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ORIGINAL PAPER

# Substitutions of 2S and 7U chromosomes of *Aegilops kotschyi* in wheat enhance grain iron and zinc concentration

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**Abstract** Biofortification through genetic manipulation is the best approach for improving micronutrient content of the staple food crops to alleviate hidden hunger, namely, the deficiency of Fe and Zn affecting more than two billion people worldwide. An interspecific hybridization was made between *T. aestivum* line Chinese Spring (CS) and *Aegilops kotschyi* accession 3790 selected for high grain iron and zinc concentration. The CS × *Ae. kotschyi* $F_1$ hybrid with low chromosome pairing was highly male and female sterile. This was backcrossed with wheat cultivars to get seed set. The selfed $BC_1F_1$ and $BC_2F_1$ plants with high grain iron and zinc concentration were selected in subsequent generations. The selected derivatives showed 60–136% enhanced grain iron and zinc concentration and 50–120% increased iron and zinc content per seed as compared to the recipient wheat cultivars. Thirteen cytologically stable, fertile and agronomically superior plants with high grain iron and zinc concentrations were selected for molecular characterization. The application of anchored wheat SSR markers, transferable to *Ae. kotschyi*, to the high grain iron and zinc containing derivatives indicated introgression of group 2 and group 7 chromosomes of *Ae. kotschyi*. GISH and FISH analysis of some derivatives confirmed the substitution of chromosomes 2S and 7U for their homoeologues of the A genome, suggesting that some of the genes controlling high grain micronutrient content in the *Ae. kotschyi* accession are on these chromosomes.

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## Introduction

More than half of the world's population suffers from iron and zinc deficiency (White and Broadley 2009; World Health Organization 2002), popularly called as hidden hunger. Biofortification of staple foods is the most promising strategy to alleviate micronutrient deficiency (Brinch-Pederson et al. 2007; Johns and Enzaguirre 2007; Welch and Graham 2004). There are several approaches to biofortify crops, including agronomic biofortification (Cakmak 2008; Rengel et al. 1999), genetic engineering (Brinch-Pederson et al. 2007; Lonnerdal 2003; Lucca et al. 2006) and conventional or molecular breeding (Graham et al. 1999; Mayer et al. 2008; Nestel et al. 2006; Welch and Graham 2004). Among these approaches, breeding for micronutrient enhancement has been considered as the best strategy due to low and non-recurrent expenditure and higher public acceptability (Nestel et al. 2006; Ortiz-Monasterio et al. 2007; Yip 1997). Wheat is currently the primary staple food of almost one-third of the world's population (FAO 2004). Most of the bread (*Triticum aestivum* L.) and durum wheat [*T. turgidum* L. ssp. *durum* (Desf.) Husn.] cultivars have low grain iron and zinc content (Cakmak et al. 2000; Monasterio and Graham 2000; Rawat et al. 2009a). The related wild *Triticum* and *Aegilops* species with useful variability for high grain iron and zinc

content (Cakmak et al. 2000; Chhuneja et al. 2006; Rawat et al. 2009a) can be utilized for biofortification of wheat cultivars. Concentration effect due to small seed size and lower harvest index of wild species have been often cited as the reasons for their high grain micronutrient content (Calderini and Monasterio 2003a; Oury et al. 2006). However, our preliminary work on fertile early generation derivatives and synthetic amphiploids with bold seeds have demonstrated proof of the concept that the *Aegilops* species possess a distinctive genetic system for high grain micronutrient content (Rawat et al. 2009a, b).

Several related wild progenitor and non-progenitor species have been used for development of alien addition, substitution and translocation lines and transfer of useful variability (Friebe et al. 1996, 2000; Qi et al. 2007; Raupp et al. 1995) in wheat. A number of genes showing resistance against various diseases and pests have been introgressed from related species into wheat and commercially exploited (Kuraparthy et al. 2007a, b; Marais et al. 2005; Raupp et al. 1993; Schneider et al. 2008).

This article deals with the introgression and molecular characterization of *Ae. kotschyi* chromosomes conferring high grain iron and zinc concentration and content to wheat cultivars.

## Materials and methods

### Plant materials

*Aegilops kotschyi* accession 3790 having very high grain iron and zinc concentrations (Rawat et al. 2009a) was obtained from the Wheat Germplasm Collection of Punjab Agricultural University, Ludhiana, India. It was crossed as the male parent with bread wheat line 'Chinese Spring' containing $Ph^1$ (Chen et al. 1994), a suppressor of the wheat *Ph1* gene (Aghaee-Sarbarzeh et al. 2002), obtained from Dr. B.S. Gill of Kansas State University. The $F_1$ hybrid was backcrossed with wheat cultivars WL711 and UP2338. The $BC_1F_2$ and $BC_2F_1$ plants thus obtained were screened for high grain iron and zinc concentration. The plant material was grown each year in the experimental fields of the Indian Institute of Technology Roorkee, India, in rows of 2 m length, with plant-to-plant distance of 10 cm and row-to-row spacing of 30 cm, with application of fertilizers and irrigation as recommended for wheat cultivation.

$BC_1F_3$ and $BC_2F_2$ seeds were used for rigorous micronutrient analysis to select the derivatives with significantly higher micronutrient concentration than the recipient wheat cultivar WL711. These selected derivatives were characterized for morphology, cytology, microsatellite markers and genomic in situ hybridization (GISH) and fluorescent in situ hybridization (FISH).

