RESEARCH NOTE

Genetic variation in colchicine-treated regenerated plants of *Eucalyptus globulus* Labill.

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Introduction

Eucalyptus globulus Labill. is one of the most important *Eucalyptus* species around the world in terms of current annual increment of wood (Li *et al*. 2000). It is widely grown in Australia, Portugal, Spain, Chile, China, Colombia, Ethiopia, Peru, USA (California) and several other countries. Nowadays, tissue culture of *E. globulus* is successful, and the seedlings from tissue culture are widely used in afforestation programmes (Li *et al*. 2000). Plant cells can be induced into polyploidy during cellular division by using colchicine, which can inhibit chromosome segregation during mitosis.

Various commercial plants have been induced into polyploidy with colchicine, because polyploid plants are usually larger, hardier, faster growing, and in general more desirable than the normal diploid parents (Kim *et al*. 1997; Eeckhaut *et al*. 2002). However, there is still no report about the induced polyploidy of *E. globulus*. The simplest way to identify polyploidy is the observation of chromosome number. Karyotyping is simple and rapid, but has the disadvantage of limited information about genetic variation (Zhang *et al*. 2006). Random amplified polymorhic DNA (RAPD) is one of the simplest and fastest of DNA-based techniques for genetic variability studies (Zhang *et al*. 2005). RAPD also has the advantage that the material is processed by an efficient and inexpensive technique without requiring prior knowledge of the genome. Many scientists have used RAPD markers to study polymorphism in various plants (Ortiz *et al*. 1997; Ranade *et al*. 2002; Rout and Das 2002; Samal *et al*. 2003; Zhang *et al*. 2005, 2008). The aim of the present study was to detect the number of chromosome and to evaluate the applicability of RAPD markers to assess genetic variability in regenerated plants of *E. globulus* treated by colchicine.

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Materials and methods

Plant materials

Tissue culture of *E. globulus* was performed as described by Li *et al*. (2000). After rooting, colchicine treatment was carried out on 150 plantlets, which were regenerated by organogenesis from the same callus. The plantlets were placed into 100-mL solution containing 750 mg/L colchicine and 20 mL/L of dimethyl sulphoxide (DMSO), and the tops were immersed 3 cm under the surface of the solution. Light of 1000 lux was supplied and the plantlets were treated at a temperature of 23–24°C for 5 h. At the end of the treatment, the plantlets were taken out and their bottoms were washed thoroughly with running tap water, and then planted into pots kept at a temperature of about 20°C for two weeks. They were then transplanted to the field. Six months later, the top branches were harvested from the field, and planted into pots kept at a temperature of about 30°C for three weeks for rooting. Finally, 90 plants of *E. globulus*, whose leaves were thicker, larger and darker green, were selected for further analysis.

Chromosome observation

Root tip meristematic cells from planted branches were used as a source of mitoses. Excised roots were treated with 2 mM of 8-hydroxyquinoline for 1–4 h at room temperature, fixed in a 3:1 (v/v) mixture of methanol and glacial acetic acid, and stored at −20°C until required. Excised roots were washed in 0.01 M citric acid-sodium citrate buffer (pH ~ 4.8) for 20 min and digested enzymatically for 1–1.5 h at 37°C in a mixture of 1% (w/v) cellulase (Calbiochem, San Diego, USA), 1% (w/v) Onozuka R10 cellulase (Serva, Heidelberg, Germany) and 20% (v/v) pectinase (Sigma, St Louis, USA). After separation from the non-meristematic parts, root tips were squashed in a drop of 45% acetic acid and the prepara-

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tions were frozen. Cover slips were removed, and the preparations were postfixed in chilled 3:1 (v/v) ethanol: glacial acetic acid, followed by dehydration in absolute ethanol and air-drying. All images were acquired using an Olympus Camedia C-4040Z digital camera (Olympus, Tokyo, Japan) attached to a Leica DMRB microscope(Leica, Wetzlar, Germany). Image processing was done by using Micrografx PicturePublisher (Corel, Dallas, USA) software.

RAPD analysis

Eucalyptus globulus genomic DNA was extracted from young leaves following Zhang *et al*. (2006). Amplification was performed in volumes of 0.02 cm^3 containing 0.002 cm^3 of the 10× buffer, and 100 mM each of dNTPs, 0.4 mM primer, 25 ng genomic DNA, and 1 unit of polymerase (Sangon, Shanghai, China). The reaction mixture was overlaid with 0.02 cm^3 mineral oil. Amplifications were carried out by using a 9600 Perkin-Elmer (Perkin Elmer, Connecticut, USA) thermal cycler programmed for 40 cycles as follows: 15 s at 94°C, 30 s at 36°C, 60 s at 72°C, with an initial melting of 2 min at 94°C, and a final extension of 10 min at 72°C.

