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ORIGINAL PAPER

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Genomic constitution and variation in five partial amphiploids of wheat–*Thinopyrum intermedium* as revealed by GISH, multicolor GISH and seed storage protein analysis

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Abstract Genomic in situ hybridization (GISH) and multicolor GISH (mcGISH) methodology were used to establish the cytogenetic constitution of five partial amphiploid lines obtained from wheat × *Thinopyrum intermedium* hybridizations. Line Zhong 1, 2*n*=52, contained 14 chromosomes from each of the wheat genomes plus ten *Th. intermedium* chromosomes, with one pair of A-genome chromosomes having a *Th. intermedium* chromosomal segment translocated to the short arm. Line Zhong 2, 2*n*=54, had intact ABD wheat genome chromosomes plus 12 *Th. intermedium* chromosomes. The multicolor GISH results, using different fluorochrome labeled *Th. intermedium* and the various diploid wheat genomic DNAs as probes, indicated that both Zhong 1 and Zhong 2 contained one pair of *Th. intermedium* chromosomes with a significant homology to the wheat D genome. High-molecular-weight (HMW) glutenin and gliadin analysis revealed that Zhong 1 and Zhong 2 had identical banding patterns that contained all of the wheat bands and a specific HMW band from *Th. intermedium*. Zhong 1 and Zhong 2 had good HMW subunits for wheat breeding. Zhong 3 and Zhong 5, both 2*n*=56, possessed no gross chromosomal aberrations or translocations that were detectable at the GISH level. Zhong 4 also had a chromosome number of 2*n*=56 and contained the complete wheat ABD-genome chromosomes plus 14 *Th. intermedium* chromosomes, with one pair of *Th. intermedium* chromosomes being markedly smaller. Multicolor GISH results indicated that Zhong 4 also contained two pairs of reciprocally translocated chromosomes involving the A and D genomes. Zhong 3, Zhong 4 and Zhong 5 contained a specific gliadin band from *Th. intermedium*. Based on the above data, it was concluded that inter-genomic transfer of chromosomal segments and/or sequence introgression had occurred in these newly synthesized partial amphiploids despite their diploid-like meiotic behavior and disomic inheritance.

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Introduction

The wheatgrass, *Thinopyrum intermedium* (Host) Barkworth and D. R. Dewey (2*n*=6*x*=42) [syn.=*Agropyron intermedium* (Host) Beauvoir=*Elytrigia intermedia* (Host) Nevski], is an important source of genetic variability for improving cultivated wheat. It has been used extensively for hybridization with bread wheat and durum wheat, and numerous useful genes, particularly those for leaf and stem rust resistance, have been successfully transferred to wheat (Fedak 1999; Fedak et al. 2000; Fedak and Han 2004). Many derivatives have been produced from wheat-*Th. intermedium* hybrids, such as octoploid amphiploids, hexaploid amphiploids, partial amphiploids and alien addition lines (Wienhues 1966; Cauderon et al. 1973; Chi et al. 1979; Sun 1981; Schulz-Schaeffer and Haller 1988; He et al. 1988; Friebe et al. 1992; Han and Li 1995; Larkin et al. 1995).

Chi et al. (1979) and Sun (1981) reported the production of the Zhong series of partial amphiploids from the hybrids of common wheat × *Thinopyrum intermedium*; these were designated as Zhong 1, Zhong 2, Zhong 3, Zhong 4 and Zhong 5. Following crosses between different lines of the Zhong series, meiotic pairing in the F_1 hybrids was examined by different investigators (He et al. 1988; Banks et al. 1993; Han 1994; Gao et al. 1999). Based on these studies, the five partial amphiploids have been classified into two types: type I includes Zhong 1 and Zhong 2, which presumably contain one of the wheatgrass genomes; type II includes Zhong 3, Zhong 4 and Zhong 5, which presumably contain another wheatgrass genome (He et al. 1988). Nevertheless, the completeness and identity of these added wheatgrass genomes remain controversial topics (Banks et al. 1993; Fedak et al. 2000; Chen et al. 2003). It is accepted that the partial amphiploids play an important role in transferring alien genes into wheat. For example, Zhong 1 and Zhong 2 provided the first source of resistance to both wheat streak mosaic virus and its vector, the wheat curl mite (Chen et al. 2003). Two sets (for a total of 14) of wheat-*Th. intermedium* alien addition lines have been established using the Zhong series (He et al. 1988). Furthermore, several translocated chromosomes derived from these addition lines have been established and a series of high-quality bread wheat varieties released (He et al. 1993).

