

Karyotyping of *Brassica oleracea* L. based on rDNA and *Cot-1* DNA fluorescence *in situ* hybridization

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Abstract To explore an effective and reliable karyotyping method in *Brassica* crop plants, *Cot-1* DNA was isolated from *Brassica oleracea* genome, labeled as probe with Biotin-Nick Translation Mix kit, *in situ* hybridized to mitotic spreads, and where specific fluorescent bands showed on each chromosome pair. 25S and 5S rDNA were labeled as probes with DIG-Nick Translation Mix kit and Biotin-Nick Translation Mix kit, respectively, *in situ* hybridized to mitotic preparations, where 25S rDNA could be detected on two chromosome pairs and 5S rDNA on only one. *Cot-1* DNA contains rDNA and chromosome sites identity between *Cot-1* DNA and 25S rDNA was determined by dual-colour fluorescence *in situ* hybridization. All these showed that the karyotyping technique based on a combination of rDNA and *Cot-1* DNA chromosome landmarks is superior to all but one. A more exact karyotype of *B. oleracea* has been analyzed based on a combination of rDNA sites, *Cot-1* DNA fluorescent bands, chromosome lengths and arm ratios.

Keywords *Brassica oleracea* L., rDNA, *Cot-1* DNA, fluorescence *in situ* hybridization, karyotyping

1 Introduction

Recently, a genome project of the genus *Brassica* represented by *Brassica rapa* L. (A genome) (Yang et al., 2005) and *Brassica oleracea* L. (C genome) (Ayele et al., 2005) has been in progress. This is followed an upsurge in research for plant genomes. Oil crops and vegetable crops, as the important crops in the genus *Brassica*, have been becoming

more and more important in people's lives. As another kind of important vegetable crop other than *Brassica rapa*, *Brassica oleracea* includes all kinds of ecotypes and many kinds of artificial hybridizing breeds. Whether from the evolutionary point or breeding point, all kinds of cytogenic research should be done. However, it is very difficult to exactly identify all the chromosomes because the chromosomes of *Brassica oleracea* are small, and their lengths and arm ratios are very similar.

The current research on the identification of the chromosomes and the karyotyping of *Brassica oleracea* are the basis of the 45S (or 25S) and 5S rDNA numbers and sites in the chromosomes (Snowdon et al., 1997; Hasterok et al., 2001). 45S rDNA loca were located on two chromosome pairs of *Brassica oleracea* L., where the repetitive copy number of 45S rDNA core sequences on the short arms of the seventh chromosome pair was much higher, followed by the fourth (Cheng et al., 1995; Fukui et al., 1998). In some breeds, 45S rDNA loca have also been detected on the short arms of the chromosome pair 2; presently, 5S rDNA loca have only been detected on the long arms of the chromosome pair 2, but the repetitive copy number of the core sequences was much lower. Thus, only 3 chromosome pairs could be identified from nine chromosome pairs with rDNA. On the other hand, six chromosome pairs could be identified from ten chromosome pairs in *Brassica rapa*, another diploid plant, of which 45S rDNA loca existed on five chromosome pairs, 5S rDNA loca existed on three chromosome pairs and no rDNA existed on four chromosome pairs. In other words, six chromosome pairs can be identified (Koo et al., 2004), and more karyotype studies of *Brassica rapa* have been done compared to *Brassica oleracea* L. Kamisugi et al. (1998) identified five chromosome pairs of *Brassica oleracea* L. with two cDNA probes and rDNA probes; Armstrong et al. (1998) identified only five chromosome pairs with three repetitive sequences.

Hence, a new chromosome identification technique is needed to be explored to exactly identify all the nine chromosome pairs of *Brassica oleracea*.

