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GENETICS AND GENOMICS

R. M. Devarumath · S. Nandy · V. Rani
S. Marimuthu · N. Muraleedharan · S. N. Raina

RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *assamica* (Assam-India type)

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Abstract An efficient in vitro propagation method using enhanced axillary branching cultures produced plants from nodal explants of three mature, elite tea clones: diploid UPASI 26 and UPASI 27 (2*n*=2*x*=30) representing *Camellia sinensis* (China type) and triploid UPASI 3 (2*n*=3*x*=45) representing *C. assamica* ssp. *assamica* (Assam-India type). The genetic fidelity of the micropropagated plants of these three tea clones was assessed by analysing their nuclear, mitochondrial (mt), and chloroplast (cp) genomes using multiple molecular DNA markers. A total of 465, 446 and 462 genetic loci were produced with RFLP, RAPD and ISSR fingerprinting in the micropropagated plants and the corresponding mother plant of *C. sinensis* clone U (UPASI) 26, and *C. assamica* ssp. *assamica* clones U3 and U27, respectively. RFLP fingerprinting was performed using six restriction endonuclease digests and 14 mt and cp gene probes in 84 enzyme-probe combinations. For PCR fingerprinting, 50 RAPD and SSR primers were used for amplifications. The micropropagated plants of both the U3 and U27 clones revealed complete stability in the 462 and 446 genetic loci analysed. In comparison, 36 (7.7%) of the 465 loci were polymorphic among micropropagated plants of the U26 clone. The observed polymorphic loci were not restricted to a particular genome (nuclear or organellar), although a relatively low (7.43%) level of polymorphism was observed in the nuclear as compared to the mt genome (16.3%). ISSR fingerprinting (12.8%) detected more polymorphic loci than RAPD fingerprinting (4.28%). No polymorphism was observed in the cp genome of the micropropagated plants of the three tea clones. The rigorous screening of nuclear and two organellar genomes has demonstrated, for the first time, subtle genetic variation at the DNA sequence level in organized meristem-derived micropropagated plants of tea. Clearly, this is another example demonstrating that organized meristem cultures are not always genetically true-to-type. The genomic changes in tea clones are genotype dependent rather than culture condition dependent.

Keywords *Camellia sinensis* (China type) · *Camellia assamica* ssp. *assamica* (Assam-India type) · Enhanced axillary branching · Molecular DNA markers · Somaclonal variation

Abbreviations *BA*: Benzylaminopurine · GA_3: Gibberellic acid · *IBA*: Indole-3-butyric acid · *ISSR*: Inter-simple sequence repeat · *RAPD*: Random amplified polymorphic DNA · *RFLP*: Restriction fragment length polymorphism · *U*: UPASI

Introduction

Tea is one of the most important beverages in the world (Charles 1981) and is a major cash crop in a number of developing countries, including India (Wachira et al. 1995; Akula and Dodd 1998). India is the largest producer of tea, and after Sri Lanka and China, it is the third largest exporter of tea in the world (Anonymous 2000). Although the cultivated teas have been taxonomically classified as *Camellia sinensis* (L) O. Kuntz, *C. assamica* ssp. *assamica* (Masters) Wight and *C. assamica* ssp. *lasiocalyx* (Planch Ms) –being endemic to China, Assam (India) and the Indo-China regions of Southeast Asia, respectively – they are also commonly referred to as China type, Assam type and Southern or Cambod type, respectively, of *C. sinensis* (L.) O. Kuntz (Barua 1965). The

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R.M. Devarumath · S. Nandy · V. Rani · S.N. Raina (✉)
Laboratory of Cellular and Molecular Cytogenetics,
Department of Botany, University of Delhi, Delhi 110 007, India
e-mail: snraina@satyam.net.in
Tel.: +91-11-7666402, Fax: +91-11-7666541

S. Marimuthu · N. Muraleedharan
UPASI Tea Research Institute, Nirardam BPO, Valparai 642 127, Coimbatore, India

cultivated tea species are self-sterile, allogamous diploids (2*n*=2*x*=30). The spontaneous occurrence of triploid, tetraploid and hexaploid cytotypes have also been reported (Ellis 1995). The outbreeding nature coupled with frequent spontaneous hybridization that takes place between different types of tea has resulted in highly heterogeneous hybrid plants extending from the 'China type' to the 'Assam type'. The hybridization is so extreme that the existence of a basic archetypical taxon is often debated (Barua 1965; Banerjee 1992). At the present time commercial plantations around the world consist mostly of hybrid clones between *C. assamica* and *C. sinensis* – popularly known as 'Indian hybrid tea' (Singh 1979). However, hybrids at many places are still generally referred to as Assam, China or Cambod type depending upon their morphological proximity to the main taxon (Banerjee 1992).

To avoid the extensive genetic non-uniformity resulting from seed propagation and, consequently, dilution of a quality product from a particular clone, tea clones are traditionally multiplied by cuttings and grafting (Satyanarayana et al. 1992; Prakash et al. 1999). An alternative method that has come into operation lately is in vitro micropropagation of organized meristem cultures of nodal/shoot-tip explants and somatic embryogenesis (Raj Kumar and Ayyappan 1992; Wachira and Ogada 1995; Akula and Dodd 1998). In tea, in vitro culture is considered to be the most effective method for mass clonal multiplication.

