Chapter 10

RAPD Analysis of Callus Derived plant of Ophiorrhiza eriantha and its Mother plant
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10.1. Introduction

Plant cells in culture show genetic instability, which can lead to the recovery of variant plants. Somaclonal variation, a common phenomenon in plant cell cultures includes all types of variation among plants or cells and derives from all kinds of tissue cultures (Skirvin et al., 1993). It is also called tissue or culture-induced variation (Kaeppler et al., 2000). Somaclonal variation is somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture conditions and is expected to generate stable plants carrying interesting heritable traits (Soniya et al. 2001).

The processes underlying somaclonal variation (Larkin and Scowcroft, 1981) are believed to require multiple genetic and/or epigenetic events that affect patterns of expression, or result in gene mutation. Various molecular mechanisms may be responsible for the DNA mutation and genetic instability. Such mechanisms include DNA damage and mutation, alteration to the cell’s ability to repair DNA damage, or to the genes responsible for cell-cycle control, repeated DNA sequences can rapidly be amplified or reduced in copy number, altered methylation patterns can be inherited, DNA replication is disturbed by altered nucleotide pools, and active genes can be silenced or silenced gene may be activated by mutations in associated non-coding regions (Scowcroft et al., 1985). The mechanism of somaclonal variation involves extensive genomic flux e.g., Epigenetic factors are also involved making it more complex (Kaeppler et al., 2000). Somaclonal variation has been related to growth regulators, cultivar variability, cultivation period, number of subcultures, cultivars age in culture, ploidy level, explant source and other culture conditions (Skirvin et al., 1994; Evans and Sharp 1986).

Methods for detection of somaclonal variation have been explored for many years (Noval, 1980). Scoring changes in plant morphology can be useful in some studies, but there is limited diversity and trait may be affected by environmental influences. Cytological assessment is not often used and can be difficult in many species like Proteaceae where chromosomes are difficult to
observe. Analyses of secondary metabolites and isozyme patterns have also been used, but they are limited in their sensitivity (Morell et al., 1995). Molecular techniques like Random Amplified Polymorphic DNA (RAPD) are often favoured over traditional phenotypic or cytological measurements, and generally assess even small variations of the genome.

Molecular markers, which can detect modifications at the DNA level, are increasingly being used to access the fidelity of in vitro propagated plants. Among these, random amplified polymorphic DNA (RAPD)-based fingerprinting is being used by several workers to detect molecular alterations in in vitro regenerated plants, and different rates of variation were reported according to the species and the regeneration system adopted. RAPD markers have been used widely in studying the genetic diversity of somaclonal variations in various plant species (Maria and Garcia, 2000; Soniya et al., 2001).

Ophiiorrhiza eriantha is one of the most important anticancer drug, camptothecin (CPT) and its derivative (10-hydroxyl camptothecin 10-HCPT) producing plant. Production of these high value drugs through tissue culture method is an alternative approach. Successfully we established in vitro cell cultures of O.eriantha and enhanced these secondary metabolites by various biotechnological methods. We also established an in vitro clonal propagation protocol for efficient multiplication of Ophiiorrhiza eriantha multiple shoots from callus cultures. For RAPD analysis we selected multiple shoots showed superior CPT production. In the present study RAPD has been used to evaluate genetic diversity or stability of in vitro plants of Ophiiorrhiza eriantha originated from callus through indirect organogenesis.

10.2. Materials and methods

10.2.1. Plant material
The shoot cultures used in the present study were established from callus by MS medium supplemented with different concentrations of BA (1-5 mg/l) as described in materials and methods section 2.2.7. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C and 15 lbs for 20 min. The cultures
were incubated under a 16 hrs photoperiod in cool white fluorescent light
25µmol m⁻²s⁻¹ and maintained at a constant temperature of 25 ± 2°C.

10.2.2. Plant material for DNA extraction
The mother plant collected from Mannavanchola, Idukki, Kerala, India and
was maintained in our garden and the fresh leaves from this were used as a
source of DNA. DNA from in vitro regenerated plantlets in MS medium
supplemented with 5 mg/l BA was also extracted.

10.2.3. Preparation of template DNA
Leaves of *O. eriantha* wild plant (genotype from which the explants were
collected) and randomly selected *in vitro* cultured plant leaves before
hardening stage were collected and set in ice. The leaves cut into pieces and
adding the liquid nitrogen. It was then grounded into fine powder using a
mortar and pestle at full speed. Immediately after homogenization, the plant
tissue powder was transferred up to 100 mg to a microcentrifuge tube. Keep
the sample on ice for immediate use or freeze as -70°C. DNA isolated from
these powder of plant tissues by using Sigma’s GenElute™ Plant Genomic
DNA Mini prep Kit (Sambrook et al., 1989; Birren and Lai, 1993) supplied by
sigma (USA). The extracted DNA was subjected to PCR analysis.

10.2.4. DNA amplification
RAPD-PCR amplification was carried out using five randomly selected
primers. The primers were 10 random bases long.

