Evaluation of somaclonal variation during repetitious subcultures of tobacco (Nicotiana tabacum L.) callus

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Abstract

In this study for the purpose of evaluating the somaclonal variation during repetitious subcultures of tobacco (Nicotiana tabacum cv. chopogh), we cultured the leaf and stem explants of tobacco plants in a medium containing 4 mg/l 2,4-dichlorophenoxyacetic acid and 0.5 mg/l kinetin. After the growth of calluses, they were sub-cultured in the same medium. After the fourth subculture, calluses were transferred to regeneration MS medium containing hormone concentrations 4mg/l kinetin, 1 mg/l zeatin. After regeneration of seedlings, total genomic DNA was extracted from the primary and regenerated plant. Somaclonal variation of the samples was analyzed using 20 Random RAPD Primer. The electrophoresis pattern of 3 Random Primers including OPC-09, OPR-12, OPA-10 indicated the polymorphism in amplified DNA band. This polymorphism resulted from production of somaclonal variation during subcultures of tobacco callus.

Keywords: RAPD-PCR; somaclonal variation; tobacco callus

Abbreviations:
CTAB: Cetyl Trimethyl Ammonium Bromide; MS: Murashige and Skoog; RAPD: Random Amplified Polymorphic DNA; RFLP: Restriction Fragment Length Polymorphism; 2, 4-D: 2,4-dichlorophenoxyacetic acid


Introduction

Tissue culture is a common way for plant mass production. All plants regenerated from cell or tissue culture have a genetic constitution identical to that of the original one, but in the study of biotechnology for the sake of long-time culture in the medium, phenotypic variation was observed in the regenerated plants. This variation is called a somaclonal variation and has been defined as a genetic and phenotypic variation among clonally propagated plant materials (Rasheed et al., 2005). Somaclonal variation is a common phenomenon in plant cell culture and may be produced and affected by factors derived from in-vitro culture conditions. Furthermore, plant regeneration via organogenesis or protoplasts often leads to somaclonal variation.

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(Mohanjain et al., 1997). The mechanisms of somaclonal variation may be due to extensive genomic flux especially altered methylation patterns which can be inherited. DNA replication might be disturbed by altered nucleotide pools. This can make the active genes silenced, make silenced genes activated by mutations in association with non-coding regions (Bordallo et al., 2004; Rasheed et al., 2005; AL-Zahim et al., 1999; Leroy et al., 2000) and also make changes in the structure of chromosomes, activate transposable element, and cause mitotic crossing over. Moreover, the extent of variation depends on genotype, age of the donor plant, cytogenetic changes and explant type (Mohanjain et al., 1997). Epigenetic factors are also involved in making it more complex. On the other hand, somaclonal variation has been related to plant growth regulators. The correlation between the culture time-length and the accumulation of chromosomal variations was first documented in Daucus carota (Bordallo et al., 2004). Also, Mohanjain et al. reported a shift in the morphogenetic pattern of differentiation from shoot bud to embryonic regeneration during the long-term culture of callus of Citrus grandis. The length of culture period also influences somaclonal variation (Mohanjain et al., 1997).

Many strategies can be used to evaluate plant genetic structure from in-vitro derived plant clones, but most of them have limitations. Karyology analysis can not reveal alteration in specific gene or small chromosome arrangements. Analysis of secondary metabolites and isozyme patterns have also been used, but they are limited in their sensitivity (Ehsanpour et al., 2007). RAPD analysis using PCR which is associated with short arbitrary primers, has been demonstrated to be sensitive in detecting variation among individual subjects (Bordallo et al., 2004). In this method, DNA sequence is amplified with oligonucleotide primers and is frequently used to identify somaclonal variation with greater precision and less effort than karyological and phenotypic analysis (Chuang et al., 2008; Qin et al., 2007; Anuntalabhochai et al., 2006). Molecular techniques such as RFLP or RAPD are often favoured over traditional phenotypic or cytological measurements and generally assess even small variations of the genome. The use of the PCR-based RAPD technique to detect somaclonal variation has been applied successfully to several monocotyledonous species, such as Lolium and Allium sativum as well as woody dicotyledonous species, such as Picea abies. It has also been applied for tomato and potato (Sonya et al., 2001; Ehsanpour et al., 2007; EL-Aref and EL-Lithy., 2003; Ngezahayo et al., 2007). In this study we employed RAPD-PCR technique for evaluation of the possible somaclonal variation by tobacco callus subcultures.

