A Robertsonian translocation from Thinopyrum bessarabicum into bread wheat confers high iron and zinc contents

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With 2 figures and 2 tables

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Abstract
Development of wheat–alien translocation lines has facilitated practical utilization of alien species in wheat improvement. The production of a compensating Triticum aestivum–Thinopyrum bessarabicum whole-arm Robertsonian translocation (RobT) involving chromosomes 6D of wheat and 6E of Th. bessarabicum (2n = 2x = 14, E6E) through the mechanism of centric breakage–fusion is reported here. An F2 population was derived from plants double-monomosomic for chromosome 6D and 6E from crosses between a DS6E(6D) substitution line and bread wheat cultivar ‘Roushan’ (2n = 6x = 42, AABBDDB) as female parent. Eighty F2 genotypes (L1–L80) were screened for chromosome composition. Three PCR-based Landmark Unique Gene (PLUG) markers specific to chromosomes 6D and 6E were used for screening the F2 plants. One plant with a T6E-S.6DL centric fusion (RobT) was identified. A homozygous translocation line with full fertility was recovered among F2 families and verified with genomic in situ hybridization (GISH). Grain micronutrient analysis showed that the DS6E(6D) substitution line and T6E-S.6DL stock have higher Fe and Zn contents than the recipient wheat cultivar ‘Roushan’.

Key words: biofortification — grain Fe and Zn — PCR-based Landmark Unique Gene marker — Triticum aestivum

Common bread wheat, Triticum aestivum L. (2n = 6x = 42, AABBDDB), has many relatives in the tribe Triticeae that are valuable sources for broadening genetic diversity and may provide genes for quality and yield improvement (Gill et al. 2006, Ma and Tomita 2013, Farkas et al. 2014).

The genomes of species belonging to the tertiary gene pool of wheat are homoeologous, and their chromosomes have similar gene content and high synteny levels that enable them to replace each other in a compensating manner (Riley et al. 1968). Different approaches have been proposed for gene transfer from alien species to wheat depending on the evolutionary relationships of the species involved (Qi et al. 2007). These strategies usually involve the production of compensating wheat–alien translocations which can be produced for targeted chromosomes through the mechanism of centric breakage–fusion (Sears 1950).

Robertsonian translocations (RobTs) arise by centromeric misdivision of univalents at anaphase I followed by segregation of the derived telocentric chromosomes to the same nucleus, and fusion of the broken ends during the interkinesis of meiosis II (Friebe et al. 2005). Centric breakage–fusion can occur at different positions within the primary constriction without affecting the behaviour of resulting wheat–alien RobTs in mitosis or meiosis (Zhang et al. 2001). The frequency of recovery of wheat–alien RobTs ranges from 4% to 20% depending on the chromosomes involved, genetic background and environmental conditions (Łukaszewski 1997, Vega and Feldman 1998, Friebe et al. 2005, Qi et al. 2011).

The majority of the world population who depend on starch-rich cereals and tubers as staple food sources are subject to iron (Fe) and zinc (Zn) deficiency (Zimmermann and Hurrell 2007). The levels of these essential minerals in cereal grains are low, and the levels of genetic variation in Fe and Zn contents are limited among T. aestivum L. cultivars and landraces (Rawat et al. 2009b). Various wild wheat relatives possess higher grain iron and zinc contents (Cakmak et al. 2000, Tiwari et al. 2008, Rawat et al. 2009a), and attempts are being made to exploit them for biofortification of wheat.

