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Note

Cytological characterization of an interspecific hybrid in *Jatropha* and its progeny reveals preferential uniparental chromosome transmission and interspecific translocation

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Genetic variation in *Jatropha curcas*, a prospective biodiesel plant, is limited, and interspecific hybridization needed for its genetic improvement. Progeny from interspecific crosses between *J. curcas* and *Jatropha integerrima* can be used to improve agronomic characters and to increase oil content and yield. However, these hybrids have not been characterized cytologically. The present study was aimed at the analysis of chromosome behavior during meiosis and chromosome composition of S_1 plants derived from an interspecific F_1 hybrid using genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH). Bivalents that formed as a result of interspecific pairing were frequently observed, suggesting the presence of homoeologous chromosomes from the two species. Almost half of microspores were derived from the reduction division; GISH analysis indicated random transmission of the parent chromosomes to microspores. Male fertility measured as pollen staining with acetocarmine was 48.4%. In contrast, GISH analysis of S_1 plants revealed preferential transmission of *J. curcas* chromosomes. We also found segment exchange between chromosomes of the two species (interspecific translocation) by GISH and FISH analyses. Introgression of *J. integerrima* chromosome segments into the *J. curcas* genome would help to improve *Jatropha* cultivars for mass production.

Key Words: fluorescence *in situ* hybridization (FISH), genomic *in situ* hybridization (GISH), interspecific hybrid, interspecific translocation, *Jatropha curcas*, *Jatropha integerrima*, preferential transmission.

Introduction

Jatropha (*Jatropha curcas* L., Euphorbiaceae; $2n = 2x = 22$; Miller and Webster 1962, Perry 1943) is a highly branching and semi-evergreen tree, and an oil-bearing plant. Jatropha is thought to be a promising plant for biodiesel production because of its higher oil content (40%–60% in seeds), rapid growth, ease of propagation, drought tolerance, and pest resistance. Because of its assumed environmental benefits, more than 10^6 ha of jatropha plantations have been established in Asia (especially in India and China), Africa, and Latin America (Singh *et al.* 2014) over the last 10 years, with a prospective seed yield of 4–5 Mg ha^{-1} yr^{-1}. However, the actual seed yield (0.5–2 Mg ha^{-1} yr^{-1}) was considerably lower than expected owing to underperformance under field conditions in various countries (Edrisi *et al.* 2015). To commercialize jatropha, genetic improvement of oil yield and quality is required.

Assessment using molecular markers such as random amplified polymorphic DNA (RAPD), inter simple sequence repeat (ISSR), and simple sequence repeat (SSR) markers indicated a modest level of inter-accessional variability in *J. curcas* (Basha and Sujatha 2007, Tanya *et al.* 2011). Using 54 SSRs and 120 single-nucleotide polymorphisms, Montes *et al.* (2014) found that only 7 of 70 *J. curcas* accessions from Asia, Africa, and Latin America had more than 10% of markers with multiple alleles per locus, suggesting a high level of self-fertilization. Thus, evaluation

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and utilization of interspecific hybridization is an option in jatropha breeding programs to enhance genetic variability in this crop. Approximately 175 diverse *Jatropha* species are known, which are widely distributed in the Old and New World tropics (Dehgan 1984). Species with better traits than *J. curcas* might be used for jatropha breeding. Almost all species are diploid ($2n = 2x = 22$ chromosomes). The same chromosome number in *J. curcas* and wild relatives may be advantageous for breeding owing to regular meiosis in the progeny of interspecific crosses (Dahmer *et al.* 2009).

