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# Identification of Elite Somaclonal Variants from Tissue Cultured Grand Naine Banana (*Musa* spp. AAA) Types Using RAPDs

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## ABSTRACT

*In vitro* propagation of Grand Naine banana (*Musa* sp. 'AAA') is gaining importance in the banana industry. However, the occurrence of somaclonal variants is at present limiting the use of tissue cultures plants in spite of several advantages. Hence a brief study was carried to characterize the somaclonal variants of tissue-cultured Grand Naine from the farmers' fields around Bangalore, India. Eleven positive variants were characterised based on their morphological and yield characters. To confirm the variants at the DNA level, Random Amplified Polymorphic DNA (RAPD) analysis was conducted to identify differences in the banding patterns. Forty three primers were used for the analysis of which OPF-09 differentiated the variants and the normal Grand Naine bananas. A band size of 320 bp was produced in all the normal samples but was absent in the variants tested. In the present study RAPD markers proved to effectively and precisely confirm the variants identified using molecular characters. Of the eleven superior variants analysed, variants GNV-04, GNV-08 and GNV-10 showed positive phenotypic characters which could be used in developmental programmes of Grand Naine banana.

**Keywords:** liable region, OPF-$09_{320}$ markers, phenotypic characters, tissue culture

## INTRODUCTION

Banana is an important fruit crop that has replaced other tropical fruit crops in terms of production and productivity. The genus *Musa* comprises members that are important as food and cash crops in the humid tropics. Its centre of origin is thought to be the Indo-Malaysian axis (Simmonds 1960), but it has spread to most tropical and subtropical regions of the world. The rise in banana productivity during recent times can be attributed to the use of *in vitro* plants, along with drip irrigation and other improved agronomical practices (Shiddlingeswara *et al*. 2004). Somaclonal variants obtained by tissue culture can also provide a rapid and reliable approach for plant improvement. Plantains and bananas have become the subjects of intense improvement programmes in which modern biotechnological methods had contributed significantly to their genetic improvement (Gordian and Philip 2007; Tripathi *et al*. 2007). Apart from faster multiplication rates in lab conditions, tissue-cultured bananas pose many advantages like regular availability, earliness, synchronized blooming and comparatively higher yields. However, the technique has been reported to predispose plant materials to chromosomal instability which does not preclude the genomic instability that ordinarily arises due to cryptic chromosomal rearrangements, somatic crossing over with sister chromatid exchanges, transposable elements, and gene amplification/diminution phenomena (Mantell 1985; Hartwell *et al*. 2000).

But, the disencouraging reports of somaclonal variations among *in vitro* plants produced by many biofactories are limiting the expansion in the use of tissue-cultured bananas. Unsatisfied farmers have even approached consumer courts seeking compensation for the heavy losses incurred in the fields due to dismal performance of somaclonal variants among tissue-cultured plants which are rather higher in cost (Shiddlingeswara *et al*. 2004). The phenomenon of somaclonal variation can be defined as a genetic variability generated during *in vitro* culture (Larkin and Scowcroft 1981). Several features of *in vitro* techniques may raise the rate of somaclonal variations observed in plants management and other poor agronomic practices followed by the farmers. By doing so they are denying the contributions of pre-existed genetic variability and over-exploited *in vitro* techniques. Hence the present study was conducted to confirm the genetic variability using RAPD in somaclonal variants in order to prove that the somaclonal variations among tissue cultured plantlets are the results of either pre-existing genetic variability of over-exploited *in vitro* techniques and not due to managerial problems.

In *Musa* sp., somatic mutations (Samson 1982) and somaclonal variations (Vuylsteke *et al*. 1988; Sandoval *et al*. 1991) have been implicated in genome instability. Furthermore, viral particles have been reported to interact with the *Musa* genome to destabilise it, especially under *in vitro* culture environments. Naturally, changes occur in the genomes of plants, but their rates are slow and natural selection removes deleterious ones from the *milieu*. However, *in vitro* systems quicken the mutation rate because additional selection pressure is placed on the cultured material manifesting itself as somaclonal variations. Somaclonal variations are not altogether undesirable since some may serve as novel raw material for further crop improvement (Larkin and Scowcroft 1981).

