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Plant regeneration *via* protoplast electrofusion in cassava



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Abstract

Protoplast electrofusion between callus protoplasts of cultivar TMS60444 and mesophyll protoplasts of cultivar SC8 was performed as an approach for the genetic improvement of cassava. The fusion products were subsequently cultured in protoplast culture medium (TM2G) with gradual dilution for approximately 1–2 months. Then the protoplast-derived compact calli were transferred to suspension culture medium (SH) for suspension culture. The cultured products developed successively into embryos, mature embryos, and shoots on somatic embryo emerging medium (MSN), embryo maturation medium (CMM), and shoot elongation medium (CEM), respectively. And the shoots were then rooted on Murashige and Skoog (1962) medium (MS). Sixty-six cell lines were obtained and 12 of them developed into plantlets. Based on assessment of ploidy level and chromosome counting, four of these plantlets were tetraploid and the remaining eight were diploid. Based on assessment of ploidy level and simple sequence repeat (SSR) analysis, nine tetraploid cell lines, one diploid variant plant line and nine variant cell lines were obtained. The diploid variant plant line and the nine variant cell lines all showed partial loss of genetic material compared to that of the parent TMS60444, based on SSR patterns. These results showed that some new germplasm of cassava were created. In this study, a protocol for protoplast electrofusion was developed and validated. Another important conclusion from this work is the confirmation of a viable protocol for the regeneration of plants from cassava protoplasts. Going forward, we hope to provide technical guidance for cassava tissue culture, and also provide some useful inspiration and reference for further genetic improvement of cassava.

Keywords: tissue culture, chromosome counting, DNA loss, ploidy analysis

1. Introduction

Cassava (*Manihot esculenta* Crantz), a perennial shrub of the Euphorbiaceae family, is cultivated throughout the lowland tropics (Howeler *et al.* 2013). Because of its tolerance to adverse environmental conditions, adaptation to poor soils and flexible harvesting time, cassava is a vital component in food security in many developing countries

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(Zhang *et al.* 2018). Cassava is extensively used for food, feed, and raw material for starch production and commercial bioethanol production (Liu *et al.* 2011; Mongomake *et al.* 2015; Chavarriaga-Aguirre *et al.* 2016).

The potential for cassava improvement by traditional breeding is constrained because of its high heterozygosity, highly outcrossing nature, lack of synchronization of flowering among progenitors, and the interest in erect plant architecture which, by default, results in late and scarce flowering (Raemakers *et al.* 1993; Li *et al.* 1998; Ceballos *et al.* 2015). Cassava breeding in China faces the problem of a short growing cycle which further limits the production of botanical seed. Conventional cassava breeding, therefore, is harder to achieve in China.

Protoplast fusion is an effective complementary method to overcome sexual barriers and break the limitations of traditional breeding. For this, a highly efficient system of protoplast regeneration is a prerequisite. However, cassava is very recalcitrant to plant regeneration from protoplasts (Sofiari *et al.* 1998). Although regeneration of cassava *via* mesophyll protoplast culture had been reported previously (Shahin and Shepard 1980), this result has never been repeated by other scholars since then (Anthony *et al.* 1995; Sofiari *et al.* 1998). With the establishment of a friable embryogenic callus system in cassava (Taylor *et al.* 1996), protoplasts isolated from suspension cultures of the friable embryogenic callus (FEC) were cultured and developed into plants (Sofiari *et al.* 1998). Nevertheless, regeneration remains a conundrum because of the low frequencies of mature and germinated embryos (Sofiari *et al.* 1998).

In recent years, we have established the regeneration system of callus protoplast for cassava cultivar TMS60444 (Wen *et al.* 2012). On the basis of the study of Sofiari *et al.* (1998), in the process of embryogenesis, the compact calli derived from protoplasts were transferred to somatic embryo emerging medium (MSN) to develop into embryos under light conditions. Globular, torpedo-shaped, and green cotyledonary embryos emerged gradually, while calli proliferated as well (Wen *et al.* 2012).

Studies on protoplast manipulation technology of cassava have lagged behind the other major food crops. There is still no report on protoplast fusion of cassava. The objectives of this work were to i) develop a protocol for protoplast fusion; ii) adapt and improve existing protocols for the regeneration of viable plants from protoplasts; and iii) analyze the genetic constitution of the material generated. This work has relevance for the generation of new genetic variability in cassava. Plant regeneration from protoplasts is also a key step for genetic engineering and cell engineering that offer great advantages for the genetic enhancement of cassava in the future.

2. Materials and methods

2.1. Plant materials

Cassava cultivars TMS60444 and SC8 were used as fusion parents. Friable embryogenic callus (FEC) of cassava genotype TMS60444 was kindly induced by Dr. Zhang Peng from the Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. FECs were sub-cultured every 3 weeks in a medium containing Gresshoff and Doy (1974) vitamins and salts, 20 g L⁻¹ sucrose, 8 g L⁻¹ micro agar, and 12 mg L⁻¹ picloram (GD medium). Cell suspension cultures were initiated by transferring approximately 1 g of FEC into a 100-mL flask with 30 mL of suspension culture medium (SH) containing Schenk and Hildebrandt (1972) vitamins and salts, 60 g L⁻¹ sucrose and 12 mg L⁻¹ picloram. The liquid medium was refreshed every 2–3 d. Cultures were agitated on a rotary shaker at 110–130 r min⁻¹. All cultures were kept in a growth chamber with a photoperiod of 12 h, an irradiance of 45 $\mu\text{mol}^{-2} \text{s}^{-1}$, and a temperature of 25°C. Suspension cultures of 5 d in SH were used for protoplast isolation.

