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Genetic analysis of cotyledon derived regenerants of tomato using AFLP markers

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MS medium supplemented with 15 μM zeatin was used for direct shoot regeneration from cotyledonary explants. DNA samples obtained from the regenerated shoots and parent cotyledonary explants were subjected to amplified fragment length polymorphism (AFLP) analysis to examine genetic uniformity of tissue-cultured tomato plants. Eighty-five markers were generated using the primer pair M-CAC/E-ACT and M-CTC/E-ACT. Identical fingerprints were generated for each primer pair for the mother and the regenerated plants. AFLP markers proved to be an ideal tool for routine analysis of genetic fidelity of regenerated tomato plants. This is a report on evaluating the genetic fidelity of tissue culture tomato plants using AFLP.

SUSTAINABILITY of the micropropagation technique is dependent upon maintenance of the genetic fidelity of the regenerates. Changes occurring during propagation of tissue culture are either epigenetic or genetic[1]. Genetic changes are heritable and are called as somaclonal variations[1,2]. Somaclonal variations may result due to modifications in the chromosome number or methylation pattern, chromosome breaks, transposon activations, deletions, genome rearrangements, polyploidy or nucleotide substitutions[1,3,4]. The somaclonal variant frequency cannot be predicted and it depends on several factors, viz. species, explant type, and donor genotype. The other factors influencing genetic stability include composition of the culture medium, especially the concentration and type of plant growth regulators used, physical conditions and duration between successive subcultures[5]. Somaclonal variation can pose a severe threat to the genomic integrity of regenerated plants, which is particularly required during the genetic transformation experiments and to achieve genetic uniformity of the propagules. Somaclonal variation can either bring changes at the DNA level or it may induce changes in chromosome numbers. Early detection of the genetic stability provides the opportunity to reevaluate the propagation protocols, and the desired modification in the protocols could be utilized to

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achieve the genetic fidelity of the tissue culture (TC)-raised plants.

Several strategies have been adopted to analyse the genetic fidelity of the TC-raised progenies, including use of RAPD[6], comparing methylation pattern of mother plant and callus[7], studying chromosomal constitution[8] and amplified fragment length polymorphism (AFLP)[9]. DNA-based markers provide an efficient way for screening TC-induced mutations, since these markers are not affected by environmental factors, are more reliable and generate reproducible results[9]. Among molecular techniques, AFLP has been proved to be an efficient tool to establish the clonal fidelity of the TC plants[9–11]. AFLP is characterized by high reproductibility and high multiplex ratio, and does not require prior sequence information. It has wide genome coverage compared to other DNA-based markers[12]. AFLP is based on the highly stringent selective PCR amplification of restriction fragments from a total digest of genomic DNA[13]. The basic method involves restriction of the genomic DNA, ligation of oligonucleotide adaptors to the DNA fragments, and high-stringency selective amplification of a subset of all the fragments in the total digest. The ligation of oligonucleotide adaptors enables PCR to be performed for any species without prior sequence knowledge. The selective amplification uses primers of the complementary sequence to the ligated adaptor plus 1–3 additional arbitrary nucleotides. Subsequent electrophoresis of the PCR product typically reveals a complex multi-locus profile of up to 100 bands. The bands are generally dominant markers, with polymorphism detected by the presence or absence of bands. To the best of our knowledge the AFLP technique has not been previously used to evaluate the clonal fidelity of TC-raised tomato plants.

F1 hybrid seeds of the 'Red Coat' cultivar of tomato were obtained from the Yates Vegetable Seeds Co Ltd, Australia. Seeds were surface sterilized for 15 min with 1% sodium hypochlorite and rinsed with sterile water before transferring to autoclavable transparent culture tubes (25 mm × 80 mm) containing 5 ml agar–water medium consisting of 0.8% agar (Sigma Co Ltd). Cotyledons were excised from one-week-old seedlings and whole cotyledons were inoculated on the regeneration medium according to the protocol described by Bhatia[14].

Total genomic DNA was isolated from the five cotyledonary explants and leaves of three regenerated shoots from each cotyledon. A total of 20 DNA samples (5 from parent cotyledons and 15 from the regenerated shoots) were extracted using the Wizard® Genomic DNA purification kit obtained from the Promega Corporation, USA according to the manufacturer's instruction.

DNA-fingerprints were generated for the DNA from parent cotyledonary explants and the regenerated shoots using AFLP primer pairs M-CAC/E-ACT and M-CTC/E-ACT. DNA-fingerprinting was performed by AFLP according to procedures outlined by Krauss[15] and involved the following steps.