The detection of subtle somaclonal variation at phenotypic, cytological, biochemical and molecular levels among micropropagated plants in many taxa (Armstrong and Phillips 1988; Amberger et al. 1992; Rani et al. 1995, 2000, 2001; Damasco et al. 1996; Fourre et al. 1997; Rani and Raina 1998a, b, 2000, 2002; Rival et al. 1998; Gimenez et al. 2001; Rahman and Rajora 2001) has, however, brought into question the validity of the concept that complete genetic stability is retained in plants derived through organized meristem cultures (see Rani and Raina 2000, 2002). The occurrence of cryptic genetic defects arising via somaclonal variation in the regenerants can seriously limit the broader utility of micropropagation systems (Rani et al. 1995, 2000, 2001; Rani and Raina 1998a, b, 2000, 2002). Therefore, it is important to first establish the suitability of a particular micropropagation protocol developed for a particular clone with respect to the production of genetically identical and stable plants before it is released for commercial purposes. No such efforts have yet been made for tissue culture-derived plants of tea clones.

During the last few years, various molecular DNA markers which screen nuclear and organellar genomes have been profitably utilized for the fast and unambiguous assessment of the genetic fidelity of micropropagated plants (see Rani and Raina 2000, 2002). In the investigation reported here, we analysed micropropagated plants of three elite tea clones – UPASI 3 (U3) and UPASI 27 (U27), representing *C. assamica* ssp. *assamica*, and UPASI 26 (U26), representing *C. sinensis* – using RFLPs of both the mitochondrial (mt) and chloroplast (cp) genomes and RAPD and ISSR fingerprinting of the nuclear genome.

Materials and methods

Production of micropropagated plants

Efficient protocols for micropropagation through enhanced axillary branching cultures were established in tea clones U3 (2*n*=3*x*=45) and U27 (2*n*=2*x*=30) representing *Camellia assamica* ssp. *assamica* and in U26 (2*n*=2*x*=30) representing *C. sinensis*. Clone U3 is a high yielder with an excellent spread and dense plucking table, but it is susceptible to drought (Venkataramani and Sharma 1967), while U27 is a drought-hardy and moderate- to high-yielding clone. U26 is both a high-yielding and drought-tolerant diploid clone (Sharma and Satyanarayana 1989).

Nodal segments having dormant axillary primordia collected from aperiodic shoots (obtained from mother bushes sprayed with 1% zinc sulphate and 50 mg/l carbendazim) served as the source of explants. The explants were washed with running tap water for 10 min, surface sterilized with 1% sodium hypochlorite (w/v) and washed with sterile Millipore water before being treated with 0.1% $HgCl_2$ for 7 min, then rinsed thoroughly with sterile distilled water. They were then cultured on half-strength MS medium (Murashige and Skoog 1962) medium for initial establishment. After 2 months, explants were transferred to a rapid multiplication medium containing half-strength MS salts with 5 mg/l BA and 160 mg/l adenine sulphate. A 2-month incubation period with 15-day subculture intervals was sufficient to achieve a multiplication ratio of 1:15. The multiple shoots were tiny and rosette in nature. For further elongation, these were excised and cultured onto a half-strength MS medium supplemented with 5 mg/l BA and 5 mg/l GA_3. Within 45 days, the multiple shoots were 5–8 cm long. For rooting, elongated shoots were transferred to a quarter-strength MS medium fortified with 3 mg/l IBA and incubated under dark conditions. About 90% of the rooting was achieved within 4 weeks. The well-rooted microshoots were transferred to pots containing loamy soil and sand (1:1, v/v) and the pH adjusted to 5.0. The plantlets were hardened in a greenhouse before they were transferred to field conditions.

DNA isolation, restriction endonuclease digestion and Southern blotting

Total DNA was isolated from fresh leaves of 14 enhanced axillary branching-derived plants of each of three tea clones (U3, U26, U27) according to the procedure of Porebski et al. (1997). For each micropropagated plant (randomly selected from a particular batch of approximately 100 plants), DNA was extracted separately approximately 3 months after their growth in the greenhouse. For each of the three tea clones, DNA was also extracted separately (leaves excised from multiple positions and bulked) from a single mature mother plant that served as the explant source and five ramets derived from the mother ortet.

Total DNA was separately digested with six restriction endonucleases (*Hin*dIII, *Dra*I, *Bam*HI, *Eco*RI, *Eco*RV, *Xba*I) according to the manufacturer's instructions (Boehringer Mannheim, Germany) and fractionated by 0.85% agarose gel electrophoresis (AGE) in 1× TAE buffer (Maniatis et al. 1982). Lambda DNA digested with *Hin*dIII was included on each gel to provide size standards. After AGE, the DNA was blotted onto a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech, UK) by the alkaline transfer method (Reed and Mann 1985).