Jatropha integerrima Jacq. ($2n = 2x = 22$; Miller and Webster 1962) is an evergreen shrub or small tree, and is thought to be a close relative of *J. curcas* (and of *Jatropha gossipifolia*) (Sudheer Pamidiamarri *et al.* 2009a, 2009b). *J. integerrima* (peregrina) carries traits that do not exist in *J. curcas* such as setting profuse flowers with uniform blooming on the same inflorescence, woody stem and branches. Yet dwarf varieties are available allowing the breeder to obtain plants with smaller canopy and higher harvest index (Laosatit *et al.* 2014, One *et al.* 2014). Reciprocal crosses between *J. curcas* and *J. integerrima* are possible, and interspecific hybrids have been developed (Dehgan 1984, Lakshminarayana and Sujatha 2001, Muakrong *et al.* 2013, Parthiban *et al.* 2009, Rupert *et al.* 1970, Sujatha and Prabakaran 2003). BC_1F_1 plants from a *J. curcas* × *J. integerrima* cross have higher seed set, yield potential and resistance to diseases, and an average yield of several BC_1F_1 plants was three times more than *J. curcas* seed yield at the same age (Parthiban *et al.* 2009). F_2 plants from *J. curcas* × *J. integerrima* have a higher potential for biodiesel production than *J. curcas* because of their short height (135 cm), small canopy width (150 cm), high seed yield (237.1 g/plant vs. 66.2 g/plant in *J. curcas*), and high oil content (37.9%) (One *et al.* 2014a). High genetic variability in the F_2 population was suggested (One *et al.* 2014b). Although these F_2 plants still need to be improved for reliable commercial use (One *et al.* 2014a), these studies suggest that interspecific hybridization is a feasible approach for jatropha improvement.

Cytogenetic studies in this genus have been limited; in particular, chromosome behavior in interspecific hybrids during meiosis has not been reported, and the karyotypes of F_2 individuals derived from F_1 hybrids between *J. curcas* and *J. integerrima* are unknown. The current study aimed to investigate the behavior of meiotic chromosomes in an F_1 hybrid derived from a *J. curcas* × *J. integerrima* cross and chromosome variation in an S_1 population derived from self-pollination of this F_1 hybrid. For the first time in this genus, we used genomic *in situ* hybridization (GISH), a molecular cytogenetic technique for discriminating chromosomes from different genomes (Schwarzacher *et al.* 1989). The GISH analysis indicated preferential transmission of *J. curcas* chromosomes in S_1 plants and mosaic pattern of two GISH signals in *J. integerrima* chromosomes. *J. curcas* has a subtelomeric repeat, the *Jatropha curcas* satellite 1 (JcSat1), that can be used for a clear FISH marker at the chromosome ends. By using sequential fluorescence *in situ* hybridization (FISH) probed with the JcSat1 and GISH, we found interspecific translocation (an exchange of small chromosome segments) in the S_1 plants.

Materials and Methods

Plant materials

This study used *J. curcas* 'Chai Nat' (a local Thai cultivar), *J. integerrima* (a local ornamental dwarf type from Thailand), and an F_1 hybrid (plant no. F_1-4) from a cross between 'Chai Nat' as the female parent and *J. integerrima* as the male parent. Plants were grown in the jatropha research field of the Department of Agronomy, Kasetsart University, Kamphaeng Saen campus, Thailand. S_1 seeds were obtained by self-pollination of F_1-4.

Chromosome slide preparation

To observe mitotic chromosomes, we collected fresh roots from germinated seeds or potted plants, pretreated them with 0.045% 8-hydroxyquinoline at 4°C for 2 h and at 25°C for 4 h. To observe meiotic chromosomes, we collected fresh flower buds and directly fixed them in 3:1 (v/v) ethanol–acetic acid. Chromosome slides were prepared according to the squash method of Wang *et al.* (2015).

GISH and FISH

Genomic DNA of *J. curcas* and *J. integerrima* was purified from young leaves without thick veins using the cetyltrimethylammonium bromide (CTAB) method according to Kikuchi *et al.* (2010). Plasmid DNA containing the *Jatropha curcas* satellite 1 (JcSat1) sequence (Kikuchi *et al.* 2010) and 45S rDNA (pTa71; Gerlach and Bedbrook 1979) was used to prepare a FISH probe. The FISH probes (plasmid DNA) were generated by nick translation (Sigma) and the GISH probes (genomic DNA) were prepared by High Prime (Sigma). GISH and FISH were conducted according to the procedures of Wang *et al.* (2015).