### Iron and zinc analysis

For micronutrient analysis, whole grain samples were washed with N/10 HCl (Merck), dried till constant weight in a hot air oven at 80°C. Grain samples (0.5 g) were digested in a mixture of two parts of concentrated nitric acid (Merck) and one part of perchloric acid (Merck), following the standard procedure described by Zarcinas et al. (1987). Digestion was continued till a white residue was obtained. The required volume was made after the completion of the digestion process and the digests were analyzed by atomic absorption spectrophotometer (GBC, Avanta Garde M). A minimum of six replications of chemical analysis were made in each of the cultivars, *Ae. kotschyi* parent and the derivatives. The grain iron and zinc status of parents and the selected derivatives was also confirmed by inductively coupled plasma mass spectrometer (ICPMS, Perkin Elmer). All the standards used in this study were purchased from Merck, Germany. For grain ash analysis, 1 g grain samples were ashed in a muffle furnace at 500°C for 10 h. The ash was carefully collected and weighed. Further, the ash samples were processed for iron and zinc analyses following the same procedure described above for grains.

Estimation of aluminum (Al) was also done along with grain iron and zinc, as Al is the indicator of contamination during preparation and handling of samples.

### Cytological studies

For meiotic analysis, spikes of interspecific $F_1$ hybrids and of selected backcross derivatives were fixed in Carnoy's solution (6 ethanol:3 chloroform:1 acetic acid) for 24 h and transferred to 70% ethanol. Anthers at various stages of first meiotic division were squashed in 2% acetocarmine, and pollen mother cells (PMCs) were scored for chromosome number and pairing. Photographs were taken with a digital camera (Canon PC1049, No. 6934108049). Pollen stainability was recorded by staining pollen grains in iodine-potassium iodide solution.

### Phenotypic traits

Phenotypic traits such as plant height, ear shape, leaf waxiness, rachis toughness, grain color, grain yield, harvest index, etc., were recorded in the field at maturity, except for waxiness which was recorded at the time of anthesis.

### Molecular analysis

DNA was extracted from young leaves of the parents and selected $BC_2F_2$, $BC_1F_3$, and $BC_1F_4$ plants by using the CTAB method (Murray and Thomson 1980). Wheat

microsatellite markers of both arms of all the 21 chromosomes of wheat were selected from molecular maps of Röder et al. (1998), Pestsova et al. (2000) and Somers et al. (2004) and screened for transferability to *Ae. kotschyi.* Two transferable markers of each chromosome arm exhibiting polymorphism between the wheat cultivars and the *Ae. kotschyi* accession 3790 were used for molecular analysis of the 13 selected derivatives. PCR was carried out according to Röder et al. (1998). All components of PCR were from New England Biolabs. Keeping in view the fact that most of the transfers from distantly related species have been reported at the distal ends of the wheat chromosomes (Sidhu and Gill 2004), initially two distally located markers on each chromosome arm were used to identify the introgressed chromosome(s) in the selected derivatives. In case of preliminary evidence of specific candidate chromosomes, additional markers were employed to further confirm the homoeologous group of the *Ae. kotshyi* chromosome(s).

### In situ hybridization

The genomic DNAs of *Ae. longissima* ($S^lS^l$) and *Ae. umbellulata* (UU) were used to prepare genomic probes for utilization in genomic in situ hybridization (GISH) experiments. Clones pAs1 and pHvG38 were used in sequential fluorescent in situ hybridization (FISH). Clone pAs1 contains a 1-kb fragment isolated from *Ae. tauschii* which permits identification of the D-genome chromosomes (Rayburn and Gill 1987). The barley clone pHvG38 contains a 900-bp GAA-satellite sequence (Pedersen et al. 1996), which has multiple FISH sites on the B-genome and some minor sites on A- and D-genome chromosomes of wheat, as well as dispersed sites in S- and U-genome chromosomes. As a whole, the FISH pattern of this repeat is distinguishable among hexaploid wheat chromosomes and S- and U-genome chromosomes. Using both pHvG38 and pAs1 clones, all 21 chromosomes of hexaploid wheat could be identified (Pedersen and Langridge 1997).

Actively growing root tips from germinating seeds were treated for 24 h with ice water to accumulate metaphases and then fixed in 3:1 ethanol:glacial acetic acid. The root tips were stained in 1% acetocarmine and squashed in 45% acetic acid. Genomic probes were prepared using sheared genomic DNA (0.2–0.6 kb) of *Ae. longissima* ($S^lS^l$) and *Ae. umbellulata* (UU). The $S^l$-genome genomic DNA and the pHvG38 repeat DNA were labeled with fluorescein-11-dUTP (green) (Roche Applied Science, Indianapolis, IN) while the U-genome genomic DNA and the pAs1 repeat DNA were labeled with tetramethylrhodamine-5-dUTP (red) (Roche Applied Science, Indianapolis, IN) by nick translation. Labeled probes were purified using QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA). The unlabeled sheared genomic DNA of Chinese Spring wheat (100 bp–1 kb) was used as blocking DNA in a ratio of 1 ng labeled probe ($S^l$- and U genome): 100 ng of blocking DNA. Hybridization conditions, post-hybridization washes and imaging were as described by Zhang et al. (2001). After genomic in situ hybridization, the same slides were reprobed with pAs1 (red) and pHvG38 (green) repetitive DNA clones for individual identification of wheat chromosomes. Chromosomes were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI). Slides were analyzed with an epifluorescence Zeiss Axioimager M1 microscope.