Restriction of the DNA: For each sample, approximately 200 ng of DNA was digested with 1.25 units of *Eco*RI/*Mse*I restriction enzyme in a reaction volume of 12.5 µl, and incubated at 37°C for 2 h. Samples were then transferred to a 70°C bath for 15 min before being briefly cooled on ice. For the ligation of adapters, 12 µl of adapter ligation solution and 0.5 units of DNA ligase were added to the digested DNA, incubated at 20°C for 2 h and then diluted 1 : 10 with TE buffer.

Pre-selective amplification by PCR: 1.25 µl of the diluted ligation mix was combined with 10 µl pre-amplification primer solution, 1.25 µl 10 × PCR buffer for AFLP, and 0.6 units Taq DNA polymerase in a PCR plate. PCR was performed for 20 cycles of 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s. Subsequently, the pre-amplification mixture was diluted 1 : 50 with TE buffer.

Selective amplification by PCR: For each primer pair, the following was added for 2.5 µl of each diluted, pre-selective DNA sample: 7.5 ng *Eco*RI-primer, 15 ng *Mse*I-primer, 3.95 µl MilliQ water, 1.0 µl 10 × PCR buffer, and 0.25 units Taq DNA polymerase. A touchdown PCR reaction commenced with 10 min at 90°C, followed by 1 cycle of 94°C for 30 s, 70°C for 2 min and 72°C for 2 min. In subsequent cycles, the annealing temperature was reduced in 1°C steps to 61°C, followed by 23 cycles at 60°C. AFLP kits and unlabelled primers were purchased from Life Technologies, while Taq polymerase and labelled primers were purchased from Perkin-Elmer.

Amplified restriction fragments were fluorescently labelled, allowing detection by electrophoresis on the ABI 377 Automated Genetic Analysis System. Fragments were detected by laser and camera, and were accurately sized with GeneScan software by the inclusion in each lane of internal size standards that are labelled with a uniquely coloured fluorescent dye. The use of different coloured fluorescent dyes enables multiplexing of the products of three separate PCR reactions in a single electrophoresis lane. Multi-locus profiles were visualized and scored from electropherograms using GeneScan software. Each DNA fingerprint was scored using GeneScan software for the presence/absence of each strong marker (band, where peak height > 300) between 66 and 500 base pairs in length.

The primer pair M-CAC/E-ACT generated 33 markers, while M-CTC/E-ACT generated 52 markers. DNA fragment sizes generated by M-CAC/E-ACT were 68.4, 76.6, 77.5, 81.7, 92.7, 94.1, 98.4, 100, 107, 118, 122.7, 124, 133, 138, 140.3, 142.5, 177.6, 199.4, 202.2, 207.2, 227, 234, 250, 256.5, 285, 289.6, 301, 304, 320.4, 334.4, 372.8, 395.4 and 409.6. DNA fragment sizes generated by M-CTC/E-ACT were 66.1, 98.2, 76.5, 81.6, 87.5, 92.4, 94, 98.2, 99.8, 103, 107, 115, 117.6, 120, 122.7, 124, 125.5, 126.5, 130.2, 133.3, 136.5, 138.1, 140.1, 142.3, 150.3, 177, 199.5, 202.2, 207.4, 227.2, 234.2, 243, 244, 250.4, 256.5, 257.8, 263.6, 272.2, 285.2, 289.5, 301, 304, 320.2, 334.3, 342.9, 346.5, 352.2, 372.7, 391.5, 395, 409.4 and 433.5. No qualitative (presence or absence) differences were detected among