For the sequential FISH and GISH detection, the slides were first processed for the detection of JcSat1 and 45S rDNA (FISH), denatured in 50% formamide/2× SSC for 2 min on a heat block at 80°C, washed, and air-dried. GISH was then conducted as mentioned above.

Acetocarmine staining

Pollen grains were collected at flowering, incubated in 0.5% acetocarmine on a glass slide for at least 20 min, and observed under a bright-field microscope (CX41; Olympus Corp.).

Results

Chromosome number of the interspecific F_1 hybrid (F_1-4)

The somatic cells of F_1-4 contained 22 chromosomes, which came from both parents. No morphological abnormalities were detected in the metaphase chromosomes (data not shown).

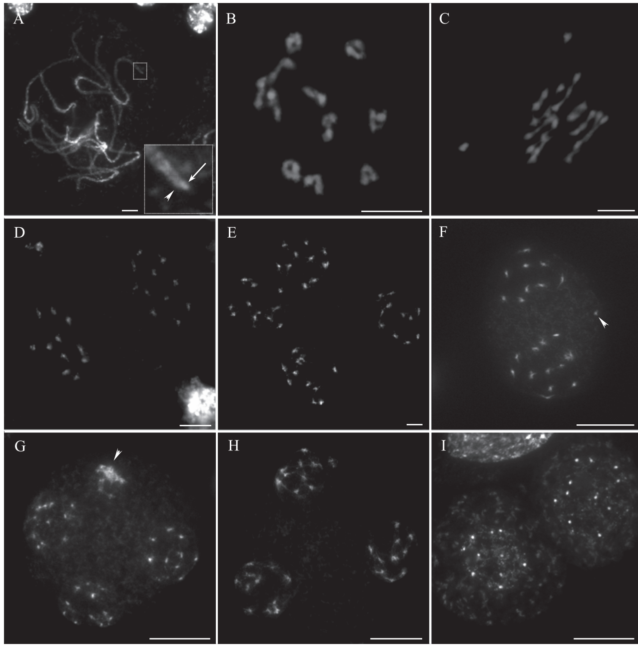


Fig. 1. Chromosome behavior during meiosis in an F_1 hybrid of *J. curcas* × *J. integerrima*. (A) Paired pachytene chromosomes. Inset: Pairing at chromosome ends with different chromatin structure. Arrow: heterochromatin. Arrowhead: euchromatin. (B) Diakinesis. Eleven bivalents (both ring- and rod-shaped chromosomes) are visible. (C) Methaphase I/Early anaphase I. Note that paired chromosomes are not arranged on the equatorial plate. (D) Telophase I/dyad stage. (E) A tetrad cell. Each daughter cell has 11 centromeric foci (chromosomes). (F–I) Abnormal cells observed at different stages (from dyad to microspore stage). (F) A dyad with a lagging chromosome (arrowhead). Upper cell has 9 chromosomes, whereas the lower cell has 11. (G) A tetrad with clumped chromosomes (arrowhead). (H) A triad. Each of the three nuclei has 10–12 fluorescence foci. (I) Microspores. The left cell has only ten chromosomes. Chromosomes were stained with DAPI. Scale bars = 5 µm.