## Results

### Phenotypic traits

Phenotypic traits of the selected plants of $BC_2F_2$, $BC_1F_3$, and $BC_1F_4$ progenies are given in Table 1. Various morphological traits associated with particular chromosomes, such as waxiness of leaves and leaf sheaths (waxy vs. non-waxy) with group 2 (Levy and Feldman 1989; McIntosh 1983), head type (square vs. spelta) with group 5 (Endo and Mukai 1988; Simons et al. 2006; Snape et al. 1985), grain color (red vs. amber) (Groos et al. 2002; Kumar et al. 2009) and fragility of rachis (brittle vs. non-brittle) with group 3 (Chen et al. 1998; Watanabe and Ikebata 2000), were taken into account for preliminary monitoring of introgression of alien chromosomes. Non-waxiness of leaves and leaf sheaths indicated the introgression of group 2 chromosome(s) of *Ae. kotschyi* in many of the selected derivatives. Other morphological traits indicated that group 3 and group 5 chromosomes of *Ae. kotschyi* were absent in all the 13 high grain iron and zinc derivatives. Red grain color in some of the derivatives could be attributed to the Chinese Spring parent. The selected plants had plant height, days to flowering and tiller number similar to that of wheat cultivar WL711. It was noteworthy that the 1,000 kernel weight of some derivatives, like 58-5-12, 58-11-(x) ($BC_2F_2$), and 117-18-17 ($BC_1F_4$), was even higher than that of WL711. Grain yield per plant and harvest index of the selected derivatives were also similar to or higher than WL711. Thus, the selected plants with requisite introgression had good background recovery.

### Grain iron and zinc content

The selected derivatives showed a wide range of grain iron and zinc concentration and content per seed (Table 2). The $BC_2F_2$ derivative 66-1-89 (Fe: 48.7 mg/kg, Zn: 46.5 mg/kg), the $BC_1F_3$ 77-36-6 (Fe: 48.5 mg/kg, Zn: 44.6 mg/kg) and the $BC_2F_2$ 63-2-13 (Fe: 42.5 mg/kg, Zn: 38.1 mg/kg), all with high harvest index and yield per plant, had very high

**Table 1** Morphological characteristics of selected plants of $BC_2F_2$, $BC_1F_3$, and $BC_1F_4$ wheat-*Ae. kotschyi* derivatives

| ID no. | Pedigree | No. of tillers | Height (cm) | Waxiness | Head type | Rachis | Grain color | 1,000 Kernel weight (g) | Grain Yield per plant (g) | Harvest Index (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | *T. aestivum* cv. WL711 | 12 | 105 | Waxy | Square | NB | Amber | 41.7 | 23.1 | 36.5 |
| Donor Parent | *Aegilops kotschyi* 3790 | 178 | 38 | Nonwaxy | Spelta | B | Red | 13.9 | NR | NR |
| $BC_2$ $F_2$ 58-5-12 | CS ($Ph^I$)/*Ae. kotschyi* 3790//WL711-4///WL711-5-12 | 15 | 120 | Nonwaxy | Square | NB | Red | 43.5 | 20.5 | 35.0 |
| $BC_2$ $F_2$ 58-5-33 | CS ($Ph^I$)/*Ae. kotschyi* 3790//WL711-4///WL711-5-33 | 10 | 128 | Nonwaxy | Spelta | NB | Red | 35.7 | 25.0 | 30.0 |
| $BC_2$ $F_2$58-11(x) | CS ($Ph^I$)/*Ae. kotschyi* 3790//WL711-4///WL711-11 | 15 | 110 | Nonwaxy | Square | NB | Amber | 43.5 | 24.3 | 32.0 |
| $BC_2$ $F_2$ 63-2-13 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2///WL711-2-13 | 15 | 120 | Waxy | Square | NB | Red | 38.5 | 23.7 | 32.8 |
| $BC_2$ $F_2$ 66-1-89 | CS ($Ph^I$)/*Ae. kotschyi*3790//PDW 274-2///PBW373-1-89 | 12 | 110 | Waxy | Spelta | NB | Red | 43.5 | 25.1 | 34.6 |
| $BC_1F_3$77-23-1 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-23-1 | 20 | 110 | Nonwaxy | Square | NB | Amber | 40.5 | 44.5 | 33.0 |
| $BC_1F_3$77-33-2 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-33-2 | 24 | 115 | Nonwaxy | Square | NB | Amber | 35.7 | 24.2 | 43.9 |
| $BC_1F_3$77-36-6 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-36-6 | 26 | 92 | Nonwaxy | Square | NB | Red | 38.5 | 21.7 | 35.0 |
| $BC_1F_3$77-46-3 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-46-3 | 10 | 130 | Nonwaxy | Square | NB | Amber | 39.7 | 19.6 | 32.0 |
| $BC_1F_3$77-50-8 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-50-8 | 29 | 132 | Nonwaxy | Square | NB | Amber | 36.3 | 20.1 | 32.6 |
| $BC_1F_3$77-50-15 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-50-15 | 43 | 150 | Nonwaxy | Square | NB | Red | 41.7 | 72.8 | 36.4 |
| $BC_1F_4$117-18-17 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-18 K-17R | 16 | 110 | Nonwaxy | Square | NB | Red | 43.5 | 29.8 | 35.7 |
| $BC_1F_4$ 117-18-22 | CS ($Ph^I$)/*Ae. kotschyi* 3790//UP2338-2R-18 K-22R | 12 | 82 | Nonwaxy | Square | NB | Red | 40.0 | 20.3 | 36.4 |

