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Different timing and spatial separation of parental chromosomes in intergeneric somatic hybrids between *Brassica napus* and *Orychophragmus violaceus*

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ABSTRACT. Experimental and newly formed hybrids and polyploids generated by wide crosses usually show varying degrees of cytological instability. The spatial separation of parental genomes and uniparental chromosome elimination in hybrid cells has been reported in many hybrids from plants and animals. Herein, the behavior of parental genomes in intergeneric somatic hybrids between *Brassica napus* and *Orychophragmus violaceus* was analyzed using genomic *in situ* hybridization (GISH). In mitotic and meiotic cells, the chromosomes from *O. violaceus* were distinguished from *B. napus* by their larger size and staining patterns. In interphase nuclei of the hybrid, *O. violaceus*-

labeled chromatin appeared as large heterochromatic blocks that were nonrandomly distributed at prophase, typically distributed toward one side of the nucleus. In pollen mother cells at prophase I of meiosis, *O. violaceus* chromosomes appeared as one or two deeply stained chromatin blocks that resolved into bivalents at a late stage, after bivalents from *B. napus* were visible. Thereafter, bivalents of *O. violaceus* congressed to the equatorial plate and segregated at anaphase I after those from *B. napus*. The different behavior of *O. violaceus* chromosomes in the hybrids indicates that they have differential condensation states at interphase and progress later through the cell cycle and meiosis than *B. napus* chromosomes. This difference in behavior may restrict or prevent the formation of bivalents of mixed genome origin. Differential gene expression of parental alleles including rDNA loci may contribute to their distinct cytological behavior and to the phenotype of hybrids.

Key words: *Brassica napus*; *Orychophragmus violaceus*; Somatic hybrids; Spatial separation; Chromosome elimination; Genomic *in situ* hybridization

INTRODUCTION

Since the phenomenon of chromosome elimination was first described in *Hordeum vulgare* x *H. bulbosum* (Kasha and Kao, 1970), uniparental chromosome elimination in hybrids has been widely demonstrated, including in plants (Kasha and Kao, 1970; Bennett et al., 1976; Gernand et al., 2005), insects (Breeuwer and Werren, 1990), fish (Fujiwara et al., 1997; Sakai et al., 2007), and mammalian cultured cells (Weiss and Green, 1967; Matsui et al., 2003). The elimination of parental chromosomes in somatically produced wide (intergeneric) hybrids can lead to irregular and incomplete chromosome elimination, which leads to asymmetric hybrids or cybrids (Liu et al., 2005). The causes of uniparental genome elimination may vary, with reports implicating asynchronous cell cycles (Gupta, 1969), formation of multipolar spindles (Subrahmanyam and Kasha, 1973), spatial separation of genomes during interphase (Leitch et al., 1991) and metaphase (Schwarzacher-Robinson et al., 1987), parent-specific inactivation of centromeres (Finch et al., 1981; Jin et al., 2004; Mochida et al., 2004), lagging chromosomes at the metaphase/anaphase transition (Sakai et al., 2007), and improper organization and function of centromeres (Jones and Pašakinskienė, 2005). The most compelling evidence that the centromere may underlie chromosome instabilities in hybrids comes from addition lines of individual maize chromosomes in oat (Jin et al., 2004) and barley hybrids (Sanei et al., 2011). In addition, in lines with Cen-H3 histone genes from maize and oat, the oat gene is dominant, and the oat Cen-H3 is incorporated into the maize centromeres where it is involved in kinetochore assembly.

The crucifer *Orychophragmus violaceus* (L.) O. E. Schulz ($2n = 24$, genomes OO), which is cultivated as an ornamental plant in China, is a tetraploid taxon that shares the common ancestor of *Brassiceae* but lacks the tribe-specific genome triplication event, suggesting a phylogenetic position outside of the tribe (Lysak et al., 2007). In the sexual intergeneric or even intertribal crosses between six cultivated *Brassica* species and *O. violaceus*, only hybrids with *O. violaceus* as the pollen parent have been obtained, and reciprocal crosses proved unsuccessful. Except for *Brassica oleracea* x *O. violaceus*, all hybrids are mixoploids.

The hybrids show the separation of parental genomes during mitotic and meiotic divisions, and chromosomes of *O. violaceus* are preferentially eliminated (Li et al., 1995, 1998; Li and Heneen, 1999; Hua et al., 2006). Chromosome behavior varies in the hybrids depending upon the *Brassica* species used in the hybridizations and is considered to be under genetic control (Li and Ge, 2007).

