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Assessment of somaclonal variation in *Plantago major* using molecular markers

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Abstract

Plantago major is a short stemmed annual herb belongs to Plantaginaceae family which is important because of its wide use in pharmaceutical, medical, healthcare and industrial purposes. The present study was conducted to evaluate the the somaclonal variations within *Plantago major* callus culture using inter simple sequence repeats markers. Callus induction was optimized using leaf and root as explants and MS medium containing various concentrations of 2,4-D and Kin. Moreover the effect of number of subcultures was considered in assessing genetic variability. Eighteen callus samples derived from different explants, media and subcultures were selected and their genomic DNA were extracted using CTAB method with some modifications. Six ISSR primers were used to estimate genetic diversity among 18 callus samples. The comparison of Banding patterns derived from 6 ISSR primers showed that the calli exhibited conspicuous somaclonal variation that could be eventually exploited for in vitro selection systems.

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Introduction

Plantago major, is one of the most important medicinal plants belonging to the the Plantaginaceae family. *P. major* originated in Eurasia and is well known as a natural source of mucilage that cultivated for their medicinal compounds. It has a short, stout and erect herbaceous stem. Leaves from basal rosette and grow up to 30 cm long (Sagar and Harper, 1964). Fruit of *P. major* is a capsule, which is 5 mm long. Large number of capsules are produced on a spike. Seeds are produced in capsules and the number of seeds per capsule is 4-15. It is wind pollinated self compatible and highly inbreeding species with very low outcrossing rate. (Kuiper and Bos, 1992). *P. major* has numerous phytochemicals in its leaves, seeds and roots, which apparently have medicinal properties and also can be used as taxonomic markers (Samulsen, 2000). Phytochemical investigations of *P. major* revealed the presence of various chemical constituents such as Flavnoids, Caffeoyl phenylethanoind gulocosides, Iridoid glocosides, Polyphenolic compounds (Zubair *et al.,* 2010). Polyphenols extracted from leaves and seeds of *P. major* have been reported to have bioactive effects especially on wound healing and to have antiulcergenic, antiflammatory, antioxidant, anticarcinogenic and antiviral activity. Considering the presence of many secondary metabolites in *P. major* and the need of pharmaceutical and cosmetic industries, propagation of this plant in large scale using in vitro culture techniques is important. Various studies have been carried out to widen the genetic makeup of *Plantago* including the induction of somaclonal variations and different environmental stresses (Li *et al.,* 2005). One of the methods for widening the genetic bases of the crop is to induce somaclonal variability in *in vitro* culture. Callus induction from hypocotyls explants of *plantago* species and regeneration of callus derived from root cultures using MS medium supplemented with IAA (Indole acetic acid) and Kinetin has been reported previously (Wakhlu and Barna, 1989). Somaclonal variation has been related to growth regulators, cultivar variability, age of cultivars in culture, ploidy level, explant source, and another culture conditions (Skirvin *et al.,* 1994). Somaclona variation result from different mutations including point mutations, chromosomal rearrangements, inversion, insertion, deletion and polyploidy. Somaclonal variation, a common phenomenon in plant cell cultures, includes all types of variations among plants or cells and derives from all kinds of tissue cultures (Skirvin *et al.,* 1993). Considering somaclonal variation occurs mainly as the result of mutations, appropriate techniques should be served for detection of occurred mutations in *in vitro* culture procedures. Although there are many techniques for detection of occurred variations, molecular markers are considered as one of the most appropriate tools due to their high accuracy and low performing time for evaluation of many samples (Martinez *et al.,* 2004). In this respect, ISSR markers, which are considered as PCR-based markers, are suitable for evaluation of somaclonal variations. Inter simple sequence repeat (ISSR) technique is a PCR based technique, reported by Ztetkiewicz *et al,* (1994), which involves amplification of DNA segments between two identical microsatellite repeat regions 'oriented in opposite direction using primers designed from microsatellite core regions. It has been revealed that extend of genetic variations is related to the number of performed subcultures and also hormonal composition of culture medium (Poorjabar *et al.,* 2009). It has been shown that PCR-based molecular markers have been effective for detection of occurred somaclonal variations. In the present investigation, we show different banding patterns for calli generated in various concenteration of 2,4-D and Kin and different subcultures and explants, based on ISSR markers.

