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Karyotypic and 2C Nuclear DNA Size Instability *in vitro* Induced Off-Types of East African Highland Banana (*Musa* AAA East Africa)

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Abstract: This study was conducted to determine chromosome number and 2C nuclear DNA content in tissue culture induced off-type banana (*Musa* AAA East Africa) landrace Uganda with tolerance black sigatoka disease, susceptibility to water stress, sparsely black-blotched pseudostems, taller pseudostems, late fruit maturation, altered inflorescence and higher fruit dry matter content. The off-type banana appeared to have higher ($p<0.05$) frequency of 31 and 32 chromosomes at 15.1 and 13.6%, respectively. Conversely, the frequency of 31 and 32 chromosomes was 12.0 and 9.6% for the micropropagation (MP) derived phenotypically normal plants and 11.8 and 9.5% for the Conventionally Propagation (CP) derived plants with no tissue culture history. Moreover, the off-type banana had lower ($p<0.05$) leaf 2C nuclear DNA amount of 1.72 pg, whilst the MP and CP derived plants had 1.81 and 1.82 pg, respectively.

Key words: Chromosome number, 2C nuclear DNA amount, somaclonal variation, East African highland banana

INTRODUCTION

Somaclonal variation in banana has commonly been linked to chromosome numerical aberrations, including high frequencies of aneuploidy and tetraploidy (Reuven and Israel, 1990; Sandoval *et al*., 1996; Shepherd *et al*., 1996; Gimenez *et al*., 2001). These chromosome numerical errors usually originate from either endoreplication or endomitosis and amitosis (Nuti-Ronchi, 1991). Moreover, *in vitro* induced variation has been associated with the reduction in nuclear DNA content (Deumling and Clermont, 1989; Harding *et al*., 1996; Price and Johnson, 1996). The nuclear DNA amount is linked to plant adaptation to environmental conditions, especially cell cycle progression, juvenility and dormancy (Bennet, 1972; Price and Johnson, 1996; Bennetzen, 2002; Gregory, 2004).

In vitro micropropagated East African highland banana landrace Uganda exhibited high incidence of off-type plants. The off-type banana plants were more tolerant to black sigatoka, susceptible to water stress, taller, latter maturing and produced altered inflorescences and fruits with higher dry matter content. The genetic bases underlying these variations are presently unknown. The objective of this study was to determine the mechanisms underlying the variation in the off-type banana based on chromosome number and 2C nuclear DNA content.

MATERIALS AND METHODS

Description of the study plant materials: Banana plants used in this study were established in the field at Sokoine University of Agriculture in Tanzania. *In vitro* suckers of East African highland banana landrace Uganda produced according to Maerere *et al.* (2003) were planted in the field. After detecting off-type plants, 15 suckers at the second ratoon crop cycle (two years from the date of planting of the *in vitro* suckers) each of the off-type banana (treatment), micropropagation (MP) derived phenotypically normal banana (control 1) and Conventional Propagation (CP) derived banana with no tissue culture history in its ancestry (control 2) were collected and planted in new plots in 2005. Planting holes of 100×100×100 cm dimension spaced at 300 m apart were prepared and filled each with 60 L of farmyard manure. The crop received optimal management, including weeding, irrigation during dry season, application of farmyard manure in subsequent years and desuckering to maintain four plants per stool. The mechanisms underlying the variation in the off-type plants was determined at the fourth ratoon crop cycle (three years from the date of planting of the *in vitro* suckers) based on root chromosome number and leaf 2C nuclear DNA amount.

Karyotypic analysis: Root collection and fixation and slide preparation were carried out according to

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Dolezel *et al.* (1998). In 2006, actively growing root tips from 10 field-grown plants each of the off-type, MP and CP derived banana were collected in 0.02% (v/v) of 14.3 M β-mercaptoethanol, treated with 0.05% (m/v) 8-hydroxyquinoline for three hours at 21°C and fixed at 4°C in freshly prepared fixative (glacial acetic acid and 99% v/v ethanol at 1:3 v/v) for 24 h. The fixed roots were rinsed in distilled water and stored in 70% ethanol at 4°C for future studies. About 10 root tips of 10 m long were washed in 7.5 mM EDTA and 75 mM KCl (pH 4.0), chopped in small pieces and digested at 37°C for 60 min in 10 mM citrate buffer (pH 4.7) consisting of 2% v/v pectinase (Sigma P-4716), 1% m/v pectolyase (Sigma P-3026) and 2% m/v cellulose (Sigma C-9422). The suspension of protoplasts was filtered through 150 μm and the pellet obtained by spinning at 750 g for 10 min was re-suspended in 500 μL of ice-cold 70% ethanol and stored at 4°C for future use.