Somatic hybrids of *B. napus* L. (2*n* = 38, AACC) and *O. violaceus* have been obtained that also produced backcrossing progenies in two generations (Zhao et al., 2008). *O. violaceus* had phenotypic and nucleolar dominance over *B. napus* in the hybrids, as the expression of only rRNA genes from *O. violaceus* was detected (Ge et al., 2009). In this study, we focused on the behavior of parental chromosomes in the somatic hybrids and their progenies by applying the method of genomic *in situ* hybridization (GISH). Different behaviors of the parental chromosomes were observed during mitotic and meiotic divisions. The possible mechanisms behind the different chromosome behaviors in sexual and somatic hybrids of these two species are discussed.

MATERIAL AND METHODS

Plant materials

An intergeneric somatic hybrid plant No. 101 between *B. napus* L. 'Huashuang 3' (2*n* = 38, AACC) and *O. violaceus* (2*n* = 24) was produced through polyethylene glycol-mediated fusions of mesophyll protoplasts (Zhao et al., 2008) and used here. The immature ovaries of young flower buds were used to determine the somatic chromosome number. Ovaries were treated with 2 mM 8-hydroxyquinoline for 3-4 h at room temperature and then fixed in 1:3 (v/v) acetic acid:ethanol. Chromosome preparations were made according to Li et al. (1995). For meiotic analysis, the young flowers were directly fixed and stored at -20°C, and then pollen mother cells (PMCs) were used to observe meiotic divisions; simultaneously, the mitotic divisions were recorded in the mitotic cells of anther walls in these flower buds without pretreatment.

DNA extraction, probe labeling, and GISH analyses

The total genomic DNA from *O. violaceus* and *B. napus* 'Huashuang 3' was labeled with digoxigenin-11-dUTP (Roche, Switzerland) and biotin-11-dUTP (Fermentas, China) by nick translation, respectively, and used as probes. The dual-color GISH was carried out in UK following the procedure of Leitch et al. (1994). For the mono-color GISH with the *O. violaceus* probe, which was carried out in China, the DNA of *B. napus* 'Huashuang 3' was shared by boiling for 15 min and used as a block. The content of probe and blocking DNA in the hybridization mixture was 3 and 20 µg/mL, respectively. Slide preparations of chromosomes for GISH mainly followed the procedures by Zhong et al. (1996), and GISH was carried out according to our procedure (Tu et al., 2008).

RESULTS

Mitoses of the hybrid and progenies

Using the method of dual-color GISH with labeled genomic DNA from *B. napus* (green) and *O. violaceus* (red) as probes, we were able to distinguish the spatial distribution

of parental chromatin in hybrid nuclei. At interphase in ovary and anther wall cells, *B. napus*-labeled chromatin usually appeared as distinct green foci of different sizes, some of which corresponded with deeply stained 4'6-diamidino-2-phenylindole (DAPI) foci (Figure 1A-F). In contrast, the chromatin labeled with the *O. violaceus* probe was more uniform in intensity and formed diffuse red patches, some of which labeled chromatin that stained brightly with DAPI (Figure 1A-F). Thus, *B. napus* chromatin occurred with distinct chromocenters and was overall more condensed than the chromatin of *O. violaceus* at the interphase.