The problem, however, is that generating somaclonal variants is unpredictable since the type and extent of variation or even synergistic processes forming them are random events. Osuji *et al*. (1997) noted that the instability at the genotype level of *Musa* compromises the conventional idea of using phenotypic characters for molecular marking of *Musa* material. Gordian and Philip (2007) worked on tagging useful chromosomal changes in *Musa* sp. Several research efforts have looked into unravelling the genotypic constitution of *Musa* plants, relying on molecular cytogenetic techniques (Kosina and Heslop-Harrison 1996; Osuji *et al*. 1997, 1998). Consequently, the present effort was aimed at screening the normal regenerants and somaclonal variants

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**Table 1** Growth characters of normal and variants of Grand Naine banana.

| Sl. № | Phenotype | Pseudostem | | № of leaves | Basal leaf | | |
|---|---|---|---|---|---|---|---|
| | | Height (m) | Circumference (cm) | | Length (m) | Breadth (m) | Area ($cm^2$) |
| 1 | GNV-01 | 2.32 | 61.50 | 11.0 | 1.71 | 0.61 | 102.15 |
| 2 | GNV-02 | 2.22 | 62.02 | 12.0 | 1.81 | 0.62 | 111.65 |
| 3 | GNV-03 | 3.80 | 84.01 | 10.0 | 2.11 | 0.75 | 157.50 |
| 4 | GNV-04 | 4.00 | 90.00 | 10.0 | 3.01 | 0.68 | 204.15 |
| 5 | GNV-05 | 2.31 | 76.00 | 09.0 | 1.90 | 0.71 | 134.75 |
| 6 | GNV-06 | 2.25 | 69.00 | 12.0 | 1.61 | 0.74 | 118.50 |
| 7 | GNV-07 | 3.81 | 62.50 | 12.0 | 1.70 | 0.71 | 120.65 |
| 8 | GNV-08 | 3.21 | 71.00 | 11.0 | 1.50 | 0.68 | 102.15 |
| 9 | GNV-09 | 2.81 | 65.50 | 09.0 | 2.11 | 0.70 | 146.50 |
| 10 | GNV-10 | 4.11 | 86.01 | 10.0 | 3.11 | 0.61 | 292.25 |
| 11 | GNV-11 | 2.65 | 65.01 | 12.0 | 2.80 | 0.64 | 182.01 |
| **Mean of Normal GN** | | 2.16 | 61.73 | 10.76 | 1.82 | 0.62 | 113.82 |
| **SEm±** | | 0.006 | 0.125 | 0.125 | 0.003 | 0.005 | 0.215 |
| **CD (5%)** | | 0.019 | 0.380 | 0.380 | 0.010 | 0.017 | 0.652 |

**Table 2** Bunch characters of normal and variants of Grand Naine banana.

| Sl. № | Phenotype | Bunch | | № of hands per bunch | № of fingers per hand | Finger | | | Peel thickness (mm) |
|---|---|---|---|---|---|---|---|---|---|
| | | Weight (Kg) | Length (m) | | | Weight (g) | Length (cm) | Circumference (cm) | |
| 1 | GNV-01 | 40.25 | 0.80 | 12.0 | 16.01 | 150.70 | 17.30 | 12.02 | 1.02 |
| 2 | GNV-02 | 41.24 | 1.75 | 10.0 | 17.01 | 154.50 | 18.03 | 10.70 | 0.82 |
| 3 | GNV-03 | 39.80 | 1.25 | 11.0 | 18.01 | 151.00 | 19.02 | 10.40 | 1.02 |
| 4 | GNV-04 | 59.75 | 2.00 | 21.0 | 20.01 | 141.50 | 16.65 | 11.70 | 1.22 |
| 5 | GNV-05 | 40.50 | 1.01 | 14.0 | 17.01 | 136.50 | 16.35 | 11.25 | 1.05 |
| 6 | GNV-06 | 39.01 | 0.91 | 10.0 | 15.01 | 152.25 | 17.45 | 12.55 | 0.97 |
| 7 | GNV-07 | 42.70 | 1.01 | 11.0 | 18.01 | 157.50 | 15.70 | 13.03 | 1.02 |
| 8 | GNV-08 | 48.21 | 1.23 | 13.0 | 18.00 | 155.50 | 16.15 | 11.90 | 1.05 |
| 9 | GNV-09 | 38.50 | 0.81 | 9.0 | 16.01 | 148.45 | 16.75 | 10.80 | 0.92 |
| 10 | GNV-10 | 50.75 | 1.75 | 18.0 | 18.01 | 151.50 | 17.70 | 12.00 | 0.82 |
| 11 | GNV-11 | 39.71 | 0.84 | 10.0 | 15.00 | 150.30 | 17.01 | 13.30 | 0.92 |
| **Mean of Normal GN** | | 36.83 | 1.27 | 10.77 | 16.39 | 154.13 | 18.98 | 13.38 | 0.93 |
| **SEm±** | | 0.034 | 0.006 | 0.023 | 0.129 | 0.613 | 0.122 | 0.094 | 0.006 |
| **CD (5%)** | | 0.105 | 0.021 | 0.071 | 0.391 | 0.858 | 0.371 | 0.285 | 0.020 |