*NR* data not recorded, *B* brittle, *NB* non-brittle

**Table 2** Introgressed alien chromosomes, grain iron and zinc concentration and content: percentage seed ash and ash micronutrient concentration in selected $BC_2F_2$, $BC_1F_3$, and $BC_1F_4$ wheat-*Ae. kotschyi* derivatives

| ID no. | Grain iron | | | | Grain zinc | | | Ash analysis | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Introgressed alien chromosomes | Concentration (mg/kg) Mean ± SE | Change over WL711 (%) | Content (μg/seed) | Concentration (mg/kg) Mean ± SE | Change over WL711 (%) | Content (μg/seed) | Ash (%) | Iron (μg/g of ash) | Zinc (μg/g of ash) |
| *T. aestivum* cv. WL711 | – | $22.4 \pm 1.4^{a}$ | – | $0.93^{a}$ | $19.7 \pm 1.7^{a}$ | | $0.82^{a}$ | $1.6^{a}$ | $1{,}708^{a}$ | $1{,}442^{a}$ |
| *Ae. kotschyi* 3790 | – | $62.8 \pm 1.6^{d}$ | 180.4 | $1.01^{a}$ | $48.6 \pm 2.2^{c}$ | 146.7 | $0.84^{a}$ | $2.8^{c}$ | $3{,}276^{c}$ | $2{,}665^{c}$ |
| $BC_2F_2$ 58-5-12 | 7 | $37.5 \pm 1.6^{b}$ | 67.4 | $1.48^{b}$ | $34.6 \pm 1.5^{b}$ | 75.6 | $1.37^{b}$ | $2.5^{c}$ | $3{,}259^{c}$ | $2{,}552^{c}$ |
| $BC_2F_2$ 58-5-33 | 7 | $38.5 \pm 1.7^{b}$ | 71.9 | $1.67^{c}$ | $35.6 \pm 1.4^{b}$ | 80.7 | $1.55^{c}$ | $1.8^{ab}$ | $2{,}781^{b}$ | $2{,}453^{c}$ |
| $BC_2F_3$ 58-11(x) | 2 + 7 | $39.9 \pm 1.0^{b}$ | 78.1 | $1.60^{bc}$ | $36.9 \pm 2.4^{b}$ | 87.3 | $1.48^{bc}$ | $1.9^{b}$ | $2{,}951^{c}$ | $2{,}386^{b}$ |
| $BC_2F_2$ 63-2-13 | 7U | $42.5 \pm 1.9^{b}$ | 89.7 | $1.52^{bc}$ | $38.1 \pm 2.2^{bc}$ | 93.4 | $1.36^{b}$ | $1.8^{ab}$ | $2{,}739^{b}$ | $2{,}092^{b}$ |
| $BC_2F_2$ 66-1-89 | 2 + 7 | $48.7 \pm 1.6^{c}$ | 117.4 | $2.12^{d}$ | $46.5 \pm 1.3^{c}$ | 136.0 | $2.02^{d}$ | $2.0^{b}$ | $3{,}126^{c}$ | $2{,}443^{c}$ |
| $BC_1F_3$ 77-23-1 | 2 + 7 | $46.6 \pm 1.8^{bc}$ | 108.0 | $1.79^{cd}$ | $43.1 \pm 3.0^{c}$ | 118.8 | $1.66^{cd}$ | $2.1^{b}$ | $2{,}919^{c}$ | $2{,}168^{b}$ |
| $BC_1F_3$ 77-33-2 | 2S | $39.3 \pm 1.9^{b}$ | 75.4 | $1.71^{c}$ | $34.5 \pm 1.6^{b}$ | 75.1 | $1.50^{bc}$ | $1.9^{b}$ | $2{,}817^{b}$ | $2{,}243^{ab}$ |
| $BC_1F_3$ 77-36-6 | 2 + 7 | $48.5 \pm 1.6^{c}$ | 116.5 | $1.96^{d}$ | $44.6 \pm 2.8^{c}$ | 126.4 | $1.81^{d}$ | $2.1^{b}$ | $2{,}894^{b}$ | $2{,}508^{c}$ |
| $BC_1F_3$ 77-46-3 | 2 | $40.7 \pm 0.9^{b}$ | 81.7 | $1.45^{b}$ | $36.4 \pm 1.2^{b}$ | 84.8 | $1.30^{b}$ | $2.0^{b}$ | $2{,}798^{b}$ | $2{,}002^{ab}$ |
| $BC_1F_3$ 77-50-8 | 2 + 7 | $47.1 \pm 2.6^{c}$ | 110.3 | $1.81^{cd}$ | $43.2 \pm 2.3^{c}$ | 119.3 | $1.66^{cd}$ | $1.9^{b}$ | $2{,}658^{ab}$ | $1{,}963^{b}$ |
| $BC_1F_3$ 77-50-15 | 2 + 7 | $45.3 \pm 2.4^{bc}$ | 102.2 | $1.80^{cd}$ | $45.9 \pm 1.8^{c}$ | 133.0 | $1.82^{d}$ | $2.0^{b}$ | $2{,}875^{bc}$ | $2{,}401^{bc}$ |
| $BC_1F_4$ 117-18-17 | 2 + 7 | $39.1 \pm 1.8^{b}$ | 74.6 | $1.42^{b}$ | $38.0 \pm 1.5^{bc}$ | 92.9 | $1.38^{b}$ | $1.9^{b}$ | $2,\ 498^{ab}$ | $2{,}086^{b}$ |
| $BC_1F_4$ 117-18-22 | 2 | $41.2 \pm 1.4^{b}$ | 83.9 | $1.47^{b}$ | $36.1 \pm 2.5^{b}$ | 83.2 | $1.29^{ab}$ | $1.8^{ab}$ | $2{,}567^{ab}$ | $2{,}193^{b}$ |

Common superscript letters on mean values denote non-significant differences among groups as based on *t* test

grain iron as well as grain zinc concentrations, approaching that of the *Aegilops kotschyi* parent (62.8 mg/kg iron and 48.6 mg/kg zinc). Grain iron and zinc content per seed was found to be much higher in these plants than that of the control WL711 (0.93 µg/seed iron and 0.82 µg/seed zinc). These derivatives, like their *Ae. kotschyi* parent, also had higher grain ash and ash iron and zinc content than the control WL711, indicating that they had higher inorganic matter per unit grain weight and higher proportion of iron and zinc in their inorganic fraction than WL711 (Table 2). Aluminum (Al) was undetected in all the samples, indicating that all the samples and analytical procedures were free from contamination.

