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Conservation and Variation of 35S Ribosomal DNA among Five *Jatropha* Species Revealed by Fluorescence *In Situ* Hybridization

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Summary To analyze the karyotypes of five *Jatropha* species (*J. cinerea*, *J. curcas*, *J. integerrima*, *J. multifida*, and *J. podagrica*), fluorescence *in situ* hybridization (FISH) with a 35S ribosomal RNA gene (rDNA) probe was conducted. FISH analysis of mitotic metaphase chromosomes showed that the numbers of chromosomes and rDNA foci in all species analyzed were conserved, although signal intensities and their locations varied. The conserved karyotypes may be advantageous for the use of *J. curcas* genome data and for breeding in the progeny of interspecific crosses.

Key words Chromosome, Fluorescence *in situ* hybridization (FISH), Ribosomal RNA gene (rDNA), *Jatropha*.

The genus *Jatropha*, Euphorbiaceae, comprises about 170 species distributed worldwide in the tropical areas (Dehgan 1984). *Jatropha curcas* is the most cultivated species in this genus for biodiesel production because of its high oil content, ease of propagation, and drought tolerance. However, its genetic variation is limited (Tanya *et al.* 2011, Montes *et al.* 2014), and thus the use of related species for interspecific hybridization is required for genetic improvement (One *et al.* 2014a, One *et al.* 2014b, Fukuhara *et al.* 2016). Several *Jatropha* species closely related to *J. curcas* are used as medicinal and ornamental plants (Muakrong *et al.* 2014, Anani *et al.* 2016).

Although karyotyping and fluorescence *in situ* hybridization (FISH) mapping of DNA repeats have been used to cytologically characterize *J. curcas* (Carvalho *et al.* 2008, Witkowska *et al.* 2009, Kikuchi *et al.* 2011, Alipour *et al.* 2013, Gong *et al.* 2013) and its whole genome has been sequenced (Sato *et al.* 2011, Hirakawa *et al.* 2012, Wu *et al.* 2015), cytological information on other *Jatropha* species is limited. FISH analysis using rDNAs is a powerful technique for karyotype analysis (Leitch and Heslop-Harrison 1992, Kikuchi *et al.* 2006, Kikuchi *et al.* 2008, Garcia *et al.* 2012). Among *Jatropha* species, it has been used only in *J. curcas* accessions and has revealed one locus of 5S and two loci of 35S (18S–5.8S–26S) rDNA in each accession (Witkowska *et al.* 2009, Gong *et al.* 2013).

Although information on the chromosome numbers of all the five *Jatropha* species is available in the chromosome counts database (CCDB; http://ccdb.tau.ac.il/home/; Rice *et al.* 2015), there was a report for *J. cinerea* (Dehgan and Webster 1979). In addition, cytological studies reported that *Jatropha* species have the same number of somatic chromosomes ($2n$=22), which are small and similar in size. However, except for *J. curcas*, no FISH-based karyotypes have been reported so far.

In this study, we first confirmed the chromosome numbers of five *Jatropha* species (*J. cinerea*, *J. curcas*, *J. integerrima*, *J. multifida*, and *J. podagrica*) and carried out FISH analysis with a 35S rDNA probe. We also used two cultivars of *J. curcas* ('Chai Nat' and 'Mexico 10') to confirm conserved karyotypes reported for this species.

Materials and methods

Plant materials

Two cultivars of *J. curcas*, 'Chai Nat' (a local cultivar in Thailand) and 'Mexico 10' (a cultivar probably introduced to Thailand from Mexico), *J. cinerea*, *J. integerrima* (a local ornamental dwarf type from Thailand; One *et al.* 2014a), *J. multifida*, and *J. podagrica* were used in this study (Table 1). Plants were grown in the *Jatropha* research field of the Department of Agron-

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Preparation of mitotic chromosome and FISH

Floral buds were collected and fixed as described in Kikuchi *et al.* (2011). Slides were prepared according to

Table 1. Origin of and 35S rDNA in two *Jatropha curcas* cultivars and four related species.

Jatropha species	Origin of the cultivars	Chromosome number ($2n$)	Number and intensity of 35S rDNA FISH signals				
			Very strong	Strong	Intermediate	Weak	Total
J. curcas 'Chai Nat'	Thailand	22	0	2	0	2	4
J. curcas 'Mexico 10'	Mexico	22	0	2	0	2	4
J. cinerea	Mexico	22	0	0	4	0	4
J. integerrima	Thailand	22	2	0	0	2	4
J. multifida	Thailand	22	0	0	2	2	4
J. podagrica	Thailand	22	0	0	0	4	4

Ranges of signal intensity: very strong (>1.20 μm^2), strong (0.81–1.20 μm^2), intermediate (0.41–0.80 μm^2), and weak (≤0.40 μm^2).