Chromosome behavior during meiosis

Meiotic chromosomes of F_1-4 showed normal and abnormal behavior (**Fig. 1**). In pachytene spreads, well-paired bivalent chromosomes were observed (**Fig. 1A**). In each pair, chromosome ends had different chromatin structure, *i.e.*, only one end carried heterochromatin (inset in **Fig. 1A**). Since *J. curcas* has a tandem repeat, JcSat1, in subtelomeric heterochromatin (Kikuchi *et al.* 2010), the pachytene chromosomes of F_1-4 might have formed by pairing of homologous chromosomes from both parents. Eleven bivalents, including rod and ring chromosomes, were present in most diakinesis cells (**Fig. 1B**). In metaphase I, mean chromosome association frequency was $0.88_{\mathrm{I}} + 10.56_{\mathrm{II}}$ per cell (**Table 1**). However, chromosomes in metaphase I frequently overlapped or were associated like multivalent (**Fig. 1C**). Although local pairing might be involved, we were unable to detect it by using the squash method. Observations of metaphase I and early anaphase I suggest that chromosomes did not show synchronized orientation on the equatorial plate; several chromosomes started moving to each pole earlier than other chromosomes (**Fig. 1C**). Normal dyads and tetrads with 11 fluorescence foci of centromeric heterochromatin were generated at frequencies of 37.5% ($n = 24$; remaining 62.5% is abnormal dyad) and 69.2% ($n = 78$; remaining 30.8% is abnormal tetrad), respectively (**Fig. 1D**, **1E**, **Table 2**).

Table 1. Chromosome pairing configurations of an F_1 hybrid between *J. curcas* and *J. integerrima*

	Chromosome number (2*n*)	No. of cells observed	Chomosome pairing configurations					
			I	II			III	TOXN[a]
				Total	Rod	Ring		
F_1-4	22	16	0.875	10.56	9.56	1.00	0	1.28

[a] TOXN = Total chiasmata / haploid chromosome number. Possible chiasma numbers of rod and ring bivalents are 1 and 2.

Several abnormalities were observed from meiotic anaphase I to the tetrad stage. One or two lagging chromosomes that could not enter daughter cell nuclei were observed (**Fig. 1F**). The average number of dyad cells with lagging chromosome(s) was 23.5% (**Table 2**). Clumping of chromosomes was observed in tetrad stages (3.9%; **Fig. 1G**, **Table 2**). In addition, 5.1% of the cells carried only three nuclei at the tetrad stage; *i.e.*, they were triads (**Fig. 1H**, **Table 2**). Because the three nuclei had 10 to 12 centromeric foci, which was similar to the haploid chromosome number (**Fig. 1H**), the triads could not have resulted from the first or second division restitution without equational cell wall formation. Lagging chromosomes in telophase II to tetrad stages were observed at a rate of 21.8% (**Table 2**). Centromeric foci revealed chromosomally variable gametes (aneuploids), which were generated because of chromosome lagging and unbalanced chromosome segregation; *e.g.*, 9 and 12 foci in a dyad in **Fig. 1F** and 10 foci in a microspore in **Fig. 1I** (left

Table 2. Frequencies of meiocytes showing abnormal chromosome behaviors and characteristics in F_1-4

Telophase I to dyad		Telophase II to tetrad			Aneuploid microspores[d]
Lagging chromosomes[a]	Un-reduction division[b]	Lagging chromosomes[a]	Clumping nuclei[c]	Triads	
23.5% (12/51 cells)	62.5% (15/24 cells)	21.8% (17/78 cells)	3.9% (3/78 cells)	5.1% (4/78 cells)	46.5% (33/71 cells)

[a] Cells with at least one lagging chromosome.
[b] Telophase I/dyad cells without expected 11 centromere foci in the daughter cell nuclei.
[c] Tetrads including clumping nuclei.
[d] Microspores without expected 11 centromere foci in the nucleus.

