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Development and characterization of *Triticum aestivum–Aegilops kotschyi* amphiploids with high grain iron and zinc contents

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Abstract

Synthetic amphiploids between *Triticum aestivum* (AABBDD) landrace Chinese Spring (*Ph*^{I}) and cultivar WL711 with different accessions of *Aegilops kotschyi* (UUS^{l}S^{l}) were developed through colchicine treatment of sterile hybrids. The F_1 hybrids and amphiploid plants were intermediate between the parents for plant morphology and spike characteristics. Meiotic metaphase chromosome analysis of the F_1 hybrids (ABDUS^{l}) showed the expected chromosome number (35) and very little but variable homoeologous chromosome pairing. The amphiploids (AABBDDUUS^{l}S^{l}), however, had variable frequency of univalents at meiotic metaphase-I. The SDS–PAGE of high molecular weight glutenin subunits of amphiploids along with the parents showed the presence and expression of all the parental genomes in the amphiploids. The amphiploids with seeds as large as that of wheat cultivars had higher grain, flag leaf and grain ash iron and zinc concentrations than the wheat parents and comparable with those of their *Ae. kotschyi* parents suggest that *Ae. kotschyi* possesses a distinctive genetic system for the micronutrient uptake, translocation and sequestration than the wheat cultivars. This could, however, be demonstrated unequivocally only with comprehensive data on biomass, grain yield and harvest index of the *Aegilops* donors and the synthetic amphiploids, which is not feasible due to their shattering and hard threshing. The use of amphiploids for the transfer of high iron and zinc concentrations and development of alien addition and substitution lines in wheat is in progress.

Keywords: *Aegilops kotschyi*; amphiploid; chromosome pairing; grain iron; grain zinc; *Triticum aestivum*

Introduction

More than two billion people, depending predominantly on starch-rich cereals and tubers as staple food, suffer from iron deficiency-related anaemia and zinc deficiency (WHO, 2002; White and Broadley, 2005; Zimmerman and Hurrel, 2007) for which there is very limited variability among *Triticum durum* (Desf.) Husn., *Triticum aestivum* L. cultivars and landraces (Cakmak *et al*., 2000; Rawat *et al*., 2009). Bread wheat originated some 10,000 years ago (Dubcovsky and Dvorak, 2007) involving three diploid species through two steps of hybridization and chromosome doubling (Kihara, 1944; McFadden and Sears, 1946; Feldman *et al*., 1997), resulting in their

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immediate isolation from the parental species due to which very limited variability among the progenitor species could get incorporated in the cultivated gene pools of tetraploid and hexaploid wheat. The germplasm of related wild progenitor and non-progenitor *Triticum* and *Aegilops* species is a rich reservoir of useful variability for resistance against biotic and abiotic stresses, quality traits, yield and yield components (Damania, 1993; Jiang *et al.*, 1994; Friebe *et al.*, 1996). Useful variability for several traits has been introgressed into cultivated wheat and exploited commercially (Dvörak, 1977; Kuraparthy *et al.*, 2007; Chhuneja *et al.*, 2008). Several diploid and tetraploid wild progenitor species have been found to possess high grain iron and zinc contents (Cakmak *et al.*, 2000; Ortiz-Monasterio and Graham, 2000; Calderini and Ortiz-Monasterio, 2003a,b; Chhuneja *et al.*, 2006), which are being used for transfer of the useful variability for biofortification of wheat for high grain iron and zinc contents. Some 'S' genome diploid and tetraploid *Aegilops* species possess variability for two to three fold higher grain iron and zinc contents (Rawat *et al.*, 2009). In addition to direct crosses between wheat cultivars and wild species, numerous synthetic amphiploids have been developed between *T. durum* or *T. aestivum* cultivars and the related wild species. The synthetic amphiploids have been used for dissecting alien genomes through the development of alien substitution and addition lines with useful variability for subsequent introgression into elite wheat cultivars using induced homoeologous pairing (Chen *et al.*, 1994; Jiang *et al.*, 1994; Aghaee-Sarbarzeh *et al.*, 2002) and molecular cytogenetics (Kuraparthy *et al.*, 2007).

This article reports the development and characterization of synthetic amphiploids between bread wheat cultivars and *Aegilops kotschyi* accessions having higher grain iron and zinc contents than the wheat cultivars.

