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Physical localization of molecular markers and assignment of the 15th linkage group to chromosome 11 of the karyotype in cassava (*Manihot esculenta* Crantz) by primed *in situ* labeling

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ABSTRACT. Physical localization of molecular markers and assignment of the 15th linkage group to chromosome 11 of the karyotype in cassava (*Manihot esculenta* Crantz) were achieved using primed *in situ* labeling. Amplified signals for both the EST507-1 and SSRY13-5 markers were consistently observed in different stages of cell division. A comparison of the length, arm ratio, and other morphological characteristics of somatic metaphase chromosomes in karyotype analysis indicated that the EST507-1 and SSRY13-5 markers were localized on the short and long arm of cassava chromosome 11 with the relative map positions of 41.67 and 23.07, respectively. The physical localization of the 2 markers on chromosome 11 of the karyotype corresponds to their positions on the 15th linkage group in cassava.

Key words: Cassava; Linkage group; *Manihot esculenta*; Molecular genetic map; Primed *in situ* labeling; Physical localization

INTRODUCTION

Integrated linkage and physical genome maps are tremendously useful for genome sequencing projects, map-based gene isolation, and comparative genome research (Klein et al., 2000; Islam-Faridi et al., 2002). The assignment of linkage groups to specific chromosomes will allow for accurate and detailed integration of cytogenetic and genetic linkage maps. Fluorescence *in situ* hybridization (FISH) has been conventionally used to integrate genetic and chromosomal maps in several plant species (Jiang et al., 1995; Dong et al., 2000; Sadder et al., 2000; Cheng et al., 2001a,b; Kulikova et al., 2001; Howell et al., 2002; Islam-Faridi et al., 2002; Kim et al., 2002; Pedrosa et al., 2002; Koumbaris and Bass, 2003; Zhang et al., 2005; Wang et al., 2008). Because of the combination of accuracy and sensitivity of polymerase chain reaction (PCR) with FISH, primed *in situ* labeling (PRINS) has been found to be a more powerful tool for localizing DNA sequences, single-copy genes (Macas et al., 2000; Kubaláková et al., 2001; Tatum and Rayburn, 2006; Kaczmarek et al., 2007; Gao et al., 2011), and restriction fragment length polymorphism loci (Zhu et al., 1995; Shi et al., 1996) in some plants (Birchler and Danilova, 2012).

Cassava (*Manihot esculenta* Crantz) is an important tuber crop extensively planted in tropical and subtropical regions (Alves, 2001; Jennings and Iglesias, 2001). As a dicotyledonous plant in Euphorbiaceae, cassava has an allopolyploid genome with 36 chromosomes in somatic cells (2N = 36), and its haploid genome is estimated to be approximately 772 Mbp long (Bennett et al., 1982; Awoleye et al., 1994). A number of molecular marker-based genetic linkage maps have been constructed (Fregene et al., 1997; Mba et al., 2001; Okogbenin et al., 2006; Kunkeaw et al., 2010a,b; Chen et al., 2010; Sraphet et al., 2011). A draft genome sequence of a single cassava accession is also available (Prochnik et al., 2012). Despite significant advances in molecular methods, few studies have examined the molecular cytogenetics of cassava and the genetic linkage groups obtained by different researchers have not been integrated into individual chromosomes. In previous studies, we successfully assigned a linkage group to a member of the cassava karyotype (Wang et al., 2013). Here, we report physical locations of molecular markers and assignment of the 15th linkage group to chromosome 11 of the karyotype in cassava (*M. esculenta* Crantz) using PRINS.

MATERIAL AND METHODS

Plant materials

A cassava variety South No. 6 (SC6), provided by Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agriculture, was used as experimental material.

Chromosome preparation

The method described by Wang et al. (2012) was used with some modifications. Root tips with lengths of approximately 1 cm were harvested and pretreated in 0.002 M 8-hydroxyquinoline for 2 h. They were then washed with double-distilled water, fixed in fresh Carnoy's solution (ethanol:glacial acetic acid, 3:1) for 18-24 h at 4°C, rinsed in double-distilled water for 30 min, and placed in an enzyme solution of 3.5% cellulose and 1.75% pectinase for 4-5 h at 37°C. The root tips were rinsed, subjected to hypotonic treatment for 30 min again

with double-distilled water, and fixed for 20-30 min. Metaphase chromosomes were placed on poly-L-lysine-coated glass slides using the flame drying technique. The slides were air-dried and stained with 5% Giemsa solution, pH 7.0, for 20-25 min.