Cot-1 DNA is enriched with highly and moderately repetitive DNA sequences. In many plant species of economic importance, repeated sequences comprise a large proportion of genome (Flavell et al., 1974; McCouch and Tanksley, 1991). These DNA sequences are distributed throughout the whole genome of the eukaryotes. Hence, it is conceivable to band and identify individual pairs from all the chromosome pairs of *Brassica oleracea* with *Cot-1* DNA.

2 Materials and methods

2.1 Plant materials

Brassica oleracea L. Heiyexiaopingtou, provided by academician Fang Zhiyuan of the Institute of Vegetable and Flowers, Chinese Academy of Agricultural Sciences, was used in the present study. The root tips of germinated seeds in room temperature were used for chromosome preparation and young plant leaves were used for genomic DNA and *Cot-1* DNA extraction.

2.2 Chromosome preparation

The chromosome preparation method was developed using the technique described in the references (Wei et al., 2000; Wei et al., 2003) but with some modifications.

2.3 Genomic DNA extraction

The extraction of the genomic DNA was performed according to the procedure described by Doyle and Doyle (1988).

2.4 Genomic DNA shearing and *Cot-1* DNA isolation

Genomic DNA shearing and *Cot-1* DNA isolation were performed as described by Zwick et al. (1997) and Wei et al. (2005). In brief, genomic DNA was diluted to a concentration of 300 mg/L in 0.3 mol/L NaCl with 5 mol/L NaCl, and each 0.5 mL sample was aliquoted into a 1.5-mL lightly Eppendorf tube, and was then autoclaved for 10 min to make 100–1000-bp DNA fragments. The sample tube was then put on ice. The DNA was denatured by placing the tube in bath water of 95°C for 10 min. The tube was removed and cooled by being swirled in ice water for 10 s, before being placed in bath water of 65°C. After it was annealed, the tube was removed from the water of 65°C and was put into ice water for 2 min, after which an appropriate amount of 10 × S1 buffer was added and the sample was mixed thoroughly. The appropriate amount of S1 enzyme (1 U/μg DNA) was added again and the solution was mixed thoroughly and then again gently. Immediately, the tube was placed in bath water of 37°C for 8 min. The reaction was stopped by immediate phenol extraction using

equal volumes of Tris-equilibrated phenol once, and then was extracted again using chloroform: Isoamyl alcohol (24:1) twice. The DNA was deposited by 2.5 volumes of absolute ethanol from the solution in –20°C. The centrifugal *Cot-1* DNA was air-dried, and the steps detailed above were performed until the *Cot-1* DNA was resuspended in TE. Samples were stored in –20°C after quantitative analysis.

2.5 Labeling of DNA, FISH and detection of the signals

Briefly, *Cot-1* DNA of *Brassica oleracea* and 5S rDNA probes were labeled with biotin-11-dUTP using a commercially available kit, the Biotin-Nick Translation Mix (No. 11745824910; Roche), and 25S rDNA probe was labeled with DIG-11-dUTP using the DIG-Nick Translation Mix (No. 11745816910). One probe was used in single-color FISH, and two probes were mixed in dual-color FISH of *Cot-1* DNA and 25S rDNA. FISH was performed according to the procedure described by Wei et al. (2003) but with some modifications. Chromosome preparations were pretreated with 100 mg/L RNase (in 2 × SSC) at 37°C for 1 h. Chromosomal DNA was then denatured by immersing the slide in 70% formamide at 70°C for 2–3 min. After dehydrating the preparation in an ice-cold 70%, 95%, and 100% ethanol series and air drying, the probe cocktail (5 mg/L labeled probe DNA, 0.5 g/L sheared salmon sperm DNA, 10% dextran sulphate, 50% deionized formamide, 0.1% sodium dodecyl sulfate (SDS), and 2 × SSC) was denatured in boiled water for 10 min before it was immediately put on ice for 10 min, and 60 μL denatured probe cocktail was added to the slide and hybridization was performed at 37°C overnight after denaturing in 90°C for 10 min. Detection of signals was performed according to the procedure described by Wei et al. (2005). Post-hybridization washes included a stringent wash in 20% formamide, a wash in 2 × SSC and then 0.1 × SSC at 42°C for 10 min after removing slightly the cover glass. In single-color FISH, the probe labeled with biotin was detected with streptavidin–Cy3 (Amersham), and the probe labeled with DIG was detected with Anti-Digoxigenin-Fluorescein (Roche); in dual-color FISH, the above isolated procedures were completed in sequence. The detecting solution per slide was prepared with 1 μL fluorescence reagent added into 50 μL PBS/1%BSA. After the detecting solution was added onto the slide and the cover glass was covered, the slides were kept at 37°C for 30–60 min. Then the slides were immersed with PBS thrice, once for 5 min. Slides were counterstained with 2 mg/L 4', 6'-diamidino-2-phenylindole (DAPI) and examined under a Leica DM IRB fluorescence microscope equipped with the DFC300 CCD software.

