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Somatic hybridization by electrofusion of banana protoplasts

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Summary

Somatic hybridization between triploid and diploid bananas was attempted by using protoplast electrofusion and nurse culture techniques. Protoplasts from embryogenic cell suspensions of 'Maçã' (*Musa* sp. AAB group) were fused with protoplasts from nonembryogenic calli of 'Lidi' (*Musa* sp. AA group). Direct somatic embryogenesis was observed when the fusion-treated protoplasts were cultured with rice nurse cells (*Oryza sativa* L. A-58 line). Somatic hybrids were identified by using random amplified polymorphic DNA (RAPD) markers. Forty-four primers were tested for polymorphism between the two parental varieties, and eleven of them showed polymorphism. Among the 13 regenerated plants from one experiment, 11 (85%) were identified as somatic hybrids by the presence of both parent bands, and 2 showed only the 'Lidi'-band patterns. From the other experiment, 5 of 11 regenerated plantlets (45%) were identified as somatic hybrids. Flow cytometric ploidy analyses showed different ploidy levels of the somatic hybrids.

Introduction

Banana is one of the most important horticultural crops in tropical and subtropical countries. One of the most serious problems of the banana plantation is the lack of varieties with high productivity and, at same time, resistance to major diseases such as Panama disease and Black Sigatoka disease. Many breeding programs have been carried out all over the world. However, only a few zygotic hybrids were obtained by conventional crossbreeding, due to the fact that most of the widely cultivated varieties are triploids and show low efficiency in seed production.

From this point of view, somatic hybridization by protoplast fusion is a promising strategy. It was first reported on *Nicotiana* species by Carlson et al. (1972) and became widely used after Melchers et al. (1978) produced somatic hybrids between well-known vegetable crops, i.e., tomato and potato. The technique is particularly useful to introduce disease-resistance of wild relatives or other species into a cultivated variety (Zuba & Binding, 1989; Hansen & Earle, 1995; Gerdemann-Knorck, et al., 1995; Yamada et al., 1997). In banana, disease-resistant varieties exist particularly in non-cultivated diploids (AA). But, the transfer of these characters to cultivated triploid bananas (AAA or AAB) is extremely difficult by using conventional breeding methods (Bakry, et al., 1997; Dantas, et al., 1997). Only a few cultivated varieties are able to cross with the diploids. New ways of developing banana hybrids are needed.

In bananas and plantains (*Musa* sp.), some reports about embryogenesis from suspension cells (Dhed'a et al., 1991; Côte et al., 1996; Grapin et al., 1996) and protoplasts (Media et al., 1993; Panis et al., 1993; Matsumoto & Oka, 1998) already exist. In the present study, we attempted to develop a protoplast fusion technique to obtain somatic hybrids between triploid and diploid bananas.

Materials and methods

Plant materials and cultures

Embryogenic cell suspensions of the triploid banana (*Musa* sp. AAB group, cv. Maçã) and non-embryogenic calli of the diploid one (*Musa* sp. AA group, cv. Lidi) were used as the source of protoplasts. The embryogenic cell suspension of 'Maçã' was obtained from a male inflorescence and maintained in a modified MS (Murashige & Skoog, 1962) liquid medium with 5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 μM zeatin (Matsumoto & Oka, 1998). They were subcultured weekly on the horizontal gyratory shaker (120 rpm) in a controlled environment room (28 °C, dark). The non-embryogenic calli of 'Lidi' were induced from a male inflorescence tip on a medium consisting of MS salts, 8p vitamins (Kao & Michayluk, 1975), 16.6 μM Picloram, 1 μM zeatin, 3% sucrose, 0.2% Phytagel (Sigma Co., USA) and pH 5.8. The induced calli were maintained on the modified MS medium without the growth regulators, supplemented with 10 mM L-proline and 0.05% activated charcoal, and solidified by 0.2% Phytagel.