Among the wild wheat relatives, Thinopyrum bessarabicum (Save ex Rayss) A Löve (2n = 2x = 14, E6E) is an important source of agronomically desirable genes for wheat breeding such as salinity tolerance and resistance to several diseases (Gorham et al. 1986, William and Mujeeb-Kazi 1995). Different wheat–Th. bessarabicum amphiploids, named Tritipyrums (2n = 6x = 42, AABBEBE6), have been produced (Alonso and Kimber 1980, King et al. 1997) and subsequently used to develop addition, substitution and translocation lines (William and Mujeeb-Kazi 1995, Qi et al. 2010, Zeinali et al. 2013). Although the micronutrient content of Th. bessarabicum has not been investigated, studies have indicated increased mineral nutrient concentration in the grain of perennial derivatives of bread wheat and Thinopyrum elongatum (Murphy et al. 2009). Our preliminary analysis also demonstrated high micronutrient contents in an amphiploid Tritipyrum and derived 6E substitution lines compared with the parental bread wheat cultivar ‘Roushan’, indicating that chromosome 6E harbours genes for higher Fe and Zn contents that could be transferred to wheat cultivars. These observations encouraged us to produce and evaluate wheat–Th. bessarabicum translocation lines in the background of the wheat cv. ‘Roushan’ which is a low Fe and Zn content cultivar (Moradi et al. 2014).

This study describes the identification of a compensating wheat–Th. bessarabicum RobT involving chromosomes 6D of wheat and 6E of Th. bessarabicum using PCR-based Landmark Unique Gene (PLUG) markers and genomic in situ hybridization (GISH). The identified RobT line, 6E addition line and substitution lines, parental wheat cultivar and amphiploid were also evaluated for grain micronutrient contents.

Materials and Methods

Plant material: The lines used in this study included T. aestivum cv. ‘Roushan’ (2n = 6x = 42, AABBDDB), Tritipyrum line Azb (2n = 6x = 42, AABBEBE6) (King et al. 1997), 6E addition line, DS6E(6D) substitution line, two Chinese Spring (CS) null-tetrasomic (N6D-T6B and N6D-T6A) and two ditelosomic (D6DLD and D6DLS) stocks. The wheat disomic chromosome substitution line in ‘Roushan’...
background, that is DS6E^6(6D), in which a 6D pair of wheat is replaced with a 6E^6 pair of *Th. bessarabicum* chromosomes was produced at the University of Kurdistan, Sanandaj (Zeinali et al. 2013).

To produce compensating RobT's involving chromosomes 6D of wheat and 6E^6 of *Th. bessarabicum*, a cross was made between ‘Roushan’ (as female parent) and the DS6E^6(6D) substitution line. F_2 plants with 42 chromosomes that were double-monomosomic for chromosomes 6D and 6E^6 were self-pollinated. The F_2 progeny were genotyped by molecular markers to identify putative RobT's.

**Molecular marker analysis:** Eighty F_2 seeds were planted in the field along with the 6E^6 addition line, DS6E^6(6D) substitution line and parental genotypes (*T. aestivum* cv. ‘Roushan’ and *Thinopyrum*) for DNA extraction and for obtaining F_2 families, in autumn of 2013. DNA was extracted from fresh leaves at the three-leaf stage by the CTAB method. The purity and concentration of DNA was assessed by comparison with 1-kb DNA ladder in 1% agarose gel and by spectrophotometry. The DNA of each plant was finally diluted to ~50 ng/μl and stored at ~20°C. Primers of PLUG markers including TNAC1674, TNAC1679 and TNAC1763 were made by Bioneer Co. (Daejeon, South Korea) and used in 20 μl PCR to identify 6E^6 chromosome arms in the lines (Table 1). These primers produce specific bands for group 6 homoeologous wheat chromosomes (Ishikawa et al. 2009, Zeinali et al. 2013). The PCR mixture contained 25 ng template DNA, 5 pmol of each primer, 2.5 mM of each dNTP, 2.5 mM MgCl_2 and 0.5 U *Taq* polymerase. Amplification was for 5 min at 94°C, followed by 30 cycles of 45 s at 94°C, 45 s at 61–63°C (depending on primers), 90 s at 72°C and a final step of 7 min at 72°C. A 10-μl aliquot of the mixture was digested overnight with 2.0 U of *HaeIII* or *TaqI* (Table 1) in incubators set at 37 or 65°C, respectively. Digested PCR products were separated by electrophoresis in 3% agarose gels.

