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Characterization of radiation induced and tissue culture derived dwarf types in banana by using a SCAR marker

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Abstract

In banana tissue cultures, the occurrence of dwarf off types is a common phenomenon and their detection become relevant. In this study, tissue culture derived (somaclonal) and radiation-induced off types in banana cultivars. Robusta and Giant Cavendish were analyzed using a SCAR-based molecular diagnostic technique. The dwarfs were initially characterized at maturity, by height, leaf shape, pseudostem height and other agronomic characters. At the molecular level, amplification with the SCAR primer resulted in an expected amplified fragment of 1500 bp in normal but not in the dwarf types. Further in a multiplex PCR, an internal positive control was included using primers for 18S rRNA which gave amplification in both the normal and dwarf types. This study shows that the dwarf off-types isolated through tissue culture and radiation-mutagenesis were true dwarfs, validated by using the dwarf-specific RAPD marker for banana.

Keywords: Banana; Dwarfs; *In vitro;* Irradiation; Multiplex PCR; SCAR marker

Introduction

Bananas and plantains are propagated vegetatively through suckers. Most of the edible bananas triploid, are nearly sterile and parthenocarpic and, hence use of conventional breeding methods are difficult and cumbersome. Owing to these constraints, mutation breeding and biotechnological methods can offer as useful tools for banana improvement. The ability to culture and manipulate a large number of totipotent plant cells provides a greater opportunity for *in vitro* mutagenesis and selection (Suprasanna et al., 2006). Somaclonal variation is a rapid and reliable approach for improvement of plants as the generated variation can be used either directly or indirectly in a breeding program aimed at crop improvement (Jain, 2000). Somaclonal variation (mutation) during banana micropropagation is a common feature, and superior quality banana clones have been produced through selection for various traits including disease resistance (Maria and Garcia, 2000; Hwang and Ko, 2004). Selection of agronomically useful somaclones requires large-scale field evaluations and often, screening of clones at the field level is a very tedious, expensive, time-consuming, and labor-intensive process (Asif et al., 2004). Additionally, the trait of interest needs to be confirmed over a number of selection cycles. In this context, development and

availability of early, reliable, and reproducible selection strategies can speed up the selection procedure and, eventually, improvement of banana.

The molecular marker technologies have become a powerful tool in crop improvement through their use in germplasm characterization and fingerprinting, genetic analysis, linkage mapping, and molecular breeding. Random amplified polymorphic DNA (RAPD) markers provide an efficient assessment of the differences in the genetic composition of related individuals (Kulkarni et al. 1999), detection of different somaclonal variants (Kaemmer et al., 1992; Damasco et al., 1996; Maria and Garcia, 2000). RAPD technique requires only small amount of DNA sample without involving radioactivity tests and are simpler as well as faster as an attractive method for the detection of somaclonal variations in banana (Sahijram et al., 2000, Martin et al., 2006, Hernandez et al. 2007). Damasco et al. (1996) examined and detected dwarfs using 66 arbitrary RAPD markers in an initial screening of which 19 revealed polymorphism between normal and dwarf plants indicating substantial variation between dwarf and wild type by the presence of a single band in the normal but not in dwarf types. Subsequently, this marker (OPJ-04) was characterized into a SCAR (sequence characterized amplified region) for use as a PCR based detection system for dwarf types and was found to amplify approx 1.5 kb band which was consistently present in all normal but absent in all dwarf plants (Damasco et al., 1997). Dwarf off types in banana are a common incidence during micropropagation and their detection at an early stage should become feasible. As part of our ongoing programm to isolate useful variants by using either radiation or tissue culture induced genetic variability, we have isolated agronomically useful variants. Herein we present results on the molecular characterization using the RAPD marker and radiation-induced dwarf off types in banana.