PRINS reaction

Using a modified method described by Gao (2012), 2 molecular markers, EST507-1 and SSRY13-5 of the 15th linkage group (LG15) map published by Chen et al. (2010), were physically located. Two pairs of primers were used, with sequences for the 2 markers as follows: 5'-GGAATGAGTCCGAATATGAA-3' (upstream primer) and 5'-ATATTTCTTGCGGC TATGAC-3' (downstream primer) for EST507-1, 5'-GCAAGAATTCCACCAGGAAG-3' (upstream primer) and 5'-CAATGATGGTAAGATGGTGCAG-3' (downstream primer).

Slides were immersed in 0.01 M HCl for 10 min, incubated in 50 µL 1 µg/mL pepsin and 0.01 M HCl at 37°C for 10 min, washed with 0.5X Tris-buffered saline solution for 10 min, rinsed with sterilized distilled water for 5 min twice, and then air-dried.

The slides were denatured for 5 min at 70°C in 70% formamide/0.1X standard saline citrate (SSC), pH 7.0, and immediately immersed in 0.1X SSC in an ice water bath for 1 min and then rinsed with sterile water in an ice water bath for 1 min. Subsequently, slides were dehydrated in an ethanol series (75, 90, and 100%) at -20°C for 3 min each and were allowed to air dry.

To each slide, we added 50 µL labeling reaction mixture and then sealed the slide with 20 µL mineral oil. The labeling reaction mixture contained 5X buffer, 25 mM $MgCl_2$, 10 mM dATP, 10 mM dGTP, 10 mM dCTP, 3.6 mM dTTP, 20 mM DIG-11-dUTP, 5 U Taq DNA polymerase, 20 mM F-primers, 20 mM R-primers, and 6 µL double-distilled H_2O. Slides without Taq DNA polymerase or primers were used as negative controls in this study. Chromosomal DNA was first denatured for 10 min at 95°C. The reactions were then thermocycled 35 times (1 min at 94°C, 1 min at 55°C, 2 min at 72°C) and the reaction was terminated with a final extension at 72°C for 10 min.

Signal detection and localization

After amplification, the coverslips were removed and slides were washed with 0.1X phosphate-buffered saline at 37°C for 5 min. The slides were placed in 100 µL 5% bovine serum albumin (0.1X SSC/Tween 20) and then covered with plastic coverslips and incubated at 37°C for 20 min. Next, the slides were incubated in 50 µL 20 mg/L anti-DIG-fluorescein with plastic coverslips at 37°C for 1 h, and followed by multiple washes in 0.1X SSC/Tween 20 for 5 min. The slides were counterstained with 1 µg/mL propidium iodide for 30 min in the dark at 4°C; the total volume was 25 µL. Observations were made using a fluorescent microscope (BX51TR-32Fa1-A03, Olympus, Tokyo, Japan) and photos were acquired using a DP72 Microscope Digital Photograph Camera attached to the microscope. The positions of amplification signal of molecular markers were analyzed based on relative map position and the percentage of the distance (in µm) from the signal site to the centromere relative to the length of the chromosome arm (in µm). Relative map position was calculated based on the average value of that measured in at least 5 individual cells.

RESULTS

Physical location of EST507-1 in the 15th linkage group of cassava

PRINS studies using the EST507-1 primers revealed the presence of the EST507-1 marker on chromosome 11. The yellow-green fluorescent signal was detected in the nuclei during different phase of cell division but the signal was absent in the negative control (Figure 1). Karyotype analysis showed that EST507-1 was located on the short arm of chromosome 11 (Figure 2).