3 Results

3.1 Shearing of genomic DNA and isolation of *Cot-1* DNA

The size of the genomic DNA obtained from *Brassica oleracea* is more than 20 kb, and after being autoclaved for

10 min, most DNA fragments obtained are within the desired size range of 100–1000 bp with only a few fragments sized more than 1 kb. The size of almost all the *Cot-1* DNA obtained is less than 1 kb (Fig. 1).

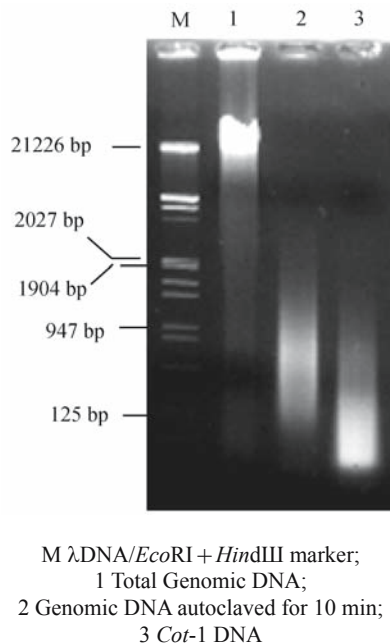


Fig. 1 Preparation of *Cot-1* DNA of *B. oleracea*

3.2 Fluorescence *in situ* hybridization

25S rDNA loca were detected on the two chromosome pairs of *Brassica oleracea*, and one of them has stronger signals (Fig. 2(a)); on the other hand 5S rDNA were detected on only one of the pairs and its signals were poorer (Fig. 2(b)). The individual chromosome pairs showed specific, strong and steady banding patterns of *Cot-1* DNA. Among them, only one chromosome pair has stronger signals (Fig. 2(c)), which were similar with the stronger signals of the hybridized 25S rDNA (Fig. 2(d)). The results of the dual-color FISH further showed the similarities of the positions of the hybridized 25S rDNA and the hybridized *Cot-1* DNA (Fig. 2(e)).