Materials and methods

Callus induction

In this study, the mature seeds were treated with 70% ethyl alcohol for 1 min, then rinsed with sterile water. The seeds were then surface sterilized in 2.5% sodium hypochlorite solution for 20 min and was followed by three washes in succession with sterile water. The sterile seeds were cultured on MS (Murashige and skoog, 1962) medium. The cultures were used as a

source of plant material for establishment of explants. The leaves and roots explants from 30 days old seedlings were cultured on sterile MS medium supplemented with 3% w/v sucrose, 7% w/v agar and containing different concentrations of plant hormones 2,4-D and KIN (Table 1). To study the effect of subculture on somaclonal variation, calli obtained from both explants were subcultured each 30 days. After 3 times subcultures, 18 calli from different explants (leaf and root) and three types of hormonal compositions, were selected (Table 2) and subjected to DNA extraction.

Table 1. Structure of testing media for callus induction of *P.mjor*.

Media code	2,4-D	Kin
A	1	0.1
B	0.8	0.1
C	2.5	0.01

2,4-D: 2,4-Dichlorophenoxyacetic acid; Kin: Kinetin.

Table 2. Type of explants, the number of subculture, media culture used in callusing stage of *P.major*.

Samples	Explants	No. of subculture	Media
1	-	-	-
2	root	1	A
3	leaf	1	A
4	root	2	A
5	leaf	2	A
6	root	3	A
7	leaf	3	A
8	root	1	B
9	leaf	1	B
10	root	2	B
11	leaf	2	B
12	root	3	B
13	leaf	3	B
14	root	1	C
15	leaf	1	C
16	root	2	C
17	leaf	2	C
18	root	3	C
19	leaf	3	C

Table 3. The sequences (5'-3') of primers used for ISSR analysis.

Primers	Sequence (5' – 3')
IS6	CACACACACACACACAG
IS15	GGATGGATGGATGGAT
IS11	ACACACACACACACACC
IS16	DBDACACACACACACACA
IS1	ACACACACACACACACyA
UBC-848	CACACACACACACACARG

DNA extraction

Genomic DNA were isolated from one normal plant and 18 calli, by CTAB method (Saghai-Maroof *et al.,*1984) with some modifications. The quality and quantity of extracted DNA were tested on 0.8% agarose gel electrophoresis.

ISSR analysis

A set of six ISSR primers was used to amplify the genomic DNA of all 19 samples. PCR amplifications were performed in 20 µl reaction volume containing: 2 µl of genomic DNA, 1.2 µl of primer, 0.4 µl dNTPs Mix, 1.5 µl $Mgcl_2$, 0.3 µl Taq DNA polymerase (5 unit/ µl), 2 µl PCR buffer and 12.6 µl double distilled water. PCR amplification was carried out with 4 min initial denaturing (to activate *Taq*DNA polymerase), followed by 35 cycles of denaturing at 94°C for 30 s, annealing (considering Tm of primers) for 45s and extension at 72°C for 2 min. This was followed by a final extension stage for 7 min at 72°C. Amplification reaction products were separated on1.5% agarose gels at 100 V until the loading dye reached to the bottom of the gel. After electrophoresis, staining performed by ethidium bromide and photographed with gel documentation.

Band scoring and data analysis

Since ISSR markers are dominant, amplified fragments were constructed by scoring 0 and 1 for absence and presence of bands, respectively. The generated data matrixes were subjected to statistical analysis using the DARwin computer software (Perrier *et al.,* 2003). Genetic similarity estimates were calculated and relationships of the genotypes were estimated from the markers data using the UPGMA clustering method based on Nei's, (1978) unbiased genetic distance(Nei, 1978). The polymorphism information content (PIC) was used to characterize the efficiency of each primer to reveal polymorphic loci. The PIC index was calculated as:

$PIC=1-\sum P^2_i$,

where P represent band frequency and q represent no-band frequency.

Results and discussion

The six ISSR primers generated 60 clear and scorable bands across 19 sampels. Analysis of banding patterns revealed 59 polymorphic bands with an average of 9.8 fragments per primer. The highest and the lowest number of polymorphic bands per assay were 13 and 7 bands, respectively (Table 4). The results showed genetic variation among different samples of *Plantago major*. Since our experiment was performed starting from a single individual plant or gynotype, the polymorphism in banding pattern reveal conspicuous somaclonal variation in samples.