For more extensive exploitation of these partial amphiploids in wheat improvement, detailed information on their genomic constitution is needed. In the investigation reported here, we used the combination of genomic in situ hybridization (GISH), multicolor GISH (mcGISH) and seed storage protein analysis to characterize the chromosomal stability or variation and genomic constitution of the two types of partial amphiploids derived from wheat-*Th. intermedium*.

Materials and methods

Plant materials

Wheat-*Thinopyrum intermedium*-derived partial amphiploids Zhong 1 to Zhong 5 were selected from crosses between common wheat varieties and *Th. intermedium*. They were distinguished as types I and II (He et al.1988; Banks et al. 1993) and kindly supplied by Dr. S.Y. Chi, Heilongjiang Academy of Agricultural Sciences, Harbin, China. The exact wheat parents that were used to establish the partial amphiploids of Zhong 1 to Zhong 5 were supplied by the Chinese Academy of Agricultural Sciences, Beijing, China. All plant materials are being maintained in our laboratory by selfing.

Genomic in situ hybridization

Seeds were germinated on moistened filter paper in petri dishes. The actively growing roots were removed from seedlings and placed in ice water for 24–28 h, fixed in Carnoy's (3:1 ethanol-acetic acid) fixative solution for 24 h and stored in 70% (v/v) ethanol. Root tips were stained with 1% w/v aceto-carmine for 0.5–2 h and squashed in 45% (v/v) acetic acid. The slides were frozen in liquid nitrogen and the cover slips removed using a razor blade. The slides were then dehydrated in 95% (v/v) ethanol for 5 min and stored at −20°C until used. Genomic DNA of *Th. intermedium* was isolated using a modified CTAB method (Kidwell and Osborn 1992), labeled with biotin-16-dUTP by the random primer method and used as a probe. Slide pre-treatment, hybridization, signal amplification and detection of the fluorescent signals were carried out as described by Han et al. (1998a, b). Briefly, the slides were first incubated in RNase A (100 ng/μl, in 2× SSC) for 1 h and pepsin (50 ng/μl, in 10 m*M* HCl) for 5–10 min, then fixed with paraformaldehyde (4%, w/v) for 10 min and dehydrated in an alcohol series (70%, 95% and 100%). The hybridization mixture was prepared to a final concentration of 5 ng/μl biotin-labeled probe in 2× SSC, 500 ng/μl denatured salmon sperm DNA, 250–500 ng/μl autoclaved genomic DNA from wheat cv. Chinese Spring as a blocker, in 50% deionized formamide. The hybridization mixture was denatured and added onto the slides and then denatured at 80°C for 6 min. After an overnight hybridization at 37°C, post-hybridization washes were carried out in 50% formamide in 2× SSC at 37°C for 10 min, 0.1× SSC at 37°C for 10 min and 2× SSC at 37°C for 10 min. The slides were placed in the BSA blocking solution (5% BSA, 100 m*M* Tris-HCl, 150 m*M* NaCl) for 5 min at 37°C. Sites of the hybridization signal for biotin-16-dUTP were detected with avidin-FITC with two rounds of amplification by biotinylated anti-avidin. The slides were washed in three times in TNT (100 m*M* Tris-HCl, pH 7.5; 150 m*M* NaCl; 0.05% Tween 20) for 10 min each time. The chromosomes were finally counterstained with propidium iodide (PI, 0.25–0.5 μg/ml) and the slides mounted in the Vectashield mounting medium (Vector Laboratories). Slides were visualized with an epifluorescence Zeiss Axioplan 2 microscope equipped with the appropriate filters, and photographs were taken on Kodak 400 color slide films.

Multicolor genomic in situ hybridization

Total genomic DNA was isolated from young leaves of *Thinopyrum intermedium*, *Triticum urartu*, *Aegilops speltoides* and *Ae. tauschii*. Total genomic DNA of *Th. intermedium* and *T. urartu* was labeled with digoxigenin-11-dUTP and total genomic DNA of *Ae. tauschii* with biotin-16-dUTP using the nick translation method. Total genomic DNA of *Ae. speltoides* was used for blocking. The slides were denatured at 75°C for 10 min; hybridization and washing conditions were exactly those of the manufacturer (Roche, Indianapolis, Ind.). Detection of the biotinylated probe was accomplished with avidin-XRITC and digoxigenin using a fluorescent antibody enhancer set (Roche). The slides were mounted in the Vectashield

mounting medium [0.5–1 μg/ml 4′,6′-diamidino-2-phenylindole (DAPI)] and examined under a Zeiss fluorescence microscope; photographs were taken on Kodak Select film ASA 400. For GISH and mcGISH observations, at least 30 well-spread metaphase cells were examined.

