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Quantitative karyotyping of three diploid *Brassica* species by imaging methods and localization of 45*s* rDNA loci on the identified chromosomes

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Abstract Chromosomes of the three diploid *Brassica* species, *B. rapa* (AA), *B. nigra* (BB) and *B. oleracea* (CC), were identified based on their morphological characteristics, especially on the condensation pattern appearing at the somatic pro-metaphase stage. The morphological features of the pro-metaphase chromosomes of the three *Brassica* spp. were quantified by imaging methods using chromosome image analyzing system II (CHIAS 2). As a result, quantitative chromosome maps or idiograms of the three diploid *Brassica* spp. were developed. The fluorescence in situ hybridization (FISH) method revealed the location of 45*s* rDNA (the 26*s*-5.8*s*-18*s* ribosomal RNA gene cluster) on the chromosomes involved. The number of 45*s* rDNA loci in the *B. rapa*, *B. nigra* and *B. oleracea* are five, three and two, respectively. The loci detected were systematically mapped on the idiograms of the three *Brassica* spp.

Key words *Brassica rapa* · *Brassica nigra* · *Brassica oleracea* · Quantitative chromosome map · Idiogram · FISH · 45*s* rDNA · Systematic mapping

Introduction

The genus *Brassica* consists of three pivotal diploid species with respective A, B, and C genomes (U 1935), as well as and several tetraploid and hexaploid species which are naturally or artificially generated by the combination of these three genomes (Karpechenko 1922; Mizushima 1980). *Brassica rapa* (2n = 20), *B. nigra* (2n = 16) and *B. oleracea* (2n = 18) represent A, B and C genome diploid species, respectively. The chromosome number of these three species were determined more than 75 years ago (Karpechenko 1922). Since then a great number of trials have been attempted to identify individual chromosomes based on their morphology. All such endeavors to identify each *Brassica* chromosome have failed regardless of the species analyzed when metaphase chromosomes were employed. Because the plant materials were pre-treated by a conventional cytological protocol, the pre-treatment condenses the chromosomes evenly to rod and/or dot-like shapes, which makes their identification almost impossible.

Fukui and Mukai (1988) found that in *Atriplex rosea* each chromosome showed a characteristic uneven condensation pattern at the pro-metaphase stage. This led to the definition of a new image parameter, CP, or density profile, along the mid-rib of a chromatid of a chromosome, which numerically represents the condensation pattern (Fukui 1989). Fukui and Iijima (1991) identified all the rice chromosomes based on this pattern and developed a quantitative chromosome map objectively based on the CP measured by imaging methods. The suitability of the condensation pattern in the identification of small chromosomes at pro-metaphase has also been proven statistically (Kamisugi et al. 1993). *Brassica* chromosomes have also been identified by using the condensation pattern (Fukui et al. 1993; Cheng et al. 1995 a; Heneen et al. 1995). The C-banding method has also been employed to identify *Brassica* chromosomes (Olin-Fatih and Heneen 1992; Olin-Fatih 1994, 1996).

In the present paper we report on the development of quantitative chromosome maps or idiograms of the three *Brassica* spp. by imaging methods using visually

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identified pro-metaphase chromosomes. We have also physically detected 45*s* rDNA (the 26S-5.8S-18S ribosomal RNA gene cluster) loci on the *Brassica* chromosomes by fluorescence in situ hybridization (FISH) and mapped the detected signals onto the idiograms. Systematic mapping (Ohmido et al. submitted) or the mapping of genes on identified chromosomes allows a unification between cytological maps and genetic maps.

Materials and methods

Plant materials

Seeds of *B. rapa* cv Nozaki 2 (AA, 2n = 20, $x = 10$), *B. nigra* var. Ni143 (BB, 2n = 16, $x = 8$) and *B. oleracea* cv Fujiwase (CC, 2n = 18, $x = 9$) were used. The seeds were sown on moist filter paper in Petri dishes and germinated at 20°C. Root tips were excised when the roots reached 2–3 cm in length and then fixed immediately in fresh fixative (ethanol : acetic acid 3 : 1) without pretreatment.