## Cytological analyses

The CS($Ph^I$)/*Ae. kotschyi* 3790 $F_1$ hybrid having 35 chromosomes showed limited homoeologous pairing, with an average of 2.86 bivalents and 0.16 trivalents (Fig. 1a). The hybrid plants were highly male and female sterile.

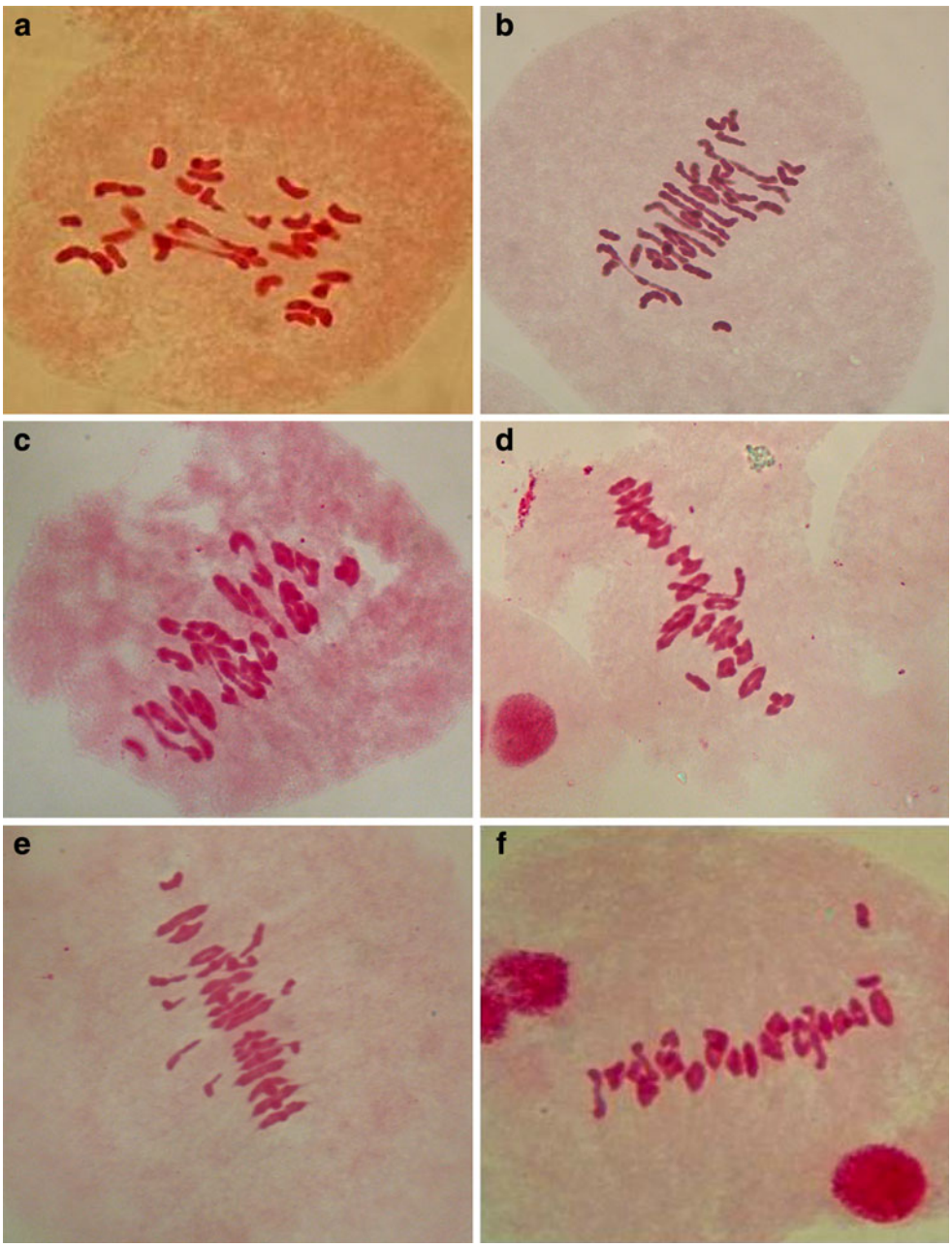


**Fig. 1** Chromosome pairing at metaphase-I of PMCs of $F_1$ hybrid, $BC_1$, $BC_2F_2$ and some selected $BC_2F_2$ and $BC_1F_3$ derivatives, **a** $F_1$ CS/*Ae. kotschyi* 3790 ($2n = 35$, 3 II + 29 I), **b** $BC_1F_1$ CS/*Ae. kotschyi* 3790//WL711-4 ($2n = 44$, 16 II + 12 I), **c** $BC_1F_2$ CS/*Ae. kotschyi* 3790//WL711-4-12 ($2n = 43$, 21 II + 1 I) **d** $BC_2F_2$ 63-2-13 ($2n = 41$, 20 II + 1 I), **e** $BC_1F_3$ 77-33-2 ($2n = 41$, 20 II + 1 I) and **f** $BC_1F_3$ 77-36-6 ($2n = 42$, 20 II + 2 I)

The chromosome number in the $BC_1$ plants varied from 39 to 56, with 8–20 bivalents and a few trivalents at meiosis (Fig. 1b). The $BC_2F_1$ and $BC_1F_2$ derivatives, with 42–46 chromosomes, had reduced univalent and increased bivalent frequency (Fig. 1c). The finally selected $BC_2F_2$, $BC_1F_3$, and $BC_1F_4$ derivatives had 41 or 42 chromosomes, with 1–4 univalents and 18–21 bivalents (Fig. 1d–f, Table 3). All the selected derivatives had pollen stainability above 90% and high seed set, indicating their normal meiotic behavior and high fertility.