To assess the genetic variation among the samples, the UPGMA dendrogram of 19 samples of *P.major* was constructed based on Nei's genetic distance matrix. The cluster, which included all the *P.major* samples, was subdivided into five groups. Somsclonal variation in the calli obtained from different culture conditions and subcultures using ISSR markers revealed different banding patterns among samples from explants of one plant in different culture and subculture conditions.

Table 4. Primers used for ISSR amplification with the number of bands, PIC and MI per primer.

Primer code	Primer sequences (5' – 3')	bands scored	polymorphic bands	PIC	MI
IS15	GGATGGATGGATGGAT	7	7	0.36	2.52
IS6	CACACACACACACACAG	12	12	0.38	4.56
IS16	DBDACACACACACACACA	12	11	0.35	3.85
IS1	ACACACACACACACACYA	7	7	0.39	2.73
IS11	ACACACACACACACACC	13	13	0.34	4.42
UBC-848	CACACACACACACACARG	9	9	0.25	2.25
Sum		60	59	-	-
Average		10	9.83	0.34	3.38

The average of PIC index was 0.34, that showed efficiency of primers to separate individuals, but Some primers were more efficient in differentiating between individuals than others. For example the maximum PIC (0.39) was observed for the primer IS-1 and the minimum PIC (0.25) was obtained with UBC-848.

Marker index (MI) was calculated for all primers, with an average of 3.38. The MI values for ISSR primers ranged between 1.92 and 4.29. This feature has been used to evaluate the discriminatory power of molecular marker systems in some plant species e.g. apricot (ISSR, MI = 3.74) (Kumar *et al.,* 2009).

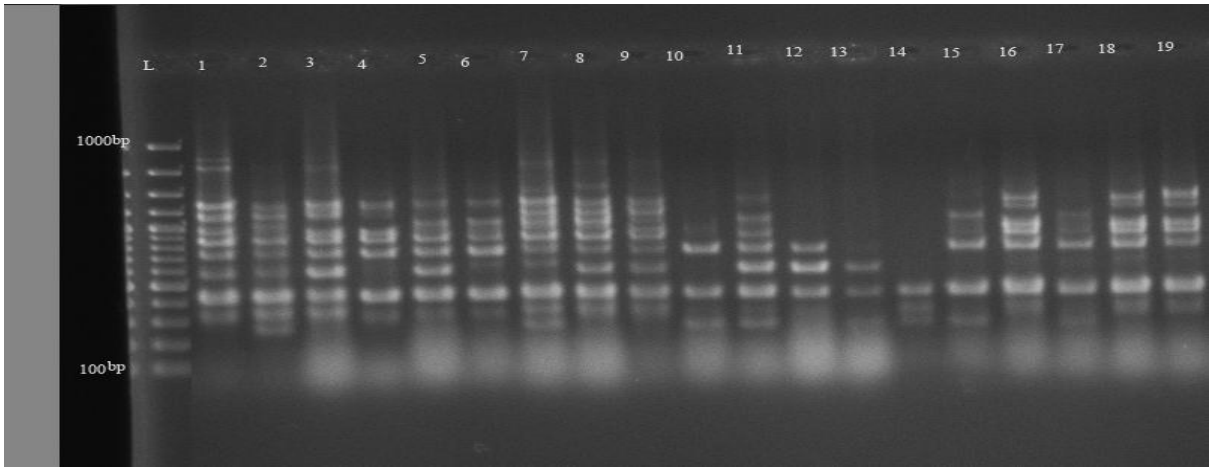

Fig. 1. ISSR banding profile generated by primer IS16.