Cytology

Chromosome samples were prepared by the EMA (enzymatic maceration/air-drying) method (Iijima and Fukui 1991; Fukui 1996) with minor modifications. The enzymatic mixtures, consisting of 4% Cellulase Onozuka RS, 1% Pectolyase Y-23 or 2% Cellulase Onozuka RS, 0.3% Pectolyase Y-23 and 0.75% Macerozyme R200, were both adjusted to pH 4.2. Then 20–40 min of maceration was applied based on the condition of the root tips. The air-dried samples were Giemsa-stained and examined by a microscope (Axiophot, Zeiss). Chromosome samples without overlapping and distorted chromosomes were selected and photographed (Neopan F, ISO 32, Fuji) under the two previously described different conditions of normal exposure and under-exposure for image analysis (Fukui and Mukai 1988; Iijima and Fukui 1991). After identification of each *Brassica* chromosome, the chromosomes were de-stained with 70% ethanol and subjected to FISH.

Image analysis

Five, ten and eight pro-metaphase chromosomal spreads of *B. rapa*, *B. nigra* and *B. oleracea*, respectively, were selected and used for image analysis. All the chromosomes were identified visually prior to image analysis. The chromosome images selected were digitally captured via a CCD camera using the chromosome image analyzing system II, CHIAS 2 (Fukui and Nakayama 1996). All the imaging procedures for the analysis of *Brassica* chromosomes have already been described in detail (Nakayama and Fukui 1997). Briefly, light distortion caused by illumination was adjusted. Then an image parameter, CP, or a density profile along a chromatid was obtained and length of the chromosomal arms was measured digitally. Differences of gray-value distribution, caused by staining, photographing and developing procedures among the chromosomal plates, were standardized. Gray values between homologous chromosomes were also adjusted. Chromosomal lengths and CPs were both averaged to obtain the standard CP or graygram. Finally, the standard CPs were delimited at two gray-value thresholds to develop a quantitative chromosome map or idiogram.

Fluorescence in situ hybridization (FISH)

The locations of 45*s* rDNA loci were detected on the chromosomes prepared by the EMA method. The probe DNA was a biotin-labelled 3.8-kb nucleotide sequence which covers a part of the coding region of 26*s* and 18*s* rRNA and the whole region of 5.8*s* rRNA (Sano and Sano 1990). The biotinylated probe was in situ hybridized to chromosomal rDNA and detected with FITC (fluorescein isothiocyanate)-conjugated avidin by a fluorescence microscope. The FISH procedure was followed the method described by Fukui et al. (1994) and Ohmido and Fukui (1995) with the minor modification of omitting most of the post-treatments except for the RNase treatment. Fluorescent signals of the 45*s* rDNA loci were digitally captured by a CCD camera (HCC-3600P, Floubel, Tokyo). The signal and chromosome images were merged into single images by using CHIAS 2 and the composite images were photographed by a color image recorder (CIR-300, Avionics, Tokyo).

Results

Figure 1 shows nuclei and chromosome complements at both the G_1 and pro-metaphase of the three *Brassica* species. It is obvious that the daughter nuclei of the