To prepare a slide, 7 μL of the protoplast suspension in 70% ethanol was dropped onto a clean ice-cold slide and the drop was allowed to spread and air-dry at room temperature of 21°C. Shortly before the drop completely dried out, 7 μL of the 3:1 fixative was added on the protoplast drop to induce spreading. The slides were rinsed in 96% ethanol before the drop completely dried, air-dried at 21°C, stained in 10% Giemsa for 30 min, washed in distilled water to remove the stain and air-dried at 21°C. A drop of paraffin oil was added on the slide prior to covering with a coverslip and sealing it with nail polish. After drying of the nail polish, a slide was mounted in a compact microscope (Zeiss Axiodkop 40) and chromosomes were observed under a 100 X/1.25 oil immersion objective. Chromosome images were captured using digital camera (Cannon PC 1064) and images were processed using software ZoomBrowser Version 4.5. Chromosome counting was carried out using 100 protoplasts with well scattered and contracted metaphase chromosomes per treatment.

Flow cytometry: In 2006, cigar leaves from 10 different plant stools each of the off-type (treatment), MP derived banana (control 1) and CP derived banana plants (control 2) were collected from the field-grown plants. Fresh leaf tissues of banana samples and calibration standard were macerated using bead beating method according to Roberts (2007). Parsley (*Petroselinum crispum* (Mill.) Nyman Champion Moss Curled') with 2C DNA amount of 4.46 pg was used as a calibration standard (Yokoya *et al.*, 2000). Briefly, 7 mg each of the test plant material and calibration standard were placed in citrate buffer in 2 mL screw-capped tubes (Heather Scientific Ltd., Glasgow) along with 10 glass silica beads each with 2.5 mm diameter (Heather Scientific Ltd., Glasgow). The sample tubes were agitated simultaneously at a speed of 6.5 m sec^{-1} for 45 sec in an FP120 FastPrep Cell Disrupter (Savant Instruments Inc, New York). Macerated leaf tissue was filtered through a 30 μm nylon mesh into a 55×12 mm test tube. The filtrate was treated with 50 μL of 3000 μg L^{-1} of RNase at final concentration of 150 μg mL^{-1}. After the above, 400 μL of the treated filtrate was added into 2000 μL of the propidium iodide staining solution, mixed thoroughly and then incubated at 20-25°C for 20 min (Hanson *et al.*, 2005). The staining solution consisted of 0.06 mg mL^{-1} propidium iodide, 56.8 mg mL^{-1} disodium hydrogen orthophosphate, 3.6 mg mL^{-1} sodium sulphate and 4.9 mg mL^{-1} trisodium citrate.

The flow cytometric analysis was carried out using CAIII flow cytometer (Partec GmbH, Munster, Germany) consisting of an argon laser light source (488 nm), a TK420 dichroic mirror, an OR610 barrier filter and a 40×0.80 quartz objective. The effectiveness of preparatory procedure was assessed from the coefficients of variation of the peaks in flow cytometer histograms, which were as low as 2.05 and 2.76% for the calibration standard and banana sample, respectively. The nuclear DNA amount of test sample in three replicates was calculated as the ratio of the fluorescence intensity of the nuclei of the test sample and the calibration standard multiplied by the nuclear DNA content of the latter.

Data analysis: Data analysis was performed using 'SPSS 15.0 software (SPSS^R, 2006). The parametric data were subjected to one-way ANOVA based on F-test and multiple means comparison was carried out based on Tukey honest significant difference (Tukey-HSD) test ($p<0.05$) (Zar, 1997). Data on chromosome frequency were analysed descriptively.

RESULTS

Chromosome number: A wide range of aneuploidy, predominantly 30, 31 and 32 chromosomes was observed in the off-type and true-to-type plants (Table 1). However,

Table 1: Chromosome frequency in the off-type banana plants of landrace Uganda

Chromosome No. (2n)	Chromosome frequency (%) (±SE)		
	Off-type banana	MP banana	CP banana
28	4.0±0.4^a	5.0±0.2^a	5.5±0.3^a
29	3.8±0.2^a	4.0±0.2^a	4.2±0.4^a
30	8.4±0.2^a	10.0±0.3^a	8.6±0.2^a
31	15.1±0.4^b	12.0±0.5^a	11.8±0.5^a
32	13.0±0.2^b	9.6±0.4^a	9.5±0.4^a
33	48.3±0.2^a	52.5±0.4^b	51.0±0.5^b
34	2.2±0.2^a	2.0±0.1^a	2.8±0.6^a
35	2.5±0.2^a	2.0±0.1^a	2.4±0.1^a

$^{a, b}$: Numbers the same letter within the row are insignificantly ($p<0.05$) different according to Tukey-HSD. SE: Standard error of the mean (n = 10)

Table 2: Nuclear DNA content of the off-type banana plants of landrace Uganda

Plant type	Leaf 2C nuclear DNA content (pg) (±SE)
Off-type plants	1.72±0.024[a]
MP plants	1.81±0.005[b]
CP plants	1.82±0.008[b]