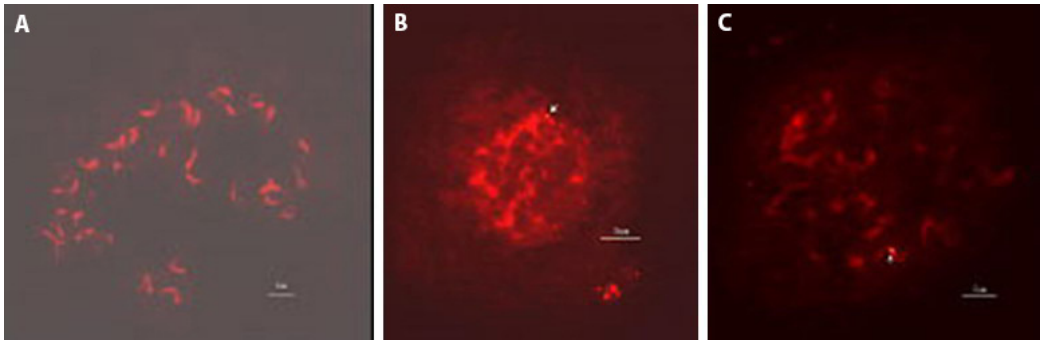

Figure 1. EST507-1 signal in cassava interphase and prometaphase nuclei. **A.** Negative control. **B.** Interphase nucleus. **C.** Prometaphase nucleus. Arrows for the yellow-green fluorescent signal of EST507-1. Scale bar = 5 μm.

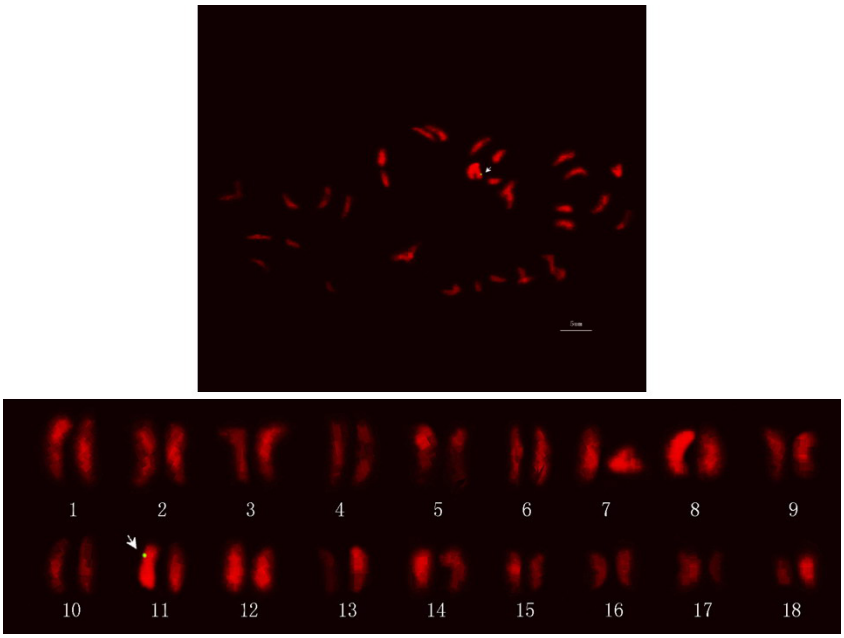

Figure 2. EST507-1 signal on cassava metaphase chromosome 11. Arrows for the yellow-green fluorescent signal of EST507-1. Scale bar = 5 μm.

Physical location results of SSRY13-5 in the 15th linkage group of cassava

PRINS labeling showed that the signal for SSRY13-5 was successfully detected in both interphase and prometaphase nuclei but not in the negative control (Figure 3). Karyotype analysis demonstrated that SSRY13-5 was amplified on the long arm of chromosome 11 by PRINS (Figure 4).