Materials and Methods

Plant materials and the establishment of in vitro cultures

Banana cultivars Giant Cavendish and Robusta belonging to genomic group AAA were used in this study. The dwarf-type variants were selected from a field evaluation of irradiation-induced variant plants of cultivar Giant Cavendish. Multiple shoot cultures (6 cultures per treatment) were irradiated at 0, 5, 10, and 30 Gy doses with gamma rays @ 20 Gy/min and were subcultured on to MS (Murashige and Skoog 1962) medium supplemented with 2 mg/l benzyl adenine and 30mg/l adenine sulphate (Ganapathi et al., 1995). This first subculture cycle was referred to as M_1V_0 and continued up to third subculture cycle (M_1V_3). Individual shoots from M_1V_3 were separated from the multiple shoot cultures and transferred to MS medium supplemented with 1 mg/l naphthalene acetic acid and. After 60-75 days of hardening in the green house, the plants were field planted in an experimental field. The tissue culture derived Robusta plants were obtained through somatic embryogenesis using scalp cultures as described earlier (Suprasanna et al., 2002). These were subsequently field planted in a small experimental field plot. The field grown plants of Giant Cavendish and Robusta were characterized at maturity by height, leaf shape, pseudostem height (pseudostem is a succulent, very juicy stem which is a cylinder of leaf-petiole sheaths and the height is measured from the base of pseudostem to the point of bunch emergence) and other agronomic characters. The data are expressed as mean $\pm$ standard error.

Isolation of DNA and analysis

Total genomic DNA was isolated from young leaves of control and dwarf off type plants of Giant Cavendish and Robusta using a modified CTAB method (Stewart and Via, 1993). The quantity and quality of DNA samples were estimated by comparing band intensities on agarose gel.

The Dwarf (Dw) specific primers were synthesized based on Damasco et al., (1996) as following: (Dw1-5´ CTGTGGTTGCATTCTCATAC3´, and (Dw2 - 5 ´ C T G A A T C A T A C T C GCGAACC3´) and used for the analysis. The PCR mix of 20 µl contained the primers (250 nM), Taq DNA polymerase (3units), 200µM of each dNTP, 1X PCR buffer (10 mM Tris-HCl, 4.5 mM $MgCl_2$, 50 mM KCl, pH 8.3) and 20-25 ng of genomic DNA as template. The thermocycler was set up at 94^0 C for initial denaturation for 5 min followed by 35 cycles of amplification with each cycle consisting of following steps: 94^0 C for 1 min, 55^0 C for 10 sec and 72^0 C for 2 minutes with a final extension for 5 min. The amplified products were analyzed using agarose gel (1.2%) electrophoresis. The gels were visualized

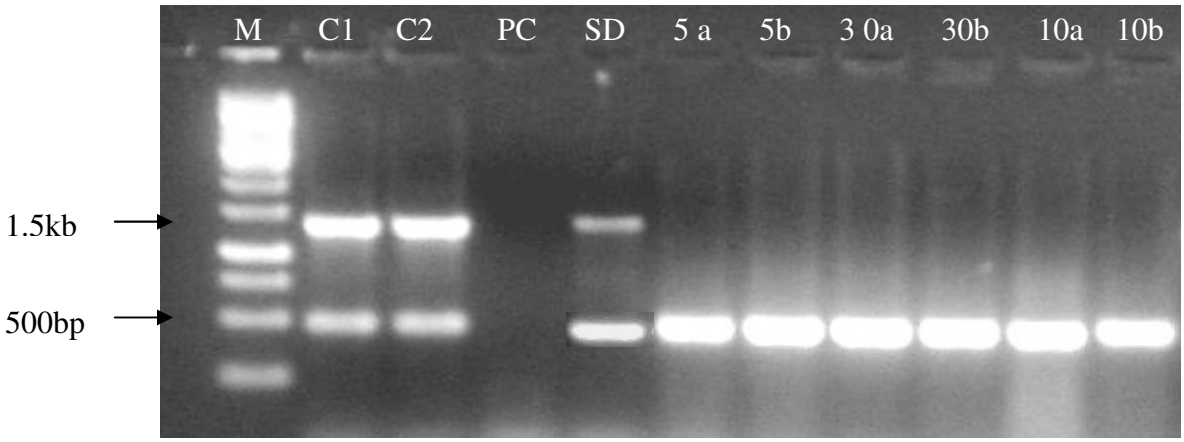

Fig 1. Characterization of radiation induced dwarf types in banana cultivar Giant Cavendish by using SCAR marker in a multiplex PCR with dwarf primer (1.5kb) and 18SrRNA primer. Lanes C1—Control; C2—Giant Cavendish, PC—PCR control, SD—Semi dwarf (5Gy), 5a, 5b – 5 Gy; 30a, 30b (30 Gy); 10a, 10b (10 Gy).