OPA 03 – 5’-AGTCAGCCAC-3’
OPC11 – 5’-AAAGCTGCGG-3’
OPC12 – 5’-TGTCATCCCC-3’
OPU 4 - 5’-ACCTTCGGAC-3’
OPU 8 - 5’-GGCGAAGGTT-3’

RAPD amplifications were performed using PCR mixture (25 µl) which
contained, 10× assay buffer (Genei, Bangalore ), 2.5 mM MgCl2, 100 µM each
dNTPS (dATP, dGTP, dCTP, and dTTP), 15 ng of the primer, 0.6 units of Taq
DNA polymerase (Bangalore Genei, India), and 10 ng of genomic DNA as template. The reaction mixture was overlaid with an equal volume of mineral oil and DNA amplification was performed in a MJ Research mini cycler PTC-150.

The amplification condition programmed for 45 cycles as follows: 1st cycle of 3 min at 94°C for template denaturation, 1 min at 37°C for primer annealing, 2 min at 72°C for primer extension; followed by 44 cycles each of 1 min at 94°C, 1 min at 37°C, 2 min at 72°C followed by one final extension cycle of 7 min at 72°C.

10.2.5. DNA electrophoresis
The 10 µl of each PCR amplification products were separated in 1.2% (w/v) agarose (SRL, Mumbai, India) gel containing 0.5 µg/L ethidium bromide and in 10x TBE buffer at 80 V and photographed under UV light. Lambda DNA/EcoR1 digested with Hind III (Genei, Bangalore, India) was used as molecular size marker.

10.3. Results
Somaclonal variation is very complex process and requires the use of several approaches to be correctly appreciated. Molecular markers including Random Amplified Polymorphic DNA (RAPD) markers have been used to detect the genetic variability of the somaclones. The genetic variability between the wild plant and cultured plants in MS medium with BA was analyzed using RAPD molecular marker technique. Alterations were detected as gains or losses in the pattern of amplified bands. Agarose gel testing of regenerated plants revealed polymorphic. Variations in patterns were due to the presence or loss of fragments and are exhibited for one of them (Fig. 10.1 ). No amplification was observed when using OPU 8 and OPA 03. Whereas, OPC11, OPC12 and OPU 4 confirmed the genetic variation from the mother plant. Individual gel banding pattern differences of the co-amplified fragments between different templates revealed polymorphisms between the genomes.
10.4. Discussion

Genetic changes are frequently observed in plants regenerated from tissue culture. This phenomenon, termed somaclonal variation. Detection of variants at the DNA level is of immense importance in order to utilize \textit{in vitro} selected lines in crop improvement.

Even single base change at the primer annealing site is manifested as appearance or disappearance of RAPD bands, it could be suggested that tissue culture conditions have induced varied amount of genetic changes in different regenerated plants.

The variations observed in the RAPD pattern may be due to different causes including loss/gain of a primer annealing, due to point mutations or by the insertion or deletion of sequence or transposable elements (Peschke \textit{et al.}, 1991). It also reported that the genetic variations induced in the regenerated plants increase with the time-period of the sub-culture Sheidai \textit{et al.}, 2010.

Explant source is also considered as one of the critical variable for somaclonal variation. Since explants may present dissimilar regeneration rates, selection procedures can differ among different explants types. For example, plants regenerated from chrysanthemum petal epidermis-induced calli showed greater somaclonal variation than those from apex-induced calli (De Jong and Custers, 1986).

In the present study, RAPD analysis of genetic variation among the \textit{in vitro} regenerated plants and mother plant was detected. It is therefore clear that \textit{in vitro} selection conditions have induced varied amounts of genetic changes among the \textit{in vitro} regenerated plants. The reported variation at the DNA level can be attributed to somaclonal variations. Cellular and molecular mechanisms behind the variations are mitotic irregularities leading to chromosomal instability, occurrence of gene amplification or deletion, gene inactivation or reactivation of silent genes, transposition and somatic crossing over, DNA methylation in case of epigenetic variation and point mutations (Larkin and Scowcroft, 1981).
The exact cause of somaclonal variation in \textit{in vitro} cultures are still unknown, although it is believed that alterations in auxin-cytokinin concentrations and their ratio, duration of \textit{in vitro} culture, \textit{in vitro} stress due to unnatural conditions, altered diurnal rhythm and nutritional conditions (Modgil \textit{et al.}, 2005) together or independently are responsible. Cultured plant tissues are also known to undergo high levels of oxidative stress due to reactive oxygen species formed within the cells and the latter is known to cause DNA damage, including that of microsatellite instability (Jackson \textit{et al.}, 1998).

It could be concluded that the \textit{in vitro} propagated plants are characterized by improved production of CPT and 10-HCPT and detection of genomic variability as compared with that of parent plants. These increased productions of these compounds may be due to genetic alternation of \textit{in vitro} propagated plants to tolerate the \textit{in vitro} condition or due to the influence of growth hormones.
Fig 10.1. RAPD profile of *O. eriantha* mother plant and regenerated plant

Lane 1- Marker DNA
Lane 2 - RAPD pattern generated OPC11 primer from genomic DNA of mother plant
Lane 3 - RAPD pattern generated OPC11 primer from genomic DNA of in vitro regenerated plant
Lane 4 - RAPD pattern generated OPC12 primer from genomic DNA of mother plant
Lane 5 - RAPD pattern generated OPC12 primer from genomic DNA of in vitro regenerated plant
Lane 6 - RAPD pattern generated OPU4 primer from genomic DNA of mother plant
Lane 7 - RAPD pattern generated OPU4 primer from genomic DNA of in vitro regenerated plant