High-molecular-weight glutenin and gliadin analysis

Proteins were extracted from crushed endosperms of single seeds. The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method for detection of high-molecular-weight (HMW) subunits and non-continuous formic acid-PAGE methods of gliadin detection as described by Zhang et al (1997 a, b) were used.

Results

GISH analysis of the five partial amphiploids Zhong 1 to Zhong 5

These five partial amphiploids have been grouped into two types—type I (Zhong 1 and Zhong 2) and type II (Zhong 3, Zhong 4, Zhong 5), presumably based on the added *Th. intermedium* genomes (He et al. 1988; Banks et al. 1993). Somatic chromosome counts indicated that line

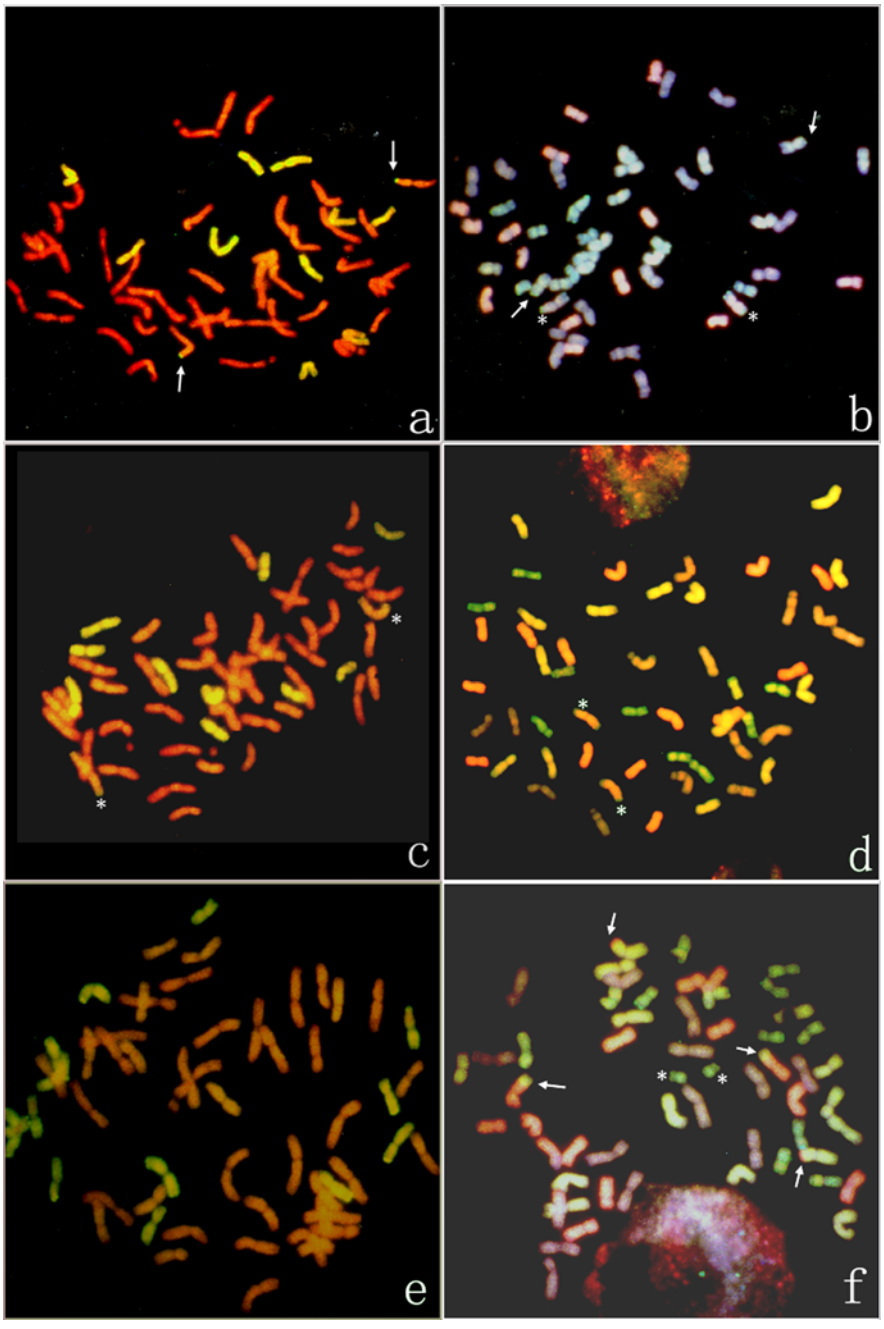

Fig. 1a–f GISH patterns of root-tip cells at mitotic metaphase (**a**, **c**, **e**) and multicolor GISH patterns (**b**, **d**, **f**) of somatic cells at metaphase. **a** GISH pattern of Zhong 1. *Arrows* indicating a pair of wheat-*Th. intermedium* translocated chromosomes. **c** GISH pattern of Zhong 2, showing the presence of 12 *Thinopyrum intermedium* chromosomes (*yellow-green*). **e** GISH pattern of Zhong 5, showing the presence of 14 *Th. intermedium* chromosomes (*yellow–green*). No gross chromosomal structural change is detectable in these partial amphiploids. **b** Multicolor GISH pattern of Zhong 1, showing the presence of 14 A-genome chromosomes (*yellow color*). *Arrows* denote one pair of wheat-*Th. intermedium* translocation chromosomes involved A genome and *Th. intermedium* chromosome segments (*yellow and green colors*, respectively), while the *asterisks* refer to another pair of *Th. intermedium* chromosomes showing a pink coloration. **d** Multicolor GISH pattern of Zhong 2. The *asterisks* indicate one pair of *Th. intermedium* chromosomes (*pink color*). **f** Multicolor GISH pattern of Zhong 4. The *arrows* indicate two pairs of reciprocal translocations involving A- and D-genome chromosomes, while the *asterisks* denote the pair of markedly smaller chromosomes of *Th. intermedium* origin