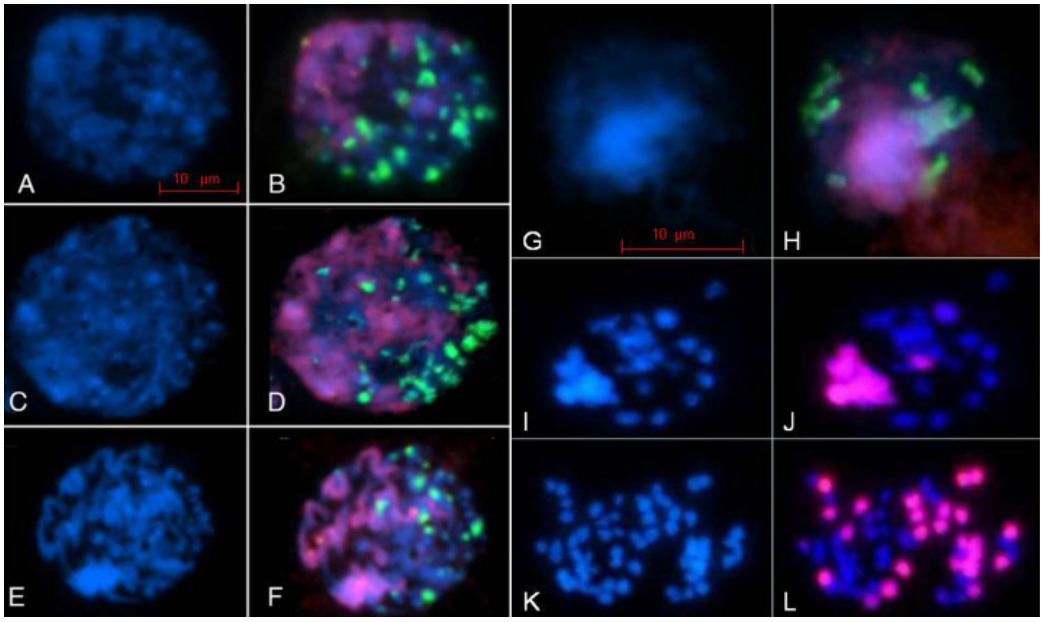

Figure 1. Mitosis and meiosis of somatic hybrids between *Brassica napus* and *Orychophragmus violaceus*. The red and green signals in merged images are from the *O. violaceus* and *B. napus* probes, respectively, and the blue color is from DAPI staining. DAPI and merged images of each cell from GISH analysis were shown. **A.-F.** The three-anther wall nuclei/cells are from anthers fixed without pretreatment. **A. B.** One interphase nucleus (A) with the chromatin of two parents being spatially separated (B). **C. D.** One interphase nucleus (C) with the chromatin of two parents being mixed but unoverlapped spatially (D). The green signals seemingly float over the red patch, suggesting that the chromatin of two parents occupies the different space in 3-D cell. **E. F.** One prometaphase cell (E) with the chromosomes of two parents positioned at distinct regions (F). In these nuclei or cells, the green signals appear as distinct green foci of different sizes, while the red signals are continuous and diffuse. **G.-L.** Three pollen mother cells (PMCs). **G. H.** One PMC at early prophase I, likely pachytene (G) with red patch at the center and green foci at periphery (H). *B. napus* chromosomes (green) are more condensed and possibly already paired, and are more advanced. **I. J.** Early diakinesis (I) with one bivalent from *O. violaceus* visible and those from *B. napus* separated (J). The clumped bivalents of *O. violaceus* possibly caused by the spatial separation from and less advanced than those of *B. napus*. **K. L.** Metaphase I/anaphase I with most of bivalents segregated and few bivalents lagged (K), the lagging bivalents and later segregated bivalents are from *O. violaceus* (L). The timing difference of parental bivalents is not obvious. Bar: 10 μm.

Whilst it is difficult to determine the three-dimensional arrangement of chromatin in a two-dimensional chromatin spread, many nuclei showed evidence of parental genome separation, with the chromatin from the two parental genomes forming domains on opposite sides of

the nucleus. This is more apparent in some cells (Figure 1B) than others (Figure 1D). We may have failed to see parental genome separation in some nuclei because of the orientation of the nucleus when it was spread or because the chromatin was interdigitated. However, even in the latter case, the chromatin domains were still largely separate from each other, i.e., there were clear sectors of each parental chromatin type.

At prophase and prometaphase, the *O. violaceus* chromosomes remained more uniformly stained than those originating from *B. napus*. The chromosomes of *O. violaceus* origin were usually longer and larger than those originating from *B. napus*. Chromosomes of *O. violaceus* origin are resolvable in prophase before the chromosomes originating from *B. napus* (Figure 1E and F).

Meioses of the hybrid and progenies

In PMCs of the hybrid at early stages of prophase I of meiosis, such as leptotene or pre-leptotene, much of the chromatin appeared as large DAPI-stained blocks (Figure 1G). This DAPI-rich area stained uniformly red with the *O. violaceus* probe. Green signals from the *B. napus* probe occurred as elongated chromatin axes or foci, usually located peripherally from the DAPI-stained block of chromatin (Figure 1H). These axes appeared aligned sometimes, perhaps representing the alignment of homeologs prior to synapsis in premeiotic interphase (Figure 1G and H).

At later stages of meiosis, bivalents from both *B. napus* and *O. violaceus* origins were visible (Figure 1I-L). Both ring and rod bivalents of *O. violaceus* chromosomes were observed, and their morphology was similar to that found in *O. violaceus* meiosis (Li et al., 1995). Once again, there appeared to be genome separation in some cells (Figure 1I and J).

GISH to meiotic material revealed no hybrid bivalents involving both *B. napus* and *O. violaceus* chromosomes. However, chromatin strands were observed between parental chromosomes in some PMCs, and these seemed not to have arisen as a consequence of homoeologous pairing. Rather, it is likely that they represent "sticky" interactions between adjacent chromosomes, perhaps caused by incomplete fixation.