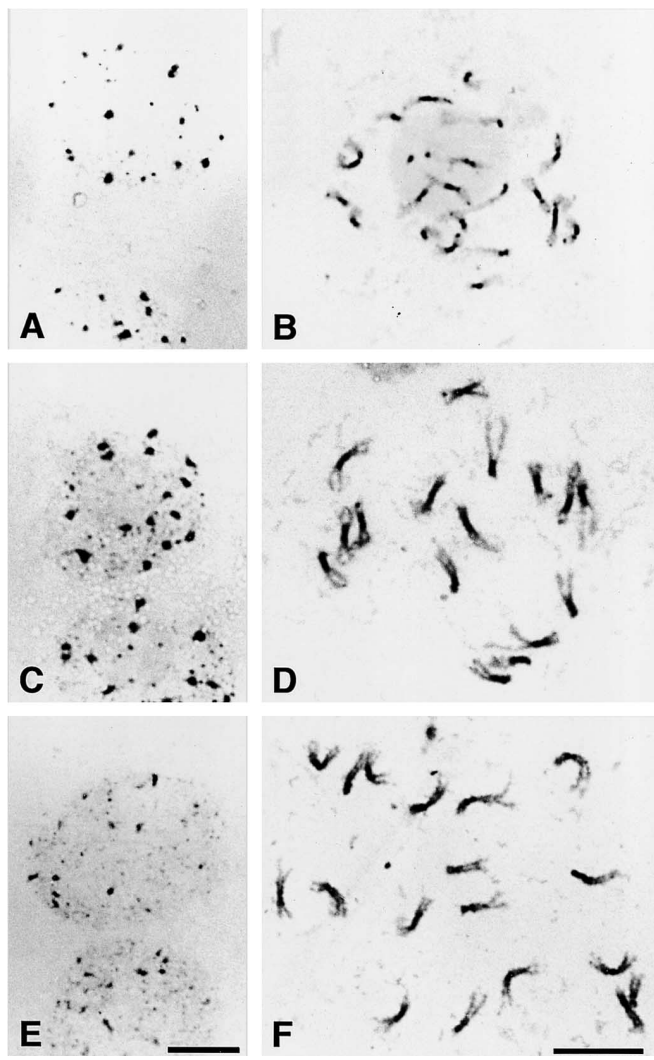


Fig. 1A–E Cell nuclei at the G_1 stage (**A, C, E**) and pro-metaphase chromosomes (**B, D, F**) of the three *Brassica* species. **A, B** *B. rapa*, **C, D** *B. nigra*, **E, F** *B. oleracea*. The scale bars indicate 10 μm

three *Brassica* spp. have prominent heterochromatic blocks at interphase. These heterochromatic blocks correspond to centromeric condensed regions of the chromosomes, which are clearly visualized at the pro-metaphase stage. The number of major blocks within a nucleus also corresponded to the number of chromosomes, although there are sometimes differences between the number of heterochromatic blocks and the chromosome number. The sizes of the heterochromatic blocks were also variable according to the stage in the cell cycle. It was observed that heterochromatic blocks of *B. nigra* were larger than those of the other two species. It was characteristic of *B. rapa* that some heterochromatic regions on the long arms were larger than those on the short arms.

All the chromosomes of the three *Brassica* spp. demonstrated clear heterochromatic blocks in the proximal regions of all the chromosomes at the pro-metaphase stage. Several chromosomes also have faint, unstable and small condensations (FUSCs) at the end of the chromosomal arm. Based on the karyotype, all the chromosomes within a complement were first classified into three major types by visual inspection. Then each chromosome was identified within a given type by considering their condensation pattern, FUSCs, and the other morphological characteristics already described in the case of rice chromosomes (Iijima et al. 1991). All the chromosomes were thus identified by visual inspection under the microscope and subjected to image analysis for the development quantitative chromosome maps as well as by FISH experiments for physical mapping of the 45*s* rDNA loci.

Figure 2 shows the 45*s* rDNA loci on the chromosomes of the three *Brassica* species detected by FISH. All the chromosomes were identified prior to FISH based on their condensation pattern. The sites of the 45*s* rDNA loci were classified into two groups regardless of their fluorescence intensity. One group includes the sites detected at the end of short arms and the other includes those detected at the interstitial regions of the chromosomes, which were observed only in *B. rapa*. Two pairs of intense and three pairs of weak fluorescence signals were clearly observed in *B. rapa*. Among five loci, four were localized at the interstitial regions and one is localized at the end of the short arm of a chromosome. One pair of large and two pairs of small signals were detected in *B. nigra*. All three loci were mapped at the end of the short arms of the chromosomes. Two pairs of medium-sized signals were observed at the end of the short arms of *B. oleracea* chromosomes.

