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Article *in* Australian Journal of Agricultural Research · January 2007

DOI: 10.1071/AR06125

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Assessment of clonal stability of *in vitro* regenerated shoots of *Macadamia tetraphylla* by RAPD analysis

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Abstract. Macadamia nuts constitute an important part of the world nut industry and are highly valued for their health-promoting properties. Macadamia is an open-pollinated crop that takes 8–12 years to bear fruit when multiplied via seeds. The yield and nut quality in seedling plantations are often highly variable, and grafting is currently the most common method for producing nursery trees with reduced variability. We have previously reported on the tissue-culture propagation of macadamia, and in the present study we assessed the clonal integrity of the regenerated shoots. The RAPD profiles of 3 macadamia stock plants and 10 *in vitro* regenerated lines from each stock plant were analysed to assess the clonal integrity of the shoots regenerated *in vitro* for micropropagation purposes. The extent of genetic variation between the stock plants and 9 randomly selected seedlings was also assessed. There was no difference in clonal identity between the stock plants and their micropropagated progeny, indicating that clonal micropropagation was possible using enhanced axillary proliferation in macadamia. In contrast, there was a large genetic variation among the seedlings and between the seedlings and stock plants, with genetic distance estimates ranging from 0.121 to 0.637 among seedlings, indicating rampant out-crossing of the macadamia plant.

Additional keywords: macadamia propagation, genetic variation.

Introduction

Australia is second only to Hawaii in the production of macadamia and is the largest exporter of macadamia nuts, contributing ~50% of the world trade. Other major producers of macadamia include Kenya, South Africa, Brazil, and Costa Rica. The world demand for macadamia nut kernels is expected to increase significantly due to its health-promoting qualities, but the expansion of macadamia plantations is limited by the inadequate supply of nursery material to prospective growers due to the slow development of uniform and productive trees and a lack of the appropriate technology to solve these problems. Macadamia is easily propagated from seeds, but seedling plantings exhibit highly variable yield and nut quality (Kermond and Baumgart 1996) due to the open-pollinated nature of the tree, and hence are of little value in commercial production. Grafting is currently the most common method for multiplying macadamia trees. The most widely used rootstocks are seedlings from *Macadamia tetraphylla* or hybrids between *M. tetraphylla* and *M. integrifolia* on which scions from *M. integrifolia* are grafted. The development of a micropropagation system for macadamia has the potential to improve the speed of clonal propagation of both rootstock and scion cultivars, as well as enhance the possibility of developing *in vitro* micrografting protocols. These technologies, which have been successful in some woody plants such as cashew (Shantha *et al*. 1999) and pistachio (Abousalim and Mantel 1992), can serve as tools in laboratory graft-incompatibility studies.

We were the first to report *in vitro* shoot multiplication of macadamia through enhanced axillary bud proliferation from single-node microcuttings (Mulwa and Bhalla 2000). The micropropagation of woody plants through axillary shoot proliferation is preferable because of the genetic stability of the resultant clonal regenerants. However, there are widespread reports of significant somaclonal variation in micropropagated plantlets, even for simple micropropagation systems such as enhanced axillary bud multiplication (Rani *et al*. 1995). Ensuring the clonal integrity of micropropagated plantlets would be greatly aided by the development of specific genetic tests, since identifying variants by phenotypic observations requires plants to be monitored until maturity. This can be especially impractical when dealing with a long-lived tree like macadamia, which takes 8–12 years to bear fruit.

Some studies have used karyotypic analysis to detect the chromosomal rearrangements in off-types in micropropagated plantlets (Bhojwani *et al*. 1986), but there are severe limitations to this method, especially where polyploidy is involved. Isozyme analysis can also be used, but the disadvantages of this technique include the small number of informative markers it generates and its sensitivity to environmental and developmental variations. The above methods also cannot detect any DNA

 10.1071/AR06125 0004-9409/07/030253

sequence polymorphisms that may be present in plants that are regenerated *in vitro*. Plants derived from tissue culture have been successfully characterised by analyses of restriction fragment length polymorphisms (Shenoy and Vasil 1992; Chowdhury and Vasil 1993), but this technique is highly complex, expensive, and involves the use of radioisotopes. Random amplified polymorphic DNA (RAPD) analysis can offset some of these problems, is quick to perform, and requires only very small amounts of genomic DNA (Williams *et al*. 1990). RAPD analysis in plants has been widely used to detect genetic and somaclonal variations (Gallego *et al*. 1997; Piccioni *et al*. 1997; Swoboda and Bhalla 1997; Rival *et al*. 1998; Jain *et al*. 1999), identify cultivars (Baum *et al*. 1998), and verify clonal lines produced from *in vitro* culture systems (Rani *et al*. 1995; Modgil *et al*. 2005).