DNA probing

A detailed description of the mt and cp probes used in the present study are given in Table 1. The gel-purified DNA probes were dena-

Table 1 Mitochondrial (mt) and chloroplast (cp) DNA probes used in the present study

Probe	Genes	Origin of sequence
Mitochondrial		
Cytochrome oxidase genes		
coxI	Cytochrome *c* oxidase subunit	Oenothera
coxII	Cytochrome *c* oxidase subunit	Pea
coxIII	Cytochrome *c* oxidase subunit	Oenothera
cob	Apocytochrome b	Wheat
Ribosomal RNA genes		
rrn18	18S ribosomal RNA gene	Wheat
rrn26	26S ribosomal RNA gene	Wheat
NADH genes		
nad3	NADH dehydrogenase subunit 3	Wheat
ATP synthase gene		
atpA	Subunit A of the ATPase complex	Pea
Membrane binding protein gene		
orf25	Open reading frame 25	Wheat
Chloroplast		
Ba5	Part of tRNI, tRNA, 23S rRNA, 4.5S and 5S rRNA and tRNN and spacer region in between these genes	Tobacco
B25-Ba4	Part of rpoC, rps2, atp1, atpH, atpF, atpA, tRNR, tRNG and spacer region in between these genes	Tobacco
B18-11a	Part of trnl, 16 S, trnv and spacer regions in between these genes	Tobacco
Ba1	Part of PsbF, PsbE, trnw, trnP, Rpl33, rps 18, rpl20, rps 22, psbB, PsbH, Pet B, rpoA, rpl14 and spacer regions in between these genes	Tobacco
B29–22c	Part of rbcL gene	Tobacco

tured and chemically labelled (overnight at 37°C) with fluorescein-11-dUTP (Amersham Pharmacia Biotech) in the presence of exonuclease-free Klenow enzyme, random monomer primers and a fluorescein nucleotide mix. The genomic DNA fragments that hybridized to the probe were detected enzymatically according to the manufacturer's (Amersham Pharmacia) protocols. A few anomalous bands, wherever observed, were not considered in the data analysis.

RAPD fingerprinting

A total of 25 arbitrary, 10-mer primers (Operon Technologies, USA) from kits A, C and K were used for the PCR amplification of DNA extracted from the mother and micropropagated plants. Each 25 µl of reaction volume contained 10 m*M* Tris-HCl (pH 9.0), 50 m*M* KCl, 2.5 m*M* $MgCl_2$ (Sigma, St. Louis, Mo.), 0.1 m*M* dNTPs (Promega, Madision, Wis.), 0.1% Triton X-100 (Sigma), 200 n*M* primer, 25 ng DNA template and 1 U *Taq* DNA polymerase (Promega). The reaction mix was overlaid with an equal volume of mineral oil (Sigma). DNA amplification was performed in a Perkin Elmer Cetus Thermal-cycler programmed for 45 cycles as described in Raina et al. (2001).

ISSR fingerprinting

Twenty-five SSR primers (University of British Columbia Biotechnology Laboratory, Vancouver, Canada) randomly selected from primer set 9 were used for PCR amplification. These primers were mostly 16–17 mer. The amplifications were carried out in 10 m*M* Tris-HCl (pH 9.0), 50 m*M* KCl, 1.5 m*M* $MgCl_2$ (Sigma), 0.1 m*M* dNTPs (Promega), 0.1% Triton X-100 (Amersham Pharmacia Biotech), 2% formamide (Sigma), 200 n*M* primer, 1 U *Taq* DNA polymerase (Promega) and 5 ng genomic DNA. The reaction mix (25 µl) was overlaid with an equal volume of mineral oil (Sigma), and PCR was performed in a Perkin Elmer Cetus 480 Thermal-cycler. Initial denaturation was for 7 min at 94°C, followed by 45 cycles of 30 s at 94°C, 45 s at a calculated annealing temperature (based on the Wallace rule; Thein and Wallace 1986), 2 min at 72°C and a final 7 min extension at 72°C.

The amplification products in both cases were size separated by standard horizontal electrophoresis on 1.4% agarose (Amersham Pharmacia Biotech) gels and stained with ethidium bromide. PCR reactions were repeated at least twice to establish reproducibility of the results.

Results and discussion

RFLP fingerprinting

Mitochondrial genome

Fifty-four enzyme (*Dra*I, *Bam*HI, *Hin*dIII, *Eco*RV, *Eco*RI and *Xba*I)–probe (atpA, nad3, rrn18, rrn26, orf25, cob, coxI, coxII and coxIII) combinations produced a total of 86, 85 and 85 bands in the mother and micropropagated plants of *C. sinensis* clone U26, and *C. assamica* ssp. *assamica* clones U3 and U27, respectively (Table 2). The number of bands produced by a single enzyme-probe combination ranged from one (*Dra*I + atpA) to five (*Hin*dIII + rrn26) in U26. The hybridized fragments ranged in size from 0.7 kb(*Hin*dIII + cob) to 23.1 kb (*Hin*dIII + rrn26) in U27 and U26, respectively. Four (*Dra*I, *Bam*HI, *Eco*RV and *Eco*RI) of the six analysed restriction endonucleases in combination with mt gene probes cob, nad3, rrn18 and rrn26 revealed 14 polymor-

Table 2 RAPD, ISSR and RFLP fingerprinting of nuclear and organelle genomes of the mother plant and micropropagated plants of *C. assamica* ssp. *assamica* (U3, U27) and *C. sinensis* (U26) clones

