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Analysis of genetic polymorphisms in somaclonal variants of strawberry by RAPD markers

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The understanding of the importance of dietary berries of maintaining human health has led to unrestrained increase of global strawberry crop production. The present work was conducted for molecular characterization and selection of genetically pure somaclonal variants of strawberry. For molecular characterization, RAPD (Random Amplified Polymorphic DNA) markers namely instead OPK-01, OPK-02, and OPK-03, showed good technical resolution and sufficient variations among the somaclonal variants. A total of 29 RAPD bands were scored, of which 27 (94.87%) by using these arbitrary primers were obtained by polymorphic amplification products. OPK-01 produced the number of polymorphic bands of 100-700 bp compared to OPK-02 and OPK-03. All the somaclonal variants produced polymorphic bands with three RAPD markers suggesting that the somaclonal variants were different from each other and genetic divergence were present among them. Thus, these markers could be used for comparison of the genetic relationships, determination of patterns of genetic variation, and measurement of genetic distance among the somaclonal variants of strawberry.

Keywords: Cluster analysis; diversity analysis; genetic distance; RAPD marker; somaclonal variants of strawberry

INTRODUCTION

Fragaria × *ananassa*, Duch. Commonly known as strawberry or garden strawberry, is a hybrid species that is cultivated worldwide for its fruit (Potter et al., 2007).Strawberry has traditionally been a popular fruit for its flavor and sweet taste. It can be consumed fresh, frozen, or processed (Sharma 2002). Cultivated worldwide, strawberry is an important non-climacteric soft fruit. Key fruit quality factors are taste, odor, textural properties, and color. Fruit quality, firmness, and market ability is important during storage and for commercial purpose of strawberry (Ahn et al., 2014; Hwang & Ku 2004; Lee et al., 2002). Total world production 4516810tons and produced in 73 countries worldwide on 241109acres (FAOSTAT, 2012).In Bangladesh, there is no statistics about the area and production of this crop.

Plant tissue culture techniques have great potential as a means of vegetative propagation of economically important species, especially for those difficult to propagate by conventional methods (seeds or cuttings).Strawberry is one of the main plants of horticultural interest, which are multiplied by micro-propagation. Tissue culture is important to establish and/or maintain a 'virus-free' stock which may be used in somatic

hybridization induction and selection of mutants and germplasm conservation (Conger1980; Vuylsteke, 1989).Several technological improvements in tissue culture have been proposed by authors working with strawberry (Damiano 1980; Drew et al., 1986; Swartz, 1987) but the highest genotypic, physiological and morphological quality of micro-propagated plants is produced by the method described by Boxus and co-workers (Boxus 1974; Jemmali et al., 1995).

In order to increase yield potential, information about genetic variability is necessary. In breeding work, the success and progress of genetic studies depend upon the magnitude of genetic variability in the available plant material. To get an idea of the genetic variability existing among varieties with regard to quantitative characters of economic importance, it becomes necessary to study them under array distinguishable environments (Biswas et al., 2008).For the design of effective breeding programs, the determination of correlation coefficients among the yield and yield components is of prime importance when selecting suitable plant types. Although in vitro culture techniques have monopolized mass propagation and are of beneficial use in various fields in plant breeding (Taji et al., 2002), phenotypic and genetic variation are reported to occur as a consequence of the in vitro propagation process, originating somaclonal variants (Garcia et al.2002). This is a common phenomenon in plant tissue culture (Skirvin et al., 1993).

In this context, the usefulness and suitability of molecular markers, such as random amplified polymorphic DNA (RAPD)-based fingerprinting, must be emphasized for the assessment of the genetic stability of micro-propagated strawberry. Using small amount of genomic DNA allows the detection of differences at the DNA level. This technique does not require any previous information on DNA sequences. In *Fragaria*species, RAPD-PCR analysis can be successfullyused to investigate genetic relationships among cultivars and genotypes (Martelli et al., 1999; Degani et al., 2001; Milella et al., 2006), to perform phylogenetic studies (Martelli et al., 2002) and to identify cultivars (Garcia et al.2003; Li et al., 2006). Knowing the fact that the economic consequences of somaclonal variation can be enormous, establishing a micro-propagation protocol developed for a particular species, in terms of the production of genetically identical plants, is extremely important (Rani and Raina, 2000).The objective of this study was to analyze the genetic variation, relationship and stability of somaclones and determine genetic diversity using RAPD marker in somaclonal variants of strawberry with concern to quality parameters.

