Synthesis and Characterization of Advanced Durum Wheat Hybrids and Addition Lines with Thinopyrum Chromosomes

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Abstract

Durum wheat (Triticum turgidum L., 2n = 4x = 28; AABB genomes) is a natural hybrid—an allotetraploid between 2 wild species, Triticum urartu Tumanian (AA genome) and Aegilops speltoides Tausch (BB genome). Even at the allotetraploid level, durum wheat can tolerate chromosomal imbalance, for example, addition of alien chromosome 1E of diploid wheatgrass, Lophopyrum elongatum. Therefore, one way to broaden its genetic base is to add a desirable chromosome(s) from diploid wild relatives. We attempted chromosomal engineering with chromosomes of a diploid wheatgrass, Thinopyrum bessarabicum—a source of resistance to some diseases including Fusarium head blight. Several advanced hybrids and alien addition lines were studied using traditional cytology, multicolor fluorescent genomic in situ hybridization, and molecular markers. Hybrid derivatives varied in chromosome number from F1 to F8 generations and in backcross generations. In advanced generations, we exercised selection against 28-chromosome plants and in favor of 30-chromosome plants that helped recover 14 addition lines in the F8 generation, as indicated by the absence of segregation for 29-chromosome plants. Disomic additions showed regular meiosis with 15 bivalents, 14 of durum wheat, and 1 of Th. bessarabicum. The addition lines will facilitate further chromosome engineering work on durum wheat for broadening its genetic base.

Key words: alien chromosome, disomic addition line, fluorescent genomic in situ hybridization (fl-GISH), homoeologous chromosome pairing, intergeneric hybrids, molecular markers, monosomic addition line

Bread wheat (Triticum aestivum L., 2n = 6x = 42; AABBD genomes) and durum wheat or pasta wheat (Triticum turgidum L., 2n = 4x = 28; AABB genomes) are among the main food sources for humankind. A large number of related, wild species have been used as donors of desirable genes for the genetic enrichment of these cultivated wheats by conventional means, sometimes combined with chromosomal engineering (Ceoloni and Jauhar 2006; Qi et al. 2007; Gill et al. 2011; Liu et al. 2011). Because of its high protein content and gluten strength, durum wheat is a cereal of choice for making pasta products (Gazza et al. 2011) and is widely grown in the Northern Great Plains of the United States. Durum wheat is an allotetraploid. Its A genome was derived from Triticum urartu Tumanian (Nishikawa 1983; Dvořák et al. 1993), and most probably the source of the B genome is Aegilops speltoides Tausch (Sarkar and Stebbins 1956; Wang et al. 1997) or a relative that may now be extinct.

Compared with allohexaploid bread wheat (with 3 genomes), durum wheat (with only 2 genomes) has a narrower genetic base and lower genetic buffering (see Jauhar 2006). However, it can tolerate chromosome engineering (Ceoloni and Jauhar 2006) and addition of alien chromosomes (Jauhar et al. 2009) or substitution of its chromosome(s) with corresponding chromosome(s) from its wild relatives (Jauhar and Peterson 2012). One way to broaden its genetic base would be to add alien chromosome(s) or chromatin from related species. Synthesis of a stable durum alien addition line by adding a chromosome 1E pair of diploid wheatgrass (Lophopyrum elongatum [Host] Ľ. Löve, 2n = 2x = 14; EE genome) (Jauhar 2008; Jauhar and Peterson 2008; Jauhar et al. 2009) opened up avenues for chromosomal engineering with chromosomes of other related wild species. Therefore, we selected a closely related diploid wheatgrass Thinopyrum bessarabicum (Sávul and Ráyss) Ľ. Löve (2n = 2x = 14;
Materials and Methods

Plant Materials

Diploid wheatgrass *Th. bessarabicum* (Sávul and Ráyss) Á. Löve (*2n* = 2x = 14; JJ genome) is perennial and is maintained by vegetative propagation in the greenhouse. Durum wheat (*T. turgidum* L., *2n* = 4x = 28; AABB genomes) cultivars Langdon and Lloyd, and the previously synthesized durum × *Th. bessarabicum* hybrids and hybrid derivatives (*Jauhar and Peterson 2006*) were grown in the greenhouse under a temperature and lighting regime of 20–24°C and 16-h light/8-h dark cycle.

Raising the *F1* and BC Generations

By crossing the durum cultivar Lloyd with *Th. bessarabicum*, F1 hybrids were raised, and these were selfed to produce F2 to F8 generations. By backcrossing the F1 with the recurrent parent, backcross generations were also derived. There was no need for embryo rescue or any hormonal treatment in these crosses.