## Molecular characterization

To identify the introgressed chromosome(s) in the wheat-*Ae. kotschyi* derivatives with high grain iron and zinc content, anchored wheat microsatellite markers were used. As CS($Ph^I$), with the $Ph^I$ gene capable of suppressing the wheat *Ph1* gene (Aghaee-Sarbarzeh et al. 2002), was used as parent in the wheat/*Ae. kotschyi* hybrid, some recombination was expected in the distal regions of wheat chromosomes (Anderson et al. 2003; Saintenac et al. 2009). On the basis of molecular analysis it was found that only group 2 and/or group 7 chromosomes of *Ae. kotschyi* were present in the selected derivatives (Table 2). Table 4 gives the list of polymorphic markers of groups 2 and 7 used along with their arm locations. Chromosome arm specific molecular markers wmc25 (2DS), gwm102 (2DS), gdm148 (2DL), and gdm539 (2DL) showed introgression of group 2 chromosomes of *Ae. kotschyi* in 9/13 selected derivatives (Fig. 2). On the basis of group 7 molecular markers (Fig. 2), 10 out of 13 derivatives showed introgression of group 7 *Ae. kotschyi* chromosomes. Plant

**Table 3** Chromosome number and pairing behavior in selected $BC_2F_2$, $BC_1F_3$ and $BC_1F_4$ wheat-*Ae. kotschyi* derivative plants

| ID no. | 2*n* | Mean ± SE (range) | |
|---|---|---|---|
| | | Univalents | Bivalents |
| $BC_2F_2$ 58-5-12 | 42 | 2.56 ± 0.34 | 19.72 ± 0.39 |
| $BC_2F_2$ 58-5-33 | 43 | 2.20 ± 0.29 | 20.40 ± 0.35 |
| $BC_2F_2$ 58-11(x) | 42 | 1.70 ± 0.29 | 20.15 ± 0.37 |
| $BC_2F_2$ 63-2-13 | 41 | 2.60 ± 0.46 | 19.20 ± 0.51 |
| $BC_2F_2$ 66-1-89 | 41 | 1.16 ± 0.21 | 20.42 ± 0.34 |
| $BC_1F_3$ 77-23-1 | 42 | 1.26 ± 0.15 | 20.37 ± 0.17 |
| $BC_1F_3$ 77-33-2 | 41 | 1.72 ± 0.29 | 20.14 ± 0.35 |
| $BC_1F_3$ 77-36-6 | 42 | 1.00 ± 0.37 | 20.50 ± 0.44 |
| $BC_1F_3$ 77-46-3 | 42 | 0.60 ± 0.42 | 20.70 ± 0.51 |
| $BC_1F_3$ 77-50-8 | 42 | 4.45 ± 0.29 | 18.76 ± 0.37 |
| $BC_1F_3$ 77-50-15 | 41 | 2.20 ± 0.31 | 19.42 ± 0.54 |
| $BC_1F_4$ 117-18-17 | 42 | 0.74 ± 0.18 | 20.63 ± 0.21 |
| $BC_1F_4$ 117-18-22 | 42 | 2.18 ± 0.19 | 19.91 ± 0.23 |

**Table 4** Anchored wheat microsatellite markers of chromosomes of group 2 and group 7 of wheat used for characterization of introgressed *Ae. kotschyi* chromosomes

| Chromosome | Arm location | Marker |
|---|---|---|
| 2D | S | cfd56, gwm102, wmc25 |
| | L | gdm148, gdm539, cfd73 |
| 7A | S | gdm350, wmc479 |
| | L | wmc809, wmc139 |
| 7B | S | barc65, wmc273 |
| | L | wmc396, gwm 344 |
| 7D | S | wmc646, cfd 41 |
| | L | wmc488, cfd 69 |

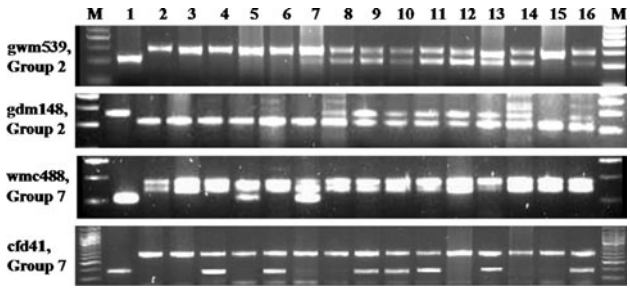


**Fig. 2** Groups 2 and 7 SSR markers on selected wheat-*Ae. kotschyi* derivatives: Lane *M*- Ladder (50 bp), *1 Ae. kotschyi* 3790, *2* Chinese Spring, *3* UP2338, *4* 58-5-12, *5* 58-5-33, *6* 58-11(x), *7* 66-1-89, *8* 77-33-2, *9* 77-36-6, *10* 77-50-8, *11* 77-50-15, *12* 77-46-3, *13* 117-18-17, *14* 117-18-22, *15* 63-2-13 and *16* 77-23-1