**Materials and Methods**

In order to create somaclonal variation in the subcultures, segments (approximately 8-10 mm) from leaves and stems of Nicotiana tabacum cv. chropogh were cultured in the MS medium containing 4 mg/l 2, 4-D and 0.5 mg/l kinetin, 30 g/l sucrose, 8 g/l Agar where the pH of the medium was set at 5.7. All cultures were maintained at a 16/8 light/dark photoperiod. Callus subculture was done every 4 weeks. After 4 subcultures, the callus was transferred for regeneration MS medium supplemented with 4 mg/l kinetine, 1 mg/l zeatin. After regeneration and growth of the regenerated plants, the primary and regenerated plants were submitted to RAPD-PCR. For DNA extraction, approximately 1 g from leaves of primary plant and regenerated plant were grounded, using a hand held grinder with liquid nitrogen. CTAB buffer was used to extract DNA for RAPD-PCR. In this work 20 random primers were used and PCR reactions carried out in total volume of 25 μl at a final concentration of 1X PCR buffer, 6 mM MgCl2, 0.4 mM dNTPs, Taq DNA polymerase enzyme (1/500 μ), 1μg DNA as a template and 0.6 mM random primer. PCR program was as follows: 94 °C for 5 min, and one cycle, 94 °C for 20 second, 32 °C for 35 second, 74 °C for 1 minute which were

![Fig. I. Grown calluses](image)
repeated in 40 cycles followed by 7 minutes extension at 72 °C. Then 10 μl from each PCR product was revealed on 1% agarose gel subjected to electrophoresis at 70 V after staining using ethidium bromide by UV light.

**Random primer sequence**

In this work 20 random primers (CinnaGen Co.) were used. Only 3 primers were able to detect somaclonal variations, which are as follows:

OPA-10 (GTGATCGCAG)
OPR-12 (ACAGGTGCGT)
OPC-09 (CTCACCGTCC)

**Results**

The grown calluses (Fig. I), were transferred to regeneration MS medium and after regenerated seedling (Fig. II), total genomic DNA was extracted from primary and regenerated plants (Fig. III).

Twenty random RAPD primers were used for evaluation of somaclonal variation. The electrophoresis pattern of three random primers indicated the polymorphism in amplified DNA bands. In OPA-10 and OPR-12 primers, the arrow indicates an extra band in the regenerated plant that is not seen in the primary plant (Figs. IV and V).

In the OPC-09, the arrow indicates two extra bands in the primary plant that were not present in the regenerated plant (Fig. VI). This polymorphism showed somaclonal variation during repetitious subcultures of *Nicotiana tabacum* callus.

**Discussion**

During frequent subcultures of callus and regenerated plants from this callus, somaclonal variation was occurred in the cells. This variation can be the result of mutation that occurred during tissue culture. This is similar to the results reported by Bordallo et al. (2004). In their study, Bordallo et al. (2004) working on Baraka cultivar of tomato showed that in the cultured calluses after 90 days somaclonal variation induction occurred. These researchers suggested that there exists an interaction among the explant, the growth regulator, and long periods of cultivation. This interaction increases variability and
frequency of chromosome aberrations (Bordallo et al., 2004).

\[ \text{Fig. V. DNA pattern after amplification using OPR-12 primer in the primary and regenerated plants. The arrow indicates a band present only in the regenerated plant.} \]

In this study we detected somaclonal variation in the regenerated plant of tobacco and compared it with the primary plant through three primers of OPC-09, OPR-12, OPA-10. Some of these primers showed additional DNA bands while in the others reduced DNA bands were observed suggesting somaclonal variation in the regenerated plant.

\[ \text{Fig. VI. DNA pattern after amplification using OPC-09 primer} \]

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