Genomic in *Situ* hybridization in intergeneric hybrids between *Raphanus sativus* and *Brassica oleracea*

CHENG Yugui[1, 2], **WU Jiangsheng**[1], **ZHANG Minghai**[2], **FANG Huaming**[2]

1. National Key Laboratory of Crop Genetic Improvement of Huazhong Agricultural University, Wuhan, 430070, China;
Email: hengygfa@hotmail.com
2. Yichang Institute of Agricultural Science of Hubei Province, Yichang Hubei, 443004, China

Abstract

Genomic in *situ* hybridization (GISH) was applied to study the meiosis of F_1 plants from intergeneric hybrids between radish (*Raphanus sativus* L., 2n=18, RR) and cabbage(*Brassica oleracea* L., 2n=18, CC). The result showed that its somatic cells had the expected chromosomes, RC, 2n=18; but that the pollen mother cells (PMCs) were different. There were three main kinds of PMCs. The first one was RC(2n=18), and the mean chromosome pairing pattern was:14.87I+1.20II+0.04III+0.06IVat Diakinesis. GISH indicated that most bivalents resulted from chromosome pairing between radish and cabbage, and that the nine chromosomes of R-genome were separated mostly in the ratio 5/4 and 6/3 at AnaphaseI, so the chromosome number and components in gametes were not in equilibrium and the gametes were sterile. The second was RRCC (2n=36) with normal chromosome pairing and separation, producing unreduced gametes. And the third was hypoploid of RRCC (2n<36=30~35). GISH showed that some radish chromosomes were lost in those PMCs, and that its gametes had nine cabbage chromosomes and partial radish chromosomes. Cytological characterization of BC_1 hybrids suggested that the unreduced gametes both RC euploid and its hypoploid could be transmitted to progenies by female parents.

Key words: *Raphanus sativus*; *Brassica oleracea*; Intergeneric hybrids; Cytogenetics; GISH

Introduction

Both radish(*Raphanus sativus*, 2n=18, RR) and cabbage(*Brassica oleracea,* 2n=18, CC)belong to *Cruciferae* crops. Attempts have been made for many years to transfer useful genes such as OguCMS and various disease and pest resistance genes from radish into cabbage or rapeseed (*Brassica napus*, 2n=38, AACC) for the improvement of *Brassica* crops (e.g. Bannerot et al., 1974; Heyn, 1977; Pan, 1999; Hagimori, 1992; Kaneko, 2003). As it provides a suitable method for chromosome discrimination, Genomic in *situ* hybridization (GISH) has been used for cytological studies of intergeneric hybrids between *Raphanus and Brassica* in recent years. This method was first applied to detect the genome components of hybrids between radish and rapeseed by Snowdow (1997); and R-genome chromosomes were monitored in natural hybrids and its progenies between *R. raphanistrum* and *B. napus* when Benabdelmouna (2004) assessed the risk of transgenic crops; Peterka (2004) determined the specific chromosome of resistant radish carrying the gene (S) for nematode resistance in rape-radish chromosome addition. However, GISH has been limited to mitotic chromosomes in above research. In this paper, GISH was applied to the meiotic analysis of F_1 and its progenies in order to discover the cytogenetic mechanism of hybrids between radish and cabbage.

Materials and methods

Plant materials

The F_1 plants used for investigation were obtained by means of embryo rescue. The female parents were Chinese radishes, *R. sativus* cvs. 'longju, 'zhedachang', 'Heqing', 'Huangzhou' (supplied by College of Plant Science, Huazhong Agricultural University); the pollen parents were *B. oleracea* var. *alboglabra* cv. zhonghua and *B. oleracea* var. *acephala* cv. Chunqiu (supplied by Wuhan Vegetable Research Institute, Chinese Academy of Agricultural Sciences). In our previous report, the hybrids were morphologically intermediate between their parents; and DNA fingerprinting of simple sequence repeat (SSR) analysis showed that the genomic DNA had fingerprint of both parents (Cheng et al., 2006).

