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Development and molecular characterization of wheat- *Aegilops longissima* derivatives with high grain micronutrients

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Abstract

Developing food crops with enhanced mineral concentrations is one of the most sustainable and cost effective approaches for alleviation of micronutrient. This article aims at development and molecular characterization of wheat- *Aegilops longissima* derivatives with high grain micronutrients (iron, zinc, copper, manganese, calcium, magnesium and potassium). *Aegilops longissima* (2n=14, S^lS^l) accession 3506 with high grain micronutrients was used for transferring these traits to elite wheat (*Triticum aestivum*) cultivars through wide hybridization. The fertile HD2687/L3506//WL711 BC_1F_3 derivatives were developed through selfing and selection for chromosome constitution, meiotic stability and micronutrient concentrations was done at each generation. Sixteen derivatives were finally selected and characterized. The selected backcross derivatives showed enhanced grain iron, zinc, copper, manganese, calcium, magnesium and, potassium concentrations over the parental wheat cultivars by up to 183.6%, 243.6%, 135.18%, 160.42%, 223.29%, 43.90% and 35.05%, respectively. Introgression of chromosomes 2, 7 and 1 from *Ae*. *longissima*, confirmed by plant waxiness,GISH, anchored wheat SSR markers and HMW glutenin subunit profiling and was found to be associated with enhanced micronutrients in the derivatives.

Key words: Alien introgression; Biofortification; Grain iron; Grain zinc; Protein.
Abbreviations: IARC- International Agricultural Research Centers, CGIAR- Consultative Group Of International Agricultural Research, DAPI- 4-, 6-diamidino- 2-phenylindole, GISH- genomic *in situ* hybridization.

Introduction

More than two billion people in the developing countries, depending largely on cereal and tuber crops as their staple food, suffer from iron and zinc deficiency commonly called as "hidden hunger" (Welch and Graham, 2004; White and Broadley, 2009). The micronutrient malnutrition results in poor mental and physical development and increased rate of mortality and morbidity (Holtz and Brown, 2004; Cakmak, 2008). Among various approaches, biofortification is considered as the most promising, cost effective and sustainable approach for alleviation of micronutrient malnutrition (Ortiz-Monasterio et al., 2007; Bouis and Welch, 2010). Various collaborative projects for the enhancement of grain micronutrients of rice, wheat, maize, cassava, beans and sweet potato have been initiated by HarvestPlus (www.harvestplus.org), IARCs of CGIAR (www.cgiar.org), and national organizations in different parts of the world (Gύmez- Galera et al., 2010). Wheat is the second most important cereal crop in terms of area and food source providing approximately 60% of the daily calorie intake in several developing countries of the world (FAOSTAT 2008; http://faostat.fao.org, Chatzav et al., 2010). Therefore, biofortification of minerals within wheat grain itself will have a positive impact on human health. Most of the wheat cultivars have very low grain micronutrient concentration and genetic variability (Monasterio and Graham, 2000; Peleg et al., 2009; Rawat et al., 2009a, b). However, a wide range of variation for grain micronutrients has been observed among wild relatives of wheat (Cakmak et al., 2004; Chhuneja et al., 2006; Chatzav et al., 2010). The useful variability for grain micronutrients has recently been transferred from some non- progenitor *Aegilops* species to elite wheat cultivars (Neelam et al., 2010; Tiwari et al., 2010). This article deals with the introgression and molecular characterization of wheat- *Ae*. *longissima* derivatives with high grain micronutrients.

Materials and methods

Plant Materials

Aegilops longissima (S^lS^l) accession 3506 with very high grain iron (59.1 mg/kg) and zinc (45.0 mg/kg) concentrations (Table 2, Rawat et al., 2009a) was obtained from the wheat germplasm collection of Punjab Agricultural University,