3.3 Karyotyping

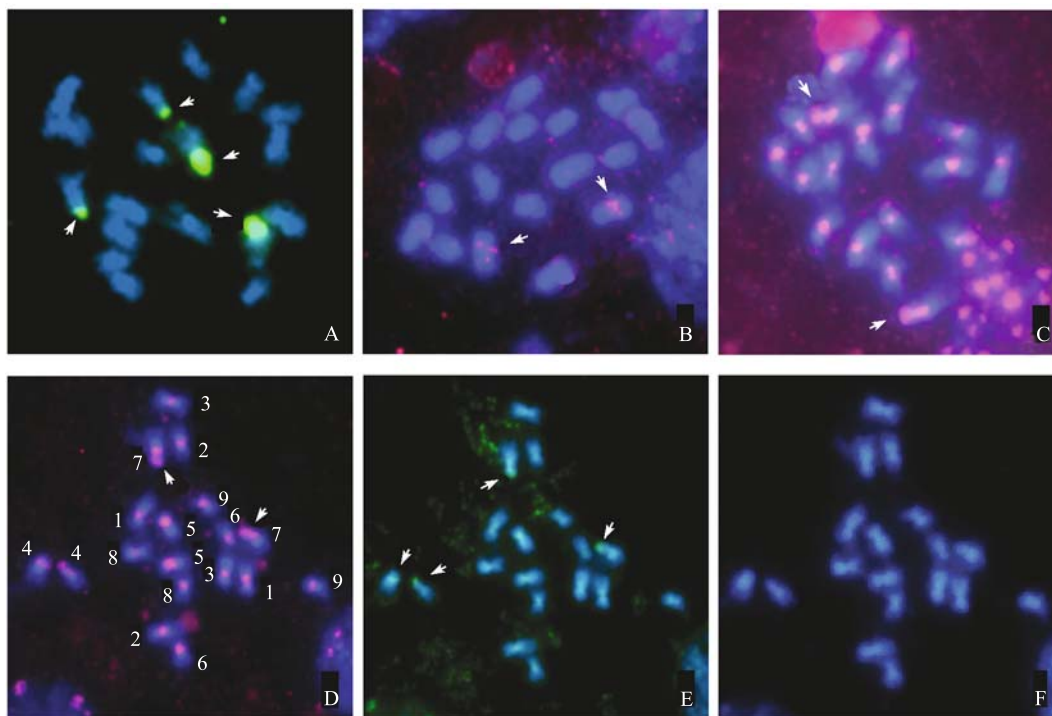
Karyotyping of *B. oleracea* was performed according to the method published by Howell et al. (Howell et al., 2002), and the chromosome pairs were arranged from the longest to the shortest (length of satellite excluded). Bands of *Cot-1* DNA were located on the long arms of the chromosome pairs 3 and 9, the short arms of the chromosome pairs 4, 5, 7 and 8, and the pericentromeric sites of the chromosome pairs 1, 2 and 6 (Fig. 3).

4 Discussion

Cot-1 DNA contains all highly and moderately repetitive DNA sequences and rDNA is one genus of the moderately

repetitive DNA sequences. Thus, the *Cot-1* DNA obtained from the genome DNA of a species certainly contains rDNA. In theory, wherever rDNA signals are detected, *Cot-1* DNA can be detected. When 25S rDNA or 5S rDNA were hybridized individually, 25S rDNA were located on the two chromosome pairs, where one chromosome pair has stronger signals (Fig. 2(a)); and 5S rDNA were located on only one chromosome pair and its signals were poorer (Fig. 2(b)). The above results are identical with the reported results (Cheng et al., 1995; Fukui et al., 1998), but the 25S rDNA sites detected on the chromosome pairs have one chromosome pair less than the results of Howell et al. (2002), which could be explained by the inability of the copies of 25S rDNA to be detected or the rDNA loca's unsteadiness among the species (Weiss et al., 2000). Otherwise, *Cot-1* DNA FISH could successfully show bands on the individual chromosome pairs of *B. oleracea*, where one of the chromosome pairs has stronger signals similar with that of the signals of the hybridized 25S rDNA hybridized (Fig. 2(c)). The dual-color FISH further proves the identity of the hybridization sites of *Cot-1* DNA and 25S rDNA (Fig. 2(c)–(e)). All these showed that the karyotyping technique based on a combination of rDNA and *Cot-1* DNA chromosome landmarks was more credible than that on the technique using only one of them. Based on the length and sites of *Cot-1* DNA and rDNA hybridization bands, the reported karyotyping result (Howell et al., 2002) and the length and arm ratio of chromosomes on all the nine chromosome pairs of *B. oleracea* were exactly identical. The idiograms of *B. oleracea*, for all the chromosomes that had the hybridization bands, were obtained for the first time (Fig. 3). This karyotyping technique was more exact, simpler and quicker in identifying all the chromosomes of certain species than any other technique.