Figure 3 shows the representative karyotype, the graygram, the false-color representation of the graygram, and the quantitative chromosome map or idiogram of the three *Brassica* species arranged in length order. Red solid arrowheads indicate the location of the 45*s* rDNA loci. The idiogram represents the morphological information from all the chromosomes of the three *Brassica* spp. Although the overall chromosomal characteristics are similar within the three species demonstrating prominent condensed regions at the centromeres, species-dependent characteristics were also detected. The most prominent species-specific characteristics that appear consistently are the relative size of the condensed regions at the centromeric regions. In the chromosomes of *B. rapa*, the larger heterochromatic regions usually occur at the proximal region of the long arms as shown in the Fig. 3. In the case of *B. oleracea*, the opposite tendency is observed in some chromosomes. The chromosomes of *B. nigra* have an almost even size of the heterochromatic blocks on both arms. The 45*s* rDNA loci are distributed on the five chromosomes of *B. rapa*. The single 45*s* rDNA locus in *B. rapa* locates at the end of a short arm. All the *B. rapa* chromosomes that have the larger heterochromatic block on the long-arm proximal region have a 45*s* rDNA locus within the heterochromatic region. *B. nigra* and *B. oleracea* carry three and two loci at the ends of their short arms.

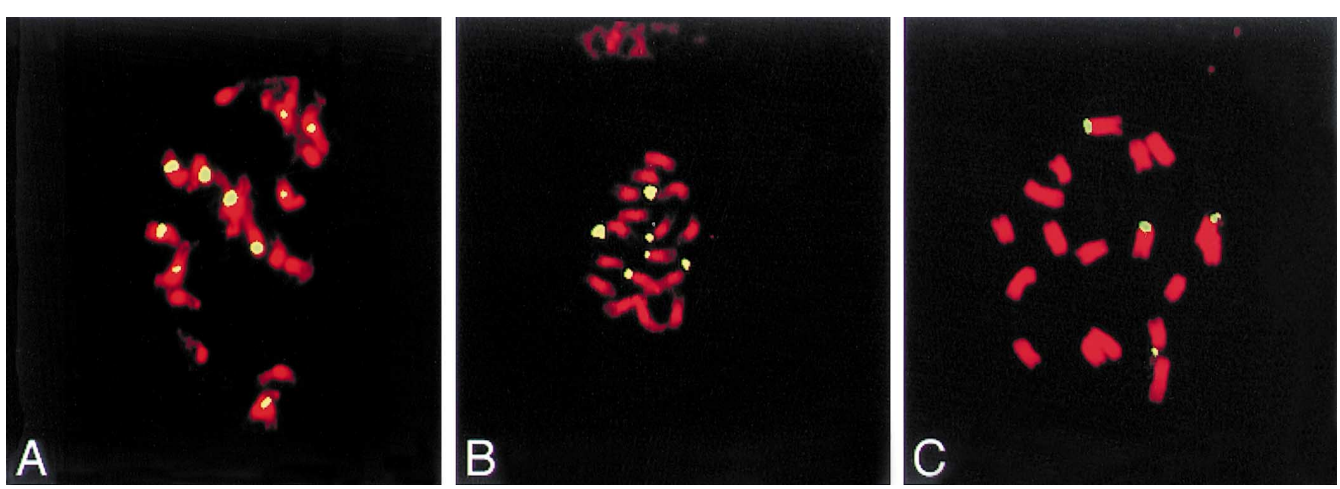
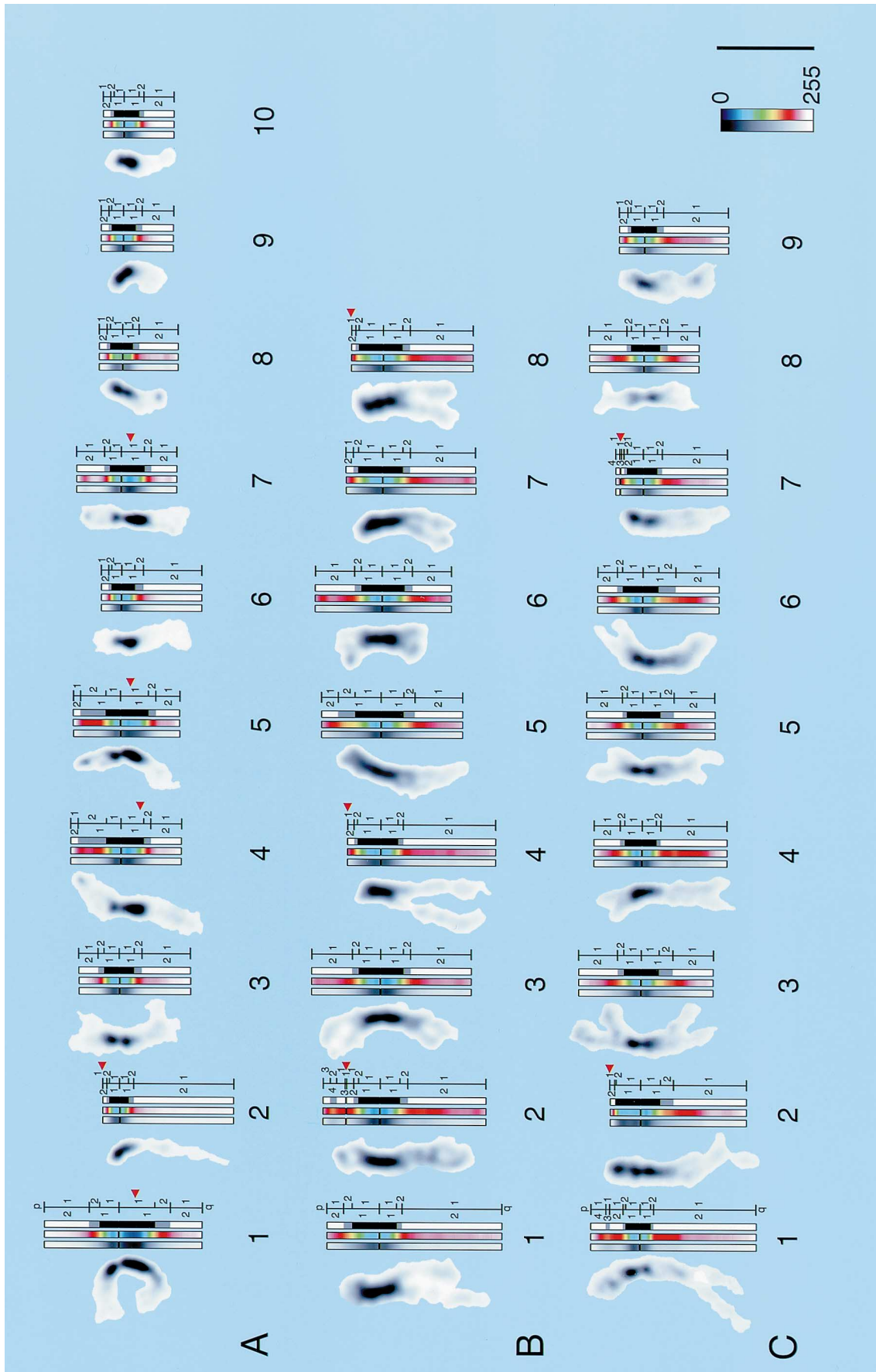


Fig. 2A–C FISH of the three *Brassica* spp. using 45*s* rDNA as a probe. **A** *B. rapa*, **B** *B. nigra*, **C** *B. oleracea*