Ludhiana, India. It was crossed as male parent with an elite wheat (*Triticum aestivum* L.) cultivar HD2687 as the female parent in 2006-07. The F_1 hybrids were partially fertile due to unreduced gamete formation giving a spontaneously developed octaploid amphiploid ($AABBDDS^lS^l$). The amphiploid was backcrossed with another wheat cultivar WL 711 to get two viable BC_1 seeds in 2007-08. The BC_1 plants ($AABBDDS^l$) were allowed to self to have eleven BC_1F_2 and sixteen BC_1F_3 backcross derivatives. The BC_1F_3 progenies were grown along with their parents in the field at Indian Institute of Technology, Roorkee in 2009-10, in rows of 2 m length, with plant to plant distance of 10 cm and row to row spacing of 30 cm with recommended fertilizer and irrigation practices as that of wheat cultivars (Package of Practices, PAU, Ludhiana). The BC_1F_3 derivatives (Table 1, Table2) were analyzed (in six replications) for grain micronutrient concentrations and characterized for morphological traits, chromosome number and pairing, GISH, and SSR markers. The details of the plant materials used are given in Table1.

Morphological traits

The data on morphological traits on 3-22 plants of different BC_1F_3 derivatives such as plant waxiness, head type, rachis toughness, grain color, number of seeds per spike was recorded in the field (Table 1).

Cytological studies

Comprehensive meiotic analysis of BC_1F_2 and BC_1F_3 plants for chromosome number and pairing was done according to the method described by Rawat et al. (2009a).

Micronutrients analysis

For micronutrient analysis, whole grain samples of BC_1F_3 derivatives were digested and analyzed according to the protocol described by Rawat et al. (2009a). A minimum of three replications of micronutrient analysis were made for each of the derivatives, cultivars and *Ae*. *longissima* accession and ultrapure grade chemicals for digestion' after standards. All of the standards used in this study were from Merck, Germany.

Statistical analysis

Student t- test was applied for testing the significance of differences among means of cultivars, *Ae*. *longissima* and BC_1F_3 derivatives.

In situ hybridization

Three of the selected BC_1F_3 derivatives viz. 79-2-1-4, 79-2-1-25 and 79-1-5-5 were subjected to GISH analysis for characterization of alien introgression. 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. The genomic probe for subsequent use in genomic *in situ* hybridization (GISH) experiments was prepared using sheared genomic DNA (0.2–0.6 kb) of *Ae*. *longissima* (SlSl). The S^l-genome genomic DNA was labeled with tetramethylrhodamine-5-dUTP (red) (Roche Applied Science, Indianapolis, IN) using nick translation following manufacturer's direction. Labeled probes were purified using QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA). In order to prevent the hybridization of labeled genomic probe with wheat chromosomes, 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-genome): 100 ng of blocking DNA. Hybridization conditions, post-hybridization washes and imaging were as described by Zhang et al. (2001). Chromosomes were counterstained with 4-, 6-diamidino- 2-phenylindole (DAPI). Slides were analyzed with an epifluorescence Zeiss Axioimager M1 microscope.

Molecular analysis

The DNA was extracted from 4-5 gram of young leaves of the parents and selected BC_1F_3 plants during early tillering stage and PCR was carried out according to Tiwari et al. (2010). For the characterization of alien introgression, the wheat anchored microsatellite markers at the distal positions of each of the 42 chromosome arms, transferable and polymorphic between the *T*. *aestivum* parents and *Ae*. *longissima* 3506 were applied on the selected derivatives with high grain micronutrients content. Further, Chromosome arm specific molecular markers gwm102 (2DS), gdm 148 (2DL) and gdm 539 (2DL) and wmc 479 (7AS), gwm 350 (7AS), wmc 139 (7AL), wmc 809 (7AL) were applied for confirming the introgression of alien group 2 and 7 chromosomes of *Ae*. *longissima* in the selected derivatives.

HMW glutenin subunit profiling

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

Results

Morphological traits

The data of various morphological traits of parental lines and sixteen fertile BC_1F_3 derivatives is given in Table 1. All of the selected plants were non-waxy with spelta head except the BC_1F_3 derivatives 79-2-1-14 and 79-1-6-5 which had square heads. The BC_1F_3 derivatives 79-2-1-24, 79-2-1-25, 79-1-5-5 and 79-1-5-6 had brittle rachis and red seed color whereas all other 12 derivatives had tough rachis and amber colored grains.