Protoplast isolation, fusion and culture

Protoplasts from the 'Maçã' suspension cells and 'Lidi' calli were isolated as described previously (Matsumoto & Oka, 1998). The protoplasts of each variety were adjusted to a 5×10^5 protoplasts·ml^{-1} with a solution of 0.6 M D-mannitol, 0.1 mM $CaCl_2$, 0.5% polyvinylpyrrolidone (MW40, 000), 3.5 mM MES and 8 mg l^{-1} bromocresol purple. The pH of the solution was adjusted to 5.7. The protoplasts were mixed at a 1:1 ratio, and 0.8 ml of the suspension was pipetted into the fusion chamber. The somatic hybridizer SSH-1 (Shimadzu Co. Ltd., Tokyo, Japan) generated electric pulses: DC pulses (1250 V cm^{-1}, 100 μsec, 3 pulses) followed an AC pulse (1 MHz, 200 V cm^{-1}, 10 sec) at room temperature. After the fusion treatment, the chamber with protoplasts was incubated in the dark at room temperature for one hour, in order to conclude the fusion process and stabilize the protoplasts. Methods for protoplast culture, colony development and plant regeneration were described previously (Matsumoto & Oka, 1998). Plantlets regenerated *in vitro* were used for DNA analysis.

Table 1. Primers used for the PCR amplification

Primers which showed polymorphism between the parental varieties	OPC4; OPAC 4–7; OPAC 9; OPAC 12; OPN 7; OPN 8; OPN 9; OPT 20
Primers which did not show polymorphism between the parental varieties	OPAC 1; OPAC 2; OPAC 8; OPAC 10, OPAC 11; OPAC 13; OPAC 14; OPN 1–6; OPN 10–20; OPT 3; OPT 5; OPT 8–12; OPT 14; OPT 19

DNA extraction

The DNA extraction was carried out according to Gawel & Jarret (1991) with the following modifications of the extraction buffer: CTAB (1%), β-mercaptoethanol (0.2%), and polyvinylpolypyrrolidone (1%). One gram of *in vitro* young leaf tissue was macerated in liquid nitrogen and transferred to 5-ml of the extraction buffer.

PCR

The PCR amplification was based on the protocol reported by Williams et al. (1990). Forty-four commercial decamer primers from Operon Technologies (Alameda, CA, USA) were used (Table 1). Amplification was performed in a 9600 Perkin Elmer DNA thermocycler (Perkin Elmer Corp., Norwalk, CT, USA). The program used was described by Vilarinhos et al. (1998) (cycle 1: 94 °C for 1 min, 35 °C for 30 s, 72 °C for 1 min; cycles 2–40: 94 °C for 15 s, 35 °C for 30 s, 72 °C for 1 min; cycle 41: 72 °C for 7 min).

Flow cytometric ploidy analysis

Some of the *in vitro* plantlets, which were identified as somatic hybrids by PCR/RAPD analysis, were submitted to ploidy analysis by flow cytometry. The methods were based on Dolezel et al.(1997) with little modification.

Results

When the protoplast suspensions from the 2 varieties were mixed and submitted to the electric pulses, the protoplasts were fused to each other. They were then cultivated by the nurse culture technique. After 40 to 60 days of culture, somatic embryos were formed (Figure 1), and plantlets were obtained by further

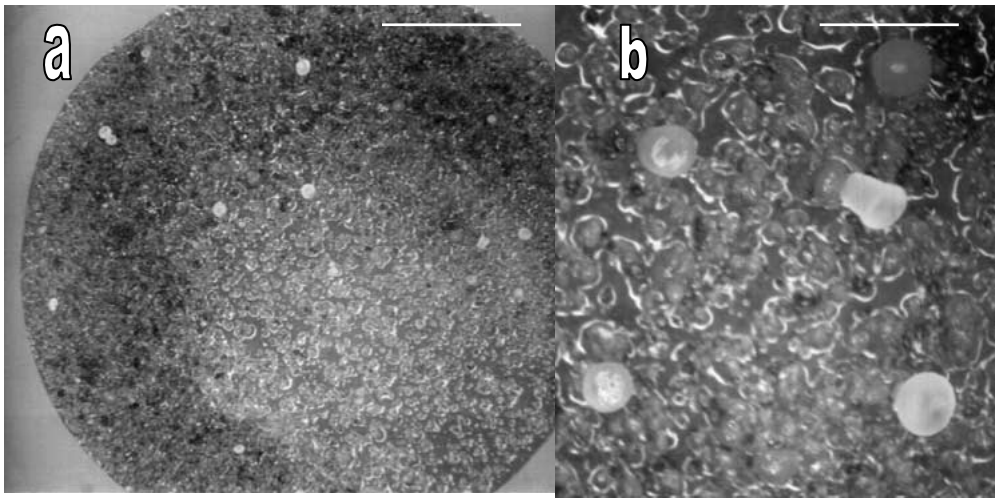

Figure 1. Somatic embryo regeneration from electro-fused protoplasts after 40 days of culture. a) Global aspect; scale bar = 5 mm. b) Detailed aspect showing the embryo format; scale bar = 1 mm.