Materials and methods

Plant material

The plant material consisted of six accessions of *Ae. kotschyi viz.*, pau3774, pau3790, pau14262 (391), pau14264 (393), pau14266 (395) and pau14267 (396) and two *T. aestivum* genotypes – landrace Chinese Spring (Ph^I) with introgressed homoeologous pairing inducing gene (Chen *et al.*, 1994), henceforth abbreviated as CS(Ph^I) and a bread wheat cultivar WL711 (Table S1, available online only at http://journals.cambridge.org) received from Punjab Agricultural University, Ludhiana. The source and origin of the *Ae. kotschyi* accessions used in this study are available from one of the authors Dr Kuldeep Singh. The plant material was grown in the experimental field of the Indian Institute of Technology, Roorkee in 2004–2005 as replicated single row of 2 m length with plant to plant distance of 10 cm and row to row spacing of 30 cm with the recommended fertilizers (50:25:25 NPK kg/acre) and irrigation practices as that of wheat. Several F_1 hybrids were produced using wheat as the female parent and the *Ae. kotschyi* accessions as the male parent (Table S1, available online only at http://journals.cambridge.org). In the following year, the F_1 seeds were sterilized with 1% sodium hypochlorite for 5 min, washed thrice with distilled water and germinated on two layers of sterilized moist filter paper in Petri plates. The chromosomes of the F_1 hybrids were doubled by treating coleoptiles of germinating seeds with 0.25% of colchicine (in 5% DMSO solution) for 5 h. The colchicine-treated seedlings were transplanted in the field. Some F_1 hybrid seeds were also grown in the field without colchicine treatment and crossed with recurrent wheat parents. The colchicine-treated and -untreated plants were grown under similar spacing and cultivation conditions as that of the wheat cultivars.

During flowering, the spikes with anthers dehiscing viable pollen grains and seed set, evidently due to chromosome doubling, were identified and tagged. Seeds (C_0 generation of amphiploids) from the doubled sectors of the tagged spikes were harvested carefully before shattering of spikes. The C_1 generation of these amphiploids was grown in the field during 2006–2007. Collection of mature spikelets and spikes of the F_1 hybrids and synthetic amphiploids had to be done repeatedly at different intervals over 2–3 weeks because of frequent shattering of spikes. Due to tough glumes and hard threshing in the amphiploids and wild donors, the grains were threshed manually. Mean number of seeds per spike was determined for each amphiploid by taking the average of number of seeds of ten spikes in each replication.

Pollen stainability

Anthers dehiscing pollen grains were collected from five spikes of each replication in the morning, and pollen fertility was determined by staining with iodine potassium iodide solution.

Seed protein electrophoresis

SDS–PAGE of high molecular weight (HMW) glutenin subunits of endosperm proteins of mature and dried seeds of parents and amphiploids was done using 10% acrylamide following the method of Smith and Payne (1984).

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Cytological studies

Spikes of F_1 hybrids and amphiploid plants were fixed for 24 h in Carnoy's solution (ethanol–chloroform–acetic acid; 6:3:1) for meiotic analysis. Spikes were transferred to 70% ethanol after 24 h of fixation. For meiotic study, the anthers were squashed in 2% acetocarmine. Pollen mother cells (PMCs) at meiotic metaphase-I were scored for chromosome number and pairing in all the crosses and synthetic amphiploids.

Micronutrient analysis

Grain analysis

For micronutrient analysis, whole-grain samples of parents and amphiploids were taken at maturity, washed with N/10 HCl to remove contaminating dust if any, and dried in hot air oven at 80°C until constant weight. Grain samples (0.5 g) were digested in a mixture of two parts of concentrated nitric acid and one part perchloric acid as per the procedure described by Zarcinas *et al.* (1987). Digestion was continued till white residue was obtained. Required volume was made after the completion of digestion process and digests were analyzed by Atomic Absorption Spectrophotometer (GBC- Avanta Garde M, Dandenong, Victoria, Australia). Seeds from each accession of wild species and the amphiploids were analyzed as three replicates to minimize the error during analysis.

Flag leaf iron and zinc contents

Flag leaves of WL711, CS(Ph^I), *Ae. kotschyi* parents and amphiploids were analyzed for iron and zinc contents before ear emergence at pre-anthesis stage. The leaves were washed thoroughly with N/10 HCl, dried at 80°C for 8 h in oven prior to digestion. Dried leaf samples were then digested as a minimum of three replications as that for grains. Iron and zinc concentrations in the digests were analyzed by AAS.