and photographed over a UV transilluminator after staining with ethidium bromide according to Sambrook *et al* (1989).

Multiplex PCR

In a multiplex PCR, a combination of RAPD primers and an extra pair of primers to amplify 18S rRNA gene (18SF 5´CATCACAGGATTTCGGTCCT3´ and 18SR 5´AGACAAATCGCTCCACCAAC3´) of *Musa acuminata* (Gene bank accession number U42083.1) were used, as described by Ramage et al., (2004). DNA amplifications were performed in a total of 20µl with 1X PCR buffer (10mM Tris-HCl, 4.5mM $MgCl_2$, 50mM KCl, pH 8.3), 0.25mM dNTP's, 500 nM B1/B2 SCAR primer, five different concentrations of *Musa* 18S primers (25, 50, 100, 125, 250 nM) and 3 units of Taq DNA polymerase. The amplification reactions were heated to 94^0 C for 5 min followed by 30 cycles each of 95^0 C for 30 sec, 56^0 C for 1 min, 72^0 C for 2 min with a final extension at 72^0 C for 3 min.

Results and Discussion

After planting in the field, phenotypic expression of dwarf off type was evident among Giant Cavendish plants, mainly by the pseudostem height and foliage development. The salient features of the dwarf types are shown in Table 1. Among the radiation-induced variants (5, 10 and 30Gy), the reduction was in the range of 58.5 to 89 inches compared to control. The 30Gy derived types displayed considerable reduction in height (about 1.5-1.7 times of the control). Some of the variant plants had more number of leaves and days to harvest.

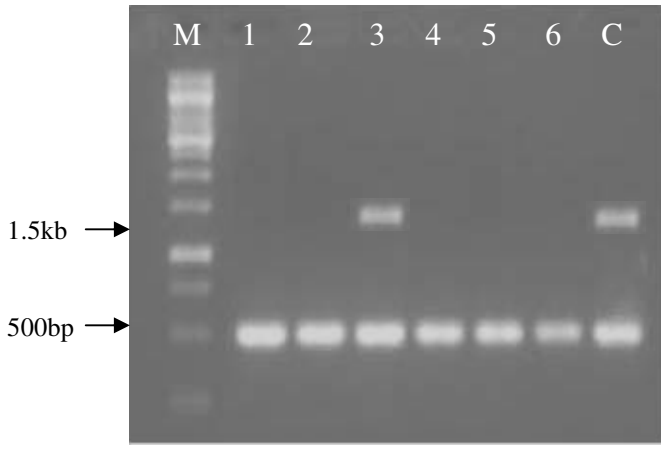

Fig 2. Characterization of tissue culture derived dwarf types in banana cultivar Robusta by using SCAR marker in a Multiplex PCR with dwarf primer (1.5kb amplification product) and 18SrRNA primer (500bp amplification product). M-molecular weightmarker 1 Kb; C-Control, 6-Dwarf, 5-Dwarf, 4-Dwarf, 3-Normal, 2-Dwarf, 1- Dwarf.