of tissue-cultured *Musa* cultivars.

One difficulty in dealing with somaclonal variation is identification of any genetic variation in the regenerated plants (Vidal and Garcia 2000). A number of different molecular techniques are currently available to detect sequence variation between closely related genomes such as those between source plants and somaclones. Representational difference analysis (RDA) has been applied to detect variation in a limited number of plant species like *Silene latifolia* (Donnison *et al*. 1996), *Musa* spp. (Cullis and Kunert 2000), *Quercus* sp. (Zoldos *et al*. 2001), date palm (Vorster *et al*. 2002) and flax (Oh and Cullis 2003). Random amplified polymorphic DNAs (RAPDs) and amplified fragment length polymorphisms (AFLPs) (Linacero *et al*. 2000; Labra *et al*. 2001) are two useful techniques when comparing DNA from any number of different samples for the differentiation of plants because of sequence variation caused by identifying random polymorphism. Because RAPD polymorphisms result from either a nucleotide base change that alters the primer binding sites or from an insertion or deletion within the amplified region (Williams *et al*. 1993), polymorphisms usually result in the presence or absence of an amplification product from a single locus (Tingey and Tufo 1993). The products of these amplifications can be polymorphic and are useful as genetic markers (Hu and Quiros 1991). In this report, we used RAPD to characterize somaclonal variants for predictive marking of *Musa* lines that may be used for incorporation into improvement programmes.

## MATERIALS AND METHODS

### Morphological data

Eleven superior variants ('GNV-1', 'GNV-2', 'GNV-3', 'GNV-4', 'GNV-5', 'GNV-6', 'GNV-7', 'GNV-8', 'GNV-9', 'GNV-10' and 'GNV-11') with four controls ('GN-1', 'GN-2', GN-3' and 'GN-4') were selected from orchards growing tissue-cultured Grand Naine bananas around Bangalore, India. Various growth parameters such as pseudostem height, pseudostem circumference, number of leaves per plant, basal leaf length, basal leaf breadth and area (**Table 1**), yield and reproductive parameters such as bunch weight and length, number of hands per bunch, number of fingers per hand, finger weight, finger length, finger diameter and peel thickness (**Table 2**) and quality parameters such as total soluble solids, reducing sugars, total sugars and titratable acidity (**Table 3**) were taken for the main crop at the bunch-harvesting stage. The mean of fifteen normal plants was used as a control for the morphological characters. Analysis,

**Table 3** Qualitative fruit characters of normal and variants of Grand Naine banana

| Sl. № | Phenotype | Total Soluble Solids ($^0$Brix) | Reducing Sugars (%) | Total Sugars (%) | Titratable acidity (%) |
|---|---|---|---|---|---|
| 1 | GNV-01 | 18.01 | 13.04 | 16.70 | 1.04 |
| 2 | GNV-02 | 19.01 | 16.33 | 17.50 | 1.04 |
| 3 | GNV-03 | 20.02 | 15.97 | 19.50 | 0.90 |
| 4 | GNV-04 | 23.55 | 16.02 | 21.01 | 0.85 |
| 5 | GNV-05 | 19.40 | 14.21 | 18.50 | 0.95 |
| 6 | GNV-06 | 21.03 | 16.13 | 19.01 | 1.01 |
| 7 | GNV-07 | 22.00 | 14.14 | 19.45 | 1.01 |
| 8 | GNV-08 | 20.01 | 15.83 | 19.02 | 1.02 |
| 9 | GNV-09 | 19.40 | 13.92 | 16.25 | 0.91 |
| 10 | GNV-10 | 21.72 | 16.23 | 20.03 | 0.80 |
| 11 | GNV-11 | 19.02 | 15.13 | 18.70 | 0.81 |
| **Mean of Normal GN** | | 20.43 | 15.52 | 19.38 | 0.84 |
| **SEm±** | | 0.058 | 0.010 | 0.072 | 0.006 |
| **CD (5%)** | | 0.177 | 0.031 | 0.217 | 0.020 |

correlation and interpretations were made by employing the RCBD method for field studies and the CRD method for laboratory studies as suggested by Fischer and Yates (1963) and Sunderraj *et al*. (1972), respectively.

