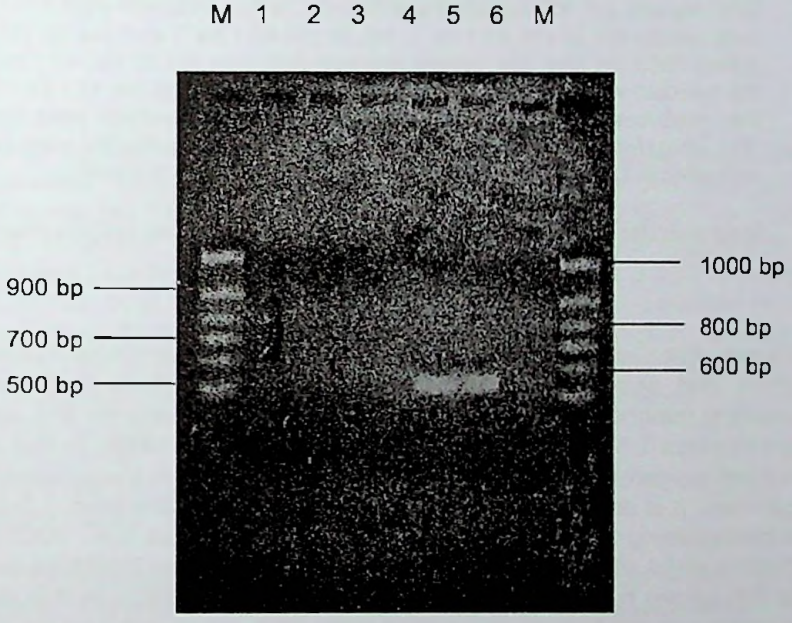

Figure 1. RAPD Fingerprinting of BSRI Stevia against primer OBP- 9 M denotes the 100bp DNA ladder