Plants of cassava cultivar SC8 were propagated by sub-culturing leafy node cuttings on MS containing Murashige and Skoog (1962) vitamins and salts, 30 g L⁻¹ sucrose, 8 g L⁻¹ micro agar, and 0.02 mg L⁻¹ naphthaleneacetic acid (NAA). Plants were placed at 25°C, 12 h light condition, and sub-cultured every 1–2 months.

2.2. Protoplast isolation and purification

The cell digestion solutions to isolate protoplasts from calli were based on Sofiari *et al.* (1998), but with some modifications. Calli protoplasts were isolated from 5 d cell suspensions. Approximately 1 g of tissue was placed in a Petri dish (9 cm diameter) with 12 mL of cell digestion solution. The concentration of Macerozyme R-10 was modified following Wen *et al.* (2012), from 200 to 400 mg L⁻¹ and, similarly, pectolyase was increased from 10 to 100 mg L⁻¹ (both enzymes from Yakult, Japan). Cellulase R-10 was maintained at a concentration of 10 g L⁻¹. The suspension tissues were incubated in the enzyme solution for 18 h on a shaker at 40 r min⁻¹ at 25°C in the dark. Mesophyll protoplasts were isolated from fully expanded but young leaves of SC8 plants. The leaves were cut into 1 mm-wide strips, approximately 1 g, which were soaked in 12 mL cell digestion solution. There was no pectolyase in the cell digestion solution. The concentration of cellulase R-10 originally suggested by Sofiari *et al.* (1998) was modified in this study from 10 g L⁻¹ to 750 mg L⁻¹. Similarly,

Macerozyme R-10 was also increased from 200 to 750 mg L⁻¹. The rest of the original solution remained unchanged. The mesophyll tissue was incubated in the enzyme solution for 16 h at 28°C in the dark.

The digested tissues were filtered through 45-μm stainless steel mesh to remove undigested cell clumps and debris. The filtrate was transferred into 10-mL centrifuge tubes and centrifuged for 6 min at 960 r min⁻¹. The supernatant was removed with a Pasteur pipet. The pellets were gently re-suspended in 1.0–1.5 mL of 13% mannitol solution containing CPW nutrients (27.2 mg L⁻¹ KH₂PO₄, 100 mg L⁻¹ KNO₃, 250 mg L⁻¹ MgSO₄, 0.2 mg L⁻¹ KI, 150 mg L⁻¹ CaCl₂, 0.003 mg L⁻¹ CuSO₄) (Frearson *et al.* 1973). Then the 13% mannitol solution containing protoplasts was slowly pipetted onto the top of 3–4 mL of 26% sucrose solution containing CPW nutrients (while avoiding mixing) and centrifuged for 6 min at 960 r min⁻¹. A band of viable protoplasts usually formed at the interface between the two layers (Fig. 1-A and D). The protoplasts were carefully removed from the interface with a Pasteur pipet and re-suspended in an appropriate amount of electrofusion solution containing 127.4 g L⁻¹ mannitol and 27.75 mg L⁻¹ CaCl₂, and then centrifuged for 6 min at 960 r min⁻¹. Purified protoplasts were gently re-suspended in the electrofusion solution, with calli protoplasts at a density of 10×10⁵ protoplasts mL⁻¹, and mesophyll protoplasts at a density of 10–20×10⁵ protoplasts mL⁻¹.

The yield of obtained protoplasts (cells g⁻¹) = $N \times 5 \times 10^4 \times V / m$; where N = number of protoplasts counted in a haemocytometer chamber; V = volume of diluted protoplasts; and m = fresh weight of plant material for protoplast isolation.

2.3. Protoplast viability test

The viability of protoplasts obtained was checked using fluorescein diacetate (FDA). A total of 12 μL of 5 mg mL⁻¹ FDA solution was added to 0.5 mL of the protoplast suspension. After 5 min, the protoplasts were examined with an Olympus IX71 inverted fluorescence microscope (green fluorescence, Olympus, Japan). The viability of obtained protoplasts (%) = $\text{Number of protoplasts with green fluorescence} / \text{Total protoplasts in the field} \times 100$. The callus protoplasts and mesophyll protoplasts were then mixed at a 1:1–2 ratio for further manipulation.