MATERIALS AND METHODS

The study was conducted at the Molecular and Biotechnology Laboratory, Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh, to characterize eighteen somaclonal variants of strawberry which were identified at the molecular level with markers as follows: G1, G2, G3, G4, G5, G6, G7, G8, G9, G10, G11, G12, G13, G14, G15, G16, G17 and G18.DNA from eighteen somaclonal variants were isolated from actively growing fresh leaves of 21 day old seedlings using CTAB (CetylTrimethayl Ammonium Bromide) method as described by Murry and Thompson (1980), later modified by Doyle and Doyle (1990). DNA was precipitated with 800 µ1 of absolute ethanol where DNA became visible as white strands by flicking the tube several times with fingers. DNA was pelleted by centrifugation and re-precipitation of the DNA solution was done by adding 400 µ1 of 70% ethanol. The pellets were then air dried and dissolved in 50 µ1 of TE buffer (10mMTris. HCl, 1Mm EDTA, pH=8.0). DNA quality was checked by electrophoresis in 0.8% agarose gel and quantified using a spectrophotometer at 260nMwavelength (Spectronic® GENESYS™, Thermo scientific, Germany). The DNA samples were evaluated both quantitatively and qualitatively using spectrophotometer.

RAPD-PCR Conditions

Seven 10-mer oligonucleotide primers (Operon Technology, USA) were randomly chosen for the study. PCR reactions were performed in a total volume of 10µL reaction mix containing 1 µL of 10x reaction buffer, containing 2 mMMgCl, 2 µL d NTP 2 at 0.2 mM, 0.1µL (0.5U) of Taq DNA polymerase (Promega,USA), 30 ng of genomic template DNA and 10 pmol primer in a preheated thermocycler (MJ Research INC, USA). PCR was initiated by a denaturation step at 92°C for 3 min and then their action was subjected to 45 cycles of 92°C for 30 sec, 35°C for 1 minand 72°C for 2 min with a final elongation step of 10 min at 72°C. In order to select the optimal conditions of the RAPD-PCR, different optimization experiments were carried out.

Visualization and Analysis of RAPD-PCR Products

Amplification products were resolved by electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV light. The presence and absence of bands between samples were scored and data were transcribed into binary format (1 and 0, respectively).The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. This was used to estimate polymorphic loci, gene diversity, population differentiation, $G_{st,}$gene flow (N_m), genetic distance (D) and to construct a UPGMA (Un weighted Pair Group Method with Arithmetic Means) dendrogram among populations using a computer program, POPGENE (Version 1.31, Canada) (Yeh et al., 1999).

RESULTS

In a breeding program, the efficient use of the germplasm is crucial for preserving the genetic relationships among breeding materials. No specific markers were found to discriminate between different somaclonal variants. In fact, the RAPD procedure exposed some degree of polymorphisms for variation study of different somaclonal variants of strawberries and also for investigating the genetic relationship among them.

RAPD and Genetic Stability Analysis

Seven primers were initially screened on 18 somaclonal variants of strawberry for their ability to produce polymorphic patterns. Three primers, OPK-01, OPK-02 and OPK-03, were selected because they gave reproducible and distinct polymorphic amplified products. DNA amplifications from all primers tested in this study were not consistently reproducible, which is a very common disadvantage of the RAPD technique. The present findings are in agreement with those of Hadrys et al., (1992) and Williams et al., (1993).In the field of genetic population research, these technical problems from amplification of the RAPD technique have been reported by many researchers (Schier water and Ender 1993; Lynch & Milligan, 1994). A total of 29 RAPD bands were scored, of which 27 (94.87%) were obtained by polymorphic amplification products using these arbitrary primers. The size of the amplification products ranged from 100-1000 bp (Table 1). The 3 primers selected produced a comparatively higher number of high intensity bands with minimal smearing, good technical resolution, and sufficient variation among different variants. A dissimilar number of bands were generated by primers, OPK-01, OPK-02 and OPK-03 (Table 1). Furthermore, the primer OPK-01 amplified the highest number of polymorphic bands (100.00%), while the primers OPK-02 and OPK-03 generated the least (90.00%) polymorphic bands. The number of RAPD markers scored for each of individual 18 variants for every primer is characterized in Table 2. A total of 206 clear and repeatable bands were amplified from three RAPD primers. The primers OPK-01, OPK-02 and OPK-03 produced 72, 63, and 71 polymorphic bands, respectively, in 18 somaclonal variants of strawberry. The present experiment produced 9.67 scorable bands per primer and 9 polymorphic bands per primer. The reason for the considerable amount of average scorable and polymorphic bands is that it consists of 60-70% GC contents (DNA base composition). Fukuoka et al., (1992) observed that increasing the GC content of the primer also increases the number of bands. The stability of the number of bands is higher when G paired with C with three hydrogen bonds than when A is paired with T by two hydrogen bonds.