Cytological analysis

The F_1 hybrids between *T*. *aestivum* cv. HD2687 and *Ae*. *longissima* 3506 were partially fertile (2n=28) with limited homoeologous chromosome pairing. The subsequent backcross with WL711 led to the development of fertile BC_1F_2 and BC_1F_3 derivatives. The BC1F2 plants with 2 to 3 fold higher grain micronutrient concentrations than those of control cultivars and nearly 18-21 bivalents were selected for further studies. The increase in bivalent frequency was observed in the selected BC_1F_2 and BC_1F_3 derivatives. The bivalent frequency in the BC_1F_2 derivatives varied from sixteen (79-2-1) to twenty-two (79-1-6) whereas the univalent frequency ranged from one (79-1-6) to seven (79-1-5, 79-2-4) with occasional trivalent and quadrivalent (Fig. 1a-d). Most of the finally selected BC_1F_3 derivatives showed 41-43 chromosomes with 18-21 bivalents and 1-6 univalents (Table 3, Fig. 1e-h).

Table 1. Morphological characteristics of the three parents and 16 selected BC_1F_3 wheat - *Ae*. *longissima* derivatives in field testing during 2010-11.

Parents/ Derivatives	Derivatives pedigree	Waxiness	Head type	Rachis	Grain color	No. of seeds per spike
L-3506	-	Non Waxy	Spelta	Brittle	Red	35.5
WL-711	S308/CHR//KAL released in 1977	Waxy	Square	Tough	Amber	48.3
HD-2687	CPAN2009/HD2329 released in 1999	Waxy	Square	Tough	Amber	45.2
79-2-1-4	BC_1F_3 HD2687/L 3506//WL711-2-1-4	Non Waxy	Spelta	Tough	Amber	20.3
79-2-1-14	BC_1F_3 HD2687/L 3506//WL711-2-1-14	Non Waxy	Square	Tough	Amber	15.4
79-2-1-19	BC_1F_3 HD2687/L 3506//WL711-2-1-19	Non Waxy	Spelta	Tough	Amber	7.5
79-2-1-24	BC_1F_3 HD2687/L 3506//WL711-2-1-24	Non Waxy	Spelta	Brittle	Red	9.0
79-2-1-25	BC_1F_3 HD2687/L 3506//WL711-2-1-25	Non Waxy	Spelta	Brittle	Red	7.2
79-2-4-5	BC_1F_3 HD2687/L 3506//WL711-2-4-5	Non Waxy	Spelta	Tough	Amber	23.5
79-1-4-2	BC_1F_3 HD2687/L 3506//WL711-1-4-2	Non Waxy	Spelta	Tough	Amber	10.7
79-1-4-6	BC_1F_3 HD2687/L 3506//WL711-1-4-6	Non Waxy	Spelta	Brittle	Amber	9.5
79-1-4-8	BC_1F_3 HD2687/L 3506//WL711-1-4-8	Non Waxy	Spelta	Tough	Amber	8.4
79-1-5-5	BC_1F_3 HD2687/L 3506//WL711-1-5-5	Non Waxy	Spelta	Brittle	Red	13.5
79-1-5-6	BC_1F_3 HD2687/L 3506//WL711-1-5-6	Non Waxy	Spelta	Brittle	Red	10.5
79-1-5-7	BC_1F_3 HD2687/L 3506//WL711-1-5-7	Non Waxy	Spelta	Tough	Amber	10.0
79-1-6-1	BC_1F_3 HD2687/L 3506//WL711-1-6-1	Non Waxy	Spelta	Tough	Amber	14.6
79-1-6-5	BC_1F_3 HD2687/L 3506//WL711-1-6-5	Non Waxy	Square	Tough	Amber	17.9
79-1-6-10	BC_1F_3 HD2687/L 3506//WL711-1-6-10	Non Waxy	Spelta	Tough	Amber	12.7
79-1-8-3	BC_1F_3 HD2687/L 3506//WL711-1-8-3	Non Waxy	Spelta	Tough	Amber	9.6

Table 2. Grain micronutrient concentrations of parents and the selected BC_1F_3 derivatives.