Grain ash analysis

One gram dried grains of each of *Ae. kotschyi* accessions, WL711, CS(Ph^I) and the seven amphiploids were cleaned thoroughly and kept for incineration at 600°C for 10 h. The ash was further processed like the grains for AAS analysis.

Results

Plant and spike characteristics and chromosome pairing of F_1 hybrids

The wheat × *Ae. kotschyi* F_1 hybrids were morphologically intermediate between wheat and *Ae. kotschyi* parents (Fig. S1; Table S1, available online only at http://journals.cambridge.org). All the F_1 hybrids were completely self sterile and had spelta heads with brittle rachis above the basal spikelet. The hybrids with CS(Ph^I) had awnless lemma and glumes, whereas those with WL711 had one glume awn and one lemma awn (Fig. S1, available online only at http://journals.cambridge.org). Chinese Spring has been known to possess awn inhibitor genes on chromosomes 4A and 6B (Sourdille *et al.*, 2002).

The rachis of F_1 hybrids disarticulated only above the basal spikelets like that of *Ae. kotschyi* (Fig. S1, available online only at http://journals.cambridge.org). The details of fertility and chromosome pairing of seven F_1 hybrids between *T. aestivum* [WL711 or CS(Ph^I)] and six accessions of *Ae. kotschyi* are given in Table 1. There was very limited intergenomic pairing in the F_1 hybrids (Fig. 1) with very high frequency of univalents (25.69–32.74), low frequency of rod bivalents (1.0–4.17) and occasional trivalents (0.09–0.32). One of the F_1 hybrids, CS(Ph^I)/*Ae. kotschyi* 396, showed higher chromosome pairing (25.69 Is, 4.17 IIs and 0.32 IIIs) when compared with other hybrids (Table 1). This may be attributed to induced homoeologous pairing due to Ph^I in CS which is epistatic to *Ph1*, the diploidization gene on the long arm of chromosome 5B (Riley and Chapman, 1958; Chen *et al.*, 1994; Jiang *et al.*, 1994; Aghaee-Sarbarzeh *et al.*, 2002). However, in the other three hybrids of CS(Ph^I) with different *Ae. kotschyi* accessions comparatively less homoeologous chromosome pairing was observed (Table 1). The F_1 WL711/*Ae. kotschyi* 393 without Ph^I also had relatively higher frequency of bivalents (up to 6 II). All the F_1 plants showed very low-pollen stainability (17.6–23.5%), no anther dehiscence and no seed set (Table 1).

The F_1 hybrid plants treated with colchicine were exactly like F_1 hybrids except for having some doubled sectors or spikes with dehiscing anthers, which could be readily distinguished from non-dehiscent sterile anthers in other spikes. These spikes/sectors with dehiscing anthers had normal seed set, whereas there was no seed set on the otherwise sterile F_1 plants without chromosome doubling. The seeds thus obtained were identified as the potential synthetic amphiploids (C_0 generation) for further studies.

Morphology and fertility of the synthetic amphiploids

Comparative morphology of the synthetic amphiploids with the parents showed their intermediate growth habit, tiller number and plant height (Table S1, available online only at http://journals.cambridge.org) like the

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Table 1. Chromosome pairing and pollen stainability in *Triticum aestivum*/*Aegilops kotschyi* F_1 hybrids

Cross	Number of PMCs studied	Chromosome number	Mean ± s.d. (range)			Pollen stainability (%)
			Univalent	Bivalent	Trivalent	
CS(Ph^I)/*Ae. kotschyi* 396	25	35	25.69 ± 6.1 (13–35)	4.17 ± 2.4 (0–11)	0.32 ± 0.6 (0–3)	23.51
CS(Ph^I)/*Ae. kotschyi* 3774	25	35	30.35 ± 1.7 (29–35)	2.04 ± 1.2 (0–4)	0.19 ± 0.6 (0–1)	18.48
CS(Ph^I)/*Ae. kotschyi* 393	25	35	31.63 ± 5.6 (26–35)	1.5 ± 2.3 (0–3)	0.13 ± 0.7 (0–1)	17.56
CS(Ph^I)/*Ae. kotschyi* 395	25	35	32.74 ± 3.9 (26–35)	1.0 ± 1.6 (0–3)	0.09 ± 0.5 (0–1)	19.72
WL711/*Ae. kotschyi* 393	25	35	30.21 ± 6.0 (18–35)	2.14 ± 2.6 (0–6)	0.17 ± 0.7 (0–1)	21.93
WL711/*Ae. kotschyi* 391	25	35	29.82 ± 0.7 (21–27)	2.35 ± 3.2 (0–3)	0.18 ± 1.3 (0–2)	17.62
WL711/*Ae. kotschyi* 3790	25	35	29.16 ± 6.5 (16–35)	2.63 ± 2.8 (0–4)	0.19 ± 0.4 (0–2)	21.09