Frequency of Polymorphic Loci and Genetic Variation

The presence and absence of bands were detected according to DNA polymorphisms. The absence of a band may be caused by the failure of primers to anneal to a site in some individuals by reason of nucleotide, by sequence differences, or by insertions or deletions between primer sites (Clark and Lanigan, 1993).

Table 1.RAPD primers with corresponding band scores and their size range together with polymorphic bands.

Primer code	Sequences (5´-3´)	Total number of bands scored	Size ranges (bp)	Number of Polymorphic bands	Proportion of Polymorphic loci(%)
OPK01	TGC CGA GCT G	9	150-700	9	100.00
OPK02	GTG AGG CGT C	10	100-720	9	90.00
OPK03	CCC TAC CGA C	10	150-750	9	90.00
Total		29		27	280.00
Average		9.67		9	93.33

Table 2. Number of polymorphic bands observed in 18 somaclonal variants of strawberry after PCR amplification on with RAPD primers OPK-01, OPK-02, and OPK-03.

Somaclonal variants	OPK-01	OPK -02	OPK -03	Total bands
G-1	6	2	4	12
G-2	6	4	4	14
G-3	1	4	6	11
G-4	1	5	4	10
G-5	5	6	4	15
G-6	5	2	3	10
G-7	5	3	5	13
G-8	1	2	3	6
G-9	4	4	3	11
G-10	6	3	4	13
G-11	6	2	3	11
G-12	3	2	3	8
G-13	1	6	5	12
G-14	4	4	7	15
G-15	2	4	5	11
G-16	4	2	3	9
G-17	4	4	3	11
G-18	7	4	2	13
Total bands	72	63	71	206

Table 3.Frequencies of polymorphic RAPD markers in 18 somaclonal variants of strawberry.

RAPD Markers	Gene frequency	RAPD Markers	Gene frequency
OPK01-1	0.0556	OPK02-7	0.1667
OPK01-2	0.3333	OPK02-8	0.1111
OPK01-3	0.1667	OPK02-9	0.1667
OPK01-4	0.5000	OPK03-10	0.1111
OPK01-5	0.5556	OPK03-1	0.0556
OPK01-6	0.5556	OPK03-2	0.0556
OPK01-7	0.7778	OPK03-3	0.1111
OPK01-8	0.5556	OPK03-4	0.5000
OPK01-9	0.3889	OPK03-5	0.3889
OPK02-1	0.0556	OPK03-6	0.8889
OPK02-2	0.0556	OPK03-7	0.3889
OPK02-3	0.6667	OPK03-8	0.9444
OPK02-4	0.7222	OPK03-9	0.6111
OPK02-5	0.8889	OPK03-10	0.0556
OPK02-6	0.5556		

Table 4.Summary of genetic diversity and Shanon information index statistics for all loci.

Loci	Gene diversity (h)	Shanon information index (i)	Loci	Gene diversity (h)	Shanon information index (i)
OPK01-1	0.1049	0.2146	OPK02-8	0.1975	0.3488
OPK01-2	0.4444	0.6365	OPK02-9	0.2778	0.4506
OPK01-3	0.2778	0.4506	OPK03-10	0.1975	0.3488
OPK01-4	0.5000	0.6931	OPK03-1	0.1049	0.2146
OPK01-5	0.4938	0.6870	OPK03-2	0.1049	0.2146
OPK01-6	0.4938	0.6870	OPK03-3	0.1975	0.3488
OPK01-7	0.3457	0.5297	OPK03-4	0.5000	0.6931
OPK01-8	0.4938	0.6870	OPK03-5	0.4753	0.6682
OPK01-9	0.4753	0.6682	OPK03-6	0.1975	0.3488
OPK02-1	0.1049	0.2146	OPK03-7	0.4753	0.6682
OPK02-2	0.1049	0.2146	OPK03-8	0.1049	0.2146
OPK02-3	0.4444	0.6365	OPK03-9	0.4753	0.6682
OPK02-4	0.4012	0.5908	OPK03-10	0.1049	0.2146
OPK02-5	0.1975	0.3488	Mean	0.3127	0.4758
OPK02-6	0.4938	0.6870			
OPK02-7	0.2778	0.4506	Standard deviation	0.1607	0.1936

The frequencies of maximum possible number of polymorphic loci were found to be high except for OPK-01 (0.7778), OPK-02(0.8889) and OPK-03 (0.9444) (Table 3).The different accessions of somaclonal variants of strawberry across the values of Nei's (1973) gene diversity and Shannon's information index for all loci are shown in Table2.The estimate of Nei's (1973) genetic diversity for entire genotypes of strawberry variants was 0.3127 and Shannon's information index was 0.4758 (Table 4). In the study, there was a high level of genetic variation of somaclonal variants of strawberry from the point of view of the proportion of polymorphic loci. The results indicated that genetic distance values of somaclonal variants of strawberry are genetically different from each other.