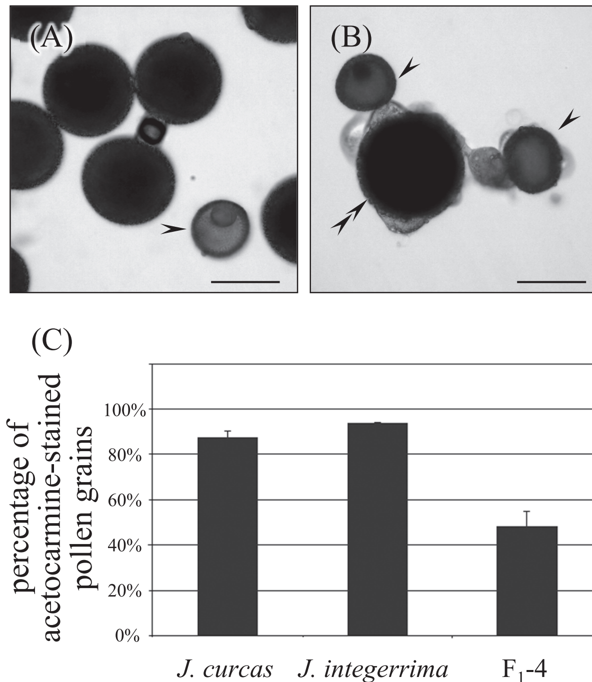


Fig. 2. Acetocarmine staining of pollen grains in the F_1 hybrid and parental species. (A) In *J. integerrima*, the cytoplasm of most pollen grains was stained; an unstained grain is marked with an arrowhead. (B) An increase in the proportion of unstained pollen grains (single arrowheads) was observed in the F_1 hybrid. A double arrowhead indicates a pollen grain that was slightly larger than the pollen grains of the *J. integerrima* in **Fig. 2A**. Scale bars = 50 µm. (C) Percentage of acetocarmine-stained pollen grains in the F_1 hybrid and the parental species. Data; *J. curcas*, *J. integerrima* and F_1-4 are means 87.5% ± 2.7% (n = 1127 pollen grains), 93.8% ± 0.3% (n = 1083 pollen grains), 48.4% ± 6.4% (n = 680 pollen grains), respectively.

cell). We detected 46.5% of aneuploid microspores with 10 to 12 chromosomes (**Table 2**).

Pollen formation in F_1-4

The frequency of pollen grains stained with acetocarmine was 87.5% ± 2.7% (out of 1127 pollen grains) in *J. curcas*, 93.8% ± 0.3% (out of 1083) in *J. integerrima*, and 48.4% ± 6.4% (out of 680) in F_1-4 (**Fig. 2**). Unstained pollen grains were empty. In addition, the size of the pollen grains of F_1-4 varied (**Fig. 2B**), suggesting variability in their DNA content.

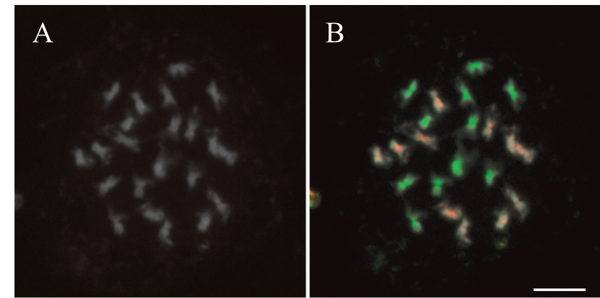


Fig. 3. GISH analysis of parental chromosomes in the F_1 hybrid. (A) DAPI staining. (B) GISH probes clearly show 11 chromosomes derived from *J. curcas* (red) and *J. integerrima* (green). Scale bar = 2.5 µm.

GISH analysis of meiotic metaphase I in F_1-4 and somatic chromosomes in S_1 plants

GISH analysis allowed us to identify the parental chromosomes in hybrid cells (**Fig. 3**). Heterochromatic condensation was observed in the proximal regions of all chromosomes derived from both parents (**Fig. 3A**); GISH signals appeared mainly at the centromeres (**Fig. 3B**), suggesting the presence of species-specific repeats in these regions. GISH signals in the heterochromatic subtelomeric regions of *J. curcas* chromosomes corresponded to JcSat1, whereas the subtelomeric regions of *J. integerrima* lacking heterochromatin showed no prominent GISH signals. GISH analysis also confirmed that bivalents were formed by interspecific pairing between chromosomes of the two species (**Fig. 4**). We also examined whether the paired parent chromosomes underwent reduction division. Aneuploid microspores were removed, and 20 microspores with 11 centromeric foci (haploid chromosome number) were used for GISH analysis to determine the composition of each chromosome (**Fig. 5A**). The average number of *J. curcas* and *J. integerrima* chromosomes per microspore were 5.3 and 5.7, respectively, and did not differ significantly from the expected number (5.5 chromosomes from each parent) by chi-square test ($P > 0.05$); therefore, reduction division occurred in male meiosis.