Fig. 1. Chromosome pairing in metaphase-I of wheat/*Aegilops kotschyi* F_1 hybrids, (a) F_1 CS(Ph^I)/*Ae. kotschyi* 396 (6 II + 23 I), (b) CS(Ph^I)/*Ae. kotschyi* 3774 (3 II +29 I), (c) F_1 CS(Ph^I)/*Ae. kotschyi* 393 (1 III +2 II +30 I), (d) F_1 WL711/*Ae. kotschyi* 393 (1 II +32 I), (e) F_1 WL711/*Ae. kotschyi* 391 (1 III +3 II +27 I) and (f) F_1 WL711/*Ae. kotschyi* 3790 (2 II +31 I).

F_1 hybrids. The amphiploids displayed some of the characteristics of the *Ae. kotschyi* parent such as spelta head, brittle rachis and red seed colour and other characteristics of the wheat parents such as 1000 grain weight. The number of spikelets per spike exceeded both the parents. Most of the spike characteristics like the number and length of awns of glumes and lemmas were again intermediate to both the parents. *Ae. kotschyi* accessions had 5–7 glume awns against none and single awn in CS(Ph^I) and WL711, respectively. The glumes of amphiploids with WL711 had single awn, while those with CS(Ph^I) were awnless and had a tooth only. The long lemma awn of WL711 was replaced by small awns in the amphiploids, whereas *Ae. kotschyi* had two lemma awns (Fig. S1, available online only at http://journals.cambridge.org).

Pollen stainability and seed set in the amphiploids varied within the season. The early flowering spikes had non-dehiscent anthers, low-pollen stainability and less seed set, whereas the late flowering tillers had

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Table 2. Chromosome number, meiotic pairing and seed set in *Triticum aestivum/Aegilops kotschyi* synthetic amphiploids (C_1)

Amphiploid	No. of PMCs studied	Chromosome number (range)	Mean ± s.d. (range)			Pollen stainability (%)	Average seed set per spike
			Univalent (I)	Bivalent (II)	Trivalent (III)		
Amphi. CS (*Ph*^{1})–*Ae. kotschyi* 396	25	35–70	8.63 ± 2.03 (2–21)	24.25 ± 2.91 (2–34)		62.6	5.6
Amphi. CS (*Ph*^{1})–*Ae. kotschyi* 3774	25	37–70	2.92 ± 0.71 (1–10)	32.80 ± 0.58 (27–34)	0.27 ± 0.14 (0–1)	81.8	17.5
Amphi. CS (*Ph*^{1})–*Ae. kotschyi* 393	25	42–70	12.40 ± 0.75 (10–14)	25.80 ± 1.86 (21–29)	–	72.5	14.2
Amphi. CS (*Ph*^{1})–*Ae. kotschyi* 395	25	39–69	2.60 ± 0.81 (1–5)	32.80 ± 0.58 (31–34)	–	76.6	15.3
Amphi. WL711–*Ae. kotschyi* 393	25	47–68	8.51 ± 0.97 (2–15)	23.17 ± 2.14 (16–32)	–	79.0	15.9
Amphi. WL711–*Ae. kotschyi* 3790	25	57–68	3.56 ± 1.78 (4–12)	27.75 ± 0.97 (24–30)	–	70.5	8.3
Amphi. WL711–*Ae. kotschyi* 391	25	39–67	11.00 ± 2.81 (2–18)	20.43 ± 3.76 (12–32)	–	57.4	4.1

dehiscing anthers, higher pollen stainability and good seed set. Pollen stainability varied from 62.6 to 81.8% in different amphiploids of CS(*Ph*^{1})–*Ae. kotschyi* accessions, while in amphiploids of WL711–*Ae. kotschyi* accessions it ranged from 57.4 to 79.0% (Table 2). Variation in the seed set was observed for different combinations of bread wheat lines and *Ae. kotschyi* accessions. Maximum seed set was observed in the amphiploid CS(*Ph*^{1})–*Ae. kotschyi* 3774 (17.5 seeds/spike) and least in WL 711–*Ae. kotschyi* 391 (4.1 seeds/spike). Seeds of the amphiploids were longer, red and had 1000 grain weight comparable with those of the wheat parents (Table S1; Fig. S1, available online only at http://journals.cambridge.org).