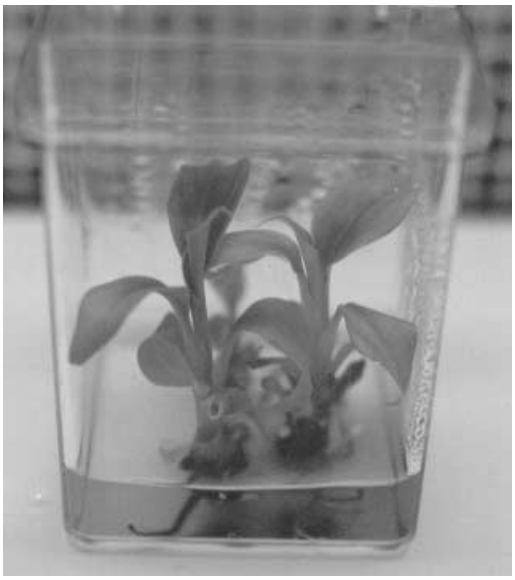

Figure 2. Plantlets regenerated from embryos derived from fused protoplasts after 2 months of embryo culture.

culturing for 2 months on the regeneration medium (Figure 2). Leaves and pseudostem of the *in vitro* plantlets were submitted to the PCR/RAPD analyses to identify the somatic hybrids.

Among the 44 primers tested, eleven primers showed polymorphism between the fusion parents (Table 1). Two of these are shown in Figure 3. The primer sequences were GTTAGTGCGG (opac5) and GACCAATGCC (opt20). Some plantlets were identified as hybrids by the presence of both parent bands (line 1-3, 5-8 and 12 in Figure 3-b) using only one primer. Others were identified so by analyzing 2 or more primers when the plantlets showed one parent-specific band by one primer and another parent specific band by another primer. The plantlets of line 4 and lines 9 to 10 of Figure 3 were identified as somatic hybrids by the latter method. Lines 11 and 13 always showed 'Lidi' band patterns only. Eleven of the 13 regenerated plants (85%) were then identified as somatic hybrids (PCR-identified hybrids). The hybridization experiments were repeated. About 200 plantlets from the second experiment without PCR analyses were transplanted to the field for the evaluation of agricultural characters. But, all of them were killed by severe drought. In a third experiment, five of the 11 regenerated plantlets (45%) were identified as somatic hybrids by PCR analysis.

Shoot apexes of the PCR-identified somatic hybrids were again cultivated *in vitro*. Rooted plantlets were acclimated in a greenhouse. Morphologically normal (similar to one of the parents or intermediate) and abnormal (differing from both parents, having short, thick pseudostems, narrow leaves and/or fan-like leaf insertions) plantlets were observed (Figure 4). Presently, 9 PCR-identified-hybrid lines are in the field (Figure 5).

Among the PCR-identified hybrids, 13 plantlets were submitted to flow cytometric ploidy analysis. The results showed that 4 plantlets (31%) were identi-