After amplification, samples were loaded and electrophoresed on 1.5% agarose gels and stained with ethidium bromide. A GeneRule 100-bp Plus DNA Ladder (MBI Fermentas, Vilnius, Lithuania) was used as size marker. Gels were visualized and photographed on an Image Master VDS system (Pharmacia Biotech, Uppsala, Sweden). Among the 67 primers screened, 14 showed reproducible patterns and were selected for further amplifications (see table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). The amplifications were repeated twice using these primers. When a polymorphic fragment was detected, the whole experiment was repeated to confirm the results.

Results and discussion

Results of chromosome observation showed that the chromosome number in 85 accessions were 44, that in another four were 42, and in another one was 40. Figure 1 shows the typical chromosome number variation in the *E. globulus* plants treated by colchicine. Genetic variation rate was 5.6%. Results showed that the method of doubling of chromosome of *E. globulus* was very successful, and the chromosome numbers of almost all the selected plants had been doubled. It is possible that colchicine can also induce chromosome doubling as well as chromosome loss (Traut and Sommer 1976), or before the doubling of chromosome numbers there may be one or two chromosomes missing in some of the accessions.

RAPD analysis in 90 micropropagated plants of *E. globulus* was performed with a total of 115 amplified reproducible bands per plant produced from 14 random primers. The number of bands per primer ranged from five in OPAK-19 to 11 bands in OPAK-10. The size of amplified bands varied from 300–3000 bp (see table 1 in electronic supplementary material). The average number of bands per primer was 8.2. Results revealed that 11 (12.2%) of the analysed plants showed at least one variation, and the number of changes per regenerated plant ranged from 1 to 15. The distribution of the variability was not uniform, and a few regenerated plants accumulated large numbers of changes, whereas some plants showed no changes. The polymorphic bands generated by different primers were different, ranging from two (OPAA-16) to eight (OPAK-10) (table 1). For example, the RAPD amplification profiles obtained by using primers

Table 1. List of chromosome numbers and polymorphic DNA fragments amplified with different primers among the regenerated plants of *E. globulus* treated by colchicine.

Plant number	Chromosome number	No. of polymorphic bands	Polymorphic bands
3	44	2	OPAA-16_{600}(+),OPAK-10_{1400}(−)
10	44	1	OPAA-16_{900}(+)
15	42	15	OPAC-19_{1300}(−),OPAJ-06_{650}(−),OPAJ-06_{1600}(−),OPAJ-06_{2000}(−),OPAK-10_{850}(−), OPAK-10_{700}(−),OPAK-19_{900}(−),OPAL-17_{1500}(−),OPAN-07_{700}(−),OPAN-08_{2100}(−), OPAN-15_{1800}(−),OPAN-15_{1100}(−),OPAO-03_{1400}(−),OPAQ-05_{1500}(−),OPAR-06_{1800}(−)
17	44	1	OPAC-19_{900}(−)
21	44	2	OPAC-19_{2000}(+),OPAJ-06_{1300}(+)
26	42	10	OPAC-19_{750}(−),OPAJ-06_{700}(−),OPAK-10_{900}(−),OPAK-10_{800}(−),OPAK-19_{1400}(−), OPAL-17_{1300}(−),OPAN-07_{1600}(−),OPAP-17_{2000}(−),OPAR-06_{1500}(−),OPAR-17_{2300}(−)
38	42	8	OPAJ-06_{850}(−),OPAK-10_{1500}(−),OPAK-19_{950}(−),OPAL-17_{700}(−),OPAO-03_{1700}(−), OPAQ-05_{1900}(−),OPAR-06_{1000}(−),OPAR-17_{800}(−)
45	44	2	OPAO-03_{1100}(+),OPAR-17_{1200}(−)
71	42	7	OPAC-19_{2400}(−),OPAK-10_{550}(−),OPAK-19_{1700}(−),OPAN-15_{1900}(−),OPAP-17_{2100}(−), OPAR-06_{1200}(−),OPAR-17_{2100}(−)
83	44	1	OPAO-03_{750}(+)
88	40	8	OPAJ-06_{1500}(−),OPAK-10_{1200}(−),OPAL-17_{600}(−),OPAN-07_{1400}(−),OPAN-08_{1200}(−), OPAN-15_{1600}(−),OPAO-03_{850}(−),OPAR-06_{1350}(−)

(+) indicates present in the planta; (−) indicates absent in the plant.