Fertility of F_1 investigation

Pollen stain ability was determined as the percentage of pollen grains stained with 1% aceto carmine. More than 1000 pollen grains from 5 flowers were counted for each plant. Normal pollen grains were fully round and densely stained, and they were easily distinguished from shrunken and lightly stained ones under the microscope. Meanwhile, the numbers of setting seeds were counted with the flowers of the main stem self pollinating and branches open pollinating.

Classical cytological analysis

Young ovaries were immerse in ice water(0°C) for 6h, treated with 2mM 8-hydroxyquinoline for3.5~4h and then fixed in carnoy`s solution for 24h, stored in 70% ethanol at 4°C for determining the chromosome numbers and GISH. They were hydrolysed in 1N HCl for 8 min, squashed in a drop of 10% modified carbol fushsin and observed under oil. To observe pollen mother cells (PMC) for meiotic analysis, buds from the terminal inflorescence were fixed immediate after collection in fresh Carnoyˋs solution for 24h. One anther was dissected out off bud and hydrolysed in 1N HCl for 2 min, squashed in a drop of 10% modified carbol fushsin and observed; the others in suitable divisive stage then stored in 70% ethanol at 4°C for GISH.

GISH probe

For GISH probe, genomic DNA was extracted from radish, using the CTAB- extraction method of Doyle and Doyle (1990), labelled with biotin-11-dUTP by nick translation (the kit supplied by Roche Company) until the lengths of probe fragments, determined by agarose-gel electrophoresis, averaged approximately 500bp. For blocking DNA, genomic DNA was extracted from *B. oleracea* var. *alboglabra*, produced by putting in boiling water for 30 min to generate fragment of approximately 300~500bp in length. Both probe and blocking DNA were stored at -20°C for use.

Cytological preparations and in situ *hybridisation*

Ovaries and anthers were digested in a cellulase- pectinase mix (enzyme solution) at 37°C until the material were soft, followed by 20-min washing in enzyme buffer. Chromosome preparations were made in 45% aqueous acetic acid on chromic acid cleaned slides. Slides were checked by phase-contrast microscope and with an inadequate divisive stage or unacceptable backgrounds were discarded. Suitable preparations were treated with RNAase, fixed with formaldehyde and denatured, and hybridisation procedure was performed according to Leitch et al. (1994), preparations incubated overnight at 37°C with hybridisation mixture including 1ug/mL probe DNA and 10ug/mL blocking DNA. After post-hybridisation washing with 20% formamide solution, hybridisation signals were detected using streptavidin-Cy_3, chromosomes were counterstained with 4 ´ 6-diamidino-2-phenylindole. Photographs were taken under fluorescence microscope (LEICA DMLB); pseudo-coloration and merging of images were done with Adobe Photoshop ver.7.0.

Results

GISH in meiotic cells of parents

When *situ* hybridizations with R-genome DNA as probe were performed to the meiotic cells of parents at metaphaseI, the 9 bivalents of radish had distinct hybridisations signals with six signals in the centromeric regions and the others at the telomeres; while cabbage chromosomes had no obvious signal except a very weak signal at the telomere in one bivalent. This result showed that all radish chromosomes could be clearly distinguished by GISH with labelled R-genome DNA in the hybrids.

The chromosome number and component of F_1

The somatic cells of F_1 contained 18 chromosomes as expected and 9 signals were detected in situ hybridisations. This conveyed the genome component of $F_1$was RC, 2n=18.

Meiosis of F_1

The fertilities of F_1 were very poor in common. No plants had selfed seed, but some plants set in open pollinating. The numbers of setting-seeds varied from 0 to 350, the pollen stainabilty were 0~37.5%. The fertility was related with the chromosome number of PMC. All sterile plants had 18 chromosomes in PMCs, but there were three kinds PMCs of 2n=18, 2n=36 and 2n⩽36=30~35 in the partly fertile plants. It expressed some PMCs were duplicated naturally. The PMCs of 2n=30~36 were named duplicated PMCs in present paper. The frequencies of duplicated PMCs varied from 0 to 34% in different plants (Table 1). Most buds had no or a few duplicated PMCs, but we also found out a bud in plant 9, whose PMCs were all duplicated with 15~18 bivalents.