In this study, we used RAPD analysis to rapidly evaluate the clonal integrity of shoots regenerated *in vitro* from *Macadamia tetraphylla* and to estimate the genetic variation among a random selection of seedlings produced from a single seed lot.

Materials and methods

Plant material

Fully expanded leaves from fresh growth were collected from 3 randomly selected 3-year-old grafted stock plants (named MI, MII, and MIII) and 9–12-month-old seedlings (numbered 1–9). The seedlings and grafted plants were grown under glasshouse conditions. Leaves were also collected from 30 micropropagated shoots (10 micropropagated shoots per mother plant).

Genomic DNA isolation

Total genomic DNA from fresh leaf tissue was isolated following the protocol of Doyle and Doyle (1990), with minor modifications. Specifically, 0.2 g of tissue from each sample were ground in liquid nitrogen to a fine powder in chilled mortars. The material was incubated for 45 min at 60°C in 2 mL of pre-heated DNA isolation buffer [4% cetyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.2% 2-mercaptoethanol, 20 mM EDTA, and 100 mM TRIS-HCl (pH 8.0)]. The homogenate in each tube was extracted once with equal volumes of 24 : 1 v/v chloroform/isoamylalcohol with thorough but gentle mixing followed by centrifuging at 14 000 rpm for 15 min at room temperature. The aqueous phase was carefully removed and transferred to a clean tube, 2/3 of its volume of cold isopropanol was added, and the mixture incubated at −20°C for 30 min to precipitate the DNA. The DNA was pelleted following centrifugation at 5000 rpm (4°C) for 10 min and then washed in 5 mL of 70% ethanol. The tubes were then centrifuged at 5000 rpm (4°C) for another 10 min and the nucleic acid pellets air-dried at room temperature to get rid of any residual ethanol. The pellets were re-suspended in 200 µL of TE buffer [10 mM TRIS-HCl (pH 7.4) + 1 mM EDTA] followed by an overnight RNase-A (1 µL of 40 µg/mL) treatment at 5°C. The quality of DNA was checked by electrophoresis using 0.8% agarose gels and quantified using a GeneQuant II spectrophotometer (Pharmacia Biotech). The final concentrations of the genomic DNA were adjusted to 50 ng/µL.

RAPD-PCR conditions

Forty RAPD primers (decamer oligonucleotides; Operon OPA-01 to OPA-20 and OPK-01 to OPK-20) obtained from Operon Technologies Inc. (Alameda, CA, USA) were tested as single primers for the amplification of genomic DNA. These were first tested on DNA samples from 3 seedlings and the 3 stock plants. PCR reactions were performed in a total volume of 20 µL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 25 µM primer, 0.25 U Taq DNA polymerase (Gibco-BRL), and 50 ng template DNA. Of the primers tested, 8 (OPA-04, OPA-09, OPA-10, OPA-11, OPA-17, OPK-07, OPK-15, and OPK-17) (Table 1) were selected for further experimentation based on very clear, scorable, and reproducible bands.

In subsequent experiments, PCR reactions were carried out using the selected primers and DNA samples from the 9 seedlings and the 3 stock plants. PCR products from these reactions were separated using 0.8% agarose gels to compare banding patterns from the stock plants and the seedlings. In another set of experiments the primers were used to amplify DNA from each stock plant and 10 of its micropropagated progeny to check for any polymorphisms among them. All the PCR amplifications were performed in a GeneAmp PCR System 2400 thermocycler (Perkin-Elmer). The PCR amplification conditions used were an initial strand separation step at 94°C for 4 min, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 2 min, plus a final extension at 72°C for 10 min. The reactions were performed in duplicate to ensure reproducibility of results. PCR products were separated on 0.8% agarose gel electrophorsed at 65 V/cm. Gels were visualised after staining with ethidium bromide on a UV transilluminator (Fotodyne) and photographed. *Hind*III/*Eco*RI-digested lambda DNA was used as a molecular marker to estimate the size of amplification products.

