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CELL BIOLOGY AND MORPHOGENESIS

Use of SSR markers to determine the anther-derived homozygous lines in coconut

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Abstract Anther culture was used to obtain dihaploid (DH) coconut plants and their ploidy level was determined by flow cytometric analysis. Simple sequence repeat (SSR) marker analysis was conducted to identify the homozygous diploid individuals. Ploidy analysis showed that 50% of the tested plantlets were haploid and 50% were diploid. Polymorphic fragments of the mother palm and their segregation patterns in anther-derived plantlets were used to determine the origin of the diploid plantlets. Using a diagnostic SSR marker (CNZ43), all the diploid plantlets tested were identified as being derived from microspores (i.e. were homozygous) and were thus candidates for use in coconut breeding programs.

Keywords Coconut · Androgenesis · Dihaploid · Flow cytometry · SSR marker

Abbreviations

BAP	6-Benzylaminopurine
DAPI	4′-6-Diamidino-2-phenylindole
2, 4-D	2, 4-Dichlorophenoxyacetic acid
GA_3	Gibberellic acid
SSR	Simple sequence repeats
WBS	Weeks before splitting

Introduction

Coconut is one of the most widely-grown plantation crops in the world covering an extent of 12 million hectares, 85% of which are in Asia Pacific region (Batugal et al. 2005). Among the numerous uses, coconut is cultivated mainly for the production of oil and desiccated coconut.

All forms of coconut assessed to date are diploid ($2n = 2x = 32$). Even though the male and the female flowers are located close together on each inflorescence, the Tall coconut (var. Typica) is, in general, allogamous (outbreeding) as pollen is shed before the female flowers become receptive. Consequently, each individual palm is highly heterozygous (Perera et al. 1998, 2001). Production of new coconut varieties by conventional breeding is mainly done by crossing individuals, resulting in segregating progeny. Hybrid breeding is not practical via this approach.

Creation of homozygous inbred lines is the first step in a hybrid breeding program. Using homozygous lines, it is

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possible to produce pure F_1 hybrids. At least 60 years of backcrossing and selfing would be required to get homozygous coconut lines by breeding. Generation of dihaploid (DH) plants would result in rapid production of homozygous lines, shortening the process to 1 or 2 years. Recessive alleles in the parents are easily uncovered in DH plants and therefore desirable mutations can be easily detected. They provide a wide spectrum of traits for selection in breeding programs.

Induction of haploids, bypassing sexual reproduction, is possible via anther/microspore culture (Peachan and Smykal 2001). For the first time, the production of coconut haploids via anther culture was reported and the protocol was developed by optimising the major critical factors (Perera et al. 2008). In general, coconut is a difficult crop to apply in vitro culture techniques, since it is a monocotyledonous tree crop. Due to very poor response of coconut tissues to in vitro conditions, it is classified as one of the most recalcitrant species to regenerate in vitro (George and Sherrington 1984). The performance of each palm is affected by its genotype and there is a variation in the success of different explants such as immature inflorescence, plumule, immature embryo and ovary that have been used for clonal propagation of coconut (Fernando et al. 2007). Plants were regenerated either by directly producing embryos from a pollen grain or indirectly via a callus phase (Perera et al. 2008). Since the anthers consist of both haploid and diploid cells, a selective cell division is impossible. Therefore, anther-derived structures containing a mixture of ploidy levels could be resulted.

Characterization of DH plants based on the homogeneity of agronomic traits has been done in the past (Kernan and Ferrie 2005). However, this approach would delay their use and increase the cost of labour and space since an additional generation of field evaluation is needed (Chen et al. 1998). Furthermore, evaluation of agronomic traits under field conditions is subjected to environmental variation and therefore unequivocal identification of DH lines using morphological markers is difficult (Chen et al. 1998). For a perennial tree crop like coconut, a minimum of 15 years for field evaluation would be required for discrimination of the homozygous lines using morphological markers. Alternatively identification of microspore-derived plants at an early stage of development using molecular markers would provide a fast, efficient and cost-effective method compared to field evaluation for the identification of DH lines (Chen et al. 1998).