Zhong 1 had a mitotic chromosome number of 2*n*=52. Occasionally, plants with a chromosome number of 2*n*=51 were detected, but the great majority of Zhong 1 plants were stable with a chromosome number of 2*n*=52. When we probed Zhong 1 with *Th. intermedium* genomic DNA using Chinese Spring (genomes ABD) genomic DNA as a block, 42 chromosomes were red and ten chromosomes were green or yellow, indicating that Zhong 1 contained 42 wheat chromosomes and ten *Th. intermedium* chromosomes (Fig. 1a). In addition, one pair of wheat chromosomes showed a distinct greenish-yellow coloration at the terminal position compared to the background PI red coloration of the remaining 42 chromosomes (Fig. 1a, arrowed), denoting that this pair of chromosomes contained a translocated chromosome segment from *Th. intermedium*. Chromosome counts showed that the chromosome number of Zhong 2 was 2*n*=54 (Fig. 1c). GISH analysis revealed a stable chromosome number of 2*n*=54, with 42 wheat chromosomes and 12 *Th. intermedium* chromosomes. Multiple individuals of this amphiploid sampled at intervals from 1996 to 2003 all showed this identical genomic constitution (data not shown), suggesting genomic stability of this amphiploid through generations.

The three type II partial amphiploids (Zhong 3, Zhong 4, Zhong 5) all had a chromosome number of 2*n*=8*x*=56 that included 42 wheat chromosomes (red) and 14 alien chromosomes (green) (Fig. 1e). There were no gross structural changes detectable at the GISH level of the wheat genome chromosomes in the type II amphiploids, but Zhong 4 contained one pair of *Th. intermedium* chromosomes that was markedly smaller than the normal *Th. intermedium* chromosomes (Fig. 1f).