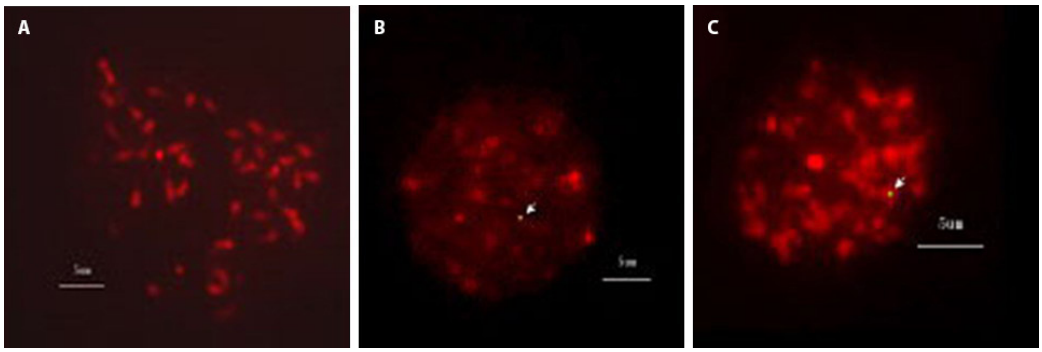

Figure 3. SSRY13-5 signal in cassava interphase and prometaphase nuclei. **A.** Negative control. **B.** Interphase nucleus. **C.** Prometaphase nucleus. Arrows for the yellow-green fluorescent signal of SSRY13-5. Scale bar = 5 μm.

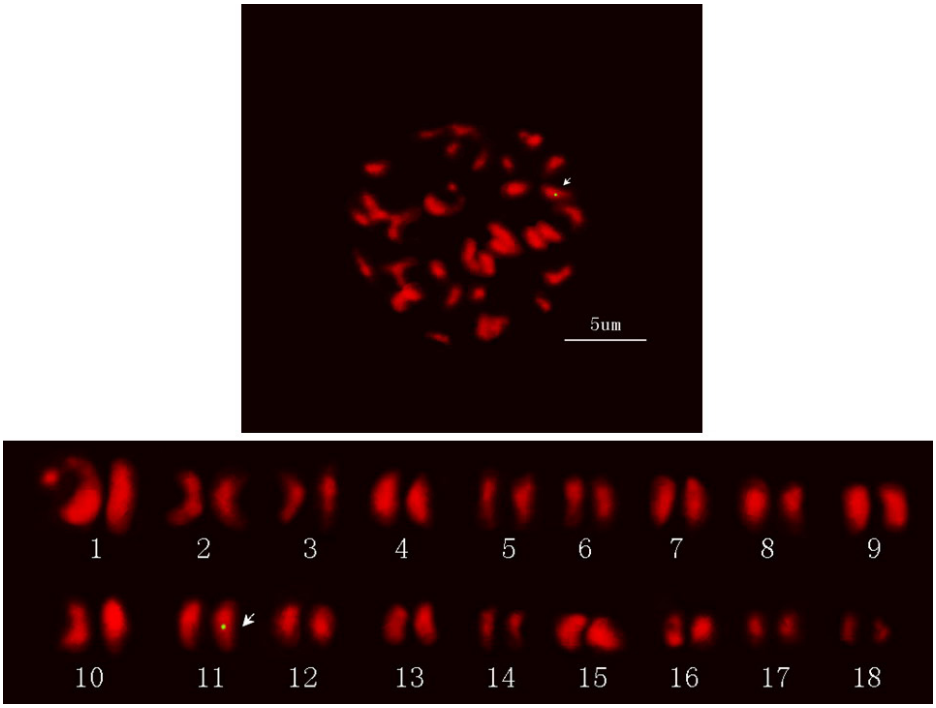

Figure 4. SSRY13-5 signal on cassava metaphase chromosome 11. Arrows for the yellow-green fluorescent signal of SSRY13-5. Scale bar = 5 μm.

Assignment of the 15th linkage group to chromosome 11 of the karyotype in cassava

Based on the length, arm ratio, and other morphological characteristics of somatic metaphase chromosomes, a standardized karyotype (ideogram) (Figure 5) for cassava was established, allowing us to distinguish individual chromosomes. Karyotype analysis and comparison of the chromosomes physically mapped with 2 molecular markers showed that the EST507-1 and SSRY13-5 markers were located on the short and long arms of cassava chromosome 11 with relative map positions of 41.67 and 23.07 (Figures 5 and 6), respectively. Both the EST507-1 and SSRY13-5 markers were mapped to the 15th linkage group constructed by Chen et al. (2010). Therefore, the 15th linkage group can be assigned and integrated to chromosome 11 of the karyotype in cassava.