Cytological Studies and Raising Alien Addition Lines with 2n = 29 or 30 Chromosomes

The hybrid derivatives with 29 or 30 chromosomes are called, respectively, monosomic addition or disomic addition lines. For somatic chromosome counts, seeds from hybrid derivatives involving Lloyd or Langdon were germinated in Petri dishes, and chromosome numbers were determined from fixed root tips stained in carbol-fuchsin (*Jauhar 2003*). Chromosome numbers were counted in at least 10 cells per plant to isolate monosomic and disomic alien addition lines with *Th. bessarabicum* chromosomes. The gminated seeds with the desired chromosome numbers of 29 or more were planted in the greenhouse. For meiotic analyses, spikes were fixed in Carnoy’s fluid (6 parts ethanol:3 parts chloroform:1 part glacial acetic acid) when the spike was approximately 1–2 mm above the node in the flag leaf. Chromosome pairing was studied from pollen mother cells from the squashed anthers (*Jauhar et al. 2004; Jauhar and Peterson 2006*). Seed from the desired plants was harvested to raise subsequent generations.

Multicolor Fluorescent Genomic In Situ Hybridization

We used the fl-GISH protocol (*Jauhar and Peterson 2006*) to identify the durum and *Th. bessarabicum* chromosomes in the intergeneric hybrid derivatives and to identify alien addition lines. A hybridization mixture of 50 μL formamide, 20 μL 50% dextran sulfate, 10 μL 20× SSC, and 20 μL of labeled probe (400 ng) and blocking DNA (8000 ng) was used on 4 slides. *Th. bessarabicum* genomic DNA was sheared with a sonicator to ~300 bp and was directly labeled using nick translation with fluorescein isothiocyanate (FITC). Autoclaved Langdon genomic DNA was used as the blocking DNA. After hybridization, the slides were counterstained with 20 μg/mL propidium iodide (PI). The durum chromosomes stained red, but the 7 *Th. bessarabicum* chromosomes fluoresced green. The parental chromosomes could thus be tracked during meiosis.

For multicolor fl-GISH, fluorescent probes were prepared for the A genome and the J genome independently. The hybridization mixture was the same as used previously with the exception of an additional probe and higher blocking DNA concentrations. 400 ng of *T. urartu* genomic DNA directly labeled with rhodamine was used to detect the A-genome chromosomes; 400 ng *Th. bessarabicum* genomic DNA directly labeled with FITC was used to detect the J-genome chromosomes, whereas 16 000 ng of autoclaved *Ae. speltoides* genomic DNA was used as the blocker. The preparations were then counterstained with 3 μg/mL 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Antifade solution was applied to all slides covered with glass coverslips and viewed with a fluorescent microscope. The *T. urartu* genomic DNA labeled with FITC probed the A-genome chromosomes of durum wheat; *Th. bessarabicum* genomic DNA labeled with rhodamine probed J genome of *Th. bessarabicum*, whereas the B genome was blocked by the *Ae. speltoides* DNA. Individual images were captured for each of the probes and counterstain. A composite was generated by overlaying the probe images onto the counterstained image using stringent transparency settings, thereby masking the counterstained chromosomes. Thus, the chromosomes of the 3 genomes A, B, and J could be studied during meioisis.

Extraction of DNA and Use of Molecular Markers

Using the cetyltrimethylammonium bromide method (*Doyle and Doyle 1990*) or a modified minipreparation (*Dellaporta et al. 1983*), DNA was extracted from the collected leaf samples. Modification to the minipreparation was primarily in the tissue grinding. After freeze drying approximately 0.3–0.75 gm of new leaf tissue, a mixer mill was used to pulverize the tissue into a fine powder. This facilitated rapid processing of a large number of samples.