Table 1. Distinctive features of the selected dwarf banana cultivars. Giant Cavendish and Robusta

Plant No	Plant Height (inch)	Pseudostem diameter (inch)	No. Of suckers	No. Of Leaves	No. Of Days to harvest	Bunch weight (Kg)
Cultivar Giant Cavendish						
Control	99	25	8	11	348	30
5Gy-a	89	26	6	8	350	30
5 Gy-b	88	24.5	8	14	349	28
10Gy-a	85.5	23.5	8	12	295	15
10Gy-b	88	23.5	8	14	332	15
30Gy-a	62.5	25.5	6	10	386	18
30Gy-b	58.5	15.5	5	9	390	20
Cultivar Robusta						
Control	210	66	5	15	120	20.2
Dwarf	175	72	3-4	10	132	25

Among the selected six dwarf types of cultivar Robusta, there was variation in height and other characters in comparison to control. The six somatic embryo raised progenies exhibited reduction in plant stature (175± 20 cm) and increased yield under experimental conditions.

The dwarf plants of the radiation-induced and tissue culture-derived treatments were analyzed at the molecular level using the SCAR marker (Figure 1, 2). A characteristic pattern with an expected amplified fragment of 1500 bp was observed in normal, semi dwarf but not in the dwarf plants of cultivars, Giant Cavendish (Figure 1) and Robusta (Figure 2). However, in order to authenticate that the absence of amplification was not due to failure of PCR amplification conditions, an internal control by using 18SrRNA primer was used in a multiplex PCR. The optimal concentration of 18S primer was determined using five different primer concentrations ranging from 25-250nM while using a constant primer concentration of 500nM in case of Dw1 and Dw2. A concentration of 125nM provided the most reliable identification of both normal and dwarf off type plants (Ramage, 2004). The amplified product of 500bp was seen in case of the control and dwarf types. The results indicated that the dwarf specific SCAR primer validated the dwarfs of banana cultivars Giant Cavendish and Robusta (AAA genomic group).

Asexual multiplication *in vitro* can induce genetic changes that can often lead to somaclonal variation, which can hamper success in propagation of commercially important clones. In banana, dwarf or giant variants are a common occurrence under field conditions and mostly the dwarf types do not revert to normal types (Smith and Drew, 1990). In the present study, morphoagronomic characteristics useful for clone evaluation were assessed in field and for the dwarf types and the characteristics were useful. The dwarf variants were found to be featured by shorter pseudostem and leaves compared to normal type. Because the dwarf plants had the same number of leaves than the controls, photosynthetic ability may not be reduced and therefore the bunch size could almost remain identical. In addition, reduced plant height can prevent it from lodging during heavy winds or rain. Early identification of dwarf off types is difficult and most effective when normal plants reach 18-20 cm in height and ready for field establishment (Smith and Hamill, 1993). However, selection is most effective when the plants are grown under uniform vigorous growing conditions.

In this study, the dwarf plants derived from tissue culture and radiation-mutagenesis were analyzed at the molecular level using the SCAR marker. Slight modifications in the PCR conditions were made as per the requirement of the banding pattern by SCAR as well as the internal control.

Using the SCAR primer, an amplified fragment of 1500 bp was observed in the normal, semi dwarf type but not in the dwarf types. This banding pattern was expected as desribed earlier by Damasco et al., (1998) and Ramage et al., (2004). That the absence of amplification was not due to failure of PCR amplification conditions, but only due to the dwarf trait was further authenticated by using an internal primer in a multiplex PCR. The amplified product of 500bp was seen in case of the control and dwarf types. Molecular analyses of banana dwarf types have been carried out with the sole objective to detect them at an early stage in development. Reports on the profiling of dwarf and normal types with differential AFLP, TE-AFLP and MSAP patterns also indicated different levels of polymorphism (around 25%) depending on the method, the primer combination, and the genotype (Engelborghs et al., 2007). For each variety, a distinction could be made between the dwarf and normal type. SCAR diagnostic test provides a useful and reliable technique and reproducible method for detection of dwarf off-types. These clones could be useful in understanding mechanism of mutations affecting gibberellin biosynthesis or signalling. Somaclonal variations affecting banana plant height are mostly associated with modifications in GA metabolism (Sandoval et al., 1995). In summary, use of SCAR marker and the multiplex PCR provides an improved, simple and reliable method for the rapid detection of dwarf- off type plants in banana.

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