A
B
C
1
2
3
4
5
6
7
8
9
10
0
255

Discussion

Comparative characteristics of the condensation patterns observed in the *Brassica* species

The condensation patterns observed at the pro-metaphase stage of the *Brassica* spp. were similar to those in the chromosomes of rice (Iijima et al. 1991), soybean (Yanagisawa et al. 1991), sugarcane (Ha et al. submitted), etc. Although a comparable condensation pattern usually appears in plant chromosome with small sizes (Fukui 1996), the degree of condensation is much more pronounced in *Brassica* than the other species, as typically exemplified by the nuclei and chromosomes in Fig. 1, due to the volume of the heterochromatin throughout the cell cycle. The preliminary results of the identification of *Brassica* chromosomes based on the condensation pattern were reported by Fukui et al. (1993).

The results currently obtained are consistent with these recently reported by Cheng et al. (1995 a). In *B. rapa*, the median-type chromosome 1, the two submedian-type chromosomes 5 and 8, and the two subterminal chromosomes 9 and 10 according to Cheng et al. (1995 a) correspond to chromosome 1, 3, 8, 2 and 10, according to length order, respectively. Chromosomes 2, 3, 4, 7 and 8 of Cheng et al. (1995 a) may be chromosomes 5, 4, 7, 9 and 10 as currently designated. In the case of *B. oleracea*, chromosomes 1, 2, 3, 4, 7, 8 and 9 (Cheng et al. 1995 a) correspond to chromosomes 3, 5, 8, 1, 9, 7 and 2. Chromosomes 5 and 6 in Cheng et al. (1995 a) may be chromosomes 4 and 6 in terms of length order. However, matching of the chromosomes was sometimes difficult because of the brief description of chromosomal characteristics and the difference of the material employed in the two studies.

The chromosomes of the three *Brassica* species show prominent heterochromatic blocks at the proximal regions. Because these blocks are observed at the interphase stage and the number of the major heterochromatic condensation sites corresponds to the chromosome number of the species, the condensed regions could reasonably be regarded as constitutive heterochromatin. Harrison and Heslop-Harrison (1995) isolated the 342-bp and 350-bp repetitive nucleotide sequences from *B. oleracea* and *B. campestris*, respectively. Their FISH experiment revealed that the sequences localized at the centromeric regions only. Although there is no report on *B. nigra*, it is quite probable that related sequences also exist at the centromeric regions in *B. nigra* considering the similar heterochromatic patterns observed both in the interphase nuclei and pro-metaphase chromosomes.

Fig. 3 Idiograms of the chromosomes of the three *Brassica* species. **A** *B. rapa*, **B** *B. nigra*, **C** *B. oleracea*. The scale bar indicates 5 μm

Localization of 45*s* rDNA in the *Brassica* chromosomes

FISH experiments revealed five, three and two 45*s* rDNA loci in *B. rapa*, *B. nigra* and *B. oleracea*, respectively, as shown in Fig. 2. The number of 45*s* rDNA loci in *B. rapa* is consistent with the result obtained by Maluszynska and Heslop-Harrison (1993). On the other hand, Maluszynska and Heslop-Harrison (1993) detected two and three 45*s* rDNA loci in *B. nigra* and *B. oleracea*. Cheng et al. (1995 b) reported two 45*s* rDNA loci in *B. oleracea*, which is consistent with the results currently obtained. The morphological characteristics described above and the results from FISH using *B. napus* (Kamisugi et al. submitted) supported the finding that the 45*s* rDNA number of *B. rapa* and *B. oleracea* was five and two in the material currently used. The difference in the number of 45*s* rDNAs may reflect a differentiation of *Brassica* chromosomes within a species, which has been shown by a studies on the segregation of rDNA RFLP markers (Kianian and Quiros 1992 a, b). Deleseny et al. (1990) and McGrath et al. (1990) also reported the presence of two and three 45*s* rDNA loci in *B. oleracea* by analyses using molecular markers.