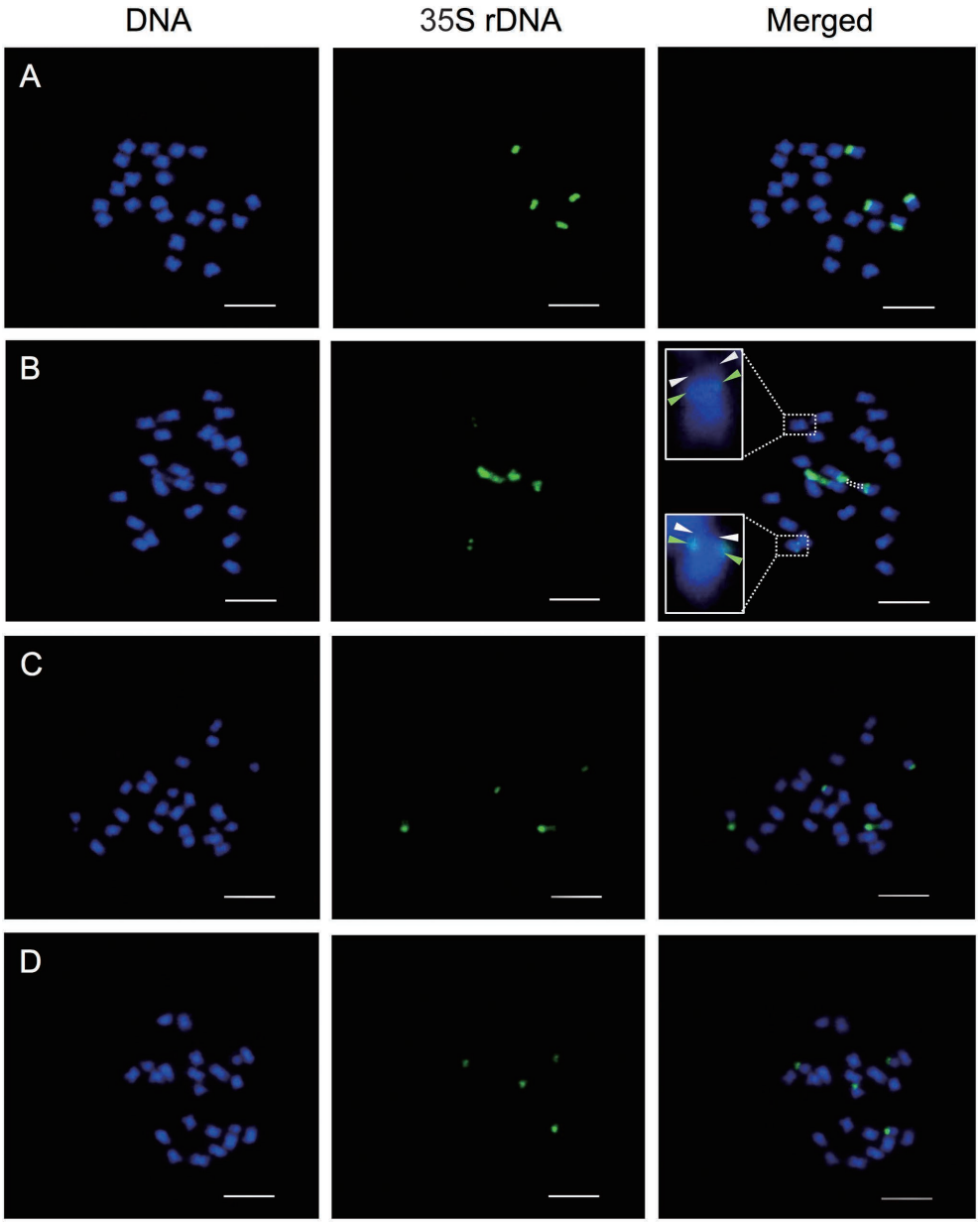

Fig. 1. FISH detection of 35S rDNA in the four *Jatropha* species. (A) *J. cinerea*, (B) *J. integerrima*, (C) *J. multifida*, and (D) *J. podagrica*. In (B), the insets show magnified chromosomes with weak 35S rDNA signals up to approximately 3.7 times by the original; green dotted lines indicate the nucleolus organizer region. Green arrowheads: 35S rDNA signals. White arrowheads: telomere end of the chromosome arm. Scale bars=5 μm.

the squash method of Fukuhara *et al.* (2016).