[a, b]: Numbers bearing the same superscript letter within the column are insignificantly, ($p<0.05$) different according to Tukey-HSD. SE: Standard error of the mean (n = 10)

the off-type plants had higher ($p<0.05$) frequency of cells with 31 and 32 chromosomes of 15.1 and 13.6% compared with 12.0 and 9.6% of the MP derived plants and 11.8 and 9.5% of the CP derived plants, respectively. Moreover, the off-type banana had lower ($p<0.05$) frequency of euploidy (2n = 3x = 33) of 48.3% compared with 52.5 and 51.0% of the MP and CP derived plants, respectively.

Nuclear DNA content: The off-type plants had lower ($p<0.05$) leaf 2C nuclear DNA content of 1.72 pg compared with 1.81 and 1.82 pg of the MP and CP derived normal plants of landrace Uganda, respectively (Table 2).

DISCUSSION

The presence of a wide range of aneuploidy in the off-type plants as well as MP derived phenotypically normal plants indicates that tissue culture process induced the karyotypic instability. Several studies have also reported substantial chromosome numerical errors, mostly aneuploidy in Cavendish banana maintained in tissue culture for a long duration (Reuven and Israel, 1990; Sandoval *et al*., 1996; Shepherd *et al*., 1996).

The leaf 2C nuclear DNA amount of landrace Uganda of 1.82 pg suggests that it is triploid (2n = 3x = 33) and is comparable with that of the triploid acuminata bananas, which are putative precursors of East African highland bananas (Mukasa and Thomas, 1970). Depending of laboratories, the leaf 2C nuclear DNA content of banana cv. Gros Michel varies from 1.81 to 1.88 pg, while that of Cavendish cv. Grand nain ranges from 1.80 to 190 pg, (Lysak *et al*., 1999; Kamate *et al*., 2001). The loss in nuclear DNA amount in the off-type plants was probably due to increased frequency of descending aneuploidy or elimination DNA sequences. Many reports have associated the reduction in nuclear genome size with an elimination of either entire chromosomes through endomitosis and amitosis or segments such as transposable elements and DNA repeat sequences (Nuti-Ronchi, 1991; Price and Johnson, 1996; Petrov; 2001; Bennetzen, 2002; Gregory, 2004; Vinogradov, 2004). A substantial loss in nuclear DNA content has been reported in callus-derived Siberian squill (*Scilla siberica* Andr.) (Deumling and Clermont, 1989), grape vine (Harding *et al*., 1996) and oil palm (Rival *et al*., 1997). The reduction in nuclear DNA amount in the off-type plants was probably induced by tissue culture stress, especially the growth regulators used to enhance morphogenetic transition. Benzylaminopurine in the growth media induced the reduction of copy number of DNA repetitive sequences in carrot callus (Pluhar *et al*., 2004; Arnholdt-Schmitt *et al*., 1991). Similarly, tobacco exposed to menadione-mediated stress (Reichheld *et al*., 1999) and sunflower grown under short photoperiodic conditions (Price and Johnson, 1996) exhibited a reduction in nuclear DNA content.

The reduction in chromosome number and nuclear DNA amount could probably underlie the observed variation in the off-type plants, particularly male bud shape, persistence of fruit floral parts, delay in crop maturation (apparent juvenility) and changes in adaptation to biotic and abiotic stresses. Many reports have also linked the loss in nuclear DNA amount to juvenility, loss in winter dormancy in temperate plants, flower abnormality in oil palm and increased *in vitro* proliferation in carrot tissue (Deumling and Clermont, 1989; Arnholdt-Schmitt, 1991; Rival *et al*., 1997; Zhao, 2004). For instance, callus-derived regenerants of Siberian squill with partial non-winter dormancy had lower nuclear DNA content of 28.3 pg compared with 123.0 pg of the donor parent (Deumling and Clermont, 1989). Similarly, Zhao (2004) reported that conventionally propagated rhubarb line CP49 with non-winter dormancy had lower nuclear DNA content of 8.2 pg in comparison with 9.9 pg of its progenitor Timperley Early.

CONCLUSION

The observed variation in the off-type plants from micropropagated landrace Uganda appear to be associated with losses in entire chromosomes, or segments. Such genetic/epigenetic defects might be frequent, but under-reported in micropropagated plant species, because propagators are more interested in the control of somaclonal variation than its mechanisms. As karyotypic analysis is relatively complex in banana, tissue culture induced off-types from landrace Uganda could easily be detected at the nursery stage based on leaf 2C nuclear DNA amount using flow cytometry. More importantly, flow cytometry would be useful for the detection of somaclonal variation observed after the plant vegetative stage such as flower, bunch and fruit characteristics.

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