Chromosome pairing in the synthetic amphiploids

Chromosome number in the amphiploids was highly variable ranging from 35 to 70 chromosomes in CS(*Ph*^{1})–*Ae. kotschyi* 396, 39–69 in CS(*Ph*^{1})–*Ae. kotschyi* 395, 42–70 in CS(*Ph*^{1})–*Ae. kotschyi* 393 and 37–70 in CS(*Ph*^{1})–*Ae. kotschyi* 3774 (Table 2; Fig. 2). There was only a small proportion of PMCs in all the amphiploids with the expected double chromosome number (70) of the F_1 hybrids (35). Comparatively, higher number of bivalents and lower number of univalents in the amphiploids CS(*Ph*^{1})–*Ae. kotschyi* 395 (32.8 II, 2.6 I), CS(*Ph*^{1})–*Ae. kotschyi* 3774 (32.8 II, 2.92 I) and WL711–*Ae. kotschyi* 393 (23.2 II, 8.5 I) might have resulted in higher seed set (Table 2) in these amphiploids, whereas irregular meiotic behaviour of CS(*Ph*^{1})–*Ae. kotschyi* 396 and WL711–*Ae. kotschyi* 391 with very wide range of chromosome number, higher frequency of univalents and lower bivalent frequency was associated with low-seed set percentage (4.1 seeds per spike; Table 2).

HMW glutenin subunit profiles of amphiploids

The SDS–PAGE profiles of the HMW glutenin subunits of CS(*Ph*^{1}), *Ae. kotschyi* accessions and the CS(*Ph*^{1})–*Ae. kotschyi* amphiploids are given in Fig. 3. *T. aestivum* cultivars PBW343, Kalyan Sona and landrace CS and CS(*Ph*^{1}) were taken as the control. CS and CS(*Ph*^{1}) had similar subunit pattern for *Glu 1B*-controlled 7 + 8 subunits and *Glu 1D*-controlled 2 + 12 subunits of HMW glutenins. All the accessions of *Ae. kotschyi* (UUSS) expressed 3–5 novel subunits of HMW glutenin subunits. Two of the slowest migrating *x* subunits had lower electrophoretic mobility than the Glu-D1 subunit 5, while the faster migrating two *y* subunits were slower than the subunit 7. HMW glutenin subunits of

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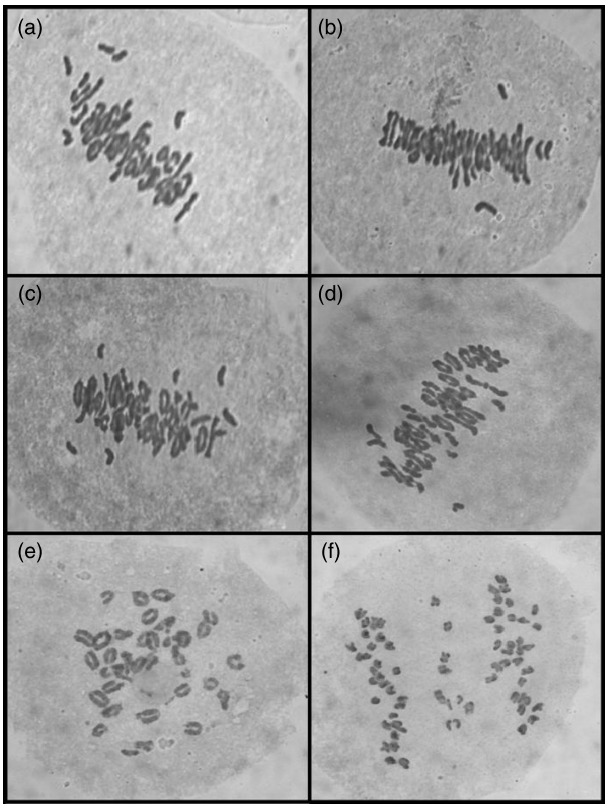