$BC_2F_3$ 63-2-13 showed introgression of group 7 *Ae. kotschyi* chromosome, as indicated by molecular markers wmc646 (7DS) and gwm344 (7BL). It was found that markers of only wheat chromosome 2D showed introgression of group 2U/2S of *Ae. kotschyi*, whereas markers of all the three wheat group 7 homoeologous chromosomes showed introgression of 7U/7S. The derivatives 77-23-1, 77-36-6, 77-50-8, 77-50-15 ($BC_1F_3$), 66-1-89 ($BC_2F_2$), and 117-18-17 ($BC_1F_4$) showed introgression of both group 2 and 7 alien chromosomes. It was also seen that the derivatives which had introgression of both group 2 and group 7 chromosomes of *Ae. kotschyi* had higher grain iron and zinc concentrations than those which had either a group 2 or a group 7 chromosome introgression (Table 2). Using additional distinctly polymorphic markers of both long and short arms of the homoeologous chromosomes involved (Table 4), only complete chromosomes were found to be introgressed in all the derivatives. This was also confirmed by in situ hybridization analyses (see ahead). Thus, it seems that no homoeologous recombination involving the critical chromosomes occurred, or that its products were eliminated as a result of selection of fertile derivatives with high iron and zinc concentration.

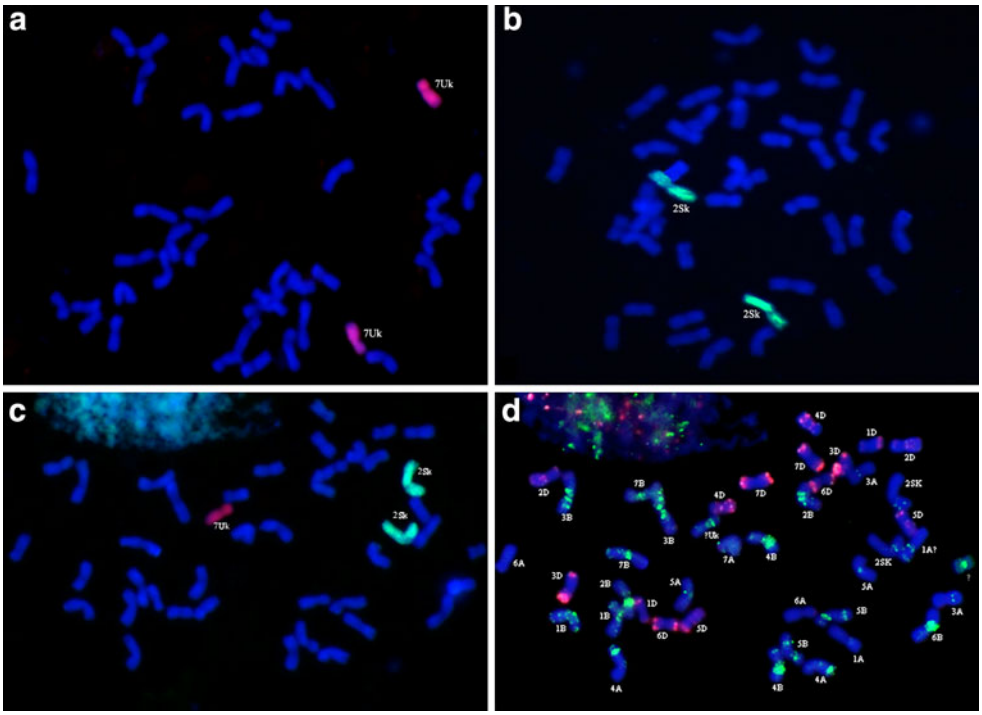


**Fig. 3** Mitotic chromosomes of wheat-*Ae kotschyi* derivatives (**a**) Somatic chromosomes of 63-2-13 showing the presence of one pair of $7U^k$ chromosome (*red*) after GISH, (**b**) Somatic chromosomes of 77-33-2 showing the presence of a pair of $2S^k$chromosome (*green*) after GISH (**c**, **d**). Somatic chromosomes of 77-36-6 (**c**) showing the presence of a pair of $2S^k$ (*green*) and a single chromosome $7U^k$ (*red*) after GISH, (**d**) the same chromosomes of 77-36-6 as in **c** after the sequential FISH using pAs1 (*red*) and pHvG38 (*green*). The chromosomes were counterstained with DAPI

### In situ hybridization

To identify the genome of the introgressed *Ae. kotschyi* chromosomes and the substituted wheat chromosome, only three representative wheat-*Ae. kotschyi* derivatives, namely, $BC_1F_3$ 77-33-2, $BC_2F_3$ 63-2-13, and $BC_1F_4$ 77-36-6, having introgression of group 2, group 7 and both groups 2 + 7 *Ae. kotschyi* chromosome(s), respectively, were selected for GISH and FISH. In 63-2-13 derivative, the U genome probe strongly hybridized with a pair of chromosomes (Fig. 3a) and sequential FISH with pAs1 and pHvG38 probes revealed that a 7U chromosome pair of *Ae. kotschyi* had substituted the 7A chromosome pair of wheat. In the 77-33-2 derivative, a pair of S genome chromosomes was identified using GISH (Fig. 3b), and the sequential FISH ascertained that in this derivative the wheat 2A chromosome pair was substituted with a 2S chromosome pair of *Ae. kotschyi*. In the 77-36-6 plant, however, the S and U genome probes hybridized strongly with one pair of chromosomes and a single chromosome, respectively (Fig. 3c). The FISH patterns of clones pHvG38 and pAs1 highlighted the substitution of 2A chromosomes of wheat by a pair of 2S and the substitution of a single 7A chromosome by one 7U chromosome (Fig. 3d). Additionally, one modified chromosome was observed in $BC_1F_4$ 77-36-6, which, based on the pHvG38 hybridization pattern, seemed to be a modified 6B chromosome of wheat (Fig. 3d).