**Genomic in situ hybridization:** Actively growing roots at 1–1.5 cm in length were cut and pretreated in ice water for 24 h. Root tips were then fixed in ethanol : acetic acid (3 : 1) for 48 h at 4°C. Chromosome preparations were carried out by the squash method as previously described (Mirzaghadari 2010). GISH was performed as described by Mirzaghadari et al. (2011) to detect E^6 chromatin. *Thinopyrum bessarabicum* genomic DNA was labelled with biotin-16-dUTP by nick translation and purified by ethanol precipitation. Unlabelled, genomic DNA was labelled with biotin-16-dUTP by *Th. bessarabicum* a cross was made between *T. aestivum* cv. ‘Roushan’ and *Thinopyrum*, 6Eb addition line, DS6Eb(6D) substitution line and the F_3 seeds of the identified T6E^6S.6DL translocation line were planted in autumn 2014 in a randomized complete block design with three replications at the Dushan Research Station of the University of Kurdistan (35°15’N, 47°01’W). Each line was sown in a 1.5-m row of 12 plants. Morphological characters were recorded in the spring of 2015. Tiller number per plant, height, spikelet number per main spike and 1000-grain weight were measured on ten plants in each plot. For micronutrient analysis, one gram of grain samples was digested in a mixture of two parts concentrated HNO_3 and one part HCl according to Zarcinas et al. (1987). Digestion was continued until a white residue was obtained. The required volume was made up after completion of the digestion process, and digests were analysed using an atomic absorption spectrophotometer (GBC 902 AA, Australia). Iron and zinc concentrations were expressed as mg/g (ppm) on a dry weight basis. Three biological replications from each genotype were analysed. For grain ash analysis, 1 g of dried grain of each line was cleaned thoroughly and incinerated at 600°C for 10 h. The ash was further processed like the grains for micronutrient analysis.

**Results**

**Validation of chromosome group 6 arm-specific markers**

To ensure correct assignment of specific PLUG marker alleles to wheat chromosome group 6 arms, we amplified them in nulli-tetrasomic and ditelosomic lines. The data showed that TNAC1674 and TNAC1679 were specific markers for short arm and TNAC1763 was specific for the long arm of chromosome 6D (Fig. 1). As TNAC1679 generated an additional band specific for 6E^6L (Fig. 1), it was also used in preliminary screening of 6E^6L in the wheat background.

**Identification of a wheat–*Thinopyrum bessarabicum* compensating RobT**

Of 80 F_2 individuals analysed using the three PLUG markers specific for 6DS, 6DL and 6E^6L chromosome arms, 65 amplified the markers for both 6DS and 6DL indicating they probably carried a complete chromosome 6D and 14 amplified neither marker indicating they lacked chromosome 6D. One plant (L48) amplified TNAC1763 specific for chromosome 6DL but lacked the specific TNAC1674 and 1679 alleles for 6DS, hence indicating the presence of only 6DL and suggesting it carried a Robertsonian translocation (Fig. 1). The TNAC1674 and TNAC1763 markers amplified only wheat alleles, whereas TNAC1679 amplified different bands for 6DS and 6E^6L and simultaneously detected both arms.

**GISH analysis**

The putative 6E^6S.6DL translocation (L48) identified with molecular markers was characterized by GISH using total genomic DNA of *Th. bessarabicum* as the probe. This confirmed that L48 is a homozygous 6E^6–wheat centric fusion and that a relatively small chromosome was involved (Fig. 2). This plant had 2n = 42.

<table>
<thead>
<tr>
<th>Primer sequence (5' → 3')</th>
<th>Enzyme producing polymorphic PCR product</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCT AGA TGG CAC ACC AAG TG</td>
<td><em>TaqI</em></td>
</tr>
<tr>
<td>CCA CCA CAG AAG CAG ATG AAT</td>
<td><em>HaeIII</em></td>
</tr>
<tr>
<td>TTC CAA ACC ACC CAG TGT GTA</td>
<td>No digestion</td>
</tr>
<tr>
<td>TAT TGG CTC AAC CAA CCA TTC</td>
<td></td>
</tr>
<tr>
<td>CGA TTG GCC GTA CAA CTT TC</td>
<td></td>
</tr>
<tr>
<td>Table 1: Primer sequences for PCR-based Landmark Unique Gene markers specific for wheat chromosome 6D and primer/enzyme combinations producing polymorphic PCR products</td>
<td></td>
</tr>
</tbody>
</table>
Phenotypic characterization