2.4. Protoplast electrofusion and culture

The fusion procedure was based on Guo and Deng (1998) using a somatic hybridizer SSH-2 instrument (Shimadzu Somatic Hybridizer-2, Japan) equipped with a 1.6-mL FTC-04 type electrofusion chamber. Mixed protoplasts of 1.6 mL were put into the sterile fusion chamber. The electrofusion

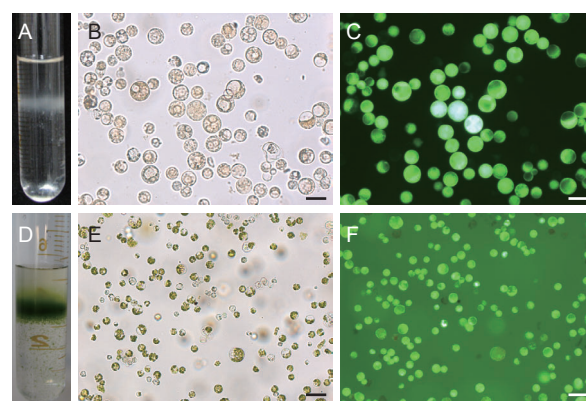


Fig. 1 Protoplasts isolation and viability tests of TMS60444 and SC8. A and D, protoplast gradient centrifugation. B and E, protoplasts under the bright field after fluorescein diacetate (FDA) staining. C and F, protoplasts under green fluorescence after FDA staining. A–C, protoplasts of TMS60444; D–F, protoplasts of SC8. Bar=20 μm.

parameters resembled those reported for citrus protoplast fusion (Guo and Deng 1998; Xiao *et al.* 2014). The electrofusion parameters were as follows: alternating current (AC) 100 V cm⁻¹, AC duration 60 s, direct current (DC) 1250 V cm⁻¹, DC duration 45 μs, DC pulse interval 0.5 s, DC pulses five times. After fusion treatment, the mixture was incubated in the electrofusion chamber for 10–20 min before being transferred to centrifuge tubes and suspended with protoplast culture medium (TM2G) (Shahin 1985; Sofiari *et al.* 1998; Wen *et al.* 2012) medium of 0.36 mol L⁻¹ glucose and centrifuged at 800 r min⁻¹ for 10–15 min. The supernatant was discarded and the fusion products were re-suspended in TM2G with 0.36 mol L⁻¹ glucose at a density of 5×10⁵ protoplasts mL⁻¹, which was based on that of the callus protoplasts in the products. The fusion products were cultured in 1.5 mL TM2G with 0.36 mol L⁻¹ glucose in a 6-cm plastic Petri dish by liquid thin layer culture, in the dark at 28°C. The medium was refreshed every 10 d, two times by TM2G with 0.33 mol L⁻¹ glucose; then twice in a medium with reduced levels of glucose (0.30 mol L⁻¹), and refreshed again two times with even lower glucose levels (0.25 mol L⁻¹ glucose).

2.5. Establishment of cell lines and plant regeneration

After cultured for approximately 1–2 months, protoplast-derived compact calli were transferred to suspension culture medium (SH) liquid medium, cultured for 2–4 weeks on a rotary shaker at 110–130 r min⁻¹, and then subsequently transferred to induce embryo development on MSN (Zhang and Puonti-Kaerlas 2000), into mature embryos on embryo maturation medium (CMM) (Zhang *et al.* 2000), into shoots

on shoot elongation medium (CEM) (6-BA 1.0 mg L⁻¹) (Zhang *et al.* 2000) and finally rooted on MS. In the process of embryogenesis on MSN, compact calli developed into embryos and high quality friable embryogenic callus (FEC (cell lines)) simultaneously. FEC (cell lines) were proliferated and conserved on GD solid medium.

2.6. Ploidy analysis, genomic DNA extraction, and SSR analysis

Ploidy analysis was carried out using a Partec flow cytometer (Partec, CYFLOW space, Germany) according to the previous report (Xiao *et al.* 2014) with minor modifications. Tissues from a few calli or leaves of regenerated plants were chopped in a plastic Petri dish containing 0.5 mL Partec HR-A buffer. After filtering, the sample was stained with 1 mL of HR-B buffer and the relative fluorescence of total DNA was measured. Each histogram was generated by analyzing at least 3 000–5 000 nuclei.

Total genomic DNA was extracted from the two parents (leaves of SC8 and callus tissue of TMS60444) and from protoplast-derived cell lines and regenerated plant lines as described by Cheng *et al.* (2003). SSR analysis was conducted according to the procedure of Guo *et al.* (2006) with minor modifications. The 130 primer pairs used were randomly selected from Mba *et al.* (2001) and Luo (2005) (Shanghai Sangon Co., China). Approximately 25 ng of genomic DNA, 1.5 mmol L⁻¹ MgCl₂, 0.25 mmol L⁻¹ dNTPs, 0.75 μmol L⁻¹ of each primer, and 1 U *Taq* DNA polymerase (Biostar), were mixed in a reaction volume of 20 μL. PCR cycles were programmed as follows: one initial denaturing cycle at 94°C for 4 min; 35 cycles of 1 min at 94°C (denaturing), 45 s at 55°C (annealing), and 45 s at 72°C (elongation); and a final cycle of 5 min at 72°C. The products were analyzed on 6.0% (w/v) denaturing polyacrylamide gels, and the gels were silver-stained according to the protocol described in the technical manual of Promega Corporation (USA).

2.7. Chromosome analysis

All regenerated plant lines were maintained on MS. Root tips from the regenerated plant lines (about 3–5 mm long) were successively collected. Chromosome analysis was carried out according to a previous report (Lan *et al.* 2016). The root tips were pretreated in a saturated aqueous solution of paradichlorobenzene for 2 h at 20°C, fixed in 3:1 ethanol/glacial acetic acid (v/v) for 24 h, and then stored at –20°C in 70% ethanol solution until use. Rinsed tissues were macerated in an enzyme mixture containing 0.25% pectinase (Sigma, Japan), 0.25% pectolyase Y-23 (Yakult), and 0.5% cellulase RS (Onozuka, Japan) for 80–90 min at

37°C. Then, the meristem was mashed using a fine needle with a drop of 60% acetic acid, and the slide was flame-dried with a drop of fixation solution.