Table1 Frequencies of different PMCs and fertilities of intergeneric hybrids between radish and cabbage

Cross	Plant code	Pollen stainability(%)	Number of set-seeds	Frequency of different PMCs(%)		
				2n=18	2n=30~35	2n=36
longju×zhonghua	1	0	0	100	0	0
	2	3.0	6	96.6	3.4	0
	3	30.3	45	94.9	4.3	0.8
zhedachang ×chunqiu	4	0	0	100	0	0
heqing×chunqiu	5	0	0	100	0	0
	6	0	2	98.4	1.6	0
heqing ×zhonghua	7	0	0	100	0	0
	8	1.4	4	94.7	5.1	0.2
	9	37.5.	205	83.3	5.6	11.1
huanzhou×zhonghua	10	0	0	100	0	0
	11	0	22	97.5	5.6	7.9
	12	11.8	130	66.0	18.6	11.4
zhonghua ×longju	13	0	0	100	0	0

Table 2 The numbers and percents of PMCs with different chromosome-pairing configurations in intergeneric hybrids between radish and cabbage

Configuration	18I	16I+1II	14I+2II	12I+3II	10I+4II	Others	Total
Number of PMCs	110	44	60	34	18	20	286
Percentage	38.5%	15.4%	21.0%	11.9%	6.3%	7.0%	
	The mean chromosome- pairing pattern			14.87I+1.20II+0.04III+0.06IV			

Table 3 The PMC numbers and frequencies with different chromosome separation ratios at AnaphaseIin intergeneric hybrids between radish and cabbage

Ratio	14/4	12/6	11/7	10/8	9/9	Others	Total
Number of PMCs	10	25	98	60	8	15	230
Percentage(%)	4.3	10.9	32.6	26.1	13.0	6.6	

Table 4 The PMC numbers and frequencies with different separation ratios of nine R-genome chromosomes at AnaphaseIin intergeneric hybrids between radish and cabbage

Ratio	5/4	6/3	7/2	8/1	Others	Total
No. of PMCs	85	45	35	5	30	230
Percentage	38.0%	32.6%	15.2%	2.2%	13.0%	

The chromosomes in PMCs of 2n=18 could not pair normally. More than 15 pairing configurations were observed(Table2), these mainly 18I, 16I+1II, 14I+2II, 12I+3II, 10I+4II at MetaphaseI. Most chromosomes existed as univalent, while a few multivalent with 3 and 4 chromosomes appeared too; the mean chromosome pairing pattern was 14.87I+1.20II+0.04III+0.06IV. GISH indicated that most bivalents resulted from homoeologous paring between radish and cabbage chromosomes. All bivalents consisted of radish and cabbage chromosomes in PMCs with configurations of 16I+1II and 14I+2II(Fig.1a); while in PMCs of 12I+3II and 10I+4II, one bivalent came from self pairing of cabbage chromosomes, the others resulted from chromosome pairing between radish and cabbage(Fig1b). Chromosomes could not depart regularly at AnaphaseI, with four main ratios of 9/9, 10/18, 11/17,12/6(Table3). GISH suggested that the nine radish chromosomes separated mostly in the ratios 5/4 and 6/3, with a respective percent of 38.0% and 32.6%, so the chromosome number and components in gametes were not in equilibrium and the gametes were sterile (Table4; Fig1e.).

The genome components were RRCC in PMCs of 2n=36. In some of those cells, the chromosomes paired and separated regularly, producing euploid gametes of RC (2n=18) (Fig.1d, f); in other PMCs, multivalent was observed at Diakinesis and asynchronous chromosomes appeared at MetaphaseI. This indicated that some chromosomes could be lost at AnaphaseI, producing hypoploid gametes of RC (2n⩽18).

PMCs of 2n⩽36 were hypoploid of RRCC. The chromosome behaviours were complicated. Most chromosomes existed as bivalents and a few univalents at Diakinesis, and there were different separation ratios of 17/17, 17/16, 16/16, 16/15, 16/14, 15/15, 15/14,1 4/14 at AnaphaseI. GISH showed that some radish chromosomes were lost in those PMCs, and its gametes had nine cabbage chromosomes and partial radish chromosomes (Fig.1c).