Biotin-nick translation mix (Sigma-Aldrich) was used to label wheat 35S rDNA (pTa71; Gerlach and Bedbrook 1979). Chromosome slides were treated with 100 ng μL^{-1} RNase A at 37°C for 60 min, and then re-fixed in 1% paraformaldehyde in 1×PBS for 15 min. Hybridization and detection were performed as described by Wang *et al.* (2015). All FISH images were captured with an Olympus BX53 fluorescence microscope equipped with a CCD camera (CoolSNAP MYO; Photometrics). FISH signal areas on condensed mitotic chromosomes of 10 cells (20 foci from sister chromatids) were measured using ImageJ (https://imagej.nih.gov/ij/).

Results

We confirmed that all of the five species, including *J. cinerea*, have $2n=22$ chromosomes (Figs. 1 and 2, Table 1). Each species examined had two 35S rDNA focus pairs (Figs. 1 and 2, Tables 1 and 2). In *J. integerrima* (Fig. 1B) and *J. multifida* (Fig. 1C), two strong signals and two weak signals were detected; this distribution was similar to that in *J. curcas* (Witkowska *et al.* 2009, Kikuchi *et al.* 2011, Gong *et al.* 2013) (Tables 1 and 2). In contrast, in *J. cinerea* (Fig. 1A) and *J. podagrica* (Fig. 1D) two pairs of 35S rDNA foci had similar signal intensity (Tables 1 and 2).

Most rDNA foci were located at the ends of chromosomes. However, the weak 35S rDNA signals in *J. integerrima* were located in the interstitial regions of the short arms (inset in Fig. 1B). Note that the weak 35S rDNA signals in *J. integerrima* (0.21 μm^2) were smaller than those in *J. curcas* (0.28 μm^2; Table 2), whereas the strong 35S rDNA signals in *J. integerrima* (1.88 μm^2) were larger than those in the *J. curcas* cultivars (0.99 and 1.03 μm^2; Table 2).

One weak and one strong pair of 35S rDNA signals were detected in the two *J. curcas* cultivars. Their inten-

Table 2. Mean 35S rDNA FISH signal area in five *Jatropha* species.

Jatropha species	Mean signal area (S.D.), μm^2				
	Very strong	Strong	Intermediate	Weak	Total in haploid
J. curcas 'Chai Nat'	—	0.99 (0.03)	—	0.28 (0.02)	1.27^{b} (0.10)
J. curcas 'Mexico 10'	—	1.03 (0.06)	—	0.28 (0.02)	1.30^{b} (0.06)
J. cinerea	—	—	0.56 (0.02)	—	1.11^{c} (0.04)
J. integerrima	1.88 (0.07)	—	—	0.21 (0.01)	2.09^{a} (0.07)
J. multifida	—	—	0.56 (0.03)	0.18 (0.01)	0.75^{d} (0.03)
J. podagrica	—	—	—	0.29 (0.01)	0.59^{e} (0.02)
F-test					**
cv (%)					5.05

Ranges of signal intensity: very strong (>1.20 μm^2), strong (0.81–1.20 μm^2), intermediate (0.41–0.80 μm^2), and weak (≤0.40 μm^2). Different superscript letters indicate significant differences at $p \leq 0.01$.