3. Results

3.1. Protoplast isolation and viability test

Purified calli and mesophyll protoplasts were counted individually in a hemocytometer chamber and then the yields of calli and mesophyll protoplasts obtained were calculated individually. The average yield obtained from calli was 3.0×10⁶ protoplasts g⁻¹ fresh weight, whereas, mesophyll tissue yielded 1.0×10⁷ protoplasts g⁻¹ fresh weight. The viability of protoplasts obtained was assessed by FDA. Ten fields were assessed and the average was calculated. The viabilities of protoplasts from calli and mesophyll tissue were both 90%. The purification, population, and viability of protoplasts are shown in Fig. 1.

3.2. Process of protoplast electrofusion

Randomly distributed parental protoplasts (Fig. 2-A) self-organized into parallel pearl chains under the AC field (Fig. 2-B). Then protoplasts were fused under DC pulses (Fig. 3-C–F). Calli and mesophyll protoplasts were fused (e.g., two cells, red arrows; multiple cells, black arrows in Fig. 2). In addition, two or multiple callus protoplasts could also fuse with each other. It took longer for multiple cells to fuse into one bigger cell.

3.3. Culture of fusion products and plant regeneration

The first, second, and third cell divisions (Fig. 3-A–D) could be observed 1 d after culture. Small colonies formed 3 d after culture and those derived from two fused cells from different parental origins were obviously green (Fig. 3-E), compared with pale ones derived from callus protoplasts which were not green. The colour of small colonies derived from fused cells and callus protoplasts were hard to distinguish after 8 d of culture, when they were all pale.

After cultured for 4 weeks on MSN, compact calli started to develop into embryos (Fig. 3-G). After that, a large number of embryos emerged continually. Meanwhile, compact calli proliferated as well. Embryos developed into mature green cotyledonary embryos after cultured for 1–4 weeks on CMM (Fig. 3-H). Mature cotyledonary embryos developed into adventitious shoots after cultured for 1–2 months on CEM (Fig. 3-I). Finally, the adventitious shoots rooted on MS (Fig. 3-J and -K). The fusion products regenerated into plants after 6–7 months of culture. In the

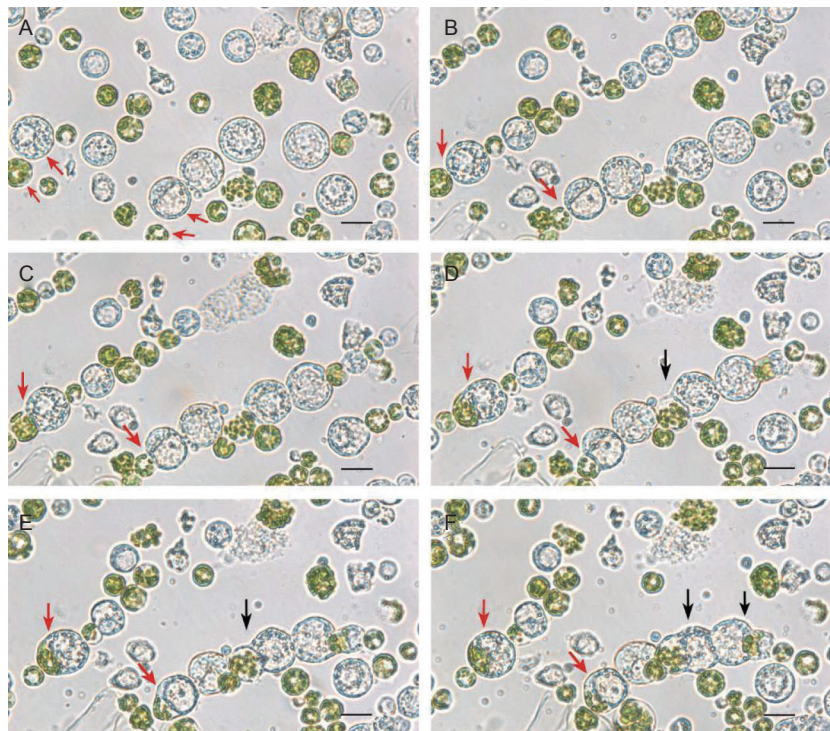


Fig. 2 Process of electrofusion between calli protoplasts and mesophyll protoplasts. A, randomly distributed protoplasts before fusion treatment. B, protoplasts formed pearl chains under alternate current. C, parental protoplasts were fused under direct current. D–F, serial process of fusing cells. Bar=20 μm . Red arrows indicate two parental cells fused into a binuclear heterokaryon; black arrows indicate multiple parental cells which were fused.

present study, 66 cell lines were obtained and 12 of them developed into plantlets.