Source	Clone U3	Clone U27	Clone U26		
	Total no. of loci[a]	Total no. of loci[a]	Total no. of loci	Number of polymorphic loci	Percentage polymorphism
Nuclear genome					
RAPD markers (25 primers)	159	183	187	8	4.28
ISSR markers (25 primers)	119	111	109	14	12.8
Total	278	294	296	22	7.43
Mitochondrial genome					
RFLP markers (54 enzyme-probe combinations)	85	85	86	14	16.3
Chloroplast genome					
RFLP markers (30 enzyme-probe combinations)	83	83	83		
Total	168	168	169	14	8.28
Grand total	446	462	465	36	7.74

[a]All monomorphic

Table 3 Polymorphic RFLP, RAPD and ISSR markers among the mother plant and micropropagated plants of *Camellia sinensis* clone U26

Probe/primer	Restriction enzyme	Number of bands	Size range (kb)	Number of monomorphic bands	Number of polymorphic bands
Mitochondrial gene probes					
cob	*Bam*HI	2	5.0–5.5		2
cob	*Dra*I	2	2.0–4.1		2
nad 3	*Eco*RI	2	1.5–9.0	1	1
rrn 26	*Bam*HI	3	6.1–19.7	1	2
rrn 18	*Eco*RV	3	5.5–23.1	1	2
rrn 26	*Eco*RV	4	3.3–23.1	2	2
nad3	*Eco*RV	4	2.3–10.2	1	3
RAPD primers					
OPA 07		9	0.55–1.54	5	4
OPK 03		6	0.59–1.62	4	2
OPC 04		4	0.65–1.52	2	2
SSR primers					
UBC 844		4	0.92–1.3	1	3
UBC 859		7	0.99–1.23	1	6
UBC 845		7	0.72–1.16	3	4
UBC 848		7	0.63–1.41	6	1

phic bands among the micropropagated plants of U26 with a polymorphism frequency of 16.3% (Tables2, 3; Fig. 1a–d); the remaining 72 bands were commonly shared between the mother and micropropagated plants. In comparison, *C. assamica* ssp. *assamica* clones U3 and U27 were devoid of polymorphic hybridized fragments, and all 85 bands were monomorphic among the mother and micropropagated plants of both clones (Table 2). Probing of DNA samples of the five ramets and the mother ortet of each of the three analysed tea clones with *Dra*I, *Bam*HI and *Eco*RI in combination with probes cob, nad3 and rrn26 gave identical profiles, indicating that the observed variation in the micropropagated plants of *C. sinensis* clone U26 was not due to any residual heterozygosity in the explant source but was induced by the cultural conditions. Both differences (Shimron-Abarbanell and Brieman 1991; Shirzadegan et al. 1991; Hartmann et al. 1992; De Verno et al. 1994; Rani et al. 2000) as well as complete uniformity (Shenoy and Vasil 1992; Rani and Raina 1998b) in mt DNA RFLPs between the mother and meristem-culture derived micropropagated plants have been observed in various plant taxa.

Chloroplast genome

Thirty combinations of six restriction enzymes (*Dra*I, *Bam*HI, *Hin*dIII, *Eco*RI, *Eco*RV, *Xba*I) and five cp DNA probes (B29–22c, Ba1, B18–11a, Ba5 and Ba25-Ba4) produced 83 monomorphic bands each in the mother and micropropagated plants of all three clones (Table 2). The number and size of the hybridized bands ranged from

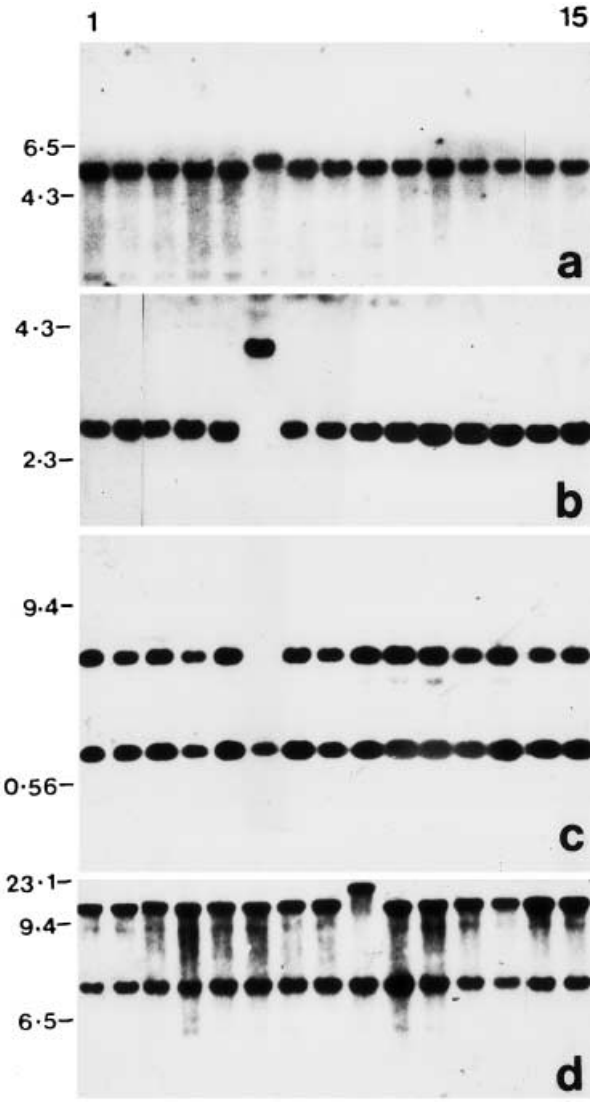

Fig. 1 *Bam*HI (**a**, **d**), *Dra*I (**b**) and *Eco*RI (**c**) restriction fragments that hybridized to mt gene probes cob (**a**, **b**), nad3 (**c**) and rrn26 (**d**) in the field-grown mother plant (*lane 1*) and enhanced axillary branching-derived plants (*remaining lanes*) of *Camellia sinensis* clone U26