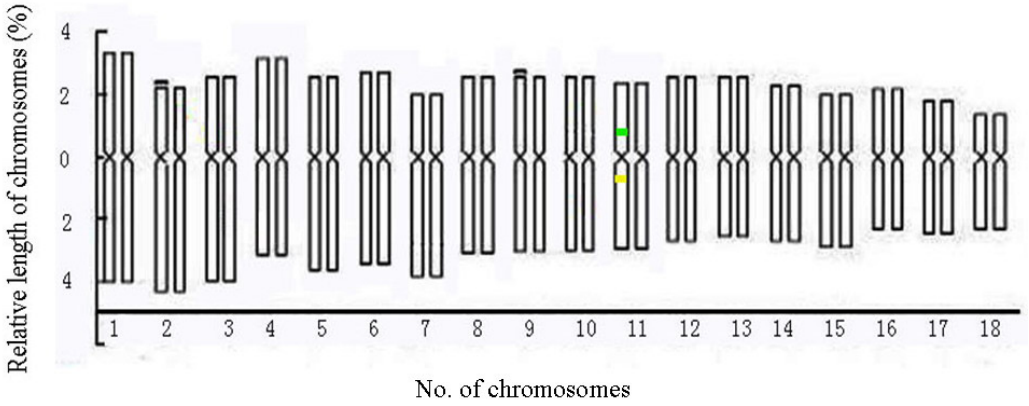

Figure 5. Idiogram of cassava metaphase chromosomes.

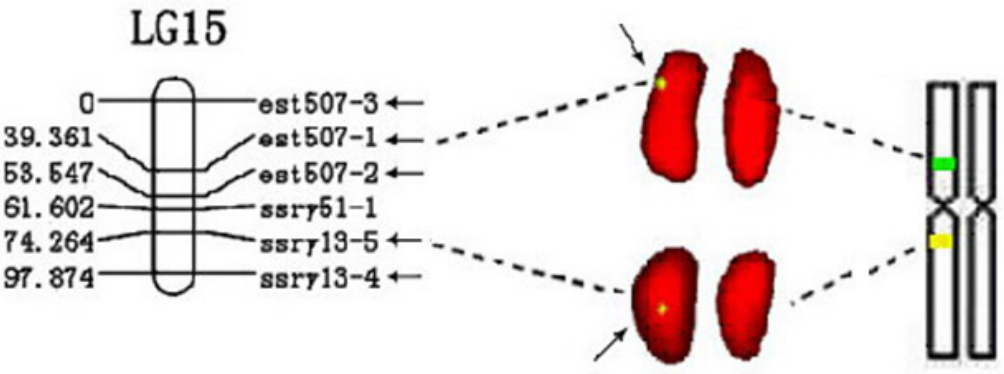

Figure 6. Comparison of the 15th linkage group (left), labeled metaphase chromosome 11 (middle) and the diagram chromosome 11 (right) of cassava. Arrows denote amplified signals.

DISCUSSION

The assignment and integration of linkage maps to individual chromosomes of the karyotype of cassava is very useful for gene cloning and genomic investigations. PRINS

can be used to detect relatively small chromosomal regions, DNA sequences, and molecular marker loci that cannot be observed using the FISH technique (Zhu et al., 1995; Shi et al., 1996; Macas et al., 2000; Kubaláková et al., 2001; Tatum and Rayburn, 2006; Kaczmarek et al., 2007). In this study, we demonstrated the feasibility of determining the physical location of molecular markers and assigning a linkage group to an individual chromosome of the karyotype in cassava using 2 markers by PRINS. The physical location of the 2 markers on chromosome 11 of the karyotype was consistent with their positions on the 15th linkage group.

We found that using poly-L-lysine-coated glass slides was critical for preventing the dropping of target cells and for improving the efficiency of *in situ* PCR amplification, and 0.1X phosphate-buffered saline solution washing for 5 min at 37°C was necessary to obtain a clean background and to obtain stronger signals. Furthermore, the steady occurrence of amplified signals in different stages of cell division ensured the authenticity of the signals.

It should be also noted that pachytene chromosomes are uniform in chromosome packaging and condensation, and are generally longer than somatic metaphase chromosomes (McClintock, 1929; Ramanna and Prakken, 1967; Peterson et al., 1999), and thus it would be advantageous to use 2-color or multicolor simultaneous mapping techniques and pachytene chromosome spreads as they allow for precise and fine-resolution physical mapping. Our results can be applied to study the molecular cytogenetics of cassava.

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