Random amplified polymorphic DNA (RAPD) analysis has been used for this purpose in flax anther culture (Chen et al. 1998). However, among the various DNA marker methods, SSR has become one of the most informative molecular marker systems in cultivar finger printing, genetic diversity assessment, molecular mapping and marker-assisted breeding due to their abundance, hypervariability and random distribution in eukaryotic genomes (Wang et al. 2005; Chen et al. 1998). SSR markers are codominant, meaning both alleles of a heterozygous locus can be identified (Chani et al. 2000). Thus, if sufficient polymorphic SSR loci in an anther donor can be found, their use offers an efficient means of distinguishing homozygous from heterozygous anther-derived diploids (Chani et al. 2000). SSR markers have been used for distinguishing microspore-derived plants in potato (Chani et al. 2000). The present study was undertaken to demonstrate the use of SSR markers for identification of dihaploid coconut.

Materials and methods

Anther culture

Anthers consisting microspores at late uni-nucleate stage were collected from a single palm and androgenesis induction was done as described by Perera et al. (2008). Anthers were given a heat shock at 38°C for 6 days prior to inoculation and cultured in modified Eeuwens Y_3 (Karunaratne et al. 1985; Eeuwens 1976) liquid medium supplemented with 100 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1% (w/v) activated charcoal (BDH acid washed, UK) and 9% (w/v) sucrose as described earlier by Perera et al. (2008).

Ten anthers were cultured in ten Petri plates (100 × 10 mm), each containing 25 ml of culture medium. Cultures were maintained in the dark for 9 months at 28°C. The calli and embryos produced were sub-cultured into the somatic embryo induction medium (modified Eeuwens Y_3 solid medium with 66 μM 2,4-D) for 4 weeks followed by somatic embryo maturation medium (modified Eeuwens Y_3 solid medium without any hormones) for 4 weeks. The embryogenic structures were then transferred and maintained in germination medium (modified Eeuwens Y_3 solid medium supplemented with 5 μM BAP, 0.1 μM 2,4-D and 0.35 μM GA_3). After embryo germination, the cultures were exposed to light (16 h photoperiod; PAR; 25 μmol m^{-2} s^{-1}). Detailed anther culture protocol for coconut has been published elsewhere (Perera et al. 2008).

Flow cytometyric analysis

Root or leaf samples from 20 regenerated plantlets were used for ploidy analysis. Well developed calli and embryos (five samples from each) obtained after 3 months of culture initiation were also analysed. Extraction of nuclei and the analysis were done according to the protocol described by Pathirana and Eason (2006). Tissue samples (approx. 500 mg) were chopped in 2 ml of extraction buffer (2.1 g citric acid per 100 ml deionised water, 0.5% Tween 20), to release the intact nuclei. The suspension containing the

nuclei was then filtered through a 30 μm nylon mesh to eliminate cell debris. The cell nuclei were then stained with 4′-6-diamidino-2-phenylindole (DAPI, 0.02 mg ml^{-1} in saturated $Na_2HPO_4 \cdot 2H_2O$), an intercalating fluorochrome, according to the manufacturer's protocol. Nuclear DNA content was determined using a Partec PA-II Flow cytometer with UV excitation. Samples were analysed using the leaves of embryo-cultured Sri Lanka Tall coconut plants as the control and hexaploid *Hieracium* that has almost twice the amount of DNA than coconut as the known standard.

Simple sequence repeats (SSR) marker analysis

Diploid plantlets derived from anthers were tested by SSR marker analysis for homozygosity. Leaf tissues were collected from plantlets for DNA extraction. Furthermore, a random sample of 22 calli or embryos derived from the anthers of the same mother palm was also tested to check the occurrence of any heterozygous diploid. The banding patterns of the samples were compared with the donor palm.