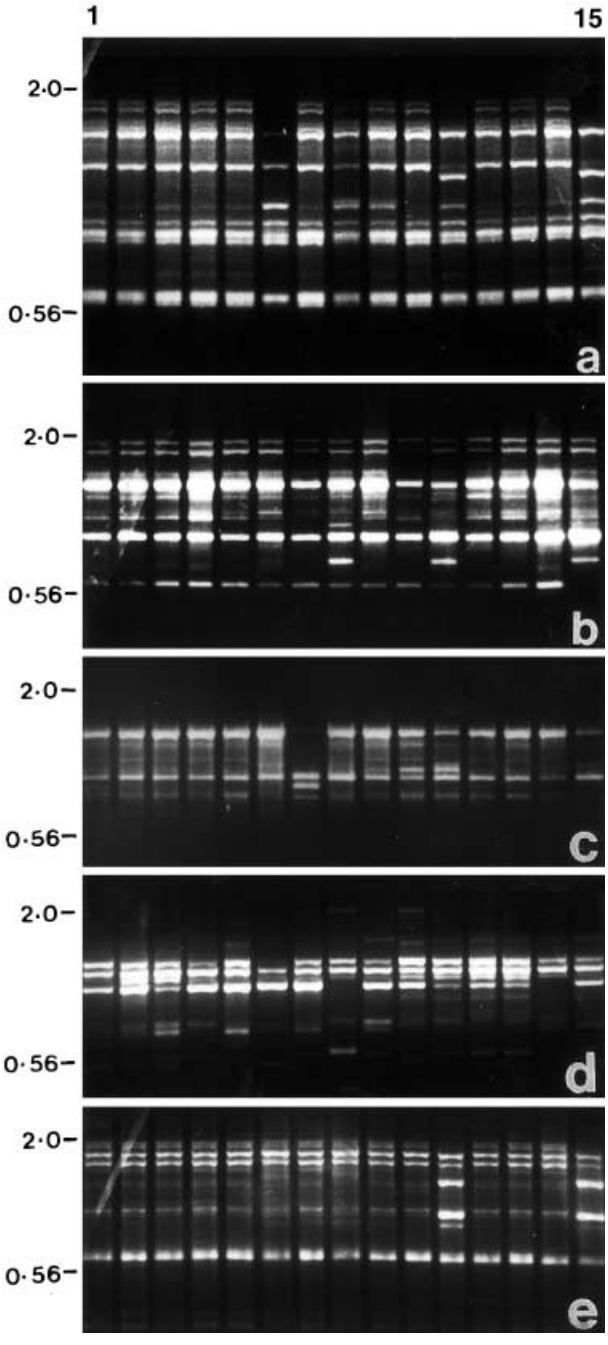

Fig. 2 Gel electrophoresis of amplification products in the field-grown mother plant (*lane 1*) and enhanced axillary branching-derived plants (*remaining lanes*) of *C. sinensis* clone U26 with RAPD and SSR primers OPA 07(**a**), OPK 03 (**b**), UBC 844 (**c**), UBC 859 (**d**) and UBC 845 (**e**)

one (*Dra*I + B29-22c) to six (*Eco*RI + Ba5) and from 0.7 kb to 23.1 kb (*Eco*RI + Ba5), respectively. The cp genome has been found to remain stable under in vitro conditions (Karp 1991; Rani et al. 2000) in most plant species.

RAPD fingerprinting

Of the 30 primers initially screened, 25 produced clear and scorable amplification products in the three tea clones. Clones U3, U26 and U27 micropropagated plants, and the corresponding mother plant produced a total of 159, 187 and 183 bands with an average frequency of 6.36, 7.48 and 7.32 bands per primer, respectively. The number of bands produced by a single primer ranged from 1 (OPA13) to 15 (OPC02). The amplified products ranged in size from 380 bp (OPA07) to 3,990 bp (OPC04). All 159 and 183 amplification products in U3 and U27, respectively, were found to be monomorphic across the micropropagated plants and corresponding mother plant (Table 2). In U26, of the 187 bands, eight (4.28%) were polymorphic. The polymorphic amplification products were

produced by primers OPA07, OPC04 and OPK03 (Tables 2, 3; Fig. 2a, b). The RAPD fingerprints of five primers (OPA07, OPA13, OPC02, OPC04 and OPK03) did not reveal any variation in the five naturally growing ramets and the mother ortet of each of the three analysed tea clones. RAPD markers have been shown to detect somaclonal variation among micropropagated plants of *Coffea arabica* (Rani et al. 2000), *Populus deltoides* clones (L34 and G3) (Rani et al. 1995, 2001), banana (Damasco et al. 1996; Gimenez et al. 2001), *Picea glauca* (Isabel et al. 1996) and oil palm (Rival et al. 1998). In contrast, RAPD markers have indicated the maintenance of genetic integrity among meristem culture-derived plants in *Picea mariana* (Isabel et al. 1993), *P. abies* (Fourre et al. 1997), date palm (Javouhey et al. 2000), *Eucalyptus tereticornis* and *E. camaldulensis* (Rani and Raina 1998b), *Picea deltoides* clones (D121, G48 and S7C15) (Rani et al. 2001) and several other plant species (see Rani and Raina 2000, 2002).