A summary of various agronomic trait measurements and grain micronutrient contents is provided in Table 2. The AD6E\(^b\) addition line had a significantly lower number of tillers per plant (7.4) and spikelets per main spike (14) than the wheat parent. The T6EbS.6DL (RobT) line had lower grain weight, higher ash content and higher iron and zinc contents than ‘Roushan’. There were about 9% and 13.5% increases in Zn and Fe, respectively, in the translocation line relative to ‘Roushan’. The amphiploid, but not the addition line, also had higher ash, Fe (39%) and Zn (71%) contents than ‘Roushan’ (Table 2).

Discussion

The PLUG markers used in the present study amplified wheat alleles, but not the 6E\(^b\)S chromosome arm. However, the presence of a TNAC1763 allele specific for 6DL and lack of the specific wheat allele for 6DS in line L48 indicated the presence of only 6DL. GISH using total genomic DNA of Th. bessarabicum as probe verified that L48 is a disomic Robertsonian translocation (Fig. 2).

The homeologous RobT was identified among 80 F\(_2\) plants, corresponding to a recovery rate of 1.2%. Various publications reported frequencies of 4–20% (Lukaszewski and Gustafson 1983, Lukaszewski 1997, Friebe et al. 2005, Qi et al. 2011). In a previous study, we recovered RobTs involving chromosome 2B of wheat and 2E\(^b\) of Th. bessarabicum at a higher frequency of about 5% (Ghazali et al. 2015).

Plant breeding offers a sustainable, low-cost way to increase micronutrient contents in grain (Graham et al. 2007, Velu et al. 2014). We observed that Th. bessarabicum can also be used as a genetic source to improve the micronutrient content of wheat, as the genotypes T6EbS.6DL and DS6Eb(6D) had significantly higher Fe and Zn contents than the wheat genotype ‘Roushan’.

The fact that the chromosome 6Eb in AD6Eb addition line has not significantly increased grain Fe and Zn, while the DS6Eb(6D) substitution and T6EbS.6DL translocation increased it, suggests a role of 6DS in suppressing Fe and Zn accumulation.

The most successful and widespread RobTs used in wheat improvement are the wheat-rye T1RS.1BL and T1RS.1AL translocations (Zeller 1973, Rabinovich 1998). Different useful RobTs have been derived from Dasypyrum villosum; for example, T4VS.4DL conferred resistance to wheat spindle streak mosaic virus (Zhang et al. 2005), T1DL.1V#3S increased gluten strength (Zhao et al. 2010) and T6AS.6V#3L conferred resistance to Puccinia graminis tritici race Ug99 (Qi et al. 2011). Translocation T2E\(^b\)S-2BS.2BL derived from Th. bessarabicum had higher yield per plant, suggesting that the alien segment carries yield-related genes (Qi et al. 2010).

Most of wheat cultivars have low grain Fe and Zn contents relative to their wild relatives (Cakmak et al. 2000, Rawat et al. 2009b). Different Aegilops and Triticum species with various genomic constitutions are potentially important sources of Fe and Zn for wheat improvement, and different chromosomes are thought to be involved in grain Fe and Zn contents (Tiwari et al. 2008, Genc et al. 2009, Neelam et al. 2011, Rawat et al. 2011, Farkas et al. 2014). Peleg et al. (2009) mapped 82 QTL for 10
Table 2: Morphological characteristics, grain Fe and Zn contents in Tritipyrum and its derivatives