Data analysis

Only scorable and reproducible bands were considered for analysis. Bands were scored 1 if present and 0 if absent. Genetic similarity was determined by pair-wise comparison of individuals based on both unique bands as well as bands in common, and computed using Nei and Li (1979) index of genetic similarity, according to the following equation:

$$S_{AB} = \frac{2 \times \text{the number of shared bands (in A \& B)}}{(\text{No. of bands in A} + \text{No. of bands in B})}$$

Table 1. RAPD primers used and their sequences

Primer	Sequence
OPA-04	AATCGGGCTG
OPA-09	GGGTAACGCC
OPA-10	GTGATCGCAG
OPA-11	CAATCGCCGT
OPA-17	GACCGCTTGT
OPK-07	AGCGAGCAAG
OPK-15	CTCCTGCCAA
OPK-17	CCCAGCTGTG

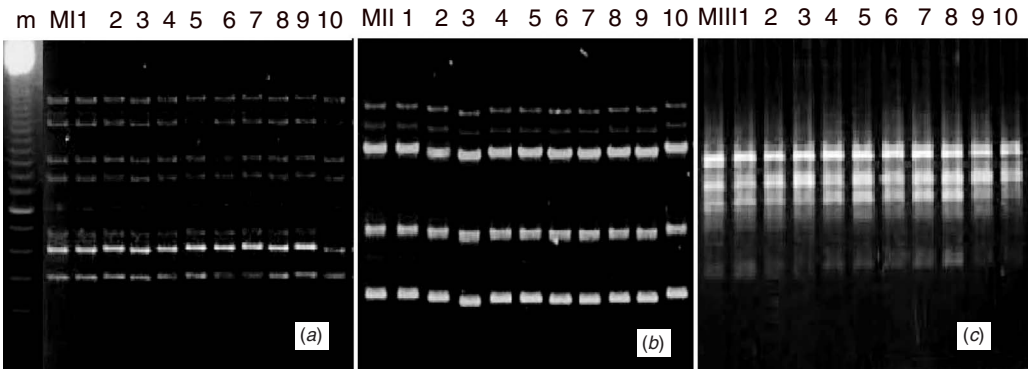

Fig. 1. RAPD profiles of mother stock plants and their *in vitro* regenerated progeny. Lane m, molecular marker; lanes MI, MII, MIII, the 3 mother stock plants; lanes 1–10, the *in vitro* regenerated shoots; (*a*), (*b*), (*c*), primers OPA-10, OPA-17, and OPK-17, respectively.

where S is similarity index and A and B are samples A and B, respectively.

Genetic distance was calculated using the formula: $d = 1 - S_{AB}$ where d is genetic distance. To investigate variation among the *in vitro* micropropagated shoots, RAPD profiles from the *in vitro*-derived shoots and the stock mother plants were examined for polymorphisms.

Results

Each of the 8 primers used for PCR amplifications produced 5–12 scorable bands per sample. In total, 211 bands were amplified for the experiments comparing stock plants with their *in vitro*-derived progeny, and experiments with the seedlings and stock plants yielded 107 bands. No polymorphic banding patterns were detected when stock plants were compared with their tissue cultured progeny, indicating the presence of complete clonal identity. Figure 1*a*, *b*, and *c* depicts the uniform and monomorphic banding patterns produced by the 3 stock plants and their *in vitro* progeny using primers OPA-10, OPA-17, and OPK-17, respectively.

Of the 107 bands scored from PCR reactions comparing individual seedlings and stock plants, 90 were polymorphic. All 8 primers tested produced polymorphic bands with the seedlings. However, the 3 stock plants produced monomorphic bands with all the primers. Figure 2 illustrates some of the polymorphisms in the seedlings and the monomorphic bands for stock plants obtained using primer OPA-09.

Estimation of the genetic distance estimate(s) for the individual seedlings and the tissue-culture stock plants revealed wide variation between them. The stock plants produced monomorphic bands, indicating the absence of genetic variation. The stock plants also registered the same genetic distance with each of the seedlings in the analyses (Table 2). These results indicate that the 3 stock plants were clones. The genetic distance between the individual seedlings ranged from 0.121 (between seedlings 5 and 6) to 0.637 (between seedlings 2 and 5) (Table 2).

Discussion

Although variations in seedling plants are expected in cases where plants are naturally outcrossing, as is the case for macadamia, micropropagation by tissue culturing is aimed at the rapid production of clonal plant materials. However, somaclonal variation is also known to occur in tissue-culture systems due to mutations caused by repeated subculturing and/or extreme culture conditions, such as the high concentrations of nutrients and growth regulators to which explants are exposed (Deverno 1995). Somaclonal mutations frequently appear in culture systems that involve plant regeneration from callus or via somatic embryogenesis (Isabel *et al*. 1993; Ostry *et al*. 1994). However, simple micropropagation techniques that involve enhanced axillary branching have also been reported to produce somaclonal variants in woody plants (Rani *et al*. 1995; Modgil *et al*. 2005). If clonal plant multiplication is an ultimate goal, this indicates the need for methods that can rapidly ascertain the clonal integrity of micropropagated plants.