ISSR fingerprinting

Twenty five SSR primers including di-, tri- and tetranucleotide repeat motifs were individually used to amplify DNA from the mother and micropropagated plants of the three tea clones. A total of 119, 109 and 111 amplification products with a size range of 430 bp (UBC 834) to 3,085 bp (UBC 808) were scored in the U3, U26 and U27 clones, respectively. Out of the 109 bands scored in U26, 14 (12.84%) bands generated by primers UBC 844, UBC 845, UBC 848 and UBC 859 were observed to be polymorphic between the mother and a few micropropagated plants of U26 (Tables 2, 3; Fig. 2c-e). The remaining 95 bands in U26, and all 119 and 111 bands produced in the U3 and U27 clones, respectively were found to be monomorphic among the analysed plants (Table 2). Five SSR primers (UBC 844, UBC 845, UBC 848, UBC 859 and UBC 876) produced identical amplification products among the five ramets and the mother ortet of each of the three tea clones. ISSR fingerprinting has been previously found to be useful for detecting somaclonal variation among micropropagated plants of *Achillea* species (Wallner et al. 1996) and coffee (Rani et al. 2000). The amplification of relatively robust and distinct bands by the dinucleotide repeat motifs in tea is in line with previous observations made in conifers (Tsumura et al. 1996), wheat (Nagaoka and Ogihara 1997) and coffee (Rani et al. 2000). The tea genomes also seem to be abundantly rich in dinucleotide repeat motifs like (CT)n and (TG)n, which could serve as useful markers for the detection of genetic polymorphism.

Our comprehensive study has explicitly revealed that the enhanced axillary branching-derived plants of U3 and U27 tea clones are genetically true-to-type; all of the 446 and 462 genetic loci, respectively, originating from the nuclear, mt and cp genomes remained unchanged under the cultural conditions described herein. Such was, however, not the case with the U26 clone, wherein 36 (7.74% of the total) out of 465 genetic loci were found to be polymorphic. This clearly indicates that complete genetic fidelity is lacking among the regenerants. The observed polymorphism, which was limited to the nuclear and mt genomes, was relatively low (7.43%) in the former compared to the latter genome (16.3%). Similar observations have been reported in somatic embryo-derived plants of *Coffea arabica,* wherein the level of somaclonal variation in the mt genome (41%) was much higher than in the nuclear genome (4.38%) (Rani et al. 2000). Modifications in the mt genome during in vitro culture might arise through rearrangements, homologous recombination and selective amplification, all of which take place mostly at non-coding hyper-variable regions (Shirzadegan et al. 1991). Of the two PCR-based assays used to screen the nuclear genome, ISSR markers, as in *Coffea arabica* (Rani et al. 2000), were more effective in detecting polymorphism among the micropropagated plants of the U26 clone.

Various factors, such as the in vitro process and its duration, auxin-to-cytokinin concentration and their ratio (hormone balance), other nutritional conditions and in vitro stress, all known to induce somaclonal variation, were exactly the same during the culture of micropropagated plants in these three tea clones. Hence, in the light of complete genomic stability in *C. assamica* ssp. *assamica* clones U3 and U27 versus somaclonal variation in *C. sinensis* clone U26 under the same set of conditions, it can be inferred that it is the genetic constitution in each clone which determined the stability/variability of the micropropagated plants under the given cultural conditions. It would appear that *C. sinensis* is inherently unstable under in vitro conditions and/or more prone to in vitro-induced stress leading to somaclonal variation. Similar results have been obtained in micropropagated plants of eucalypts and poplars: using the same micropropagation protocol, somaclonal variation was detected in micropropagated plants of *Eucalyptus tereticornis,* while micropropagated plants of *E. camaldulensis* were found to be genetically stable (see Rani and Raina 1998b). Similarly, micropropagated plants of two (L34, G3) out of five clones (L34, G3, G48, D121, S7C15) of *Populus deltoides* exhibited profound somaclonal variation (Rani et al. 1995, 2001). A species/genotype-specific response to genetic stability/instability in tissue culture conditions is not uncommon and has been reported in many other plant taxa as well (Zehr et al. 1987; Mohmand and Nabors 1990; see Rani and Raina 2000, 2002).