mcGISH analysis of the five partial amphiploids

We further analyzed the five partial amphiploids by mcGISH using various total genomic DNAs to determine the genomic identity of the wheat chromosomes as well as possible inter-genomic structural changes. Using this technique, the A-, B- and D-genome chromosomes were respectively revealed as having yellow, brown/gray and red/pink fluorescence, while the alien chromosomes of *Th. intermedium* were labeled as a green fluorescence. Apparently, Zhong 1 contained the complete set of wheat chromosomes—namely, 14 for each of the A, B and D genomes (Fig.1b). In addition, Zhong 1 contained one pair of translocated chromosomes involving an A-genome chromosome with a terminal *Th. intermedium* segment on the short arm. These observations confirmed the results of GISH analysis (Fig. 1a). A closer inspection, however, revealed a slight difference between the GISH and mcGISH patterns with respect to the Zhong 1 chromosome constitution: whereas the GISH patterns indicated the existence of ten *Th. intermedium* chromosomes in Zhong 1 (Fig. 1a), the mcGISH pattern clearly showed only eight *Th. intermedium* chromosomes (green) —with the remaining two chromosomes being a pair that showed a pink coloration in the proximal regions and green coloration in the terminal regions (Fig. 1b). This result suggests that one pair of *Th. intermedium* chromosomes may be distantly homoelogous to the wheat D-genome chromosome(s).

mcGISH showed that Zhong 2 contained the complete 42 chromosomes of wheat (14 from each of the A, B and D genomes) and 12 *Th. intermedium* chromosomes (Fig.1d). Notably, one pair of chromosomes exhibited the faint green/pink coloration suggestive of some affinity to the wheat D genome, similar to that shown by Zhong 1 (Fig. 1d). No gross structural changes in Zhong 2 were detected by mcGISH (Fig. 1d).

The mcGISH results revealed that lines Zhong 3 and Zhong 5 contained 14 A-genome chromosomes, 14 B-genome chromosomes and 14 D-genome chromosomes plus 14 *Th. intermedium* chromosomes and that there were no gross structural changes (data not shown). Zhong 4 also contained 14 A-genome chromosomes, 14 B-genome chromosomes and 14 D-genome chromosomes plus 14 *Th. intermedium* chromosomes. It is interesting that two pairs of reciprocal translocations involving the A- and D-genome chromosomes occurred in Zhong 4 (Fig. 1f). All of the wheat parental cultivars involved in the production of these partial amphiploids were checked by mcGISH, and no structural changes in any of these wheat cultivars were detected (data not shown).

Gliadin and HMW glutenin analysis of the five partial amphiploids

The electrophoretic profiles of the gliadin and HMW glutenin subunits of the five partial amphiploids, Zhong 1 to Zhong 5, were analyzed. Zhong 1 and Zhong 2 had identical banding patterns for both gliadin and HMW glutenin (Figs. 2, 3). One *Th. intermedium*-specific HMW glutenin band that was lacking in all wheat parents was present in Zhong 1 and Zhong 2. In addition, Zhong 1 and Zhong 2 contained several gliadin bands that were not present in either the wheat parents or in more than ten random individual plants of *Th. intermedium* (Fig. 2, arrowed), implying that they were the products of either inter-genomic coordinated expression and/or physical genomic changes. It is worth mentioning that Zhong 1 and Zhong 2 contain a 1, 7+9 and 5+10 HMW subunit composition in the Glu-A1, Glu-B1 and Glu-D1 regions, respectively, and, consequently, these two partial amphiploids should possess excellent breadmaking qualities according to the standard for bread-making qualities of bread wheat (Payne et al. 1988). Zhong 3, Zhong 4 and Zhong 5 had identical gliadin banding patterns with one specific band from *Th. intermedium* (Fig. 4). These type II partial amphiploids also contained identical HMW glutenin banding patterns (data not shown).

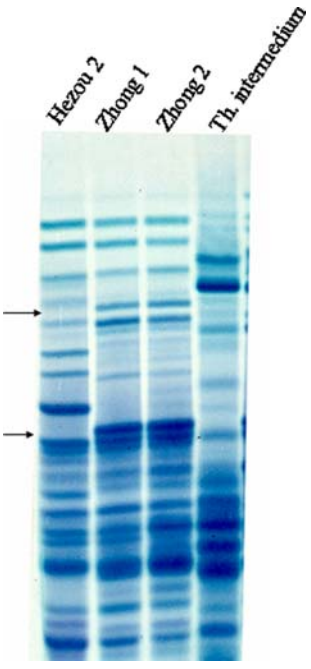

Fig. 2 Gliadin electrophoretograms of partial amphiploids Zhong 1, Zhong 2 and their parents. *Arrows* indicate novel bands only present in the partial amphiploids