DNA extraction

Genomic DNA was extracted using the CTAB method, as previously described by Doyle and Doyle (1987) with some modifications, described by Perera et al. (1998). Small pieces of tissue (about 1 g) was ground in liquid nitrogen and put into an eppendorf tube containing 1 ml of extraction buffer (2% CTAB, 2% PVP-40000, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, 2% Monothioglycerol; pH 8.0). The mixture was incubated at 65°C in a water bath for minimum of 30 min. Proteins were extracted into 200 ml chloroform:isoamyl solution (24:1) by centrifugation (Jouan SA, France) at 13,000 rpm for 2 min and the procedure was repeated twice. Pre-chilled (at −20°C) isopropanol (0.6 volume) was added to the supernatant and kept for 1 h to precipitate DNA. DNA was pelleted by centrifugation at 13,000 rpm for 2 min. DNA concentration was estimated by comparing with the fluorescent intensity of a series of standard DNA solutions.

PCR analysis

The primers used in this study (Table 1) were purchased from TIB MOLBIOL Syntheselabor GmbH, Eresburg-straβe 22–23, D-12103 Berlin, Germany. Polymerase chain reaction (PCR) was performed in 10 μl reaction mixture, containing 1 μl of 10× PCR buffer (10 mM Tris–HCl, 50 mM KCl and 0.1% Triton®X-100; pH 9.0 at 25°C), 1 μl of 200 μM dNTPs, 1.5 μl $MgCl_2$ (25 mM), 0.2 μl (1 unit/μl) of Taq polymerase (Promega; Catalog # M1865), 0.3 μl sterilized distilled water, 1.5 μl of forward and reverse primers and 3 μl genomic DNA (about 50 ng) as template. The PCR was performed [in a DNA Thermal Cycler (BIO-RAD; MyCycler™)] in 35 cycles with the following conditions: denaturing at 94°C for 1 min, annealing step at 54°C for 2 min and extension/polymerization step at 72°C for 1.5 min, followed by one cycle (5 min) at 72°C at the end of amplification. Each PCR sample (10 μl) was electrophoresed on 6% (w/v) polyacrylamide gel using 1× TBE buffer (89 mM Tris–HCl; pH = 8.3, 89 mM boric acid, 2 mM EDTA) at 55 W for 1.5 h. The gel was stained with 0.15% (w/v) silver nitrate for 15 min followed by dipping in a solution of 0.6% (w/v) NaOH and 0.5% (v/v) formaldehyde.

Results and discussion

Both direct and callus-mediated embryogenesis could be observed under the culture conditions described. Embryos or calli were produced from the anthers after 3 months of culturing. One hundred and twenty-five embryos or calli per 100 anthers were obtained and 27% of those were germinated. Among the regenerated plantlets, 92% were derived from embryos and the rest from calli. Embryos were distinguished from calli by their heart or round shape. Maturing of the embryos was identified by a change in translucent to opaque appearance (Fig. 1a). If the concentration of auxin is high enough, a somatic embryo may instead give rise to new somatic embryos that is called as secondary or repetitive embryogenesis instead of proceeding to next stage of

Table 1 The code numbers and sequences of the primers tested

Code number	Forward primer (5′–3′)	Reverse primer (5′–3′)
CNZ04	TATATGGGATGCTTTAGTGGA	CAAATCGACAGACATCCTAAA
CNZ06	ATACTCATCATCATACGACGC	CTCCCACAAAATCATGTTATT
CNZ10	CCTATTGCACCTAAGCAATTA	AATGATTTTCGAAGAGAGGTC
CNZ12	TAGCTTCCTGAGATAAGATGC	GATCATGGAACGAAAACATTA
CNZ29	TAAATGGGTAAGTGTTTGTGC	CTGTCCTATTTCCCTTTCATT
CNZ40	CTTGATTGCATTCTCAAATGG	CTGAGACCAAATACCATGTGT
CNZ43	TCTTCATTTGATGAGAATGCT	ACCGTATTCACCATTCTAACA
CNZ44	CATCAGTTCCACTCTCATTTC	CAACAAAAGACATAGGTGGTC