Whether the genetic changes observed in the present study influence traits of interest or not remains to be seen, but what does matter most is that, of the two most important tea species, *C. sinensis* has been found to be prone to genomic changes in the given micropropagation protocol, leading to the conclusion that investigators have to proceed with caution when micropropagating clones of the China type. The results of the present study in conjunction with earlier reports (Armstrong and Phillips 1988; Shimron-Abarbanell and Breiman 1991; Amberger et al. 1992; Kawata et al. 1995; Rani et al. 1995, 2000, 2001;

Damasco et al. 1996; Franzone et al. 1996; Isabel et al. 1996; Kaeppler et al. 1998; Rani and Raina 1998a, b, 2000, 2002; Rival et al. 1998; Gimenez et al. 2001; Rahman and Rajora 2001) has convincingly emphasized that plants derived even through organized meristem culture may not always be genetically true-to-type. Further, the present comprehensive analysis indicates that two important tea clones, U3 and U27, exhibit complete stability during in vitro propagation through enhanced axillary branching culture method, and therefore, in the context of the commercial production of micropropagated plants by this method, no significant differences with respect to tissue culture-induced genetic variation are to be expected.

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References

Akula A, Dodd WA (1998) Direct somatic embryogenesis in a selected tea clone, TRI 2025, *Camellia sinensis* from nodal explants. Plant Cell Rep 17:804–809

Amberger PV, Shoemaker RC, Palmer RG (1992) Inheritance of two independent isozyme variants in soybean plants derived from tissue culture. Theor Appl Genet 84:600–607

Anonymous (2000) Report of International Tea Association, UK

Armstrong CL, Phillips RL (1988) Genetic and cytogenetic variation in plants regenerated from organogenic and friable embryogenic tissue cultures of maize. Crop Sci 28:363–369

Banerjee B (1992) Botanical classification of tea. In: Wilson KC, Clifford MN (eds) Tea cultivation to consumption. Chapman and Hall, London, pp 25–52

Barua DN (1965) Classification of the tea plant: species hybrids. Two Bud 12:13–27

Charles HB Jr (1981) Seed to civilization: the story of food. Freeman, San Francisco

Damasco OP, Graham GC, Henry RJ, Adkins SW, Godwin ID (1996) Random amplified polymorphic DNA (RAPD) detection of dwarf off-types in micropropagated Cavendish (*Musa* spp. AAA) bananas. Plant Cell Rep 16:118–123

De Verno LL, Charest PJ, Bonen L (1994) Mitochondrial DNA variation in somatic embryogenic cultures of *Larix*. Theor Appl Genet 88:727–732

Ellis RT (1995) Tea *Camellia sinensis* (Camelliaceae). In: Smartt J, Simmonds NW (eds) Evolution of crop plants, 2nd edn. Longman, Essex, UK, pp 22–27

Fourre JL, Berger P, Niquet l, Andre P (1997) Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. Theor Appl Genet 94:159–169

Franzone PM, Suarez EY, Solari RM, Favert EA, Rios RD, Diaz-Paleo AH (1996) Somaclonal variation in three Argentinean varieties of *Triticum aestivum* with different karyotype variability. Plant Breed 115:89–93

Gimenez C, Garcia ED, Enrech NXD, Blanca I (2001) Somaclonal variation in banana: cytogenetic and molecular characterization of somaclonal variant CIEN BTA-03. In Vitro Cell Dev Biol Plant 37:217–222

Hartmann C, De Buyser J, Henry Y, Morere-La Paven MC, Dyer TA, Rode A (1992) Nuclear genes control changes in organization of genome in tissue cultures derived from immature embryos of wheat. Curr Genet 21:515–520

Isabel N, Tremblay L, Michaud M, Tremblay FM, Bousquet J (1993) RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis derived populations of *Picea mariana* (Mill.) B.S.P. Theor Appl Genet 86:81–87

Isabel N, Bovin R, Levasseur C, Charest PM, Bousquet J, Tremblay FM (1996) Occurrence of somaclonal variation among somatic embryo-derived white spruces (*Picea glauca*, Pinaceae). Am J Bot 83:1121–1130

Javouhey M, Daguin F, Letouze R (2000) Somatic embryogenesis, an efficient tool for date palm (*Phoenix dactylifera* L.) industrial micropropagation, characterization and genetic stability of original offshoots and regenerated plantlets by RAPD markers. Acta Hortic 530:237–241

Kaeppler SM, Phillips RL, Olhoft P (1998) Molecular basis of heritable tissue culture-induced variation in plants. In: Jain SM, Brar PS, Ahloowalia DS (eds) Somaclonal variation and induced mutations in crop improvement. Kluwer, Dordrecht, pp 465–484

Karp A (1991) On the current understanding of somaclonal variation. Oxf Surv Plant Mol Cell Biol 7:1–58

Kawata M, Ohmiya A, Shimamoto Y, Oono K, Takaiwa F (1995) Structural changes in the plastid DNA of rice (*Oryza sativa* L.) during tissue culture. Theor Appl Genet 90:364–371

Maniatis T, Fritsch EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Springer Harbor Laboratory Press, Cold Springer Harbor, N.Y.