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Spikelets/main tillers/plant</th>
<th>Spikelet height (cm)</th>
<th>1000-grain weight (g)</th>
<th>Ash (%)</th>
<th>Fe (µg/g dry weight)</th>
<th>Zn (µg/g dry weight)</th>
<th>% increase in ash Fe over Roushan</th>
<th>% increase in ash Zn over Roushan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphiploid</td>
<td>7.8 ± 0.5</td>
<td>72 ± 1.3</td>
<td>34.1</td>
<td>34.3 ± 1.4</td>
<td>0.3* ± 0.1</td>
<td>0.3** ± 0.1</td>
<td>7.3 ± 0.5</td>
<td>10.1 ± 0.4</td>
</tr>
<tr>
<td>DS6Eb(6D)</td>
<td>9.0 ± 0.1</td>
<td>72 ± 0.1</td>
<td>34.0</td>
<td>34.5 ± 0.5</td>
<td>0.3* ± 0.1</td>
<td>0.3** ± 0.1</td>
<td>7.2 ± 0.5</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>T6EbS.6DL</td>
<td>8.5 ± 0.8</td>
<td>72 ± 1.5</td>
<td>34.1</td>
<td>34.5 ± 1.4</td>
<td>0.3* ± 0.1</td>
<td>0.3** ± 0.1</td>
<td>7.2 ± 0.5</td>
<td>10.0 ± 0.4</td>
</tr>
</tbody>
</table>

* indicates significantly different from Roushan at α = 0.05 and α = 0.01, respectively.

Acknowledgements

The wheat nulli-tetrasomic and ditelosomic lines were kindly provided by Dr. H. Shahsavand and Dr. G. Fedak from AgriFood, Ottawa, Canada. The Tritipyrum allohexaploid were originally from Cereal Research Department of John Innes Centre, UK, and provided by Dr. H. Shahsavand Hasani, Shiraz University. The authors also thank to Ms. P. Shahidi for help in the atomic absorption spectrophotometer analyses. This research was financially supported by the University of Kurdistan, Sanandaj. The authors declare that they have no competing interests.

References


different minerals with most of the positive alleles contributed by wild emmer wheat, suggesting the importance of wild wheat relatives in genetic biofortification of wheat. QTL for Fe and Zn grain contents exhibited significant overlap, implying that the alleles for Zn and Fe deposition in the grain cosegregate or are pleiotropic and therefore that Zn and Fe can be improved simultaneously (Velu et al. 2014).

Little information is available about *Thinopyrum* spp. which may also be valuable for micronutrient biofortification. Murphy et al. (2009) reported increased grain Fe and Zn contents in perennial wheat lines derived from interspecific crosses between bread wheat and *Th. elongatum* (2n = 2x = 14; EE), a close relative of *Th. bessarabicum*. In the present study, the 1000-grain weight of Tritipyrum (34.1 g) and ‘Roushan’ (34.2 g) was nearly equal. The Tritipyrum amphiploids, in spite of having seed weight almost equal to that of bread wheat ‘Roushan’, had higher grain Fe and Zn contents, suggesting that the iron and zinc contents of the grain of Tritipyrum are not due to seed size but could be due to higher uptake and accumulation in seeds. A similar result was reported for *T. durum–Aegilops longissima* amphiploids (Tiwari et al. 2008). Higher Fe and Zn contents in the seeds of the translocation T6E5S.6DL suggest that the introgressed 6E5S chromosome might have contributed to grain micronutrient content. In wild emmer, wheat (*T. dicoccoides*) gene *Gpc-B1* for grain protein was shown to be critical in the regulation of rate of senescence and grain protein, as well as Zn and Fe contents (Uauy et al. 2006).

In the present study, a T6E5S.6DL Robertsonian translocation was developed and confirmed using molecular markers and GISH. Assays of the parental stocks showed high grain micronutrient content in the wheat–*Th. bessarabicum* amphiploids, but no increase in the 6E5 addition line. The translocation line showed a moderate increase in the grain micronutrient content, suggesting that 6E5S has a positive effect on Fe and Zn accumulation. However, other factors such as the interaction effect between the genes of the wheat and the introduced alien segment may be involved.

The T6E5S.6D Robertsonian translocation might be useful as a new genetic resource for improvement of grain micronutrient content of modern wheat cultivars. Further improvement could be achieved by directed chromosome engineering (Qi et al. 2007) aimed at shortening the *Th. bessarabicum* segment and further characterization of the T6E5S chromosome.


Zhang, P., B. Friebe, A. J. Lukaszewski, and B. S. Gill, 2001: The centromere structure in Robertsonian wheat-rye translocation chromosomes indicates that centric breakage-fusion can occur at different positions within the primary constriction. Chromosoma 110, 335–344.