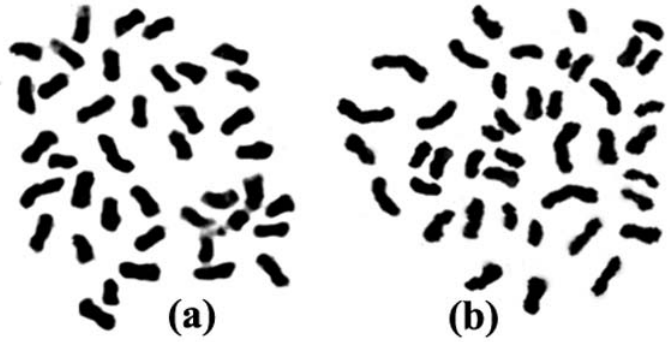

Figure 1. Chromosome number variation of the micropropagated plants of *E. globulus* treated by colchicine. (a) Chromosome number of accession 17, the chromosome number was 40; (b) chromosome number of accession 43, the chromosome number was 42.

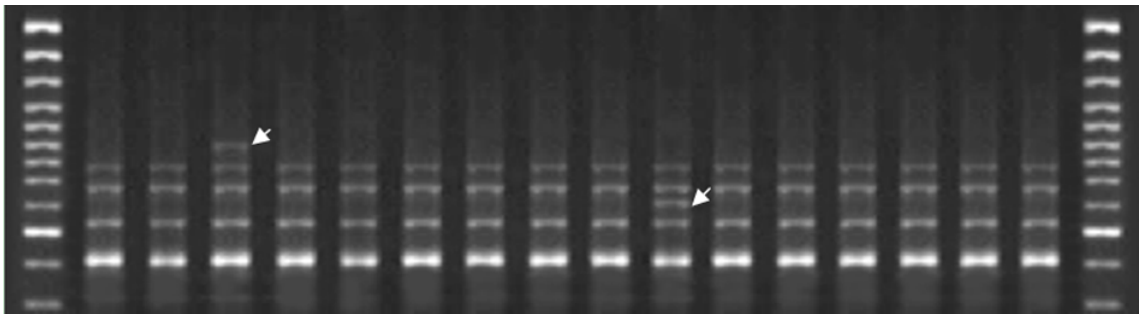

Figure 2. Amplifications produced by using primer OPAA-16. Lane M, DNA marker; lanes 1–16, different accessions of *E. globulus* treated by colchicine; arrows in the figure indicate polymorphic bands.

OPAA-16 in 90 micropropagated shoots showed identical bands except for two plants. These two plants showed two additional bands, and portion of gels showing typical amplification products are shown in figure 2. These unique bands showed a molecular size of approximately 600 bp and 900 bp.

It was worth noting that the polymorphic bands were all missing in those plants that accumulated large numbers of changes, and the polymorphic bands were generated or missed in those just accumulated small numbers of changes. Results also showed that those plants that accumulated large numbers of changes were just the five whose chromosome numbers were 42 or 40 (table 1). Results confirmed that there may be one or two chromosomes missing before the doubling of chromosome numbers. The other six genetic change plants had just one or two polymorphic bands, indicating that there was genomic change during tissue culture. The proportion of polymorphic fragment number did not correlate with the number of the chromosome lost (table 1). The reason of such phenomena was probably that we did not use enough number of primers to cover the whole genome. The results of RAPD analysis revealed that the different RAPD markers were lost in the different plants (table 1), indicating that different chromosomes were lost in the different plants.

In this study, some of the polymorphism was due to somaclonal variation (Larkin and Scowcroft 1981), because polymorphic bands were found among plants regenerated from the same bud explant, an observation not limited to any particular group of plants. Polymorphism was also due to colchicine treatment, because colchicine could also induce chromosome doubling as well as chromosome loss. Till date, the RAPD technique has been applied in a few studies of regenerated plants (Feyissa *et al*. 2007). Now RAPD analysis has revealed as a reliable and efficient method for identification of genetic variation in *E. globulus* regenerated plants treated by colchicine.

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