We also analyzed the chromosome composition of 26 randomly selected S_1 individuals derived from F_1-4 (**Figs. 5B**, **6A–6C**). No aneuploids were observed in any of them, and each S_1 plant had 22 chromosomes in its somatic cells. In contrast to the chromosome composition of microspores (**Fig. 5A**), all S_1 plants (except No. 327) had more

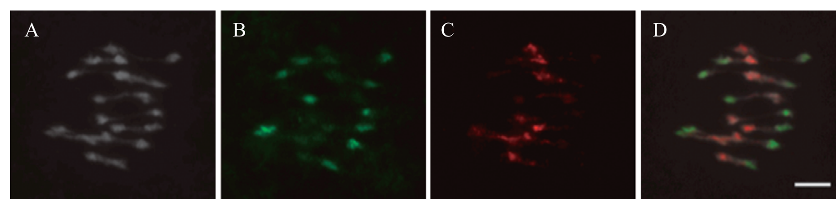
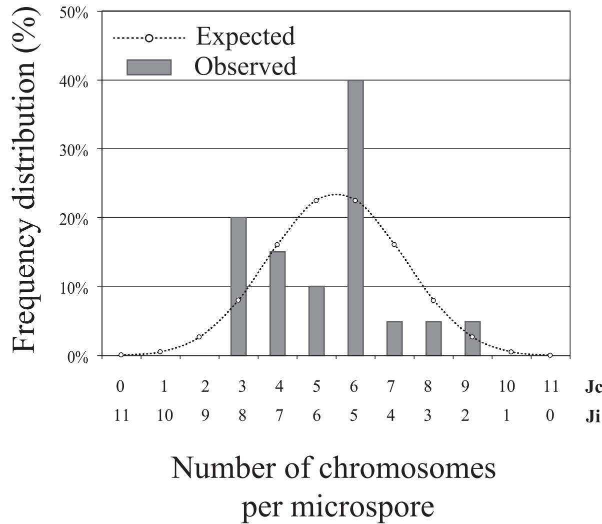


Fig. 4. GISH analysis of meiotic metaphase I in the F_1 hybrid. (A) DAPI staining. (B) *J. integerrima* genomic DNA. (C) *J. curcas* genomic DNA. (D) Merged images shown in A–C. Scale bar = 5 µm.



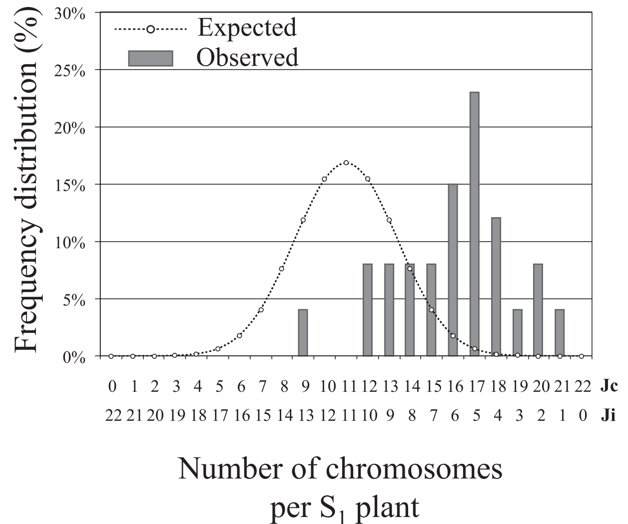


Fig. 5. Expected and observed numbers of parental chromosomes. (A) Microspores. Frequency distribution of the 12 combinations of parental chromosomes showed a random distribution. (B) Twenty-six S_1 plants. Most S_1 plants had more *J. curcas* chromosomes (Jc) than *J. integerrima* chromosomes (Ji). Expected values were calculated assuming a binomial distribution.