It is interesting that only *B. rapa* carries four chromosomes with a 45*s* rDNA locus at the long-arm proximal region among the ten chromosomes within the haploid complement. It is reasonable to assume that these interstitial 45*s* rDNA loci are transcriptionally inactive because they locate in the middle of the heterochromatic region, as shown in Fig. 3. This may explain the specific abundance of the 45*s* rDNA loci in *B. rapa* compared with the other two species *B. nigra* and *B. oleracea*, which have three and two 45*s* rDNA loci, respectively. Because the localization of 45*s* rDNA reflects the larger size of the long-arm proximal heterochromatin, it should also be pointed out that the *B. rapa* chromosomes with the interstitial 45*s* rDNA can be used as a marker chromosome indicating a *B. rapa* origin for amphidiploid species consisting of A-genome chromosomes, such as *B. juncea* (2n = 36, AABB) and *B. napus* (2n = 38, AACC). In fact, four pairs of chromosomes with an interstitial 45*s* rDNA site were identified as of *rapa*-origin by the analysis of the condensation pattern in *B. napus* (Kamisugi et al. submitted). The localization of the 45*s* rDNA loci at interstitial regions may thus provide an insight into the origin and differentiation of the diploid *Brassica* species.

Systematic mapping in FISH

Quantitative chromosome maps are essential for localizing genes and nucleotide sequences systematically to specific regions on the chromosomes. The identification of chromosomes, and even chromosomal regions, prior to FISH would offer a basis for a practical and precise physical mapping. Systematic mapping of genes would

also provide essential information for the unification of linkage or genetic maps and cytological or chromosome maps. The orientation of RFLP maps can be confirmed by the systematic mapping of unique nucleotide sequences that have been identified on the RFLP maps in rice (Ohmido et al. submitted).

Quantitative chromosome maps themselves are the essential basic information not only for the genome project, but also in a variety of biological fields such as evolutionary, phylogenetic, and taxonomic studies. Unification of the linkage or genetic maps and the cytological or chromosome maps would provide a comprehensive whole view of the genetic constitution of plant genomes.

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References

Cheng BF, Heneen WK, Chen BY (1995 a) Mitotic karyotypes of *Brassica campestris* and *Brassica alboglabra* and identification of the *B. alboglabra* chromosome in an addition line. Genome 38 : 313–319

Cheng BF, Heneen WK, Pedersen C (1995 b) Ribosomal RNA gene loci and their nucleolar activity in *Brassica alboglabra* Bailey. Hereditas 123 : 169–173

Deleseny M, McGrath JM, This P, Chevre AM, Quiros CF (1990) Ribosomal RNA genes in diploid and amphidiploid *Brassica* and related species: organization, polymorphism, and evolution. Genome 33 : 733–744

Fukui K (1989) Application of image analysis methods in plant chromosome research. In: Deyuan H (ed) Plant chromosome research 1987. Acad Sinica, Beijing, China, pp 195–200

Fukui K (1996) Plant chromosomes at mitosis. In: Fukui K, Nakayama S (eds) Plant chromosomes: laboratory methods. CRC Press, Boca Raton, Florida, pp 1–17

Fukui K, Iijima K (1991) Somatic chromosome map of rice by imaging methods. Theor Appl Genet 81 : 589–596

Fukui K, Mukai Y (1988) Condensation pattern as a new image parameter for the identification of small chromosomes in plants. Jpn J Genet 63 : 359–366

Fukui K, Nakayama S (1996) Analysis of chromosome information. In: Fukui K, Nakayama S (eds) Plant chromosomes: laboratory methods. CRC Press, Boca Raton, Florida, pp 241–255