Parents/ derivatives	Fe (mg/kg) ± S.E	Zn (mg/kg)± S.E	Cu (mg/kg)± S.E	Mn (mg/kg)± S.E	Ca (mg/kg)± S.E	Mg (mg/kg) ± S.E	K (mg/kg)± S.E
WL-711	29.4^{a}±1.2	26.4^{a}±0.8	5.1^{a}±0.9	16.1^{a}±1.3	140.2^{a}±1.2	495.2^{a}±1.3	2010.4^{a}±1.6
HD-2687	31.7^{a}±1.4	28.6^{a}±1.2	5.7^{a}±1.2	16.2^{a}±0.6	100.3^{a}±1.4	520.1^{a}±2.0	1884.5^{a}±1.2
L-3506	59.1^{c}±0.8	45.0^{d}±1.1	8.0^{b}±1.5	24.0^{ab}±1.3	348.2^{d}±0.7	630.5^{b}±0.9	2604.1^{e}±0.8
79-2-1-4	57.5^{bc}±2.0	60.3^{f} ±1.6	8.4b^{c}±0.9	34.0^{d}±2.4	204.4^{b}±1.6	647.4^{b}±1.2	2440.2^{d}±1.0
79-2-1-14	77.4^{d}±1.3	70.6^{h}±1.2	9.2^{e}±1.0	36.2^{d}±0.6	200.1^{b}±0.9	650.0^{b}±1.4	2309.3^{c}±0.9
79-2-1-19	65.8^{c}±1.0	69.4^{gh}±1.0	12.2^{e}±2.0	42.0^{e}±1.3	210.8^{b}±0.5	687.3^{b}±1.5	2104.0^{b}±1.5
79-2-1-24	64.6^{c}±1.4	94.0^{h}±1.5	8.2b^{c}±1.2	36.0^{d}±1.6	208.3^{b}±0.7	600.1^{b}±1.6	2502.1^{d}±2.3
79-2-1-25	40.5^{ab}±1.8	74.1^{h}±1.8	7.8^{b}±1.4	40.1^{e}±0.8	220.4^{bc}±1.4	652.0^{b}±1.7	2293.8^{c}±2.1
79-2-4-5	75.7^{d}±2.0	68.8^{g}±1.4	13.6^{e}±1.6	36.2^{d}±1.2	388.2^{e}±0.9	689.5^{b}±1.0	2156.2^{b}±1.7
79-1-4-2	55.3^{bc}±1.3	83.8^{i}±1.7	9.3^{c}±0.9	36.4^{d}±1.5	250.6^{c}±1.2	625.1^{b}±1.3	1896.3^{a}±0.8
79-1-4-6	67.0^{d}±0.8	87.0^{i}±0.9	8.0^{b}±0.8	38.6^{de}±2.0	224.3^{bc}±0.8	639.0^{b}±0.7	1927.5^{a}±1.3
79-1-4-8	48.6^{b}±1.2	66.1^{g}±1.0	7.8^{b}±0.9	40.3^{e}±2.4	209.4^{b}±0.9	630.5^{b}±1.6	2128.0^{b}±1.4
79-1-5-5	86.1^{e}±1.0	75.0^{h}±1.6	12.2^{e}±1.2	38.5^{de}±1.0	352.1^{d}±0.7	678.0^{b}±1.0	2403.5^{d}±0.9
79-1-5-6	56.5^{bc}±1.4	86.3^{i}±2.1	11.8^{de}±1.4	37.1^{d}±0.8	342.7^{d}±1.2	710.1^{b}±2.1	2630.1^{e}±1.8
79-1-5-7	61.2^{c}±1.6	69.8^{gh}±1.3	12.0^{e}±1.9	36.4^{d}±0.7	316.5^{d}±1.2	665.4^{b}±1.7	2506.2^{d}±1.6
79-1-6-1	72.5^{d}±0.9	78.2^{i}±0.9	8.4^{bc}±2.3	35.3^{d}±1.3	348.1^{d}±1.7	683.0^{b}±0.7	2400.8^{d}±1.3
79-1-6-5	67.0^{c}±0.6	70.9^{h}±1.0	9.2^{c}±1.8	42.2^{e}±1.2	300.4^{d}±0.8	696.3^{b}±1.2	2387.0^{d}±1.0
79-1-6-10	77.5^{d}±0.7	94.9^{k}±0.7	9.4^{c}±0.9	38.6^{de}±2.0	333.6^{d}±1.4	709.5^{b}±1.6	2392.1^{c}±0.7
79-1-8-3	76.6^{d}±0.9	93.7^{k}±1.5	12.7^{e}±1.7	41.1^{e}±0.9	352.1^{d}±1.2	730.2^{bc}±2.0	2600.2^{e}±1.2

Notes: Different superscript letters on mean values denote significant differences among derivatives and control as based on t-test.