Molecular markers were used to identify the alien chromosomes in various hybrid derivatives and addition lines designated as KS1 to KS13. Based on previous experience, and using markers of *Mullan et al. (2005)*, we selected primers to characterize *Th. bessarabicum* chromosomes. The markers used to characterize the J-genome chromosomes are listed in Table 1. Primers were used to amplify DNA of selected lines.
Polymerase chain reaction (PCR) was done according to the method standardized earlier (Jauhar et al. 2009) and used in subsequent research (Jauhar and Peterson 2011). We used a 25 µL PCR prepared as follows: 10× PCR buffer containing 1.5 mM MgCl₂ pH 8.3 (5 µL), forward primer 50 ρmol (1 µL), reverse primer 50 ρmol (1 µL), dNTP mix 2.5 mM (4 µL), sterile water (34.6 µL), Taq DNA polymerase 2 units (0.4 µL) (Roche Applied Science, Mannheim, Germany), and 800 ng genomic DNA in Tris/ethylenediamine tetraacetic acid (TE) buffer pH 7.4 (4 µL of 200 ng/µL genomic DNA). A Dyad thermocycler (MJ Research, Waltham, Massachusetts) running a touchdown program was used: 94°C for 2 min to denature followed by 94°C for 30 s, 65°C for 40 s; this step was decreased by 0.3°C each cycle, 72°C for 1 min, and cycled 35 times. The final extension step of 72°C was run for 7 min, and after this step, the reaction was held at 4°C. Five µL of bromophenol blue tracking dye was added and samples loaded onto 8 cm × 8 cm, 1% acrylamide gels, and run at 70 V at 20 mA until tracking dye migrated off the gel. The gel was then stained with 5 µL GelRed (Biotium, Hayward, California) according to the manufacturer’s instructions and then visualized with a UV light box.

Results

Frequency of Monosomic and Disomic Additions in F₁ to F₈ Generations

A Lloyd × Th. bessarabicum F₁ hybrid set 2 seeds and from these, F₂ plants were obtained. These F₂ plants were crossed with Lloyd to produce new F₃. Through a series of selfing, F₁ to F₆ generations were raised (Table 2). The chromosome number in the Lloyd × Th. bessarabicum hybrids varied from the F₁ to F₈ generations, and while studying these generations, we continuously selected against 28-chromosome plants, i.e., durum plants. The number of plants studied, their chromosome numbers, and the frequency of monosomic and disomic alien additions are given in Table 2.

In the F₁ and F₂ generations, the chromosome number varied from 28 to 39, and there were some alien additions with 29 and 30 chromosomes. In F₃ to F₈ the chromosome number varied from 28 to 32 and several alien additions with 29 or 30 chromosomes were derived. As we advanced to F₁₀, F₁₅, and F₁₈ generations, we exercised further selection for 29- and 30-chromosome plants, resulting in 14 stable addition lines in the F₈ generation. Thus, in F₈ we obtained all 30-chromosome disomic addition lines but no monosomic additions (Table 2). These disomic additions showed regular meiosis forming 15 bivalents, 14 of durum wheat and 1 of Th. bessarabicum. The identity of the added chromosome(s) in these addition lines could not be determined.

Characterization of BC₁ Plants with 2n = 35 Chromosomes Using Fluorescent GISH

By backcrossing the original F₁ hybrids with the recurrent parent Lloyd or Langdon, BC₁ plants were obtained and subsequent backcrosses yielded BC₁F₂, BC₁F₃, and BC₁F₄ generations. By backcrossing the BC₁ with the recurrent parent, BC₂, BC₂F₂, BC₂F₃, and BC₂F₄, and BC₂F₅ generations were also raised (Table 3).

The 6 BC₁ plants had mostly 35 chromosomes that resulted from meiotic restitution; the unreduced 21-chromosome gamete of the F₁ hybrid fused with the reduced 14-chromosome gamete of the recurrent parent. Fluorescent GISH showed that the BC₁ plants had 28 durum chromosomes that paired as 14 bivalents (fluorescing red) plus 7 Th. bessarabicum chromosomes (fluorescing green) (Figure 1A). Single-color fl-GISH did not distinguish between the A-genome and B-genome chromosomes. However, multicolor fl-GISH analysis confirmed the presence of 7 A-genome bivalents (fluorescing red), 7 B-genome bivalents (fluorescing blue), and the added 7 J-genome univalents (fluorescing green) (Figure 1B). Thus, the chromosomal constitution of these BC₁s was confirmed by multicolor fl-GISH.

Through a series of selfing in the BC₁s we derived BC₁F₂, BC₁F₃, and BC₁F₄ plants. Further, by backcrossing the BC₁s to the recurrent parental cultivar Langdon or Lloyd, we produced BC₂F₂, BC₂F₃, BC₂F₄, and BC₂F₅ generations. As
in the BC₁, mostly 35-chromosome plants were obtained in the BC₁F₂. In this generation, no alien monosomic or disomic addition lines were obtained. However, in the advanced generations, BC₁F₃ and BC₁F₄, we obtained alien addition lines with 29 and 30 chromosomes (Table 3).