No obvious difference was found in the meiosis of F_1 in reciprocal crosses and diverse combinations except for the different frequencies of duplicated PMCs.

Morphologies and cytogenetically observations of BC_1

31 seeds were reproduced via backcrosses (The number of cross flowers in each combination was 1500) with F_1 as female parents; and 13 seedlings obtained after sowing, including 3 plants (coded with BC_1-1~ BC_1-3) from backcross of F_1 to radish, and 10 plants (coded with BC_1-4~ BC_1-13) from cross between F_1 and *Raphanobrassica* (RRCC, 2n=36). Five seeds obtained from the crosses between F_1 and cabbages were shrunken and not germinant.

BC_1-1~ BC_1-3 were morphologically inclined to radish, for example, purple flower and crinkle leaves, but with smaller flower and lower pollen stainabilities of 0~50% (Fig.2a, 2b). The plants were mixoploid of 2n=20~27, namely in the same plant, the chromosome numbers were different in different somatic cells. The main pairing configurations were 9I+9II or 7I+10II at Diakinesis; and 5~7 univalent chromosomes were observed at MetaphaseI. This showed that the genome components of BC_1-1~ BC_1-3 were triploid of RRC, in other words, RC×RR→RRC, and that the gametes came from F_1 were unreduced one. In these plants, BC_1-1 was a little different from the others, whose chromosome numbers of somatic cells were from 22 to 25, and the main chromosome pairing configuration was 5I+9II (Fig.2e, 2f). The highest chromosome number of BC_1-1 was not more than 27, which showed that BC_1-1 was hypoploid of RRC; therefore we could make a conclusion that the gamete from $F_1$was hypoploid of RC, reproducing progenies of hypoploid of RRC.

Cytological characterization of BC_1-4~ BC_1-13 demonstrated that RC×RRCC→RRCC. Those plants were morphologically similar to F_1 with bigger flower and higher pollen stainability of 10~80% and produced most PMCs with 18 bivalents except BC_1-5, whose botanical characters inclined to cabbage with deep green, complete leaves and a lot of wax (Fig.2c, 2d). BC_1-5 was also mixoploid, 2n=30~33⩽36; 15 bivalents and 3 univalents was observed in its PMCs (Fig.2g, 2h). This indicated BC_1-5 was hypoploid of RRCC, and it could be suggested that some radish chromosomes were lost, resulting in typical character of cabbage appearance.

The result above confirmed that the unreduced gametes both RC euploid and hypoploid came from F_1 could be transmitted to progenies by female parents.

Discussion

Chromosome pairing of F_1

The chromosome-pairing pattern in interspecific hybrids is usually considered to represent the relationship of two parental genomes. In former studies, homoeologous pairing were observed mostly in F_1 between *Raphanus* and *Brassica* with classical cytological methods (e.g. Richharia, 1937). Mizushima (1980) speculated that *Raphanus* genome and *Brassica* genome (including A, B and C chromosome sets) were homoeology. However, the current studies showed that the chromosomes could self-pair in haploid of cabbage (Armstrong, 1982). It is difficult to estimate that the bivalents in F_1 result from weather self pairing in one chromosome set or homoeologous pairing between two chromosome sets using classical cytological method, because both radish and *Brassica* chromosomes are small and similar. In our study, we verified that most bivalents in F_1 resulted from chromosome pairing between radish and cabbage with GISH, which confirm that *Raphanus* and *Brassica* are close relative genera and that introgression of useful genes through instant hybridisations between them is possible and valuable.