Mohmand AS, Nabors MW (1990) Somaclonal variant plants of wheat derived from mature embryo explants of three genotypes. Plant Cell Rep 8:558–560

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nagaoka T, Ogihara Y (1997) Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor Appl Genet 94:597–602

Porebski S, Bailey GL, Baum BR (1997) Modifications of a CTAB DNA extraction protocol for plant containing high polysaccharide and polyphenol components. Plant Mol Biol Rep 15:8–15

Prakash O, Sood A, Sharma M, Ahuja PS (1999) Grafting micropropagated tea [*Camellia sinensis* (L.) O. Kuntze] shoots on tea seedlings – a new approach to tea propagation. Plant Cell Rep 18:883–888

Rahman MH, Rajora OP (2001) Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*) Plant Cell Rep 20:531–536

Raina SN, Rani V, Kojima T, Oghiara Y, Singh KP, Devarumath RM (2001) RAPD and ISSR fingerprints as useful genetic markers for analysis of genetic diversity, varietal identification and phylogenetic relationships in peanut (*Arachis hypogaea*) cultivars and wild species. Genome 44:763–772

Raj Kumar R, Ayyappan P (1992) Micropropagation of *Camellia sinensis* (L.) O. Kuntze. J Plant Crops 20 [Suppl]:252–256

Rani V, Raina SN (1998a) Genetic fidelity and molecular diagnostics in micropropagation system. In: Gupta PK, Singh SP, Balyan HS, Sharma PC, Ramesh B (eds) Genetics and biotechnology in crop improvement. Rastogi, Meerut, India, pp 370–388

Rani V, Raina SN (1998b) Genetic analysis of enhanced axillary branching derived *Eucalyptus tereticornis* and *E. camaldulensis* plants. Plant Cell Rep 17:236–242

Rani V, Raina SN (2000) Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cell Dev Biol Plant 36:319–330

Rani V, Raina SN (2002) Molecular DNA marker analysis to assess the genetic fidelity of micropropagated woody plants. In: Jain SM, Ishii K (eds) Micropropagation of woody trees and fruits. Kluwer, Dordrecht (in press)

Rani V, Parida A, Raina SN (1995) Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus deltoides* Marsh. Plant Cell Rep 14:459–462

Rani V, Singh KP, Shiran M, Nandy S, Goel S, Devarumath RM, Sreenath HL, Raina SN (2000) Evidence for new nuclear and mitochondrial genome organizations among high frequency somatic embryogenesis-derived plants of allotetraploid *Coffea arabica* L. (Rubiaceae). Plant Cell Rep 19:1013–1020

Rani V, Parida A, Raina SN (2001) Chromosome number dependent genome size and RAPD fingerprinting diagnostics for ge-

netic integrity of enhanced axillary branching-derived plants of ten forest tree species. Acta Hortic 560:531–534
Reed KC, Mann DA (1985) Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res 3:7207–7221
Rival A, Bertrand L, Beale T, Combes M-C, Trouslot P, Leshermes P, (1998) Suitability of RAPD analysis for detection of somaclonal variation in oil palm (*Elaeis guineensis* Jacq.) Plant Breed 117:73–76
Satyanarayana N, Cox S, Sharma VS (1992) Field performance of grafts made on fresh tea clonal cuttings. J Plant Crops [Suppl] 20:151–156
Sharma VS, Satyanaryana N (1989) UPASI's new tea clones. In: UPASI Tea Scientific Department (ed) Handbook of tea culture. Tea Research Institute, Valparai, Coimbatore, Tamil Nadu, India, Sect. 2, p 3
Shenoy VB, Vasil IK (1992) Biochemical and molecular analysis of plants derived from embryogenic cultures of napier grass (*Pennisetum purpureum* K. Schum). Theor Appl Genet 83 :947–955
Shimron-Abarbanell D, Breiman A (1991) Comprehensive molecular characterization of tissue-culture-derived *Hordeum marinum* plants. Theor Appl Genet 83:71–80
Shirzadegan M, Palmer JD, Christey M, Earle ED (1991) Patterns of mitochondrial DNA instability in *Brassica campestris* cultured cells. Plant Mol Biol 16:21–37
Singh ID (1979) Indian tea germplasm and its contribution to world's tea industry. Two Bud 26:23–26
Thein SI, Wallace RR (1986) The use of synthetic oligonucleotides as specific hybridization probes in the diagnosis of genetic disorders. In: Davies KE (ed) Human genetic diseases: a practical approach. IRL, Oxford, pp 33–50
Tsumura Y, Ohba K, Strauss SH (1996) Diversity and inheritance of inter simple sequence repeat polymorphisms in Douglas-fir (*Pseudosuga menziesii*) and sugi (*Cryptomeria japonica*). Theor Appl Genet 92:40–45
Venkataramani KS, Sharma VS (1967) Notes on the UPASI tea clones approve by Tea Board and released for commercial planting. In: UPASI Tea Scientific Department (ed) Handbook of tea culture. Tea Research Institute, Valparai, Coimbatore, Tamil Nadu, India
Wachira F, Ogada J (1995) In vitro regeneration of *Camellia sinensis* (L.) O. Kuntze by somatic embryogenesis. Plant Cell Rep 14:463–466
Wachira FN, Waugh R, Hackett CA, Powell W (1995) Detection of genetic diversity in tea (*Camellia sinensis*) using RAPD markers. Genome 38:201–210
Wallner E, Weising K, Rompf R, Kahl G, Kopp B (1996) Oligonucleotide and RAPD analysis of *Achillea* species: characterization and long-term monitoring of micropropagated clones. Plant Cell Rep 15:647–652
Zehr BE, Williams ME, Duncan RD, Widholm JM (1987) Somaclonal variation among the progeny of plants regenerated from callus cultures of seven inbred lines of maize. Can J Bot 65:491–499