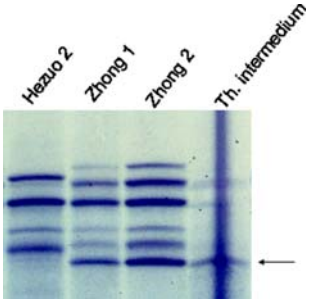

Fig. 3 Glutenin electrophoretograms of partial amphiploids Zhong 1, Zhong 2 and their parents. The *arrow* indicates the *Th. intermedium*-specific band

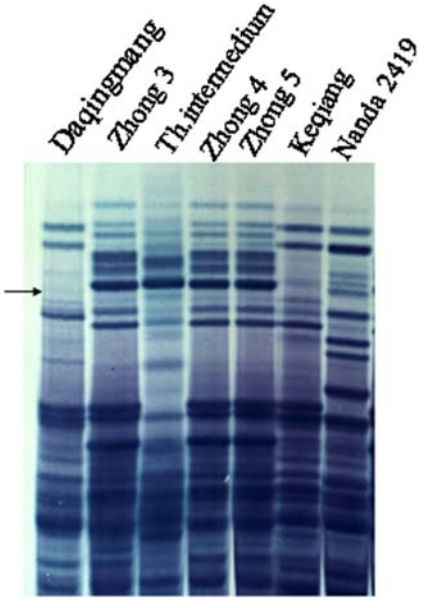

Fig. 4 Gliadin electrophoretograms of partial amphiploids Zhong 3, Zhong 4 and Zhong 5 and their parents. The *arrow* indicates the *Th. intermedium*-specific band

Discussion

Polyploidy is a process whereby two or more genomes are brought together into the same nucleus, usually by hybridization followed by chromosome doubling. Allopolyploids or amphiploids usually contain two or more complete genomes of the parental species. In this respect, the five partial amphiploids of common wheat-*Th. intermedium* analyzed in the present study are of a different type of newly formed allopolyploids in that they contain the complete genomes of wheat but an incomplete genome (a set of chromosomes) of *Th. intermedium*. These partial amphiploids were developed by backcrossing the F_1 hybrids of wheat-*Th. intermedium* with wheat as the reciprocal parent. In the many of the earlier investigations differential partial amphiploids were characterized by meiotic analysis of F_1 hybrids and by inter-crossing the different partial amphiploids (Banks et al. 1993; Han 1994; Fedak et al. 2000).

When the five partial amphiploids (Zhong 1 to Zhong 5) were released in the 1970s, many researchers believed that Zhong 2 had a chromosome number of $2n$=56, with the genomic constitution of Zhong 1 being uncertain (Han 1994). Since 1985, we have been systematically studying the Zhong series of partial amphiploids and their derivatives as well as maintaining the original plant materials by strict selfing. In the present study we used conventional GISH and multicolor GISH techniques to definitively resolve the genomic constitutions of Zhong 1 and Zhong 2 by showing that both contain the complete wheat A, B and D genomes but with 10 and 12 *Th. intermedium* chromosomes, respectively.

It is generally believed that only euploid amphiploids are genetically stable, while aneuploids are labile often resulting in loss of the added alien chromosomes (Matzke et al. 1999). Nevertheless, our results here show that some combinations of aneuploids, such as Zhong 1 and Zhong 2, can be remarkably stable. Zhong 1 and Zhong 2 do not contain the 14 *Th. intermedium* chromosomes necessary to classified as euploids; nevertheless, they are characterized by bivalent pairing, full fertility and disomic inheritance (Gao et al. 1999). Interestingly, although the partial amphiploid lines themselves can be fully fertile and show regular meiotic behavior, there is a great deal of unexplained chromosome pairing in backcrosses and inter-crosses of different partial amphiploid lines (Fedak et al. 2000). In inter-crosses of partial amphiploids, meiotic configurations containing chains of up to eight chromosomes have been observed (Fedak et al. 2000). It has been suggested that the extensive chromosome pairing may be due to multiple translocations and/or unpredictable behavior and dosages of the meiotic pairing control genes (Fedak et al. 2000).

Compared to conventional GISH, mcGISH provides a powerful technique to determine the genomic origin of translocated chromosomes originating from intergeneric hybridizations by identifying all three wheat genomes plus the alien genome (Han et al. 2003). Using this technique, we have confirmed that Zhong 1 has a pair of translocated

chromosomes between wheat and *Th. intermedium*, with the chromosome segments of *Th. intermedium* being translocated to the short arms of a pair of wheat A-genome chromosomes (Fig. 1b). For the purpose of characterizing genomic constitutions of these partial amphiploids (Zhong 1 to Zhong 5), mcGISH apparently provides a much more accurate and informative resolution than is possible with the conventional GISH.