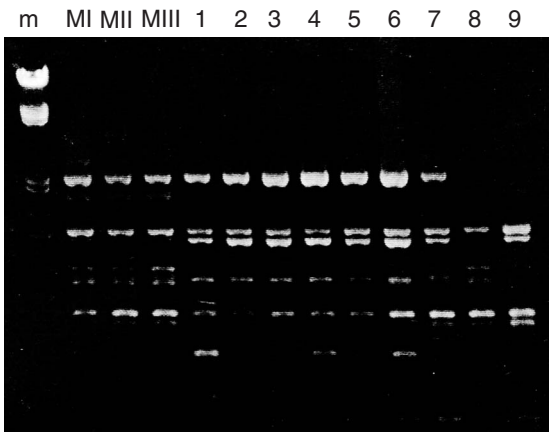

Fig. 2. RAPD profiles of 3 stock plants (lanes MI, MII, MIII) and 9 randomly selected seedlings (lanes 1–9), using primer OPA-09, showing monomorphic bands of the stock plants and polymorphic bands for the seedling plants (lane m, molecular marker).

In this study the direct analysis of DNA using RAPD markers proved to be an effective technique for rapidly evaluating the clonal integrity of the *in vitro*-derived shoots of macadamia and also for estimating the genetic variability present in a single seed lot. We found that the micropropagated

Table 2. Genetic distance estimates between stock mother plants (MI, MII, MIII) and 9 randomly selected seedlings (1–9)

	Stock plants			Seedling plants								
	MI	MII	MIII	1	2	3	4	5	6	7	8	9
MI	0.0	0.0	0.0	0.416	0.391	0.323	0.333	0.328	0.304	0.270	0.273	0.290
MII		0.0	0.0	0.416	0.391	0.323	0.333	0.328	0.304	0.270	0.273	0.290
MIII				0.416	0.391	0.323	0.333	0.328	0.304	0.270	0.273	0.290
1					0.227	0.216	0.269	0.346	0.351	0.400	0.409	0.429
2						0.131	0.245	**0.637**	0.274	0.321	0.338	0.286
3							0.631	0.271	0.197	0.289	0.252	0.339
4								0.168	0.147	0.266	0.301	0.395
5									**0.121**	0.252	0.238	0.379
6										0.259	0.246	0.353
7											0.163	0.227
8												0.215

shoots exhibited 100% genetic identity after several cycles of repetitive axillary proliferation, which lends credence to the potential of clonal micropropagation in macadamia using single nodes as starting explants. These results also concur with the observations of Deverno (1995) that explants that retain their developmental integrity in culture, such as apical and axillary meristems, rarely produce plantlets that vary from their mother plants. George (1993) also noted that nodal segments, such as those used in macadamia *in vitro* cultures, are the most reliable explants for clonal *in vitro* plant multiplication. These observations not withstanding, it appears that the genetic stability of micropropagated progeny varies with plant species. Modgil *et al*. (2005) reported significant RAPD polymorphisms in apple rootstock shoots produced by axillary proliferation from nodal explants compared with their mother stock plants. Mondal and Chand (2002) also described wide variations among micropropagated tea plantlets and their mother plants. Both of these previous studies indicate the need to perform genetic testing.

The results of RAPD analyses also showed the extent of genetic variability among the 9 seedlings sampled (Table 2). This strongly supports the assertion by Kermond and Baumgart (1996) that every seedling growing from a macadamia seed lot would have a different genetic makeup due to the high outcrossing tendency of the plant. This has significant implications in the use of seedling rootstocks in macadamia propagation due to the likely presence of variable rootstock/scion interactions. Moreover, concerns have been raised about the quality of macadamia kernels from trees grafted on seedling rootstocks. Trochoulias (1992) reported that the flavour of macadamia kernels produced on trees grafted on some seedling rootstocks was inferior to those from trees grafted on clonal rootstocks grown from cuttings, which may be attributable to the wide genetic variability evident in seedlings (as demonstrated by the present study).

In conclusion, this study shows that RAPD analysis is a useful technique for establishing the clonal integrity of tissue-cultured macadamia plants, and is also suitable for the detection of genetic variation in seedling plants of this species.

Acknowledgments

Support from the Australian Macadamia Society and Horticulture Australia Ltd is gratefully acknowledged. The authors also thank Prof. Quinsheng Wu for critical comments on the manuscript, and the Australian Government for awarding R.M.S.M. an ADCOS scholarship to study at The University of Melbourne.

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Manuscript received 21 April 2006, accepted 4 December 2006