Fig. 2. Chromosome pairing in wheat–*Aegilops kotschyi* amphiploids (a) Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 396 (Chr-64, 1 III + 28 II + 5 I) (b) Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 3774 (Chr-68, 32 II +4 I) (c) Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 393 (Chr-69, 31 II +7 I) (d) Amphi. WL711–*Ae. kotschyi* 393 (Chr-69, 32 II +5 I), (e) Amphi. WL711–*Ae. kotschyi* 3790 (Chr-64, 30 II +4 I) and (f) Amphi. WL711–*Ae. kotschyi* 391 (Chr- 67, 29 II +8 I).

both the wheat and *Ae. kotschyi* parents were present in all the amphiploids confirming the presence and expression of both the parental genomes (Fig. 3). Similar additive profile of HMW glutenin subunits was observed in the three amphiploids of WL711–*Ae. kotschyi*.

Grain and flag leaf iron and zinc concentrations of amphiploids

Table 3 shows grain and flag leaf iron and zinc concentrations of the amphiploids along with both the parents *viz.*, WL711, CS(*Ph*^{I}) and *Ae. kotschyi* accessions. The micronutrient concentration of *Aegilops kotschyi* accessions was two to three folds higher when compared with wheat parents. *Ae. kotschyi* accession 3774 had the highest grain iron (70.8 mg/kg) and zinc concentration (35.7 mg/kg), respectively. The micronutrient concentrations of wheat parents were quite low, iron being 22.8 mg/kg in WL711 and 30.2 mg/kg in CS(*Ph*^{I}) and zinc 16.6 mg/kg and 18.3 mg/kg in WL711 and CS(*Ph*^{I}), respectively. The micronutrient concentrations of amphiploids were comparable with those of *Ae. kotschyi*. The micronutrient content per seed in *Ae. kotschyi* parents was, however, similar to that of the wheat parents in spite of the fact that they had three times smaller seeds than the wheat cultivars, which could be either attributed to their distinctive genetic system for micronutrient deposition or concentration due to lower harvest index.

Ae. kotschyi also had two to three times higher flag leaf iron and zinc concentrations than the wheat parents, suggesting that their higher grain micronutrient content could be attributed to their distinctive genetic system for deposition rather than to concentration in their smaller seeds. The amphiploids also showed an increase

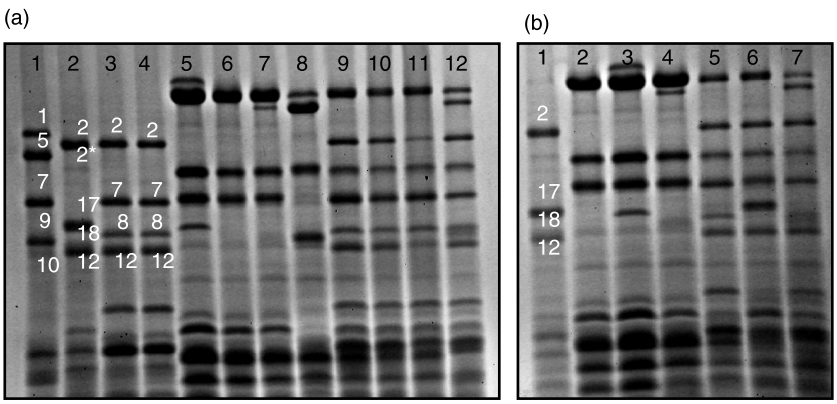

Fig. 3. HMW glutenin subunit profile of *Triticum aestivum* cultivars, *Aegilops kotschyi* accessions and the amphiploids of CS(*Ph*^{I}) and WL711 with *Ae. kotschyi* accessions. (a) Lane 1, PBW343; 2, Kalyan Sona; 3, Chinese Spring; 4, Chinese Spring(*Ph*^{I}); 5, *Ae. kotschyi* 393; 6, *Ae. kotschyi* 395; 7, *Ae. kotschyi* 396; 8, *Ae. kotschyi* 3774; 9, Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 393; 10, Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 395; 11, Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 396; and 12, Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 3774. (b) Lane 1, WL711; 2, *Ae. kotschyi* 391; 3, *Ae. kotschyi* 393; 4, *Ae. kotschyi* 3790; 5, Amphi. WL711–*Ae. kotschyi* 391; 6, Amphi. WL711–*Ae. kotschyi* 393; and 7, Amphi. WL711–*Ae. kotschyi* 3790.