### Cytological Characterization of Monosomic and Disomic Additions

The monosomic alien additions with 29 somatic chromosomes (Figure 2A) formed mostly 14II + 1I at meiotic metaphase I, the alien *Th. bessarabicum* chromosome remaining as a univalent (Figure 2B). The disomic additions with 30 chromosomes (Figure 2C) showed mostly 15II (Figure 2D), as expected. However, some 30-chromosome additions (Figure 2E) were double monosomic additions and formed 14II + 2I, the 2 alien chromosomes being the univalents (Figure 2F).

### Molecular Characterization of Monosomic and Disomic Additions

In addition to using traditional cytological tools combined with fl-GISH, we used molecular markers to characterize alien chromosomes in the durum addition lines. Various markers used to identify J-genome chromosomes of *Th. bessarabicum* are listed in Table 1. We identified markers for chromosomes 1, 2, 3, 5, 6, and 7 of the J genome in monosomic or disomic addition lines but could not find a suitable marker to characterize chromosome 4. Thus, we used Xedm74 for identifying chromosome 1J, Xedm8 for chromosome 2J and 3J (it bands both chromosomes), Xedm54 for 5J, Xedm80 for 6J, and Xedm34 for 7J. The chromosome not profiled by any of these primers can be inferred to be chromosome 4J.

We studied 10 hybrid lines derived from a 31-chromosome plant that paired as 14 bivalents and 3 univalents. These lines were screened using the set of simple sequence repeat (SSR) markers to identify the added *Th. bessarabicum* chromosomes. Figure 3 shows the gels for markers that generated a band for an added chromosome. We found that the added chromosomes were J2 or J3, J6, and J4. The marker gels that were null for bands are not shown in Figure 3.

### Discussion

Unlike hexaploid bread wheat with 3 genomes, AA, BB, and DD, durum wheat is an allotetraploid with 2 genomes AA and BB, and has a narrower genetic base. One way to broaden its genetic base is to integrate desirable alien chromosomes from the addition lines involving related species. This may appear difficult because of its allotetraploid nature that confers a limited amount of genetic buffering and hence low tolerance for added alien chromosomal material. However, we have shown earlier that durum wheat can tolerate addition of chromosomes from related wild species. The synthesis of a stable durum disomic addition line with 2n = 28 + 2 chromosomes by adding a double dose of chromosome 1E of diploid wheatgrass, *L. elongatum* (EE genome), with genes for FHB resistance (Jauhar 2008, 2011; Jauhar and Peterson 2008; Jauhar et al. 2009) opened up new avenues for chromosomal engineering with chromosomes of another desirable and related diploid wheatgrass, *Th. bessarabicum* (JJ genome). The 2 diploid wheatgrass, *L. elongatum* (EE genome) and *Th. bessarabicum* (JJ genome) are rich reservoirs of genes for agronomically desirable traits, including FHB resistance and salt tolerance (see Jauhar et al. 2009 and references therein; Zhang et al. 2002; Qi et al. 2010). The E and J genomes are closely related (Jauhar 1990), and they have been incorporated into durum wheat separately (Jauhar 1991) and together (Jauhar 1992) and should help the genetic enrichment of durum wheat.

It is interesting that the chromatin of a diploid wheatgrass can be integrated with that of durum wheat (Jauhar and Peterson 2008). Disomic *Th. intermedium* addition lines in bread wheat with resistance to barley yellow dwarf virus and rust were synthesized (Larkin et al. 1995). Shulan et al. (2012) produced and characterized wheat-*L. elongatum* (= *Th. elongatum*) addition lines that were a new source of FHB resistance. Having achieved success with durum alien addition with a *L. elongatum* chromosome pair 1E (Jauhar et al. 2009), we have been trying to develop similar alien addition lines involving *Th. bessarabicum* chromosomes to help tap the desirable traits stated earlier. *Thinopyrum intermedium* and *Th. ponticum* have been used as desirable sources of resistance to fungal and viral diseases of wheat (Li and Wang 2009; Gill et al. 2011). However, *Th. bessarabicum* is a diploid with 7 pairs...
of chromosomes and hence easier to use in gene introgression work with durum wheat.