Zhong 1 and Zhong 2 were derived from the same hybrid combination as well as the same backcrossing pedigrees (Chi et al. 1979). Nevertheless, different types of structural genomic changes have occurred in these two partial amphiploids. Since their production, apparent inter-chromosomal exchanges (translocations) have occurred in Zhong 1 (Fig.1a), whereas only the loss of a pair of *Th. intermedium* chromosomes has occurred in Zhong 2 (Fig.1c). Similarly, Zhong 3, Zhong 4 and Zhong 5 were also derived from the same pedigree, but only Zhong 4 exhibits genomic changes at the chromosomal level (Fig. 1f). These observations suggest that the wheat homoeologous pairing control genes, such as *ph1* (Riley 1960; Sears 1976), were differentially suppressed by the presence of different *Th. intermedium* chromosome(s) (reviewed in Dvorak and Dubcovsky 1995 and references therein). Consequently, limited homoeologous chromosomal recombination between the wheat chromosomes and those of *Th. intermedim* has occurred in some cases but not in others (Fedak et al. 2000). Alternatively, the genomic changes may have resulted from some non-Mendelian mechanisms like the ones proposed recently by Feldman and colleagues (Feldman et al. 1997; Levy and Feldman 2002; Liu and Wendel 2002; Feldman and Levy 2003). If the later is the underlying cause, then the genomic changes in the partial amphiploids are likely stochastic in nature or that some unidentified genomic loci responsible for genomic stability from *Th. intermedium* are differentially present in the partial amphiploids even though they share the same pedigrees. It should be noted, however, that the absence of chromosomal changes does not rule out cryptic inter-genomic introgression and genomic changes at the DNA sequence level. Indeed, genomic changes have been detected in Zhong 3 and Zhong 5 by means of restriction fragment length polymorphism analysis (Liu et al. 1999)

Apart from genomic changes, another important aspect of rapid adjustment to duplicated genome dosage is through modifications to gene expression. In wheat, inter-genomic suppression, as seen by the disappearance of storage protein subunits, was observed immediately upon the formation of a wheat allohexaploid: when the D genome was removed from hexaploid wheat, the resulting tetraploid regained the disease resistance and breadmaking quality traits (Galili and Feldman 1984). In a newly synthesized wheat allotetraploid, Kashkush et al. (2002) found that transcript disappearance was caused either by gene loss or gene silencing. In addition, the activation of new transcripts, all of which being related to retrotransposons, was also found in some new amphiploids (Kashkush et al. 2003). Therefore, intergeneric crosses in wheat do not ensure complete parental-like, additive gene expression in the progeny. This is clearly illustrated in the gliadin and HMW glutenin patterns of Zhong 1 and Zhong 2, where both the loss of wheat parental bands and the appearance of novel bands were observed (Fig. 2). Incidentally, Zhong 1 and Zhong 2 have been found to have very good breadmaking characteristics, and their use as a parent has resulted in the release of several new wheat cultivars with excellent breadmaking qualities (He et al. 1993). It is presently unclear whether this quality trait is associated with the novel expression patterns of gliadin and HMW glutenin mentioned above. Several recent studies have shown that allopolyploidy accelerates genome evolution in wheat in two ways (reviewed in Feldman and Levy 2003 and references therein): (1) allopolyploidization triggers rapid genome changes (revolutionary changes) through the instantaneous generation of a variety of cardinal genetic and epigenetic alterations; (2) the allopolyploid state facilitates genomic changes during the life of the species (evolutionary changes) that are not attainable at the diploid level. Thus, the evolutionary changes comprise structural and functional changes. The results of the present study appear to lend support to this allopolyploidy paradigm because structural changes, such as inter-genomic transfer of chromosomal segments, have apparently occurred in one of the partial amphiploids, Zhong 4, that involved the wheat A- and D-genome chromosomes (Fig. 1f). In addition, Zhong 4 also contained a pair of cytologically discernible, markedly smaller chromosomes of *Th. intermedium* origin (Fig. 1f), thus further suggesting genomic instability of this nascent partial amphiploid.

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