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Table 3. Whole grain and flag leaf iron and zinc, grain ash iron and zinc concentrations of amphiploids and their parents

	Whole grain				Flag leaf		Grain ash				
Plant material	Iron concentration (mg/kg) ± s.d.	Zinc concentration (mg/kg) ± s.d.	Iron content (μg/seed)	Zinc content (μg/seed)	Iron (mg/kg)	Zinc (mg/kg)	Grain ash (%)	Fe (μg/g) of ash	% Change in ash Fe content over WL711	Zn (μg/g) of ash	% Change in ash Zn content over WL711
Triticum aestivum cv. WL711	22.8 ± 0.8	16.6 ± 1.3	0.7	0.5	73.1	32.4	1.69	1667	–	1341	–
Chinese Spring (*Ph*^{I})	30.2 ± 0.5	18.3 ± 0.9	0.9	0.6	71.5	33.4	1.70	1867	12	1568	16.93
Ae. kotschyi 396	65.6 ± 1.4	32.3 ± 1.2	0.8	0.4	199.3	86.9	2.07	3272	96.28	2373	76.96
Ae. kotschyi 3774	70.8 ± 0.7	35.7 ± 0.7	0.9	0.5	195.6	76.2	2.02	3170	90.16	2261	68.61
Ae. kotschyi 393	58.4 ± 0.9	29.8 ± 0.8	0.7	0.3	189.3	78.8	1.98	3119	87.1	2099	56.52
Ae. kotschyi 395	63.2 ± 1.0	31.5 ± 1.5	0.7	0.4	186.2	85.9	2.05	3151	89.02	2195	63.68
Ae. kotschyi 391	63.1 ± 1.1	35.2 ± 0.5	0.7	0.4	188.8	79.4	2.11	3089	85.3	2178	62.42
Ae. kotschyi 3790	67.5 ± 0.6	30.8 ± 2.1	0.9	0.4	196.1	81.9	2.21	3176	90.52	2265	68.9
Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 396	62.9 ± 1.5	43.2 ± 1.2	2.2	1.5	126.2	54.1	1.72	2697	61.79	2013	50.11
Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 3774	59.2 ± 0.5	28.8 ± 0.6	2.2	1.1	154.3	60.8	1.88	3082	84.88	2188	63.16
Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 393	65.0 ± 1.3	36.5 ± 0.6	2.0	1.1	166.3	62.9	1.89	2687	61.19	1998	48.99
Amphi. CS(*Ph*^{I})–*Ae. kotschyi* 395	64.2 ± 1.8	29.5 ± 1.5	1.9	0.9	122.9	53.1	1.83	2712	62.69	2118	57.94
Amphi. WL711–*Ae. kotschyi* 393	64.6 ± 2.0	30.7 ± 1.3	1.8	0.9	123.5	51.4	1.86	2609	56.51	2016	50.34
Amphi. WL711–*Ae. kotschyi* 3790	65.2 ± 2.2	33.1 ± 2.0	2.3	1.1	143.5	59.1	1.76	2798	67.85	2148	60.18
Amphi. WL711–*Ae. kotschyi* 391	61.8 ± 1.7	33.1 ± 0.8	1.8	0.9	128.5	53.4	1.92	2705	62.27	2059	53.54

of up to 127% in iron and 92% in zinc in the flag leaves and 2-3 times higher iron and zinc content per seed than the parents due to the inherent distinctive micronutrient deposition system of the *Ae. kotschyi* parents.

Ash content and ash iron and zinc contents of amphiploids

The grain ash content in *Ae. kotschyi* was up to 30% higher than that of the wheat cultivars indicating their higher inorganic component, whereas for the wheat–*Ae. kotschyi* amphiploids it was intermediate between the parental species (Table 3). The grain iron and zinc contents in the amphiploids were more than double of that of the wheat parents, whereas the iron content in the grain ash was 61–85% and zinc was 50–63% higher than that of WL711 cultivar.

Discussion

The wheat/*Ae. kotschyi* F_1 hybrids as well as the amphiploids were morphologically intermediate between the wheat and *Ae. kotschyi* parents for plant height, growth habit, tiller numbers per plant, etc. However, other characters like ear shape, glume awns, hard threshing and brittle rachis were more like their *Ae. kotschyi* parents. The intermediate morphology of the F_1 hybrids and their synthetic amphiploids has been reported in several studies (Sears, 1954; Martin and Laguna, 1982; Sharma *et al.*, 1987; Oliver *et al.*, 2005). The genes controlling brittle rachis (*Br*), tenacious glumes (*Tg*) of *Ae. kotschyi* appear to be epistatic over the *Q* locus controlling square head, tough rachis and free threshing in *T. aestivum* (Endo and Gill, 1996; Li and Gill, 2006) as the amphiploids resembled their *Ae. kotschyi* parents.