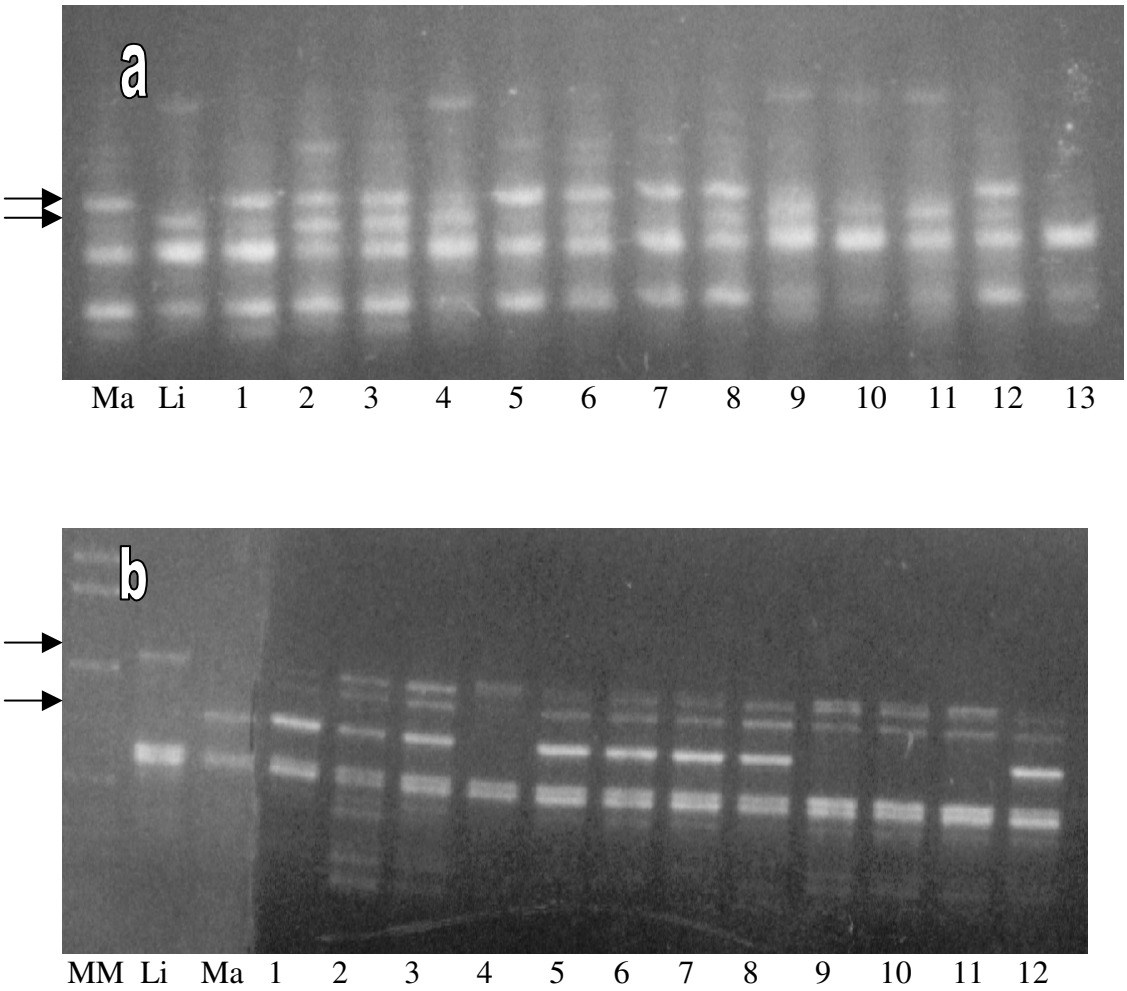

Figure 3. PCR/RAPD analysis of the putative somatic hybrids. (a) primer OPAC 5; (b) primer OPT 20. (MM) Molecular size marker, (Ma) 'Maçã', (Li) 'Lidi', (1–13) regenerated plantlet lines after the protoplast fusion. The primer OPAC 5 indicated the lines 1–9 and 12 as hybrids by the presence of both parental bands. The primer OPT 20 indicated lines 1–3, 5–8 and 12 as hybrids. Other primers were used to analyze hybridity of the lines 10, 11 and 13.

fied as pentaploids, 7 (54%) as triploids and 2 (15%) as diploids (Table 2).

Discussion

Although the efficiency of somatic hybrid acquisition was still low (1–10 hybrids / 40.000 protoplasts) and varied from one experiment to another, some hybrids were always obtained by each experiment. From this point of view, the efficiency may be considered rather high, comparing with conventional cross-pollination method. In addition, no zygotic hybrid of 'Maçã' has been obtained by the cross-pollination method (Dantas et al., 1997).

Somatic hybridization should be useful for banana plant breeding even though, in many other species, sterile plants are frequently produced and undesirable genes may be incorporated (Terada et al., 1987; Matsumoto, 1991; Narasimhulu et al., 1994; Siemens & Sacristán, 1995; Hansen, 1998). The sterility of the somatic hybrids is not a problem in banana. On the contrary, it is a desirable character, because the plant is vegetatively propagated, and a banana fruit with seeds is not edible. The problem of undesirable gene incorporation by somatic hybridization has also been reduced by the intensive diploid-banana breeding in progress for more than 30 years (Dantas et al., 1997). The bred diploid, which has solid seeds, is generally not useful itself as a cultivated variety. It is mainly use-

Figure 4. Somatic hybrids in acclimatization. (L) 'Lidi', (M) 'Maçã', (H) somatic hybrids.