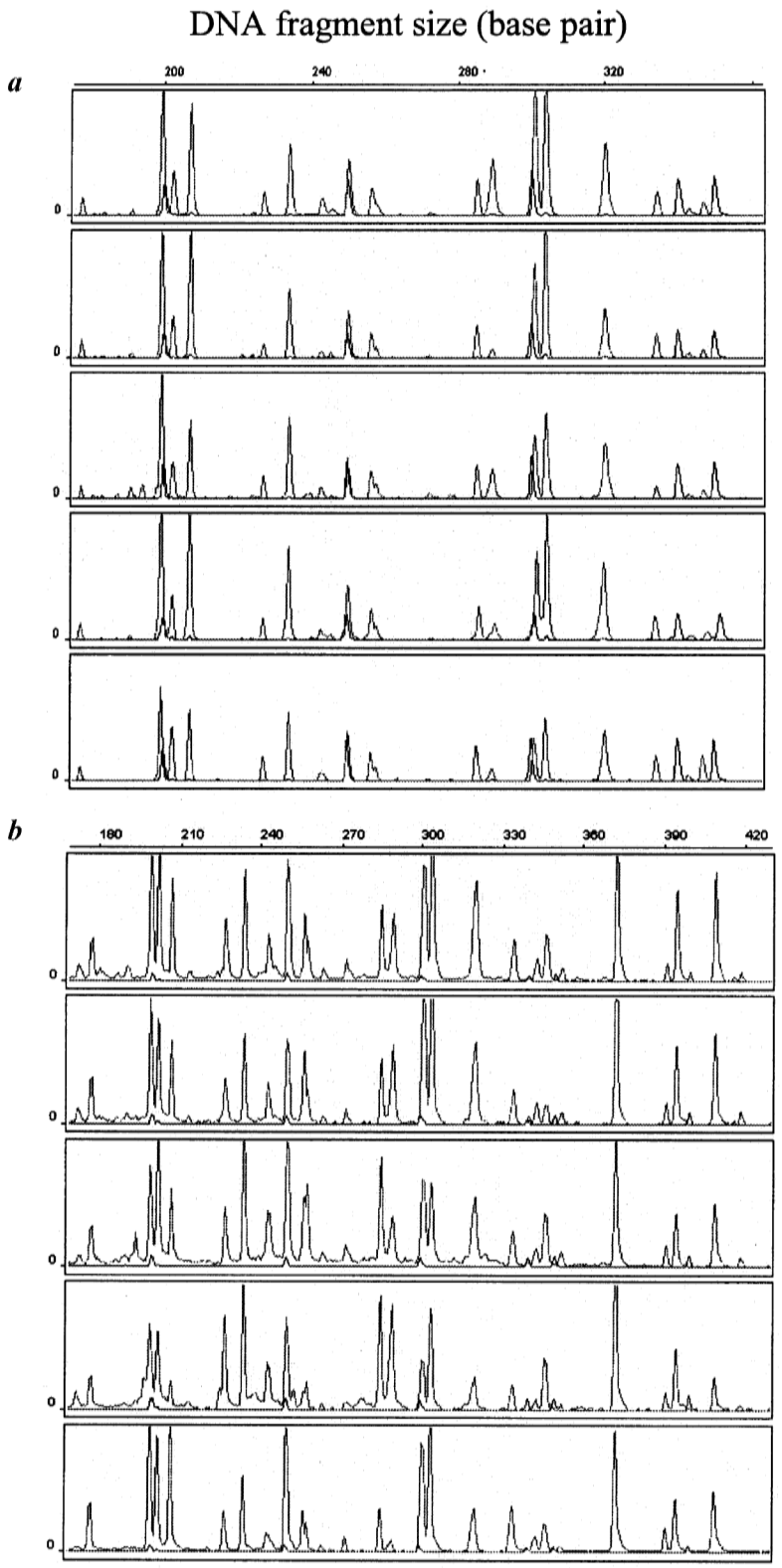

Figure 1. Part of identical AFLP DNA fingerprints generated by the primer pairs (*a*) M-CAC/E-ACT for samples of TC plant, TC plant, parent plant, TC plant and TC plant and (*b*) M-CTC/E-ACT for samples of parent, TC plant, TC plant, TC plant and TC plant.

the 20 DNA fingerprints (five of each of the two primer pairs are shown in Figure 1 *a* and *b*). For each primer pair, all DNA fingerprints were identical (20 samples for 85 markers). Cotyledonary explants from the hybrid (F1) seeds were identical to each other and to the regenerated shoots. In conclusion, we did not detect any genetic differences between any of the samples analysed by AFLP.

Genetic analysis of the regenerated and mother plant indicated no genetic differences between the two, which shows that the direct regeneration protocol used in the current study could be safely used for both micropropagation and tissue transformation purposes.

It is understood that many factors associated with culture manipulations can lead to the induction of genetic instability. It is known that DNA methylation and demethylation in higher plants play an important role in many developmental events[16]. The change in DNA methylation pattern may induce genetic variation in the TC-derived plants[17] and in AFLP fingerprints of genetically identical material[18]. Results of the current experiment could be interpreted as non-occurrence of change in the DNA methylation pattern of the regenerated plants, indicated by the absence of genetic mutation. Similar to our results, Smulders *et al.*[7] failed to detect any differences in the methylation pattern of tomato callus and leaf DNA of the parent plants. Nevertheless, Nambisan *et al.*[19] detected DNA polymorphism of tomato at the Cab locus for two of the 17 somaclones tested. Buiatti *et al.*[20] reported that single gene mutations might occur during the TC of tomato plants. When plants of cv. UC105 (regenerated from cotyledons cultured *in vitro*) were selfed, chlorophyll mutations and other morphological abnormalities were found in the progenies at a frequency of 17%, suggesting a unicellular origin of the mutation regenerated plants. Bulk *et al.*[21] also concluded that various phenotypic alterations in TC-raised tomato plants result due to the occurrence of recessive, single gene mutations. These single mutations are not dependent on explant source or prolonged culture duration. Mandal[22] reported genetic differences as well as consequent phenotypic variations in tissue-cultured tomatoes. He also reported substantial somaclonal variation for major agronomic traits such as plant height, number of primary branches, number of fruits, fruit yield per plant and resistance to wilt caused by *Pseudomonas solanacearum* in the somaclone second generation. Soniya *et al.*[6] obtained TC tomato plants through callus, which was induced using the picloram, and studied the genetic stability of tomato TC plants using RAPD. They used picloram and BAP respectively, as auxin and cytokinin sources. In their experiments, 15 non-parental bands were observed, of which three were shared and 12 were unique. Estimation of genetic similarity coefficient based on RAPD band-sharing data indicated that ten regenerated plants were more than 95% similar to the mother plant, but one was found to be distinctly different. These reports indicate that tomato is particularly prone to genetic changes if produced through callus.