The chromosome component and transmission to progenies of gametes of F_1

It is commonly realized that interspecific hybrid sterility is caused by unbalanced chromosome sets (Liu, 1999). Since Digby (1912) found a fertile plant that appeared spontaneously among the sterile one generated in a cross between two primrose species, this plant had twice the number of chromosomes of its sterile sibs; Wing (1917) speculated that speciation could occur by interspecific hybridization followed by genome duplication, because upon doubling, a proper pairing -partner would be available to each chromosome resulting in fertility. Karpechenko (1927) verified Winge`s prediction experimentally and discovered the mechanism of chromosome doubling in hybridization between radish and cabbage. He found the nucleus that have divided fuse again before meiosis in generative cells, resulting in the appearance of duplicated PMCs in F_1. We conveyed this conclusion; but the meiosis of fewer buds, like one in plant 9, show that some somatic cells in F_1 perhaps duplicated spontaneously too, resulting in all PMCs in one bud duplicating together. Meanwhile, we applied GISH to study the meiosis and component of gametes, and three main kinds of gametes were observed. The first one was reduced gametes, whose chromosome number and components were not in equilibrium, and the gametes were sterile. The second was unreduced gametes, RC euploid with whole R-genome set and C-genome set, its quantity was small, but could transmit to progenies smoothly. The third was also unreduced gametes, but hypoploid of RC with nine cabbage chromosomes and partial radish chromosomes. This kind of gametes was theoretically fertile; in fact, hypoploid of RRC and RRRCC were obtained in BC_1, which showed the gametes of RC hypoploid could be transmitted to progenies by female parents too.

About the duplicated PMCs, there was similar discovery. In F_1 between *B. campestris* (AA, 2n=20) and *R. sativus*, Wu (1998) found the somatic chromosome number were expected, 2n=19; but its flower were different, the small flower were sterile with PMCs of 2n=19 and the big one were fertile with duplicated PMCs of 2n=38 and partial duplicated PMCs of 2n=29. He thought separation of parental genomes happened in partial duplicated PMCs, resulting in unequal triad of 10-10-9. We did not observe the separation of parental genomes in F_1 between radish and cabbage. Whereas wild and cultivated allopolyploid plants are well adapted; man-made allopolyploids are typically unstable, such as chromosomal rearrangements and changes in the number. Comai (2005) reasoned that instability of newly formed allopolyploids related to homeologous recombination between the parental genomes, which hindered the normal progress of meiosis by forming, for example, trivalents and univalents. He also pointed out that perfect homologous was often a seconding adaptation, that progressive differentiation of the parental genomes prevented homeologous synapsis and recombination, forcing the establishment of a diploid-like meiosis in established allopolyploids. We presumed that gametes of RC hypoploid resulted from highly homeology between R- genome and C-genome; for multivalents and univalents at Diakinesis and asynchronous chromosomes at MetaphaseIwere observed, which suggested that some chromosome could be lost in meiosis, producing hypoploid gametes.

Changes in chromosomal number

F_1 between radish and cabbage had stable karyotype, RC, 2n=18; but the BC_1 plants were mixoploid, namely the chromosomal number were different in different somatic cells in same the plant. Segregation of euploid and aneuploid had been reported in the progenies of amphidiploid between radish and cabbage. In Benabdelmouna`s research, two descendants were obtained when interspecific hybrid F_1 between *B. napus* and *R. raphanistrum* (ACR, 2n=28) backcrossed to *R. raphanistrum*.. Benabdelmouna determinated that their chromosome number varied between 45 and 48, including 9 chromosomes from *R. raphanistrum* and 36-39 chromosomes from *B. napus*. About the unexpected chromosome combination, Benabdelmouna considered that *B. napus* genome generated unreduced gametes and meanwhile *R. raphanistrum* chromosomes eliminated. It is easy to understood that segregation of euploid and aneuploid in the progenies of amphidiploid by comai`s perspective; but it is difficult to explain the changes of chromosomal number in somatic cells with homeologous recombination in meiosis. Li (1995, 2005) found out spatial separation of parental genomes during the mitotic division of intergeneric hybrids between *Brassica* species and *Orychophragmus violaceus*, thus the hybrid became the mixoploid in nature. He thought that the abnormal chromosome behaviours in plant wild crosses, such as pseudogamy, semigamy, chromosome elimination and the mitotic and meiotic separation of parental genomes indicated the incompatibility of two parental species at gametic and chromosomal levels. We considered that homeologous recombination as well as abnormal chromosome behaviours in mitosis played a role in chromosome number instability.