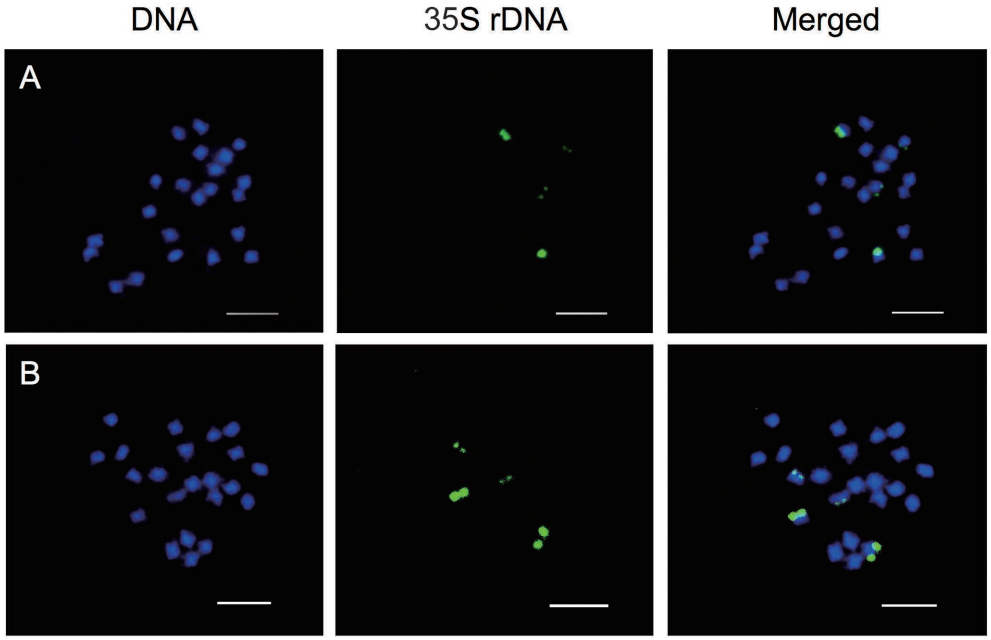

Fig. 2. FISH detection of 35S rDNA in the two *J. curcas* cultivars. (A) 'Mexico 10'. (B) 'Chai Nat'. Scale bars=5 μm.

sities and positions were similar between the cultivars (Fig. 2, Tables 1 and 2).

Discussion

Here, we documented the distribution of 35S rDNA foci on the somatic chromosomes of four *Jatropha* species. We found that the karyotypes of the four species were similar to those of *J. curcas* cultivars (Witkowska *et al.* 2009, Gong *et al.* 2013). These sat-chromosomes with 35S rDNA may be homologous-like, because Fukuhara *et al.* (2016) found sat-chromosome segments exchanged by meiotic recombination using FISH and genomic *in situ* hybridization (GISH) analysis in the F_2 plants from a cross between *J. curcas* and *J. integerrima*. Conserved karyotypes may be advantageous for the use of *J. curcas* genome data and for breeding owing to regular meiosis in the progeny of interspecific crosses.

In *J. integerrima*, we observed weak 35S rDNA foci in interstitial regions. Other species had weak 35S rDNA signals at chromosome ends, indicating that the rDNA foci of *J. integerrima* underwent repositioning. Ribosomal RNA genes may change their position without changes in the order of surrounding genes (Schubert and Wobus 1985, Dubcovsky and Dvorák 1995, Shishido *et al.* 2000), likely by transposon-mediated events (Raskina *et al.* 2004). Other mechanisms for rDNA repositioning are chromosome rearrangements such as translocations, inversions, duplications, and deletions (Maluszynska and Heslop-Harrison 1993, Hall and Parker 1995, Mohannath *et al.* 2016). DNA sequencing and FISH analysis may help us to understand polymorphisms of chromosome structure in *J. integerrima* and the repositioning events.

Signal intensity of 35S rDNA loci varied among the five *Jatropha* species (Figs. 1 and 2, Tables 1 and 2), whereas their number was conserved. Gong *et al.* (2013) reported that the weak signals in *J. curcas* remained condensed throughout the interphase and mitosis and that they were not connected with the nucleolus, suggesting an inactive state. The weak signals of 35S rDNA in both *J. curcas* cultivars and *J. integerrima* were also small and condensed (Figs. 1B and 2, Tables 1 and 2), indicating that these 35S rDNA loci may be transcriptionally inactive. Although terminal location of 35S rDNA loci is not always essential for their expression (Garcia *et al.* 2017), the weak 35S rDNA foci in the interstitial region in *J. integerrima* suggest a reduced copy number in the rDNA array. On the other hand, both 35S rDNA focus pairs in *J. cinerea* and *J. podagrica* had the same signal intensity and thus may be transcriptionally active. Molecular mechanisms that regulate the expression levels of individual rRNA genes might include DNA methylation and histone modification (Grummt and Pikaard 2003, Pontvianne *et al.* 2012); Gong *et al.* (2013) observed several patterns of DNA methylation in the two pairs of 35S rDNA loci in *J. curcas*. A position effect on rRNA gene expression has been recently reported (Mohannath *et al.* 2016).

The variations in the location and signal intensity of 35S rDNA partially reflect the phylogenic relationships. The close phylogenic relationship between *J. curcas* and *J. integerrima* suggested by the analysis of the sequences of internal transcribed spacers of nuclear ribosomal DNA (Pamidimarri *et al.* 2009a). *J. podagrica* formed a major clade with *J. curcas* and *J. integerrima* in the study (Pamidimarri *et al.* 2009a), whereas *J. podagrica* clustered with *J. multifida* in phylogenic analysis based on RAPD and AFLP fingerprinting (Pamidimarri *et al.* 2009b). Our FISH results suggest that *J. podagrica* is not close relative with *J. curcas* and *J. integerrima*.

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