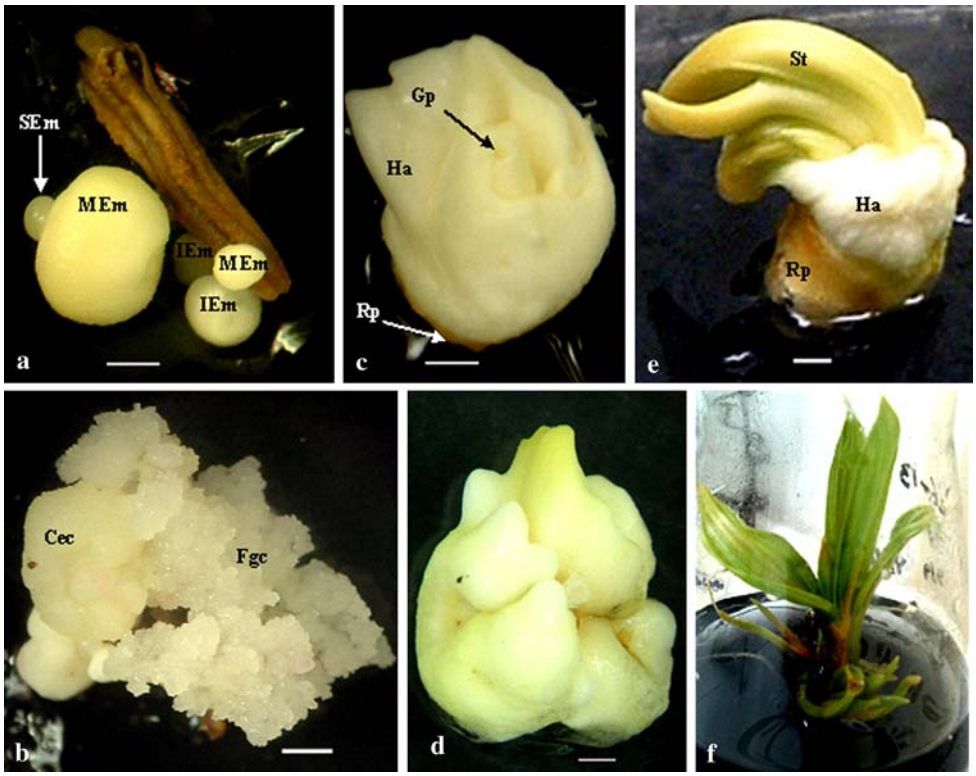

Fig. 1 Morphological aspects of anther culture in coconut (*Cocos nucifera*). **a** Anther-derived embryos (*Bar* 1.2 mm). Note four embryos at different maturity stages. Mature embryo (*MEm*) (in *opaque color*) and immature embryo (*IEm*) with translucent appearance. Secondary embryo (*SEm*) produced by the embryo derived from the cultured anther is also present. **b** A compact embryogenic callus (*Cec*) and a fast growing callus (*Fgc*) (*Bar* 2.9 mm). **c** A mature embryo (*Bar* 2 mm). Note the germination point (*Gp*) formed in the haustorium (*Ha*) and root pole (*Rp*) at the base of the embryo. **d** A callus-derived somatic embryo (*Bar* 1.5 mm). **e** Shoot development in the germinating embryo (*Bar* 1.4 mm). Note that the shoot (*St*) is emerging through the germination point of the haustorium. **f** Anther derived plantlet

development (Parrott et al. 1991). Occasionally, secondary embryos were derived from primary embryos (Fig. 1a) when they were maintained in the same androgenesis induction medium without sub-culturing.

Calli were determined by translucent and frilly appearance (Fig. 1b). Non-embryogenic fast growing callus (Fig. 1b) was also observed under the same culture conditions. All of the anther-derived structures had very low affinity to the anther wall that easily fell off. In the medium devoid any growth regulators, embryos were directly germinated (Fig. 1c) while calli gave rise to an intermediate phase of somatic embryos (Fig. 1d) before germination. Sub-culturing the germinated embryos into the medium containing 5 μM BAP and 0.35 μM GA_3 the shoot was emerged through the germination point of the haustorium (Fig. 1e). Under the present culture conditions, a total of 20 plantlets (Fig. 1f) were obtained.