3.4. Ploidy analysis

Flow cytometry analysis was conducted with callus of TMS60444 as a control, and the peak of diploid level was 50 (Fig. 4-A). Ploidy analysis was conducted on 66 cell lines, and also on the 12 plant lines to confirm that there was no change in ploidy during the regeneration process. The results showed that 11 cell lines were tetraploid with ploidy peak level of 100 (Fig. 4-B), four cell lines were chimeras with one peak of 50 and the other of 100 (Fig. 4-C) and the rests were diploid. In the 12 regenerated plant lines, eight plant lines were diploid and four plant lines were tetraploid (Fig. 4-D). In the four tetraploid plant lines, there was no change in ploidy level during the regeneration process. After subculture for 2 years, the four chimera cell lines, and two of the 11 tetraploid cell lines reverted to diploid as confirmed by flow cytometry analysis.

3.5. SSR analysis

SSR analysis was conducted for the 66 cell lines and the 12 regenerated plant lines with 130 randomly selected primer

pairs. The result showed that one plant line and nine cell lines had missing bands (Fig. 5), suggesting partial loss of genetic material compared to that of the parent TMS60444, so one variant plant line and nine variant cell lines were obtained. The banding pattern of the variant plant line derived from the variant cell line was identical with that of the variant cell line as revealed by SSR analysis. The primer pairs are listed in Table 1 (Mba *et al.* 2001; Luo 2005).

3.6. Morphological observation of regenerated plants

Four tetraploid plant lines (TPL) were regenerated. The phenotypes of two of these TPLs were similar, and the phenotypes of the two remaining lines were also similar to each other. The leaves of TPL-1 were broad and curled outward (Fig. 3-K), their roots were weak and easy to stop growing, and they did not survive after transfer into soil. The TPL-2 produced normal vigorous plants after transfer into soil (Fig. 3-L). The plant shapes (Fig. 3-L), leaves, and stems of TPL-2 (Fig. 3-O) were obviously different from those of parent TMS60444 (Fig. 3-M and N), parent SC8 and TPL-1 (Fig. 3-K). The plant shape, length of leaves and stem diameter of TPL-2 (Fig. 3-O) were smaller than those of parent TMS60444, but every lobule was wider than those of leaves from parent TMS60444. The roots of TPLs 1 and 2 were