Fukui K, Ohmido N, Kamisugi Y, Mathias RJ, Kuginuki Y, Yamabe M (1993) Analysis and utility of chromosome information. 54. Complete identification of *Brassica* A, B and C genome chromosomes. Jpn J Breed 43 (Suppl 2): 360

Fukui K, Ohmido N, Khush GS (1994) Variability in rDNA loci in genus *Oryza* detected through fluorescence *in situ* hybridization. Theor Appl Genet 87 : 893–899

Harrison GE, Heslop-Harrison JS (1995) Centromeric repetitive DNA sequences in the genus *Brassica*. Theor Appl Genet 90 : 157–165

Heneen WK, Chen BY, Cheng BF, Jonsson A, Simonsen V, Jorgensen RB, Davik J (1995) Characterization of the A and C genomes of *Brassica campestris* and *B. alboglabra*. Hereditas 123 : 251–267

Iijima K, Fukui K (1991) Clarification of the conditions for the image analysis of plant chromosomes. Bull Natl Inst Agrobiol Resour 6 : 1–58

Iijima K, Kakeda K, Fukui K (1991) Identification and characterization of somatic rice chromosomes by imaging methods. Theor Appl Genet 81 : 597–605

Kamisugi Y, Furuya N, Iijima K, Fukui K (1993) Computer-aided automatic identification of rice chromosomes by imaging parameters. Chrom Res 1 : 189–196

Karpechenko GD (1922) The number of chromosomes and the genetic correlation of cultivated Cruciferae. Bull Appl Bot Gen Pl Breed 13 : 3–14

Kianian SF, Quiros CF (1992 a) Generation of a *Brassica oleracea* composite RFLP map: linkage arrangement among various populations and evolutionary implications. Theor Appl Genet 84 : 554–554

Kianian SF, Quiros CF (1992 b) Genetic analysis of major multigene families in *Brassica oleracea* and related species. Genome 35 : 516–527

Maluszynska J, Heslop-Harrison JS (1993) Physical mapping of rDNA in *Brassica* species. Genome 36 : 774–781

McGrath JM, Quiros CF, Harada JJ, Lanbdry BS (1990) Identification of *Brassica oleracea* monosomic alien-chromosome addition lines with molecular markers reveals extensive gene duplication. Mol Gen Genet 223 : 198–204

Mizushima U (1980) Genome analysis in *Brassica* and allied genera. In: Tsunoda S, Hinata K, Gómez-Campos C (eds) *Brassica* crops and wild allies. Jpn Sci Soc Press, Tokyo, pp 89–106

Nakayama S, Fukui K (1997) Quantitative chromosome mapping of small plant chromosomes by improved imaging on CHIAS II. Gene Genet Syst 72 : 35–40

Ohmido N, Fukui K (1995) Cytological studies of African cultivated rice, *Oryza glaberrima* Steud. Theor Appl Genet 91 : 212–217

Olin-Fatih M (1994) A new method for differential staining of *Brassica* metaphase chromosomes, and karyotypes of *B. campestris*, *B. oleracea* and *B. napus*. Hereditas 120 : 253–259

Olin-Faith M (1996) The morphology, cytology, and C-banded karyotypes of *Brassica campestris*, *B. oleracea* and *B. napus* plants regenerated from protoplasts. Theor Appl Genet 93 : 414–420

Olin-Fatih M, Heneen WK (1992) C-banded karyotypes of *Brassica campestris*, *Brassica oleracea* and *Brassica napus*. Genome 35 : 583–589

Sano Y, Sano R (1990) Variation of the intergenic spacer region of ribosomal DNA in cultivated and wild rice species. Genome 33 : 209–218

U N (1935) Genome analysis in *Brassica* with special reference to the experimental formation of *B. napus* and peculiar mode of fertilization. Jpn J Bot 7 : 389–452

Yanagisawa T, Tano S, Fukui K, Harada K (1991) Marker chromosomes commonly observed in the genus *Glycine*. Theor Appl Genet 81 : 606–612