Flow cytometric analysis

The ploidy level of anther-derived calli/embryos and regenerated plantlets was evaluated by flow cytometry (Chatelet et al. 1999). The ploidy levels of all the anther-derived structures were compared with hexaploid *Hieracium* (Fig. 2a) and diploid coconut leaves (Fig. 2b).

Among the tested structures, haploids as well as diploids were present. Embryos with both haploid ($n = x = 16$) (Fig. 2c) and diploid ($2n = 2x = 32$) (Fig. 2d) DNA contents were identified. Similarly, among callus (Fig. 2e, f) and plantlets (Fig. 2g, h), there were both haploid and diploid DNA contents. Haploid state indicates that the origin of the calli/embryos and plantlets were from the pollen grains of the anthers. This was further supported by histological evidence where the origin of these structures was shown to be from the inner part of the anther (Perera et al. 2008). Among the 20 plantlets tested, 10 were haploid and 10 were diploid.

The diploid state could have arisen due to formation of DHs by spontaneous chromosome diplodisation (Ingram et al. 2000; Lionneton et al. 2001), endomitosis, endoreduplication, nuclear fusion or unreduced microspores (Ishizaka 1998). Hu and Kasha (1997) reported that in wheat, about 82.7% of microspore-derived plants were spontaneously diploidised and 78.8% of them were completely fertile. Further, diploids can originate from somatic tissues including anther wall, tepetum and filament. However, origin of the calli/embryos could be identified morphologically based on their affinity to the anther. As discussed above, the low affinity of the calli/embryos to the anther wall indicated that they were derived from pollen grains within the pollen sacs. In the present study, anther-derived plants containing diploid DNA content were identified. Diploid plants can be divided into three groups depending on their origin: plants regenerated from normal reduced gametes (microspores) followed by chromosome