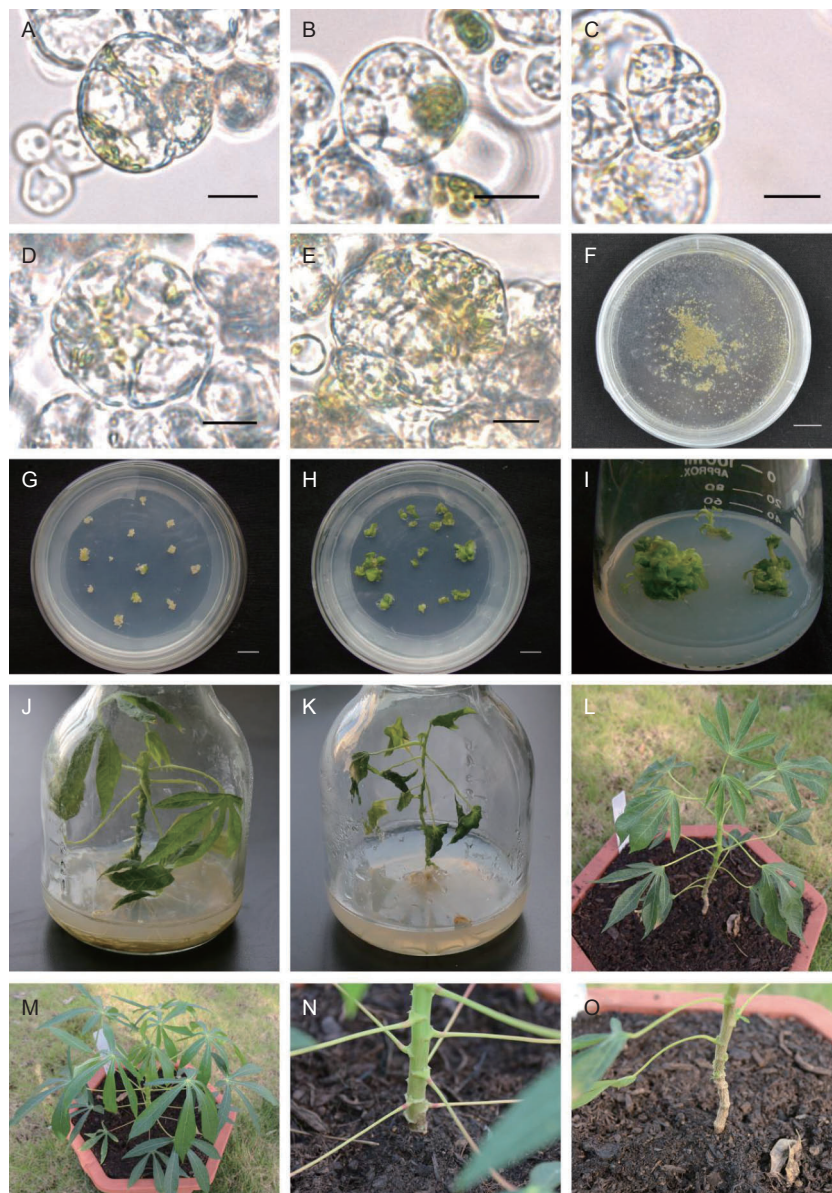


Fig. 3 Regeneration process of fusion products. First (A), second (B and C), and third (D) cell divisions after 1 d culture. E, small colony formation after 3 d. F, full view of the Petri dish after 2 mon of culture. G, somatic embryos emerged on somatic embryo emerging medium (MSN). H, green cotyledonary embryos. I, mature green cotyledonary embryos culture on shoot elongation medium for 10 wk. J, TMS60444, rooting on Murashige and Skoog (MS) medium. K, tetraploid plant line 1, rooting on MS medium. L, tetraploid plant line 2, planted in a pot. M, TMS60444 regenerated from friable embryogenic callus (FEC), planted in a pot. N, the stem of M. O, the stem of L. A–F, bar=10 μ m; G–I, bar=1 cm.

thicker than those of the diploid parent, but the performance of their roots was deficient, particularly for TPL-1.

3.7. Chromosome analysis

Thirty root cells were analyzed. Chromosome numbers of TMS60444 were, as expected, 36 (Fig. 6-A). The chromosome number of TPL-2 ranged from 69 to 71 (Fig. 6-B–D), with a higher frequency of 70. The chromosome number of TPL-2 was fewer than the expected

72. The chromosome number of TPL-1 was also fewer than the expected 72, but it was difficult to count the TPL-1 chromosomes accurately, due to their poor root system.

4. Discussion

4.1. Isolation of mesophyll protoplasts

The isolation of mesophyll protoplasts was previously reported in cassava with high yield and viability (Shahin

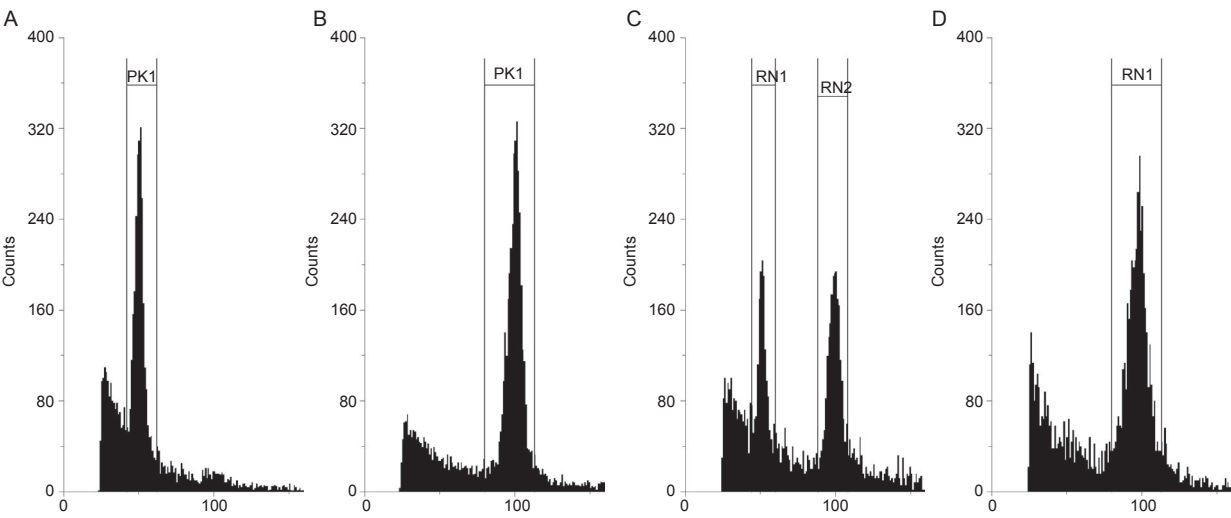


Fig. 4 Ploidy analysis of calli cell lines and plant lines. A, diploid (control). B, tetraploid cell lines. C, chimera cell lines. D, tetraploid plant lines.