In vitro tissue culture techniques are well known to be an important tool in the induction of variation leading to the development of new plant genotypes (Kuksova *et al.,* 1997; Larkin and Scowcroft, 1981). This source of variability is considered as a useful tool for geneticists and plant breeders (Amzad *et al.*, 2003). Pramanik *et al,* (1995) developed callus from hypocotyl of *P. ovata* in MS basal medium with different concentrations of 2,4-D/kinetin and NAA/BAP and subsequently regenerated the shootlets (Pramanik *et al.*,1995). In another study, in vitro culture of *Plantago major* by using MS medium supplemented with 0.2 mg L-1 of IAA and 1.0 mg L-1of TDZ has been reported (Li *et al.*, 2005). RAPD analysis has been used to detect the somaclonal variations (Jin *et al.,* 2008). The process of somaclonal variation is believed to require multiple genetic and/or epigenetic events which affect patterns of expression, or result in mutational alteration of genes. Various molecular mechanisms may be responsible for the DNA mutation and genetic instability leading to the development of variations. Such molecular mechanisms would include DNA damage and mutation, alteration of cell ability to repair damaged or mutated DNA, alteration of genes for cell-cycle control mechanisms, DNA methylation (Merlo *et al.,* 1995). Mutations detected by microsatellites primers suggest several possible alterations in the genome rapidly accessible by ISSR. Researches based on this model may be used to assess the genotoxic effects of various compounds such as herbicides. With a same approach, genomic DNA would be subjected to analysis by restriction enzymes to detect, for example, methylation status; moreover, the whole gene could be analysed through the development of amplified consensus gene markers . It is reported that tissue culture procedure leading to great cellular reprogramming level may be a consequence of increased somaclonal variations (Morcillo *et al.,* 2006). Various somaclonal variations in transgenic *Arabidopsis thaliana* plants resulted from callus formation has been reported (Labra *et al.*, 2004). According to previous studies somaclonal genetic variation results from micropropagated plant cultures (Kaeppler *et al.,* 2000). In plant tissue culture system the rate of somaclonal variation enhances with the increase of subcultures in micropropagation protocols (Gaafar and Saker, 2006).

This study clearly revealed that ISSR techniques can be used to detect somaclonal variation in *P.major*.

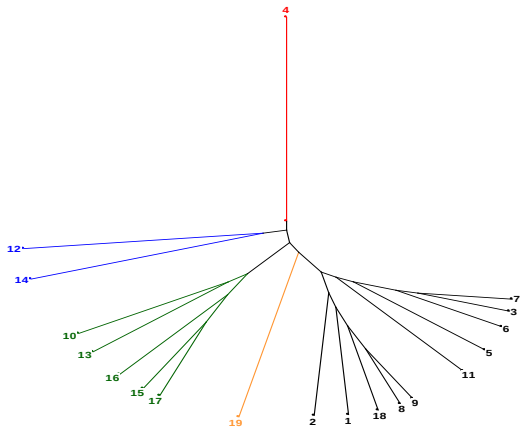

Fig. 2. Dendrograms generated with hierarchical UPGMA cluster analysis based on ISSR data.

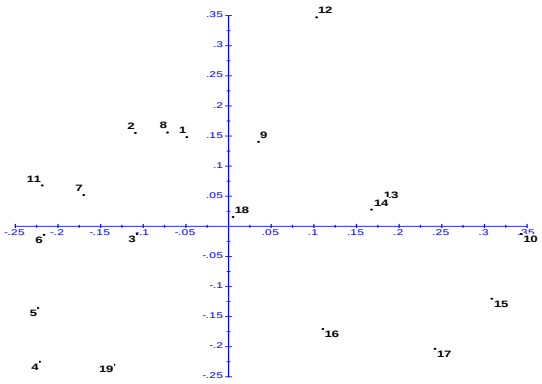

Fig. 3. Principal coordinate analysis (PCO) of the nineteen samples based on genetic distances calculated with ISSR markers.

References

Amzad H, Kunihiko K, Mineo M, Kazuhiro N. 2003. Somaclonal variation of regenerated plants in chilli pepper (Capsicum annuum L.). Euphytica **130(2),** 233-239.

Gaafar RM, Saker MM. 2006. Monitoring of cultivars identity and genetic stability in strawberry varieties grown in Egypt. World Journal of Agricultural Sciences **2(1),** 29-36.

Jin S, Mushke R, Zhu H, Tu L, Lin L, Zhang Y, Zhang X. 2008. Detection of somaclonalvariation of cotton (Gossypium hirsutum) using cytogenetics, flow cytometry andmolecular markers. Plant Cell Reports. **27(8),** 1303-1316. http://dx.doi.org/ 10.1007/ s00299-008-0557-2

Kaeppler SM, Kaeppler HF, Rhee Y. 2000. Epigenetic aspects of somaclonal variation in plants. Plant Mol Biology **43(2-3),** 179-188.

Kuiper PJC, Bos M. 1992. Plantago: a Multidisciplinary study. Springer, Berlin.

Kuksova VB, Pieven NM, Gleba YY. 1997. Somaclonal variation and in vitro induced mutagenesis in grapevine. Plant Cell, Tissue and Organ Culture. **49(1),** 17-27.