chromosomes from *J. curcas* (16.04 per cell on average) than from *J. integerrima* (5.96 per cell on average) (**Fig. 5B**). The average numbers differed significantly from the expected number (11 from each parent) by chi-square test ($P < 0.05$). Plant No. 327 had 9 chromosomes from *J. curcas* and 13 from *J. integerrima* (**Figs. 5B**, **6A**). Thus, preferential transmission of *J. curcas* chromosomes was detected in almost all S_1 plants.

In S_1 plants, we found small *J. curcas* GISH signals at the ends of *J. integerrima* chromosomes (**Fig. 6A–6C**), suggesting translocation between chromosomes of the two species. Because *J. integerrima* chromosomes did not have clear GISH signals at their ends, such mosaic pattern of two GISH signals could not be clearly observed in *J. curcas* chromosomes. Our FISH analysis revealed that *J. integerrima* does not have JcSat1 (manuscript in preparation). Sequential FISH and GISH analysis of plant No. 387 confirmed that three chromosome pairs exchanged JcSat1 between the two species, because JcSat1 signals were irregularly observed at the ends of three *J. integerrima* chromosomes (**Fig. 6D–6E**). A pair of chromosomes with translocation was identified as SAT-chromosomes carrying 45S rDNA (**Fig. 6F**). Translocation of JcSat1 found in their SAT-chromosomes might indicate that these chromosomes were originally homoeologous and exchanged segments by meiotic recombination.

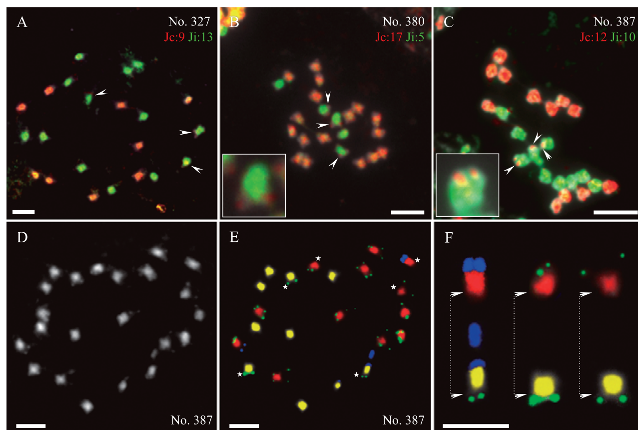


Fig. 6. GISH and FISH/GISH analysis of the somatic chromosomes of S_1 plants ($2n = 22$) derived from self-pollination of the F_1 hybrid. (A) No. 327. (B) No. 380. (C) No. 387. Red: *J. curcas* genomic DNA; green: *J. integerrima* genomic DNA. Arrowheads, chromosome segments of *J. curcas* at the ends of *J. integerrima* chromosomes. Insets: *J. integerrima* chromosomes with GISH signal of *J. curcas*. (D–F) Sequential FISH and GISH analysis of plant No. 387. (D) DAPI-staining. (E) Combined FISH/GISH image. Red: *J. curcas* genomic DNA; yellow: *J. integerrima* genomic DNA, green: JcSat1, blue: 45S rDNA. Asterisks indicate inter-species translocation. (F) A high-magnification image of E. Arrowheads with dash line indicate possible translocations. Scale bars in A–E = 2.5 μm.