## Discussion

Most of the high yielding bread and durum wheat cultivars have low grain iron and zinc (Cakmak et al. 2000; Rawat et al. 2009a), whereas the related wild *Triticum* and *Aegilops* species possess useful variability for high grain iron and zinc concentration (Cakmak et al. 2000; Chhuneja et al. 2006; Rawat et al. 2009a, b; Tiwari et al. 2008). The higher grain micronutrient concentration reported in the related wild species could be attributed to a concentration effect, because of their smaller seed size, and low grain yield and harvest index (Calderini and Monasterio 2003b). This could be verified only by dissecting the related wild species' genomes as addition, substitution or translocation lines in a wheat background. The S genome, being closely related to the B genome of polyploid wheat (Daud and Gustafson 1996; Dvörak and Zhang 1990; Faris et al. 2002), can be effectively used for transferring useful variability for high iron and zinc content into wheat. Useful variability for rust resistance has already been transferred from *Ae. umbellulata* (UU), *Ae. triuncialis* (UUCC), and *Ae. geniculata*

(UUMM) into wheat for commercial exploitation (Chhuneja et al. 2008; Kuraparthy et al. 2007a, b; Sears 1956).

On the basis of morphological and molecular analyses, as well as of GISH and FISH of the selected wheat-*Ae. kotschyi* derivatives with high grain iron and zinc concentration, chromosomes 2S and/or 7U of *Ae. kotschyi* were found to be introgressed and associated with the high micronutrient content. Tiwari et al. (2009) also reported QTL for high grain iron and zinc content on chromosomes 2A and 7A in a diploid wheat *T. monococcum* × *T. boeoticum* RIL population. Peleg et al. (2009) have recently reported three major QTL for grain zinc and iron concentrations, two on chromosome 2A and one on 7A in a *T. durum–T. dicoccoides* RIL population where the common QTL for zinc and iron was in the same marker interval on 7A. Shi et al. (2008) in a DH population of wheat detected four QTL for grain Zn concentration (mg/kg) and seven for grain Zn content (μg/grain), and the two QTL explaining 14.6 and 13.4% contribution to phenotypic variance were mapped on chromosomes 7A and 2D, respectively. All the four QTL for Zn concentration were found to be co-located with four QTL for Zn content. Recently, Lonergan et al. (2009) reported three major QTL, one on 2HS and two on 2HL of barley, controlling culm Zn content at various stages of development and seed Zn content and concentration, in the same marker intervals in a DH barley population. Barley chromosome 2H is syntenic to its wheat homoeologue (Van Deynze et al. 1995; Gale and Devos 1998; Cho et al. 2006). All these results support our findings that the homoeologous chromosomes 2S and 7U of *Ae. kotschyi* possess orthologs for grain iron and zinc content and concentration. It was also observed that the alien chromosomes have additive effects for enhanced grain iron and zinc content, as the plants with 2S and 7U chromosomes had higher micronutrient content than those having either 2S or 7U chromosomes.

The grain size, grain yield per plant, and harvest index of most of the selected derivatives were similar or even better than those of the wheat parent. This strongly suggests that their higher grain iron and zinc concentration and content were not due to concentration effect, as reported in the synthetic hexaploids by Calderini and Monasterio (2003b), but due to efficient genetic system(s) of *Aegilops kotschyi* for uptake and translocation of the micronutrients. The dissection of the *Ae. kotschyi* genome in the form of introgression lines for high grain iron and zinc content in the wheat background, and the identification of chromosomes with superior alien homoeoalleles has provided unequivocal proof of the concept for use of related wild species for biofortification of wheat. Cakmak et al. (2004) screened diverse collection of *T. turgidum* ssp. *dicoccoides* and various *T. dicoccoides* substitution lines in various wheat backgrounds and found higher grain iron and zinc contents associated with 6A, 6B, and 5B substitution lines. Subsequently, 6BS of *T. dicoccoides* (6B substitution line in Langdon durum) was found to carry a Mendelian locus, *Gpc-B1*, encoding the transcription factor *NAM-B1* which enhances grain protein, zinc, iron and manganese content through accelerated senescence, phloem loading and nutrient mobilization from leaves to developing grains (Uauy et al. 2006; Distelfeld et al. 2007). Similar accelerated senescence has not been observed in the 2S and 7U substitution lines with higher grain micronutrients. Preliminary estimation of grain protein content, however, reveals a slight increase in the grain protein content of the 2S- and 7U-containing derivatives as compared to the recipient wheat cultivars (Tiwari et al. unpublished). This might be due to enhanced mobilization of amino acid pools by genes on 2S and 7U chromosomes like that induced by *Gpc-B1*. The use of *T. dicoccoides NAM-B1* allele in combination with the biofortified wheat with 2S and 7U might further enhance the grain micronutrient content and concentration through enhanced mobilization of their reserves in leaves.

Only the complete 2S and 7U chromosome substitution lines with high grain iron and zinc were recovered among the selected derivatives. The failure to recover any recombinant with high grain iron and zinc could be due to their elimination during strict selection for higher fertility and micronutrient content. The presence of two chromosomes in the majority of the $BC_1$ derivatives further suggests that future efforts should be concentrated in getting more backcross seeds from sterile $F_1$ hybrids, which should be allowed to self and stabilize for recovery of a sufficient number of recombinants. The novel 2S and 7U substitution derivatives developed and identified in this study may not be very suitable for commercial exploitation due to linkage drag. Transfer of defined chromosomal regions containing genes controlling high grain iron and zinc concentration and content is in progress using irradiation and induction of homoeologous chromosome pairing.

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