## DNA isolation

Fifty g of young leaves were collected, pre-treated by washing with distilled water, wiped with 70% (v/v) ethanol, then air-dried prior to storage in sealed plastic bags at 4°C. DNA was extracted according to a modified Cetyl trimethyl ammonium bromide (CTAB) method following Simon *et al*. (2007). Two g of leaf sample was powdered in liquid nitrogen to extract DNA. The powder was mixed with 10 ml extraction buffer, preheated to 65°C, and containing 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl, 3% (w/v) CTAB, 2% polyvinyl pyrrolidone and 1% β-mercaptoethanol, then incubated at 65°C for 1 h. The mixture was cooled to room temperature, 6 ml cold 24:1 (v/v) chloroform:isoamylalcohol was added, and the contents were mixed well. After centrifugation at 6,500 × *g* for 8 min at 4°C, the supernatant was transferred to a fresh tube and the chloroform: isoamylalcohol step was repeated until a clear supernatant was obtained. 5 M NaCl was added to the supernatant (0.5 v/v) and mixed gently followed by the addition of 1 volume of cold isopropanol to precipitate the DNA. The mixture was incubated overnight at 4°C, and then centrifuged at 7,500 × *g* for 15 min. The resulting pellet was washed with 70% (v/v) ethanol, air-dried, and dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). Two µg RNase (Bovine pancreatic ribonuclease, Bangalore Genei, Bangalore, India) was added to each sample which was incubated for 3 h at 37°C, mixed with an equal volume of phenol and centrifuged at 7,500 × *g* for 10 min at room temperature. This step was followed by a wash with an equal volume of 1:1 (v/v) phenol:chloroform then with chloroform alone. DNA was precipitated overnight at 4°C with 0.5 vol of 5 M NaCl and 1 vol cold isopropanol, and the resulting pellet obtained after centrifugation was dissolved in TE buffer, analysed on an agarose gel and quantified using a spectrophotometer (NanoDrop Technologies, Wilmington, USA).

## PCR amplifications

PCR amplification followed the protocol of Williams *et al*. (1990) with minor modifications. Of the 60 primers screened using pooled DNA, 43 clear and distinguishable bands were selected for RAPD-PCR analysis. Reproducibility of the primers was tested by repeating the PCR amplification three times under similar conditions. PCR reactions were carried out in a volume of 25 µl containing 30 ng template DNA, 150 µM each dNTP, 1.5 mM $MgCl_2$, 1.5 units *Taq* DNA polymerase (Sigma Aldrich Chemicals, Bangalore, India), 5 pmol primer (OPA, OPB, OPC, OPD, OPE, OPF, OPG, OPH, OPI, OPJ and OPK series, Operon Technologies, Alameda, CA, US) in PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.05% (v/v) NP40 and 0.05% (v/v) Triton X-100). Amplifications were performed in a Corbett Research Thermocycler (Corbett Research Mortlake, New South Wales, Australia), programmed for an initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, primer extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR products were resolved in a 1.2% (w/v) agarose gel, visualized and documented using an Alpha Digidoc system (Alpha Innotech, San Leandro, CA, US). Each reproducible band was visually analysed for the presence and absence between the normal and somaclonal variants. The band sizes were determined by comparing with 500 bp DNA ladder (Genei, Banglore, India), which was run along with the amplified products.