All the F_1 hybrids (ABDUS^{l}) had the expected 35 chromosomes (Table 1; Fig. 1) indicating complete parental chromosome complement and chromosome stability. Low to high intergenomic homoeologous chromosome pairing was observed in different F_1 hybrids. High-chromosome pairing observed in F_1 CS(Ph^I)/*Ae. kotschyi* 396 is probably due to the presence of *Ph1*-inhibitor gene, Ph^I, transferred from *Aegilops speltoides* that is known to induce considerable amount of wheat–alien pairing even in a single dose (Chen *et al.*, 1994). Our CS(Ph^I) stock seems to be heterogeneous as some other F_1 hybrids with CS(Ph^I) had limited pairing. Intermediate homoeologous pairing in hybrids with cultivar WL711 may also be explained due to some pairing promoters in *Aegilops* species that are known to suppress or enhance pairing in Triticeae (Riley and Chapman, 1958; Sears, 1976; Jauhar, 2007). Mello-Sampayo (1973) also observed the interaction of pairing promoters that inactivate *Ph1* or *Ph1*-like genes in wheat/*Ae. speltoides* and wheat/*Aegilops longissima* hybrids.

The F_1 hybrids had too low-pollen stainability to permit anther dehiscence and hence had no self-seed set. The low- to medium-chromosome pairing permitted some of the paired chromosomes to undergo reduction division and move to anaphase poles before the large number of unpaired univalents align on the metaphase-I plate and divide. Only those paired chromosomes with intact sister chromatids would divide equationally in the second meiotic metaphase, while the univalent chromatids already separated in metaphase-I are expected to move randomly resulting in tetrads with unbalanced chromosome number and micronuclei. However, no fertile first division restitution nucleus was observed as reported for *T. durum/Aegilops tauschii* and *T. durum/Ae. longissima* crosses (Matsouka and Nasuda, 2004; Jauhar, 2007; Tiwari *et al.*, 2008). Medium to highly fertile synthetic amphiploids (AABBDDUUS^{l}S^{l}) with nearly the expected chromosome number ($2n = 10x = 70$) were obtained indicating the effectiveness of colchicine treatment for doubling the chromosome number of the F_1 hybrids.

All the amphiploids developed in the present study showed nearly additive parental electrophoretic pattern of HMW glutenin protein subunits (Fig. 3) showing the presence and expression of genomes of both the parents.

Most of the amphiploids having larger grains than the wheat parents, and nearly as high grain iron and zinc and flag leaf iron and zinc concentrations as that of the *Ae. kotschyi* parent (Table 3), suggest that the higher micronutrient content of *Ae. kotschyi* as reported earlier (Chhuneja *et al.*, 2006; Rawat *et al.*, 2009) is due to its distinctive genetic system(s) for uptake, translocation and sequestration in grains rather than due to their smaller grains or lower harvest index (McDonald *et al.*, 2008). Higher flag leaf iron and zinc, grain ash and ash micronutrient concentrations in amphiploids with seeds larger than or as large as the wheat cultivars further indicate that *Ae. kotschyi* possesses genetically distinctive micronutrient uptake, translocation or seed sequestration system(s), which could be introgressed and commercially exploited in elite wheat cultivars. Our inability to record data on grain yield and harvest index in *Ae. kotschyi* accessions and the synthetic amphiploids due to their shattering and hard threshing and compare the same with wheat in term of micronutrient concentrations continues as a major bottleneck in unequivocal demonstration of *Ae. kotschyi* possessing superior genetic control for micronutrient biofortification. *Ae. kotschyi* is still a potential source of useful variability for wheat biofortification for high grain iron and zinc in addition to other progenitor species reported earlier (Calderini

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and Ortiz-Monasterio, 2003a,b; White and Broadley, 2005; Chhuneja *et al*., 2006). The work to transfer and dissect useful variability of *Ae. kotschyi* through recurrent backcrossing and development of alien addition and substitution lines in wheat background is in progress.

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