Discussion

Chromosome polymorphism in J. curcas *and* J. integerrima

We showed that meiotic chromosomes often formed 11 bivalents in F_1-4. Therefore, the two species had homoeologous chromosome-like. The homoeologous chromosomes showed different distribution of heterochromatin at their ends; however, they could form well-paired pachytene chromosomes. Formation of ring-shaped bivalents (including two chiasmata) (**Fig. 1B**) suggested strong chromosomal (genomic) affinity. Although the distribution of heterochromatic

regions reflected the different structures of the two species, their overall karyotypes probably indicate no large-scale translocations or chromosomal inversions, as indicated by the absence of multivalent and loop structures at meiosis (**Fig. 1A**).

Disordered chromosome orientation on the equatorial plate was observed in meiotic metaphase I in F_1-4 (**Fig. 1C**). Several bivalents showed precocious chromosome segregation (**Fig. 1C**). GISH analysis suggested that it was not premature separation of sister chromatids (Petronczki *et al.* 2003). In an interspecific *Lilium* hybrid, half of the bivalents always disjoin prematurely at metaphase I (Lim *et al.* 2001). The inability of chromosomes to orient on the equatorial plate may be related to inflexible activity of kinetochore and spindle assembly checkpoint. Our observation suggests that the centromeres of *J. integerrima* were distributed at the polar side, whereas the centromeres of *J. curcas* tended to be localized on the equatorial plate (**Fig. 4**). Thus, polymorphism of centromere function may also contribute to the disordered orientation of bivalents in the F_1 hybrid.

We also observed preferential transmission of *J. curcas* chromosomes to S_1 plants (**Fig. 5B**). Because we did not examine an F_1 hybrid and its S_1 progeny from the reciprocal cross, it is not certain whether preferential transmission is affected by cytoplasmic factors. Disordered chromosome orientation on the equatorial plate during meiotic metaphase I in F_1-4 (**Fig. 1C**) suggests that centromere function is a potential cause of preferential transmission. A meiotic drive for distorted segregation was found in *Mimulus* hybrids from polymorphism of their centromeres (Fishman and Willis 2005, Fishman and Saunders 2008).

Use of interspecific hybridization for jatropha improvement

We observed variable karyotypes of S_1 plants (**Figs. 5B**, **6**). Using EST-SSR primers, One *et al.* (2014b) found high genetic variability in an F_2 population derived from an F_1 hybrid of *J. curcas* × *J. integerrima*. Dwarfness (a local *J. integerrima* trait) and erect growth (a *J. curcas* trait) segregated according to Mendelian inheritance in the F_2 population (One *et al.* 2014c). Genetic maps with 11 linkage groups were constructed on the basis of the interspecific cross (Wang *et al.* 2011, Wu *et al.* 2015). Although we observed meiotic errors (**Fig. 1**, **Table 2**) and preferential uniparental chromosome transmission (**Fig. 5B**), they are unlikely to cause severe problems for genetic analysis and construction of genetic maps. Bivalent formation, production of a sufficient number (about half) of normal pollen grains, and generation of S_1 plants with the normal chromosome number ($2n = 22$) may contribute to successful genetic analysis.

The rate of seed formation after self-pollination of the F_1 hybrid is low (Muakrong *et al.* 2014, Parthiban *et al.* 2009, Sujatha and Prabakaran 2003), and thus it is not easy to advance selection to the next generation. However, backcrossing to produce BC_1F_1 was successful, although only when *J. curcas* was used as the female parent (Muakrong *et al.* 2014). We have screened a dwarf jatropha plant with *J. integerrima* chromosome among the backcrossing plants. Thus, the backcross could easily be used for forming population and serving as good sources of variability for genetic analysis and genetic improvement of jatropha. Interspecific translocation found in this study might be useful to produce introgression lines with valuable agronomical traits of *J. integerrima* such as seed yield and oil content (One *et al.* 2014a, Parthiban *et al.* 2009), dwarfness and erect growth (One *et al.* 2014c), woody biomass (Muakrong *et al.* 2013), and ornamental qualities (Muakrong *et al.* 2014, Sujatha and Prabakaran 2003), for creation of commercial jatropha varieties.

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