## RESULTS AND DISCUSSION

The appearance of somaclonal variants may not be a desirable process in propagation (Cullis 2005). Various types of mutations have been described in somaclonal variants, including point mutations, gene duplication, chromosomal rearrangements, and chromosome number changes (Peschke and Phillips 1992; Phillips *et al*. 1994; Kaeppler *et al*. 2000). Transposable element movement and changes in DNA methylation (Smulders *et al*. 1995; Kubis *et al*. 2003; Koukalova *et al*. 2005), possibly through the function of small interfering RNA (Lippman *et al*. 2003), have also been implicated as potential mechanisms behind somaclonal variations. The trigger for all these types of changes can be described as genomic shock or plasticity, which occurs after the plant has exhausted its ordinary physiological responses to environmental stress (Cullis 1999). This genomic shock response may be a radical, but limited, genomic reorganization, which is an adaptive mechanism and can be activated under stress. The occurrence of hotspots of mutation and recurring menus of alternative alleles is consistent with this response being limited to a sub-fraction of the genome.

The clonal propagation of horticultural species and crops, such as bananas and oil palm, is intended to produce elite individuals in mass and negative variations are problematic, where even a low per cent are unacceptable for commercial use (Thomas *et al*. 2007). Significant differences were obtained in the morphological character between the Grand Naine normal and variants analysed (**Tables 1-3**). A maximum height and circumference of the pseudostem was obtained in the variants GNV-10 (4.11 m) and GNV-04 (90.0 cm), respectively. Supreme bunch weight of 59.75 kg (GNV-04), 50.75 kg (GNV-10) and 48.21 kg (GNV-08) was obtained when compared with the average weight to normal 36.83 kg. The bunch length varied from 0.80 to 2.00 m among the variants contrasting to the mean length of 1.27 m in the normal plants. The bunch of variants GNV-04 and GNV-10 were comprised of 21 and 18 hands with an average of 20.01 and 18.01 fingers, respectively. A maximum finger weight of 157.50 g in GNV-07 and a minimum weight of 136.50 g in GNV-05 were noticed. The quality

**Table 4** Correlation matrix among growth and bunch characteristics among Grand Naine variants of banana.

| | Bunch weight (kg) | Pseudo-stem height (m) | Pseudostem circumference (cm) | № leaves | Basal leaf length (m) | Basal leaf breadth (m) | Basal leaf area ($cm^2$) | Bunch length (m) | № hands/ bunch | № fingers/ hand |
|---|---|---|---|---|---|---|---|---|---|---|
| Bunch weight (kg) | 1.00 | | | | | | | | | |
| Pseudostem height (m) | 0.756* | 1.00 | | | | | | | | |
| Pseudostem circumference (cm) | -0.180 | -0.434 | 1.00 | | | | | | | |
| Number of leaves | 0.571* | 0.673* | -0.257 | 1.00 | | | | | | |
| Basal leaf length (m) | 0.371 | 0.386 | -0.083 | -0.096 | 1.00 | | | | | |
| Basal leaf breadth (m) | 0.674* | 0.746* | -0.284 | 0.922** | -0.001 | 1.00 | | | | |
| Basal leaf area ($cm^2$) | 0.090 | 0.048 | 0.066 | 0.279 | -0.345 | 0.241 | 1.00 | | | |
| Bunch length (m) | 0.376 | 0.433 | -0.187 | 0.050 | 0.467 | 0.099 | -0.845** | 1.00 | | |
| Number of hands per bunch | 0.262 | 0.363 | -0.169 | 0.468 | -0.308 | 0.452 | 0.908** | -0.631* | 1.00 | |
| Number of fingers per hand | 0.183 | 0.104 | -0.075 | 0.205 | -0.308 | 0.182 | 0.895** | -0.706* | 0.874** | 1.00 |