Kumar Sharma S, Bryan GJ, Winfield MO, Millam S. 2007. Stability of potato (Solanum tuberosum L.) plants regenerated via somatic embryos, axillary bud proliferated shoots, microtubers and true potato seeds: a comparative phenotypic, cytogenetic and molecular assessment. Planta **226(6),** 1449-1458. http://dx.doi.org/ 10.1007/ s00425-007-0583-2

Labra M, Vannini C, Grassi F, Bracale M, Balsemin M, Basso B, Sala F. 2004. Genomic stability in Arabidopsis thaliana transgenic plants obtained by floral dip. Theoretical and Applied Genetics **109(7),** 1512-1518. http://dx.doi.org/ 10.1007/s00122-004-1773-y

Lambe P, Mutambel H, Fouche J, Deltour R, Foidart J, Gaspar T. 1997. DNA methylation as a key process in regulation of or-ganogenic totipotency and plant neoplastic progression. In Vitro Cellular & Developmental Biology - Plant **33(3),** 155–162.

Li P, Chen H, Li YX. 2005. Establishment and optimization of in vitro regeneration system for Plantago major L. Chinese Journal of Biotechnology **21(6),** 916-922

Lei CP, Jiun KS, Choo CS, Singh R. 2006. Analysis of tissue culture – derived regenerants using methylation sensitive AFLP. Asia Pacific Journal of Molecular Biology and Biothechnology **14(2),** 47-55.

Morcillo F, Gagneur C, Adam H, Richaud F, Singh R, Cheah S.C, Rival A , Duval Y, Tregear J.W. 2006. Somaclonal variation in micropropagated oil palm. Characterization of two novel genes with enhanced expression in epigenetically abnormal cell lines and in response to auxin. Tree Physiology **26(5),** 585-94.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacoo tissue cultures. Physiologia Plantarum **15(3),** 473-496.

Nei M. 1978. Estimation of average heterozygosiy and genetic distance from a small number of individuals. Genetics **89(3),** 583-590.

Perrier X, Flori A, Bonnot F. 2003. Methods for data analysis. In: Hamon P, Seguin M, Perrier X, Glazmann JC (eds) Genetic diversity of cultivated tropic al plants. Science Publishers, Inc and Cirad, Montpellier, 31–63.

Pourjabar A, Mohammadi SA, Khosroshahli M, Motalebi AR, Ziai SA. 2009. Optimization of in vitro culture of Milk Thistle (Silybum marianum) and analysis of somaclonal variation by RAPD markers. JOURNAL OF AGRICULTURAL SCIENCE **19(2),** 95-106.

Sagar GR, Harper JL. 1964. Plantago major L., P. media L. and P. lanceolata L. Journal of Ecology **52(1),** 189–221.

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW. 1984. Ribosomal Spacer Length Polymorphism in Barley: Mendelian Inheritance, Chromosomal Location and Population Dynamics. Proceedings of National Academy of Science of theU.S.A **81(24),** 8014–8018.

Samuelsen AB. 2000. The traditional uses, chemical constituents and biological activities of Plantago major L. A review. Journal of Ethnopharmacology 71(1-2), 1–21. http://dx.doi.org/10.1016/S0378-8741(00)00212-9

Skirvin RM, Norton M, Mcpheeters KD. 1993. Somaclonal variation: has it proved useful for plant improvement? . II International Symposium on In Vitro Culture and Horticultural Breeding.

Stavri M, Mathew KT, Bucar F, Gibbons S. 2003. Pangelin, an antimycobacterial coumarin from Ducrosia anethifolia. Planta Medica. **69(10),** 856-859.

Tatikonda L, Wani SP, Kannan S, Beerelli N, Sreedevi TK, Hoisington DA, Devi P, Varshney RA .2009. AFLP-based molecular characterization of an elite germplasm collection of Jatrophacurcas L., a biofuel plant. Plant Science **176(4),** 505-513. http://dx.doi.org/10.1016/j.plantsci.2009.01.006

Wakhlu AK, Barna KS. 1989.Callus initiation growth and plant regeneration in Plantago ovate Forssk. Cv.Gl-2 Plant Cell Tissue and Organ Culture **17(2)**, 235-241. http://dx.doi.org/10.1007/BF00046870

Zubair M, Rumpunen K, Lindholm C, Nybom H. 2010. Genetic and phytochemical variations in Plantago major. 28th international horticultural congress. Lisboa, Portugal, August 22-27, 2010. Poster presentation.

Zietkiewicz E, Rafalski A, Labuda D. 1994. Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics **20(2),** 176-183. http://dx.doi.org/10.1006/geno.1994.1151