In tobacco, Hirochika *et al.*[23] reported that the amplification of retrotransposons could be responsible for the TC-induced variations. Genetic stability of the regenerated shoots in the present experiment could be because of the short culture duration and noninvolvement of an intermediate callus phase. In corroboration with our interpretation, Hirochika[24] and Hirochika *et al.*[23] reported that somaclonal variations in rice increase with culture duration, due to an increase in the copy of retroelements (T_{OS} 17), whose activation leads to somaclonal variations. Conclusively, direct regeneration of the 'Red Coat' cultivar of tomato shoots from cotyledonary explants results in genetically stable regenerated shoots and could be safely used for transformation/micropropagation purposes. AFLP markers could be used with high precision for detecting somclonal variation in tomato.

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Identification of sugarcane clones resistant to the sugarcane woolly aphid (*Ceratovacuna lanigera* Zehntner)

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Sugarcane woolly aphid (SWA) has become a serious pest in peninsular India, causing significant loss in cane yield and sugar recovery. The currently cultivated commercial varieties are susceptible, necessitating the need for finding resistant sources. The hybrid progeny population numbering 828, pre-selected from 45 crosses involving commercial varieties, were evaluated in field trials to isolate resistant clones with improved productivity. The hybrid progenies were evaluated over three hot spot locations for the SWA along with four commercial varieties. Nine progeny clones were totally free from aphid infestation and two clones had lower aphid load under natural infestation. When these clones were evaluated under artificial infestation, the nine clones confirmed their resistant reaction. Three clones, viz. SNK 44, SNK 61 and SNK 754 are promising for both cane and sugar yield with acceptable cane features. It is suggested to test these clones in large-scale yield trials to know their suitability for commercial cultivation as well as their utility in future breeding programmes aimed at incorporating woolly aphid resistance.

SUGARCANE woolly aphid, *Ceratovacuna lanigera* Zehntner was first reported on sugarcane in 1897 from Java[1] and it is a serious pest of sugarcane in Asia[2–9]. In India, it has been reported as a pest on sugarcane from West Bengal, Assam, Nagaland, Sikkim, Tripura and Uttar Pradesh[10–15]. Since 2002, it has attained a serious status in Maharashtra and Northern Karnataka[16–18] and has also spread to Andhra Pradesh, Tamil Nadu, Goa, Kerala (pers. commun.), Bihar, Uttaranchal and Uttar Pradesh. It has resulted in significant loss in cane yield and sugar recovery[16–22] which has led to drastic reduction in sugar production, cane area and crushing duration, ultimately affecting the economy of sugar mills[19] (through results of survey conducted by N.S.K.).

To manage this pest, several control measures, viz. chemical, biological, cultural and host plant resistance have been suggested[16–23]. Among these, host plant resistance is of paramount importance as this approach is environmentally safe, more stable and viable[18,24]. Attempts to identify resistant sugarcane germplasm have been made in Taiwan, Philippines and Indonesia. In Taiwan varietal differences for aphid incidence and its biology were studied and indicated the variety ROC 1 to offer resistance to some extent, as it was associated with longer nymphal period and fewer progenies per adult[25]. In a similar study at Philippines, varietal differences in relation to aphid biology were reported[26]. In Indonesia a resistance breeding programme was initiated for SWA[27]. There are no reports available on the level of resistance for sugarcane woolly aphid in India. However, earlier studies reported relative susceptibility of genotypes[16,19] and varieties with lower nitrogen content, total soluble solids and higher silicon were reported less susceptible[11].

The present study was envisaged to isolate resistant clones with improved productivity from large hybrid progeny population derived from 45 crosses involving diverse commercial varieties. Pre-selected (for cane yield and HR Brix%) 828 hybrid progenies were evaluated with four commercial varieties, at three hot spot locations for the

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