* Significant at 5%
** Significant at 1%

**Fig. 1 Bunch characters of somaclonal variant GNV-04.**

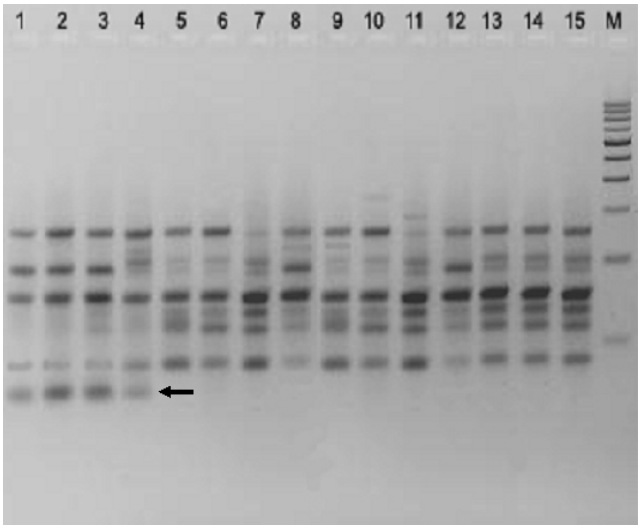


**Fig. 2 Gel profile of normal and somaclonal variants of banana using RAPD-PCR primer OPF-09.** Normal banana (Lanes 1-4): 'GN-1', 'GN-2', GN-3' and 'GN-4'. Somaclonal variants (Lanes 5-15): 'GNV-1', 'GNV-1', 'GNV-2', 'GNV-3', 'GNV-4', 'GNV-5', 'GNV-6', 'GNV-7', 'GNV-8', 'GNV-9', 'GNV-10' and 'GNV-11'. Lane M: 500 bp DNA ladder. Arrow: OPF-$09_{320}$.

parameters such as total soluble solids, reducing sugars, total sugars and titratable acidity did not show considerable variations between the normal and variant fruits. Among the variants GNV-04, GNV-08 and GNV-10 showed promising yield characters. The correlation matrix of the phenotypic characters is presented in **Table 4**. The bunch character of somaclonal variant GNV-04 is shown in **Fig. 1**.

Two g of young leaves preferably cigar leaves, were used to extract DNA, as mature leaves were highly fibrous and rich in polyphenols and polysaccharides that hindered the extraction of PCR-quality DNA. The pre-treatment of the leaves removed dust particles and external microbial contaminations. The CTAB method for DNA extraction was found to be optimal to release the nucleic acid from the cell and to remove RNA and proteinaceous contamination rendering the DNA suitable for PCR amplifications. PCR amplification procedure followed was the standard protocol reported by Williams *et al*. (1990) with minor modifications, and produced good amplifications with 30 ng of template DNA. The amplifications using 1.5 units of *Taq* DNA polymerase and 1.5 mM $MgCl_2$ produced intense and clear banding patterns. A primary screening of 60 RAPD primers resulted in selection of 43 primers that produced clear and reproducible fragment patterns. Screening is essential to save time, cost and to reject primers not informative for the analysis (Prakash *et al*. 2002).

RAPD has proved to be useful in identifying regions of the banana genome that vary between normal and somaclonal variants. The primer OPF-09 proved useful to differentiate the variants and the normal Grand Naine bananas by producing a polymorphic band of size 320 bp found in all the normal samples but was characteristically absent in the somaclonal variants tested (**Fig. 2**). This evidence is consistent with the fact that all the sequences present in the somaclonal variants are also present in the normal plants, but not *vice versa*. The production of a single marker (OPF-$09_{320}$) differentiating normal and the somaclonal variants suggests the presence of a limited set of loci being modified during the generation of variation due to *in vitro* conditions. The region is vulnerable to a large number of mutations that arise frequently during *in vitro* propagation. This variation of the sequence also makes the region a candidate for identifying single-nucleotide polymorphisms as possible markers developed through *in vitro* propagation of Grand Naine bananas. The findings of this study are consistent with the notion that there is a labile fraction of the genome that is modified during the generation of somaclonal variation in banana. The difference in the products reported (OPF-$09_{320}$) should have all the characteristics of representatives of the susceptible region of the genome.

The primer OPF-09 was tested effectively to distinguish individuals developed from commercial *in vitro* propagation. A small number of variants was successfully tested based on the amplifications produced by this primer, yet a larger number of individuals are essentially to be evaluated. The phenotype of the identified individuals should be determined to confirm the association of the marker and the specific phenotype. A large number of decamer primers could also be tested to identify the frequency of liable regions within the banana genome. However, the preliminary evidence indicates that primer OPF-09 can be used as a diagnostic key for identifying somaclonal variants in Grand Naine banana plants produced through *in vitro* propagation. The marker reported here and the primer has the potential of being developed into a robust diagnostic DNA marker to ascertain somaclonal variation. Further, the RAPD marker $OPF09_{320}$ can be cloned and sequenced to identify and tag the liable region of Grand Naine bananas. SCAR markers have efficiently been used in identifying loci related to sex in *Carica papaya* (Giovanni and Victor 2007), *Eucommia ulmoides* (Xu *et al*. 2004) and *Asparagus* (Jiang and Sink 1997). Among the eleven positive variants tested three variants (GNV-04, GNV-08 and GNV-10) showed encouraging morphological characters and could be used in breeding programmes for the development of new banana varieties.

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