Genetic Distance

The pair-wise comparison values of Nei's (1972) genetic distances between genotypes were computed from combined data for the three primers. They ranged from 0.1092 to 0.9694, which was similar to a previous study (GaafarandSaker,2006) who studied the genetic relationship among seven commercially-grown strawberry cultivars. The small number of pair-wise differences (high genetic similarity) among some genotypes was likely due to their genetical relatedness. On the other hand, the large number of pair-wise differences (low genetic similarity) was observed among those genotypes developed from somatic embryogenesis. Comparatively higher genetic distance (0.9694) was found between G-7 vs. G-15 genotypes pairs. The lowest genetic distance (0.1092) was revealed between G-2 vs. G-18 whose genotypes were morphologically very similar. For genetics distance values, the results indicated that the variants were genetically so different from each other that they could be used as distinct cultivars or be proposed for release as a new variety.

Cluster Analysis

In this study, 18 somaclonal variants of strawberry have been differentiated into three main clusters: cluster I (G_1, G_6, G_{11}, G_{12}, G_7); cluster II (G_2, G_{18}, G_9, G_{16}, G_5, G_{17}, G_{10}, G_{14}) and cluster III (G_3, G_{13}, G_8, G_{15}, G_4).Cluster III was totally different from other genotypes, showing genetic relationship was not present between genotypes of cluster I and II and genotypes of cluster III. Cluster I was divided into two sub-clusters, A and B. Sub-cluster A included G_1, G_6, G_{11}, and G_{12} as genetic relationship was present among them while sub-cluster B had G_7which was different from other genotypes. Cluster II was divided into two sub-clusters, C and D. Sub-cluster C had G_2, G_{18}, G_9, and G_{16} while G_5, G_{17}, G_{10}, and G_{14} formed another sub-sub-cluster according to their genetic relationships

DISCUSSION

For valuable cultivar identification, the results indicated that the RAPD technique is effective to develop genotype–specific banding patterns. The results verified the effectiveness and suitability of RAPD markers for strawberry cultivars identification. Landry et al., (1997) used DNA from two independent micro extractions for verified amplification profiles and polymorphism in 75 strawberry cultivars and lines, while reproducibility by repeating reactions at two or three times with eight RAPD primers to find the genetic relatedness among the nine clones were ensured by Levi et al., (1994). The author's property rights were protection as well as selection of parents suitable for creating of mapping population they have confirmed the value of RAPD markers for strawberry cultivar identification. Cultivar identification and genetic diversity analysis studies using RAPD techniques are considered as the most used techniques because it does not require previous DNA sequence information and uses a very small quantity of DNA. Therefore, for the further research program, especially for hybridization, a genotype selected from different clusters will provide maximum heterosis regarding yield. Gaafar and Saker (2006) also found three clusters in seven varieties of Egyptian strawberry which were tested for association. These results confirmed the genetic stability of the tissue culture-derived strawberry plants. Random molecular markers were better suited for discriminating between genotypes (individuals) rather than for revealing relationships among wild populations (Harrison et al., 1997a, 2000). Especially in the case of the leaves of tissue culture-derived strawberry plants in contrast to results, minor morphological variation was observed. Kroons and Hutchings (1995) specified that clones of many species have high level of morphological plasticity in response to environmental conditions.

CONCLUSION

We verified that RAPD analysis can identify sufficient polymorphism to differentiate among somaclonal variants of strawberry and that this technique is appropriate for studying their genetic relationships. The molecular characterization derived the genotypic variations are indicated that genotypes belonging to different clusters depend on their genetic components itself, but not geographical origin at all. Therefore, further research programs could accomplish that, especially for hybridization.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Bir and SH designed and performed the experiments and also wrote the manuscript. MH, UK, and RA performed experimental treatments, data collection, and data analysis. EH and KW designed experiments and reviewed the manuscript. All authors read and approved the final version. Two authors (Md. Shahidul Haque Bir and Md. SamiulHaque) equally contributed to this work and should be considered as joint first authors.

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