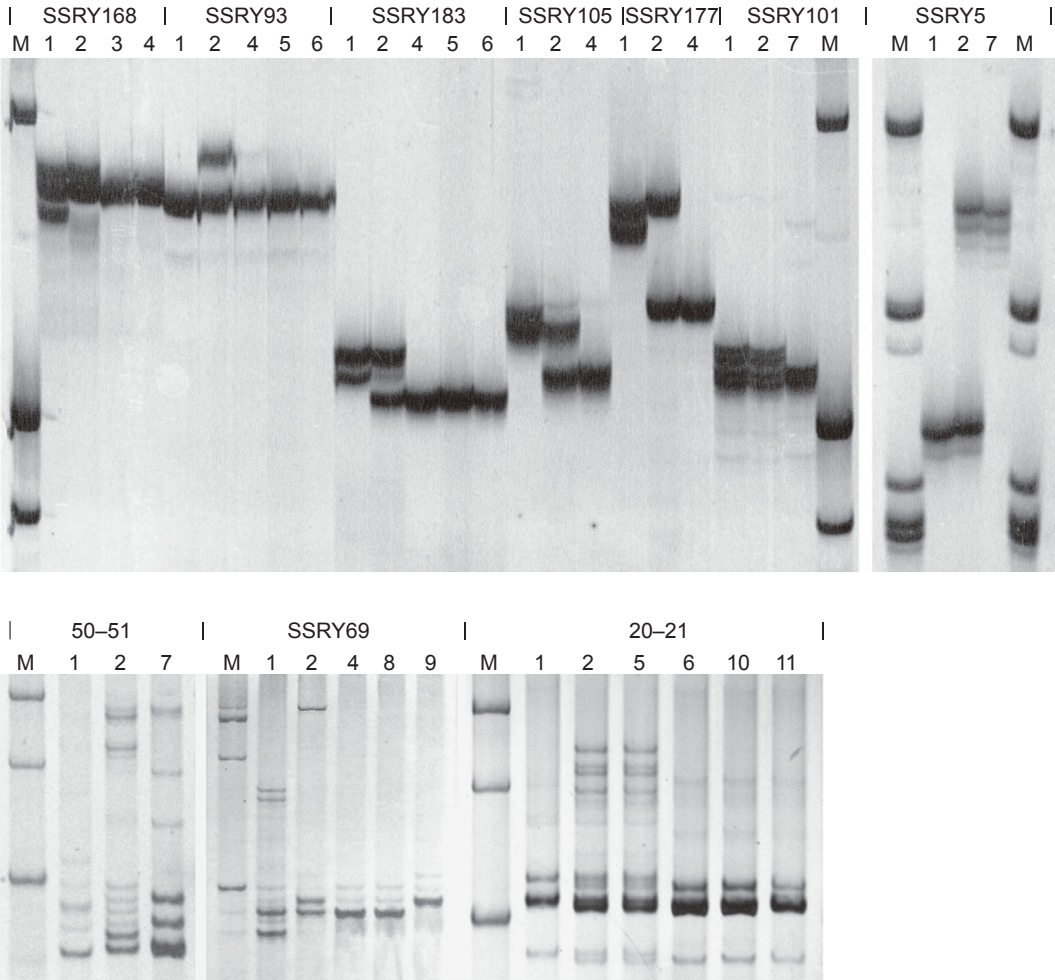


Fig. 5 SSR analysis of nuclear genomes of the plant line, the cell lines and both of their parents. M, marker; 1, SC8; 2, TMS60444; 3, regenerated plant line; 4–11, cell lines.

and Shepard 1980; Anthony *et al.* 1995). Using the enzyme solution for citrus protoplast isolation (Grosser and Gmitter 1990), a high number of viable cassava mesophyll protoplasts was also produced (Nie 2011). In the present study, the cell digestion solution for isolating protoplasts from calli was the same as that suggested by Wen *et al.* (2012). However, for isolating protoplast from mesophyll tissue, significant changes to the Sofiari *et al.* (1998) and Wen *et al.* (2012) solutions were made in the current work by increasing Macertyme R-10 and decreasing Cellulase R-10 and by not using Pectolyase. These changes produced positive responses in mesophyll protoplast yields.

4.2. Culture of fusion products

Callus protoplasts cultured in TM2G containing 0.33 mol L⁻¹ (59.4 g L⁻¹) glucose (Sofiari *et al.* 1998), or cultured in TM2G containing 0.30–0.36 mol L⁻¹ glucose (Wen *et al.* 2012) could all divide and grow. A concentration of 0.36 mol L⁻¹ glucose in the media facilitated the culture of fused protoplasts from mesophyll and calli. Diluting the concentration of glucose gradually in TM2G could stimulate the growth of small colonies. Davey *et al.* (2005) also suggested gradually reducing the medium osmolarity, thereby maintaining protoplast growth in protoplast-to-plant systems. However, it was not necessary to reduce the osmolarity of the culture medium in somatic hybridization of potato, a species with a good response to the medium, i.e., the modified VKM-medium (Binding and Nehls 1977; V, inorganic salts as in V-47, Binding 1974b; KM, organic components of medium KM, Kao and Michayluk 1975) for

fusion products (Thieme *et al.* 2008, 2010).

When fusion products were cultured, the first, second, and third cell divisions could be observed within a day. On the other hand, the first cell divisions in unfused calli protoplasts were observed only after 3–4 d in culture (Sofiari *et al.* 1998), and the second/third cell divisions only after 6 d (Wen *et al.* 2012). These results were consistent with a previous report that electrofusion could enhance cell division (Keller *et al.* 1997). After cultured in SH, compact calli became looser than before, and this was advantageous for further embryogenesis or proliferation on MSN or GD.

4.3. Ploidy, SSR, and chromosome analysis

Chimerism, polyploidization, loss of genetic material, and chromosome elimination all occurred in the present study, and were common phenomena in other protoplast fusions (Harms 1983; Mizuhiro *et al.* 2001; Cui *et al.* 2009; Thieme *et al.* 2010; Gupta *et al.* 2015; Rakosy-Tican *et al.* 2015).

In the present study, two of the 11 tetraploid cell lines, and four chimera cell lines reverted to diploid after subculture for 2 years. Perhaps the two tetraploid cell lines were initially undetected chimeras. In the six chimera cell lines, diploid cells were competitively advantageous over the tetraploid cells, since after 2 years of subculture, the chimera cell lines tended to become stable diploid cell lines. We suppose that the existence of chimeras was possibly because of the insufficiency of cytoplasmic mixings in the fused cells and the faster dividing of the fused cells than the unfused cells.