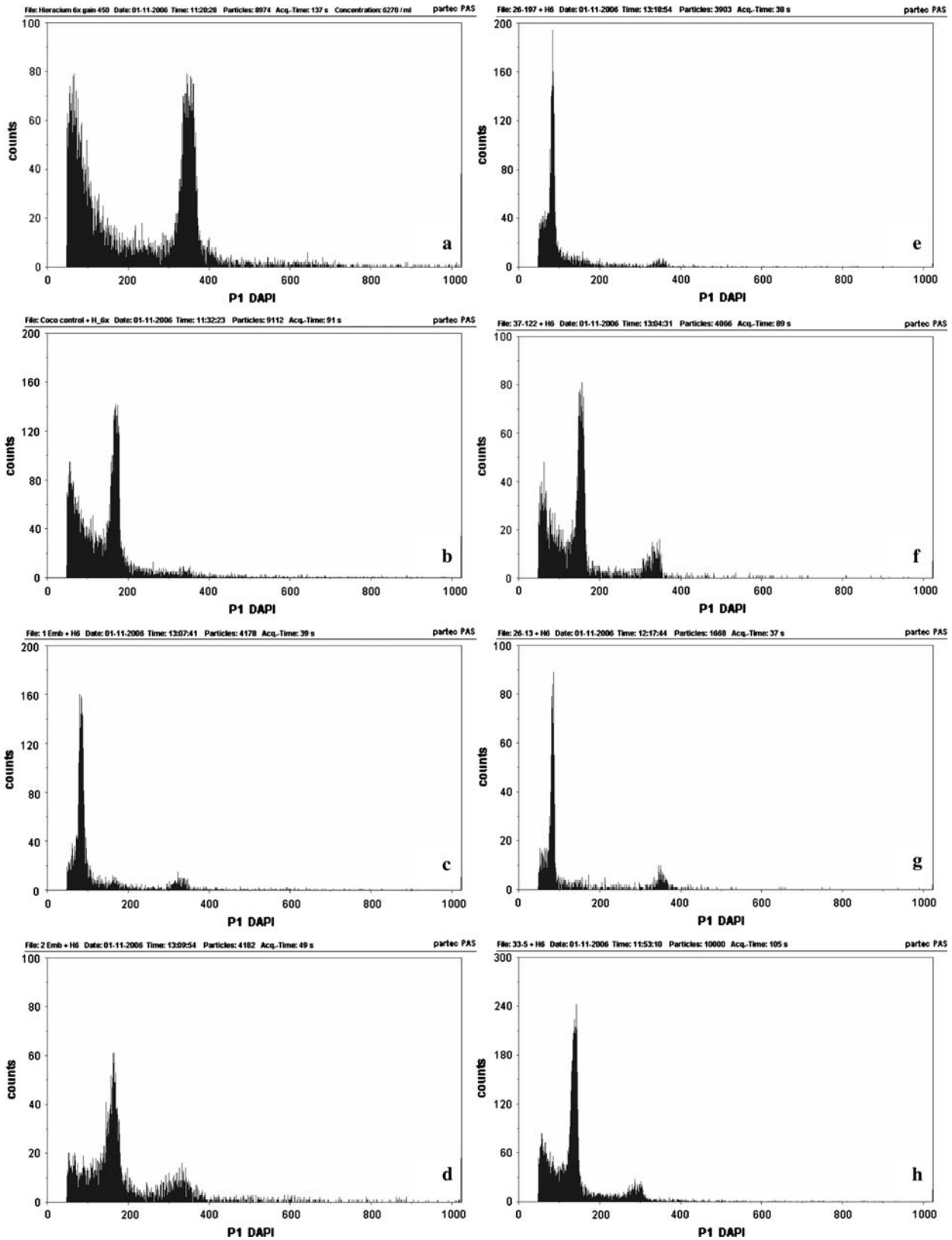

Fig. 2 Flow cytometry histograms of ploidy levels of anther-derived structures: **a** *Hieracium* (internal reference) $2n = 2x = 64$; **b** diploid control of embryo cultured coconut leaves, $2n = 2x = 32$; **c** haploid embryo; **d** diploid embryo; **e** haploid callus; **f** diploid callus; **g** haploid shoot; **h** diploid shoot

doubling during culture, plants regenerated from somatic cells of anther and plants regenerated from unreduced gametes (Chani et al. 2000). These three categories cannot be differentiated by flow-cytometric analysis. Among the diploid plants, those of the first group are equivalent to monoploids with regard to homozygosity and can be used directly in breeding whereas those in latter two groups, are heterozygous and generally considered undesirable products of anther culture and would be routinely discarded (Chani et al. 2000). Thus, it is important to determine the origin of the diploid plants before using them for breeding purposes.

SSR marker analysis

A set of coconut-specific microsatellite primer pairs has been developed by Rivera et al. (1999) using genomic DNA from the coconut variety Sri Lanka Tall and currently being used for genome mapping in coconut. From that, a total of eight SSR primers (CNZ04, CNZ06, CNZ10, CNZ12, CNZ29, CNZ40, CNZ43, CNZ44) were used to determine their feasibility as markers for the identification of microspore-derived plants in coconut (Table 1). Segregation pattern of the mother palm with the tested primers is illustrated in the Fig. 3. Two SSR primers (CNZ06 and CNZ12) were found to be non-reactive at the annealing temperature of 54°C. It was not the optimum annealing temperature for CNZ10 and CNZ29, since a number of non-specific bands have been developed. With CNZ04, CNZ43 and CNZ44, a clear banding pattern could be observed. Among them, with CNZ04, the mother palm was homozygous for the relevant loci whereas CNZ43 and CNZ44 were found to generate distinguishable and polymorphic alleles. However, a single primer with the segregating allele in the donor palm is sufficient for distinguishing the population since all the analysed structures have been derived from the pollen of the same donor palm. Thus, CNZ43 primer was selected for testing the segregation pattern of the anther-derived structures.