DISCUSSION

Different structural characteristics of parental chromosomes

Chromosomes of diploid *Brassica* species show heterochromatic blocks around their centromeres, and some also show condensed regions toward their telomeres (Fukui et al., 1998). Consequently, *Brassica* chromosomes have predominant GISH signals at centromeric and terminal regions (Hua et al., 2006). In contrast, *O. violaceus* chromosomes are similarly condensed along their length at prometaphase, lack heterochromatic blocks around centromeric or other regions (Li et al., 2005), and are uniformly stained by GISH (Hua et al., 2006; Zhao et al., 2008; present study). These differences enabled the chromosomes to be distinguished in the hybrid material examined here and in the addition and substitution lines of *B. napus* with individual chromosomes from *O. violaceus* (Ding et al., 2013). The different cytological characteristics probably represent substantially different DNA sequence compositions and/or organization between the two species. If epigenetic markers also account for the different

condensation states of the chromatin, then the global patterns are broadly maintained in the hybrid, allopolyploid, addition, and substitution lines.

Different timing and spatial separation of parental chromosomes

The *O. violaceus* chromosomes in some materials tended to be more advanced in the meiotic and mitotic cycles. This differential response may be a consequence of different affinities of the chromatin to cell cycle and meiotic regulators. Such differential activity could result from the activities of the homeoalleles, especially transcription factors, and their *cis*- and *trans*-interactions with binding sites. Such interactions may influence patterns of gene expression and modulate chromatin modification (Chen, 2007).

The spatial separation of parental genomes at metaphase has been reported in many intergeneric and interspecific hybrids from both plants (Finch et al., 1981; Gleba et al., 1987; Leitch et al., 1991; Linde-Laursen and Jensen, 1991) and animals (Zelesco and Graves, 1988; Brandriff et al., 1991). Genome separation potentially can influence chromosome behavior, gene expression, and DNA replication (Schwarzacher et al., 1992; Jackson, 2003). Previously, we observed genome separation in synthetic hybrids *B. napus* and *O. violaceus* (Li et al., 1995; Li and Ge, 2007). Here, we observed the phenomenon in synthetic allopolyploids involving these species (Figure 1). Thus, the parental genomes in these materials can behave asynchronously in both time and space.

The segregation of the parental chromosomes in the synthetic allopolyploid was regular, whilst the segregation was aberrant in backcross material and hybrids (Li et al., 1995; Ge et al., 2009). The separation of parental chromosomes in space and time potentially restricts homoeologous pairing and favors homologous pairing, resulting in balanced chromosome segregation (Figure 1K and L).

Possible mechanisms for different cytology in somatic and sexual hybrids

In F_1 sexual hybrids between *B. napus* and *O. violaceus*, the chromosomes of *O. violaceus* origin are partially or completely eliminated (Li et al., 1995), which is in contrast to their allopolyploids, which have balanced segregation of chromosomes (Zhao et al., 2008; present study). Chromosome elimination in the sexual hybrids probably occurs during the early stage of embryo development (Kasha and Kao, 1970; Mochida et al., 2004; Gernand et al., 2005) and may be caused by impaired centromere function (Laurie and Bennett, 1989) that results from the failure to recruit centromere-associated structures at the kinetochore (Mochida et al., 2004). It is possible that insufficient centromere function is caused by the silencing of centromere-associated factors of *O. violaceus* origin, similar to the rDNA gene suppression that is involved in nucleolar dominance (Jones and Pašakinskienė, 2005). If such silencing happened for centromere proteins, any incongruence between their centromeric repeats and the proteins encoded by the genes of another parent might be deleterious, as was observed for the incorporation of oat Cen-H3 in an oat-maize addition line with only one maize chromosome (Jin et al., 2004), or the loss of Cen-H3 from centromeres preceding uniparental chromosome elimination in interspecific barley hybrids (Sanei et al., 2011). In contrast, the somatic hybrid material studied here may have codominant expression of parental alleles.

In conclusion, the intergeneric or intertribal somatic hybrids and backcross progenies

display some distinctive cytological features, such as differential chromatin condensation and spatial and temporal separation of chromosomes in mitotic and meiotic cells. Such differences may be attributed to the different structural characteristics of parental chromosomes and to differential expression of the parental alleles, such as centromeric proteins. The results provide some new clues to the cytological mechanisms behind the phenotypic and genetic instability commonly displayed in synthetic allopolyploids (Comai, 2000; Chen, 2007). These hybrids and progenies are ideal materials for tracing the behavior of parental chromosomes through the cell cycle.

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