Table 2. Flow cytometric ploidy analysis using an internal standard of *Petunia* sp.

Plantlet line	Relative DNA content of banana (B)	Relative DNA content of petunia (P)	B/P	Estimated ploidy
Maçã parental	120	211	0.56	3n
diploid parental	85	207	0.41	2n
H-1*	118	212	0.55	3n
H-2	120	213	0.56	3n
H-3	118	208	0.56	3n
H-4	86	209	0.41	2n
H-5	118	220	0.54	3n
H-6	225	214	1.05	5n
H-7	90	219	0.41	2n
H-8	111	214	0.52	3n
H-9	211	211	1.00	5n
H-10	109	206	0.52	3n
H-11	205	205	1.00	5n
H-12	215	205	1.04	5n
H-13	111	213	0.52	3n

* H-1 to H-13 were PCR-identified somatic hybrids.

ful as parental material for triploid or tetraploid banana breeding. Unfortunately, many cultivated triploid varieties are, however, incompatible for cross-pollination with the bred diploids. Somatic hybridization by protoplast fusion is, in these cases, the only method to obtain hybrids.

In our previous experiments, no plantlet was regenerated from protoplasts of the non-embryogenic 'Lidi' callus (data not shown). Considering that fusion efficiency is about 30–40% (Matsumoto et al., 1992) and protoplasts from non-embryogenic 'Lidi' callus do not regenerate plantlets, the frequency of somatic hybrids in regenerated plants is theoretically estimated to be 12–15%. In our experiments, 85% of plantlets recovered from the first experiment and 45% from the third experiment were identified as hybrids without any selection (The hybrid identification was not realized in the second experiment). These results suggest that hybrids showed more vigor than parental varieties, at least in their initial growth. This phenomenon was also observed in interspecific somatic hybrids of *Datura* (Schieder, 1978), *Lactuca* (Matsumoto, 1991) and *Solanum* (Yamada et al., 1997) species. For conclusion, however, more detail experiments are needed to have a better understanding of protoplast fusion in *Musa* and a secure acquisition of somatic hybrids.

The PCR/RAPD analysis is a simple and very useful technique to identify symmetric somatic hybrids (Xu et al., 1993). In the genome combination used in our experiments (AAB + AA), DNA polymorphism was observed with many primers and the hybrid identifications were realized. However, it may be difficult for genome combinations such as AAA + AA, because

Figure 5. Somatic hybrid plants 5 months after transplantation to the field: (a) Original 'Maçã', (b) Original 'Lidi', (c – e) Somatic hybrids.

of their similarity. Easier and more precise methods for somatic hybrid identification must be developed.

Flow cytometry also is a useful tool for somatic hybrid identification. Previous studies showed that the method was efficient and rapid for ploidy analysis (Dolezel et al., 1997; Lysak et al., 1999). We applied this method for somatic hybrid detection. When we carried out a symmetric somatic hybridization between cv. Maçã (3n) and a diploid variety (2n), pentaploid (5n) plantlets were expected to be obtained. Our result showed that about 30% were identified as pentaploids and the others were triploids and diploids. Aneuploids might have existed but the method used was not able to identify them. To explain the appearance of triploids and diploids, we cannot discard the possibility of misinterpretation of the PCR analysis that identified the somatic hybrids. However, it is more likely that chromosome losses occurred before and during plantlet regeneration. This phenomenon has been observed in other species (Siemens & Sacristan, 1995; Hansen & Earle, 1995). Furthermore, when chromosome numbers of some of the hybrid plants were studied by microscopic observation, aneuploids, in addition to pentaploids, were observed (data not shown).

In banana, genetic transformation techniques have already been reported (Sagi et al., 1994; 1995; May et al., 1995; Becker et al., 2000). The application of these techniques to banana breeding depends on the molecular-level knowledge of useful genes, particularly genes related to mechanisms of resistance to the major diseases. Sexual and somatic hybridization can help to identification of the genes. On the other hand, transgenic plants with a marker gene, such as antibiotic resistance gene, make somatic hybrid identification easier. These techniques should be applied in banana breeding as complementary tools, and the somatic hybridization technique developed here should be useful to on-going banana breeding.

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