In the present study, the individual phase chromosome pair of *B. oleracea* could be stably banded by *Cot-1* DNA. Moreover, the specific and analogous banding patterns were shown on two members of the homologous chromosome pairs, and this indicated that the homologous chromosomes possessed homologous or similar repetitive DNA, whereas non-homologous chromosome pairs did not. These were the foundation of karyotyping with *Cot-1* DNA. *Cot-1* DNA contains all the highly and moderately repetitive DNA sequences of the genome DNA, so it must distributed individually or in tandem over the whole genome of *B. oleracea*. This provides the possibility that the individual chromosomes of *B. oleracea* can be banded by *Cot-1* DNA, and the hybridization band can be located on the centromeric, the long arm, or the short arm of the chromosome. This point was sufficiently tested in this paper. Hasterok and his coworkers found that the hybridization signals were mainly located on the pericentromeric sites when the C genome DNA probes of the genus *Brassica* were hybridized to the mitotic metaphase of *B. oleracea*. The results we derived didn't absolutely agree with Hasterok et al. (2005) because the hybridization signals contained not only *Cot-1* DNA but also non-*Cot-1* DNA. Also the genome of *B. oleracea* was very small and the repetitive sequences'



(a) Result of 25S rDNA hybridized, the signals were detected on the short arms of the chromosome pairs 4 and 7, and the signals on the chromosome pair 7 were much stronger; (b) Result of 5S rDNA hybridized, the signals were detected on the only chromosome pair 2, and the signals were poorer; Dual-colour fluorescence *in situ* hybridization result of *Cot-1* DNA and 25S rDNA; (c) Result of *Cot-1* DNA hybridized, and the strong signals were detected on all chromosome pairs; (d) Result of 25S rDNA hybridized, the signals were detected on the chromosome pairs 4 and 7; (e) Folded photo of C and D; F: DAPI stained of metaphase corresponding to the spread in E.

Fig. 2 FISH of 25S rDNA, 5S rDNA and *Cot-1* DNA probes to the metaphase chromosomes of *B. oleracea*

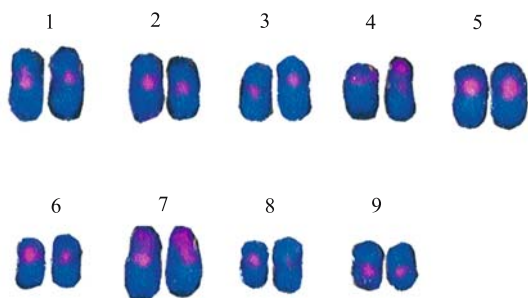


Fig. 3 Ideogram of *B. oleracea* somatic chromosomes based on the metaphase plate shown in Fig. 2E

Notes: Red bands of *Cot-1* DNA were detected on the long arms of the chromosome pairs 3 and 9, the short arms of the chromosome pairs 4, 5, 7 and 8, and the pericentromeric sites of the chromosome pairs 1, 2 and 6.

proportion was very low so that the small proportional repetitive sequences in the probes were difficult to be detected. However, since all of *Cot-1* DNA probes were the repetitive sequences, the developed hybridization efficiency made the repetitive sequences on the arm of chromosome easily detected. Otherwise, the distribution difference of the repetitive sequences among the different species needed to be further explored. Although all the nine chromosome pairs were identified based on FISH of *Cot-1* DNA and all the nine chromosomes were arranged from the longest to the shortest

(Fig. 3), it would be difficult to judge whether *Cot-1* DNA bands existed on the long arm, the short arm or the centromeric sites of the chromosomes because the emanative fluorescence signals existing on the pericentromeric sites covered the centromeres. This problem would be preliminarily solved by the confirmation of the *Cot-1* DNA hybridization bands sites based on the signal center, or the more exact analysis made on the stretched-out chromosomes in meiotic pachynema. This is our next study.

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