The meiotically restituted 35-chromosome hybrids with 28 durum chromosomes and 7 J-genome chromosomes are in essence alien addition lines with all the 7 chromosomes added to the durum chromosome complement. Multicolor fl-GISH is an excellent tool in revealing the chromosomal makeup of 35-chromosome hybrids. It clearly distinguishes the 7 A-genome bivalents in red color, 7 B-genome bivalents in blue, and 7 J-genome univalents in green (Figure 1B) and is a valuable tool in characterizing alien chromosomes introduced into wheat.

The hybrids and hybrid derivatives between durum wheat and Th. bessarabicum are vigorous and perennial and hence available for sustained chromosome manipulation. Hybrid material developed through combination of backcrossing...
and selfing yielded several addition lines ranging from 29 to 35 chromosomes. Chromosome pairing in addition lines with 29 and 30 chromosomes revealed their chromosomal makeup. The truly disomic addition lines with 30 chromosomes (Figure 2C) paired mostly as 15 bivalents, 14 durum bivalents, and 1 *Th. bessarabicum* bivalent (Figure 2D), as expected. Some apparently disomic addition lines with 2n = 30 chromosomes paired as 14 bivalents and 2 univalents (Figure 2F) and that showed their double monosomic nature. The double monosomic additions characteristically formed 14 durum bivalents plus 2 J-genome univalents.

In the F₁ through F₈ generations of the durum × *Th. bessarabicum* F₁ hybrid, the frequency of monosomic and disomic additions showed an interesting trend. Exercising selection against 28-chromosome plants, that is, the durum revertants, and in favor of the 29- and 30-chromosome alien addition lines progressively yielded more addition lines. The F₄ to F₈ generations yielded only 28- to 30-chromosome plants, whereas the F₈ generation produced 30-chromosome disomic addition lines, but no monosomic additions, as indicated by absence of segregation for 29-chromosome plants. These monosomic addition lines, as expected, formed
14 durum bivalents and 1 *Th. bessarabicum* bivalent. The BC$_1$F$_3$ and BC$_1$F$_4$ generations yielded several addition lines (Table 3). However, additional backcrosses after the BC$_1$ did not confer any benefit in producing monosomic or disomic addition lines.

In addition to cytological techniques and fl-GISH, molecular markers provide useful tools for rapid screening of alien addition lines for specific alien chromosomes. We had primers to identify all of the J-genome chromosomes except 4J. Xedm74 was used to identify chromosome 1J, Xedm8 for chromosome 2J and 3J, Xedm54 for 5J, Xedm80 for 6J, and Xedm34 for 7J. Figure 3, for example, shows results obtained in the 10 lines screened. Xedm8 generated bands in 7 plants; Xedm80 produced bands in 3 plants. Xedm74, Xedm54, and Xedm34 generated no bands. In Figure 3, for example, line KS4 a 29-chromosome hybrid derivative did not show bands with any of the molecular markers. Therefore, we inferred that the chromosome not profiled by any of these primers was chromosome 4J. In Figure 3, however, shows bands for chromosomes 2 and 6, confirming the chromosomal constitution of this 30-chromosome hybrid derivative. This indicates the double monosomic constitution of this derivative.

The usefulness of molecular markers in marker-assisted selection for induced desirable traits such as FHB resistance...
(Buerstmayr et al. 2009) and characterization of alien chromosomes in addition lines (Lapitan and Jauhar 2006; Peleg et al. 2008; Jauhar and Peterson 2011; Liu et al. 2011) is well documented. Jauhar and Peterson (2011) successfully employed specific markers to simultaneously identify homologous group-1 chromosomes 1A, 1B, 1D, and 1E in hybrid derivatives of a durum disomic alien addition line. They also showed the usefulness of marker Xdem17 in producing unique bands for chromosome 1E and consistently profiling this chromosome in L. elongatum and the durum disomic addition line with chromosome 1E. Clearly, the FL-GISH and molecular markers would help characterize alien chromosome(s) in the reconstructed durum genome.

**Conclusion and Perspectives**

The cytogenetic architecture of cultivated polyploid wheats, durum wheat and bread wheat, provides an excellent model of evolution by allopolyploidy. This is an outstanding evolutionary pathway by which the most successful polyploid wheats originated. Of the 2 cultivated wheats, durum wheat with 2 genomes, AA and BB, has a narrower genetic base. The tools of cytogenetic and chromosomal engineering described here could help broaden its genetic base. Our work has shown that durum wheat has tolerance for added alien chromosome(s). Therefore, its genomic reconstruction with chromatin of related wild species is possible and may be effective in enhancing the genetic base of this important cereal crop. The alien addition lines could form effective bridges to transfer desirable genes from the alien species into the durum genome.

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