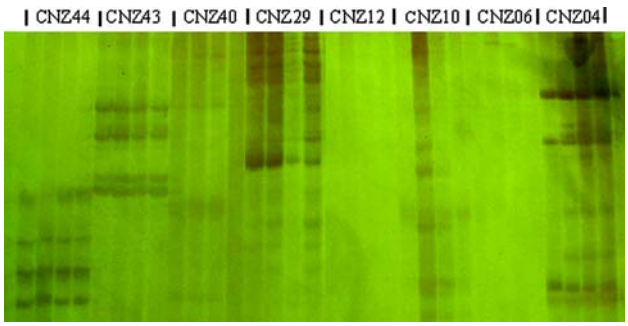

Fig. 3 Primers tested for SSR marker analysis of anther derived structures. Eight primers were used and four DNA samples from the mother palm were amplified at 54°C annealing temperature with each primer

Sixteen anther-derived coconut plantlets, including 10 diploid plantlets (identified by flow-cytometry) were tested (Fig. 4). A random sample of 22 anther-derived calli/embryos was also analysed (Fig. 5) and each of them was sampled from a cultures under similar conditions. All the anther derived-embryos/calli and plantlets were identified as being derived from microspores. None of the heterozygous diploids were found in the samples tested and all of them were either haploids or DHs.

Thus, it can be concluded that the protocol developed in the present study is effective in direct induction of pollen

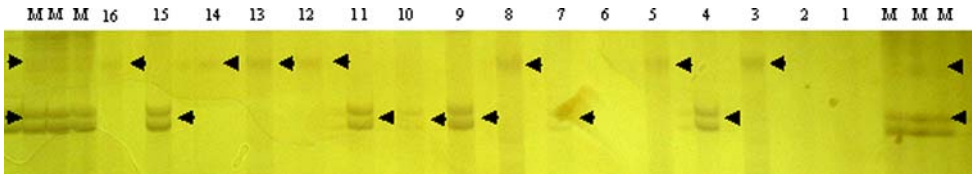

Fig. 4 Amplification of the primer CNZ43 with the DNA extracted from plantlets derived from the anthers. DNA from three samples has not amplified properly (*1*, *2* and *6*). Note the presence of either of the two bands in the samples, when compared to the mother palm (*M*)

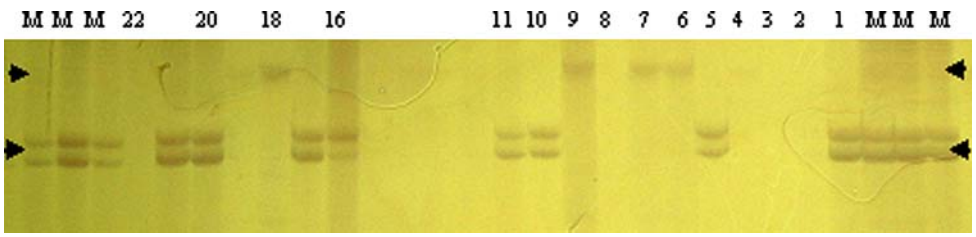

Fig. 5 Amplification of the primer CNZ43 with the DNA extracted from calli/embryos derived from the anthers. Note the similar banding pattern in these samples as well. Some of the tested samples were not amplified (however, they have amplyfied in other reactions)

embryogenesis. However, out of six palms tested, only one palm responded for androgenesis (data unpublished). Number of reasons could be affected in this regard, however mainly it could be due to high palm to palm variation (Perera et al. 2001) arisen by the heterozygous nature of the coconut palm. The research on DH plant production is continuing to get more palms responded for androgenesis. However, plant regeneration from the responded palm is continuously possible by culturing the anthers at each two months interval (data unpublished). For a crop like coconut, possibility of obtaining DHs from even a single palm is highly advantaged for gearing up the existing plant breeding program. Furthermore, the results showed the feasibility of using SSR markers to identify DH plants at a very early stage of development.

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