This study is still in its early stages and more work about GISH in hybrid progenies should be done to discover the cytogenetic mechanism of hybridization between radish and cabbage. We believe that systematic studies on chromosomal behaviors and genetics in plant hybridizations are not only needed to undermine the mechanisms responsible for the formation and evolution of new species, but also beneficial to introgression of useful genes in instant hybridization breeding.

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References

Armstrong KC, Keller WA. Chromosome pairing in haploid of *Brassica oleracea*. Can J Genet Cytol, 1982, 24:735-739

Bannerot H, Boulidard L, Gauderon Y, Jemper J. Transfer of cytoplasmic male sterility from *Raphanus sativus* to *Brassica oleracea.* In: Proc. EUCAPLA Meeting-*Cruciferae*, 1974, 52-54

Benabdelmouna A, Gueritaine G, Abirached-Darmency M, Drmency H. Genome discrimination in progeny of interspecific hybrids between *Brassica napus* and *Raphanus raphanistrum*. Genome, 2003, 46, 3:469-472

Cheng YG, Wu J S, Chen HG, Zhang M H. Studies on intergeneric hybrization between *Raphanus sativus* and *Brassica oleracea*. Chinese Journal of oil crop sciences, 2006, 28(1):1-6

Comai L. Genetic and epigenetic interactions in allopolyploids plants. Plant Molecular Biology, 2000,43:387-399

Doyle JJ, Doyle JL. Isolation of plant DNA from fresh tissue. Focus, 1990, 12:13-15

Digby L. The cytology of *Primula kewensis* and of other related *Primula* hybrids. Ann. Bot.1912, 26: 357-388

Hagimori M, Nagaoka M, kato N, Yoshikawa H. Production and characterization of somatic hybrids between the Japanese radish and cauliflower. Theor Appl Genet, 1992, 84: 819-824

Heyn F W. Transfer of restore genes from *Raphanus* to cytoplasmic male sterile *Brassica napus*. *Cruciferae* Newslett, 1977, 1: 15-16

Kaneko Y, Yano H, Bang S W, Matsuzawa Y. Genetic stability and maintenance of *Raphanus sativus* lines with an added *Brassica rapa* chromosome. Plant Breeding, 2003, 122: 239-247

Karpechenko G D. The production of polyploid gametes in hybrids. Hereditas, 1927, 9: 349-368

Leitch A R, Schwarzacher T, Jackson D, et al. In situ hybridization: a practical guide. Oxford: Bios Scientific Publishers Limited, 1994

Liu D J. Cytogenetics. Beijing: Agriculture Press, 1999. 159-161

Li Z, Liu H L, Luo P. Production and cytogenetics of intergeneric hybrids between *Brassica napus* and *Orychophragmus violaceus.* Theor Appl Genet, 1995, 91: 131-136

Li Z Y, Hua Y W, Ge×H, Xu C Y. Chromosomal behaviors in plant wide hybridizations and their genetic and evolutionary implications. Hereditas (Beijing), 2005, 27(2): 315-324

Mizushima U. Genome analysis in *Brassica* and allied genera. In Tsunlda et al. 1980: 89-96

Pan D R. Analysis of nematode resistance in BC_2 hybrids obtained from crossing between *Brassica napus*×*Raphanus sativus.* Journal of Fujian Agricultural University, 1999, 28(4): 402-406

Peterka H, Budahn H, Schrader O, Ahne R. Schiitze W. Transfer of resistance against the beet cyst nematode from radish (*Raphanus sativus*) to rape (*Brassica napus*) by monosomic chromosome addition. Theor Appl Genet, 2004, 109:30-41

Richharia R H. Cytological investigation of *Raphanus sativus*, *Brassica oleracea* and their F_1 and F_2 hybrids. J.Genet, 1937, 34: 19-44

Snowdon R J, kohler W, Friedt W. Genomic in situ hybridization in *Brassica* amphidiploids and interspecific hybrids. Theor Appl Genet, 1997, 95: 1320-1324

Wing O. The chromosomes, their number and general importance. C.R.Trav.Lab.Carlsberg, 1917, 13: 131-275

Wu YY, Luo P. Preliminary study on hybridization between *Brassica Campestris* and *Raphanus sativus* L. Var. *oleifera Makino.* Guihaia**,** 1998, 18(1):54-57