Harms (1983) explained in detail the complexity of fusion product fate after protoplast fusion: Disturbance of the cell cycle after protoplast fusion will inevitably disturb mitosis and consequently will produce segregation, maldistribution,

Table 1 SSR primer pairs used to verify the nuclear genome origin of the fusion products

Locus	Primer sequence (5'→3')	Reference
SSRY168	ACAGCCACACTTGTCTCCA CTGCAATCTCCAACAGCAAC	Mba <i>et al.</i> (2001)
SSRY93	TTTGTGCTCAGATGAAACG CAGATTCTTGTGGTGCGTG	Mba <i>et al.</i> (2001)
SSRY183	TGCTGTGATTAAGGAACCACTT TTAACTTTTCCAGTTCTACCCA	Mba <i>et al.</i> (2001)
SSRY105	CAAACATCTGCACTTTTGCG TCGAGTGGCTTCTGGTCTTC	Mba <i>et al.</i> (2001)
SSRY177	ACCACAAACATAGGCACGAG CACCCAATTACCAATTACCA	Mba <i>et al.</i> (2001)
SSRY101	GGAGAATACCAACGACAGGA ACAGCAGCAATCACCATTTT	Mba <i>et al.</i> (2001)
SSRY5	TGATGAAATTCAAAGCACCA CGCCTACCACTGCCATAAAC	Mba <i>et al.</i> (2001)
SSRY69	CGATCTCAGTCGATACCCAAG CACTCCGTTGCAGGCATTA	Mba <i>et al.</i> (2001)
20–21	CAAATTTGCAACAATAGAGAACA TCCACAAAGTCGTCCATTACA	Luo (2005)
50–51	GCTGCAGAATTTGAAAGATGG CAGCTGGAGGACCAAAAATG	Luo (2005)

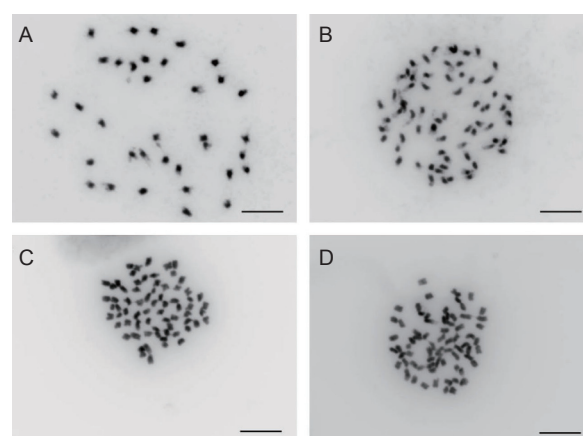


Fig. 6 Chromosome analysis. A, TMS60444 (control, 2n=36). B–D, tetraploid plant line TPL-2 (B, chromosome number=69; C, chromosome number=70; D, chromosome number=71). Bar=10 μm.

and elimination of chromosomes. There is a cytoplasmic mixing and complex coordinative process required for the development of a fusion product and this process is apparently unaffected by cellular incompatibility. It appears clear that a heterokaryocyte was formed, including both an active cycling nucleus from a protoplast isolated from rapidly growing suspension cultures and a resting nucleus from a mesophyll protoplast of the cereal species, and that the active cycling nucleus imposed a transient force on the resting ones for mitotic activity rather than an enduring stimulation. In our study, Fig. 2 showed the process of callus protoplast and mesophyll protoplast fusion into one cell and the division of fused cells was found in the process of cell culture (Fig. 3). In Fig. 5, lines 6, 10, and 11 seemed to combine the specific bands of both parents, but the specific band of the mesophyll parent was not so evident. Therefore, the fusion strategy of 'callus protoplasts+callus protoplasts' from different genotypes might be more effective in cassava.

In chromosome analysis of TPL-2, the number of chromosomes ranged from 69 to 71, with a higher frequency of 70, but the TPL-2 grew normally (Fig. 3-L). The chromosome number of TPL-2 was fewer than the expected 72, implying that chromosome elimination had occurred during either the fusion, culture, or regeneration processes. Chromosome elimination was unidirectional and random in somatic hybridization and it might have been induced either by protoclonal variation or somatic incompatibility (Harms 1983). In the somatic hybridization of potato cultivars (4x) and their wild species (2x), elimination of chromosomes or genes might be caused by: (1) genome ratio (4x:2x); (2) protoclonal variation; (3) somatic incompatibility; or (4) epigenetic effects (Rakosy-Tican *et al.* 2015). Plants regenerate from fusion products that include protoplasts which exhibit regeneration capacity and those which lack morphogenetic ability. In some cases, aneuploid chromosomal constitutions are known to interfere with the normal morphology and fertility of plants, therefore the regenerated plants exhibited reduced or absent fertility (Harms 1983). This might explain the differences of plant phenotypes and root performance between the regenerated tetraploid and the diploid parent plant lines in our study.

5. Conclusion

This is the first report of protoplast fusion in cassava. In this study, a protocol for protoplast electrofusion was developed and validated. The results showed that protoplast fusion provided a new tool for cassava genetic improvement. Another important conclusion from this work is the confirmation of a viable protocol for the regeneration of plants from cassava protoplasts. This study provides technical guidance for cassava tissue culture, and also

provides some useful inspiration and reference for future genetic engineering and cell engineering in cassava.

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