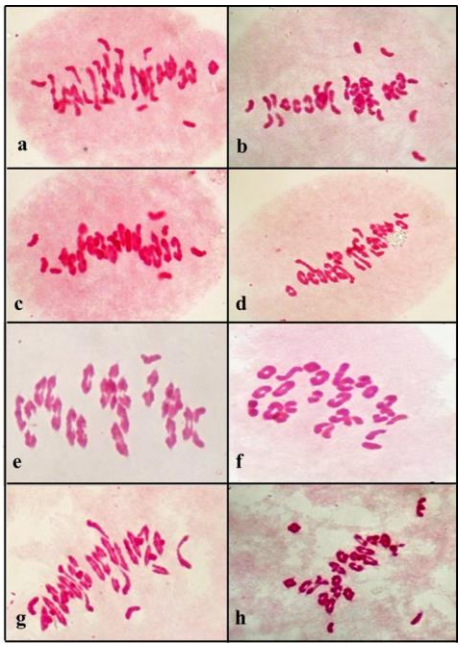

Fig 1. Chromosome pairing at metaphase-I of PMCs of some BC_1F_2 and BC_1F_3 derivatives, a. BC_1F_279-2-1(2n = 47, 1IV+2 III+16II+5I), b. BC_1F_279-2-4 (2n = 48,1III+19II +7I), c. BC_1F_2 79-1-4 (2n = 43, 18 II + 7I), d. BC_1F_279-1-6 (2n = 45, 22 II+1I), e. BC_1F_379-1-4-6 (2n = 41, 20II+ 1I),f. BC_1F_379-2-4-5 (2n = 45, 22 II+1I), g. BC_1F_379-1-4-8 (2n = 44, 21 II+2I), h. BC_1F_379-1-5-6 (2n = 43, 1III+19II + 2I).

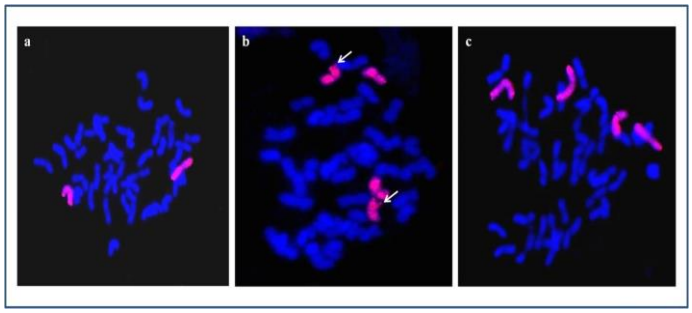

Fig 2. Genomic in situ hybridization pattern of root tip cells at mitotic metaphase of three wheat-*Ae longissima* acc. 3506 derivatives a. BC_1F_379-2-1-4 with one pair of a S^l chromosome (red), b. BC_1F_379-2-1-25 with one pair of a satellite S^l and one pair of a S^l chromosomes (red) c. BC_1F_379-1-5-5 with a pair of S^l chromosome and two other S^l chromosomes.

Grain micronutrients concentrations

The wheat cultivars *T. aestivum* WL711 and HD2687 had very low grain micronutrient concentrations (Table 2). *Ae. longissima* 3506 had almost 2.2 fold higher grain iron concentration and 3.4 fold higher grain calcium concentration whereas for most of the other minerals studied it showed nearly 1.4 fold higher concentrations than the wheat cultivars. All of the selected derivatives showed significant differences in grain micronutrient concentration over both of the wheat cultivars and also among themselves. A wide range of variation was found for concentrations of grain iron (40.5-86.1 mg/kg), zinc (60.3-94.9 mg/kg), copper (7.8-13.6 mg/kg), manganese (35.3-42.2 mg/kg), calcium (200.1-352.1 mg/kg) and magnesium (600.1-730.2 mg/kg) except for potassium. The highest increase in grain iron concentration was observed in the BC_1F_3 derivative 79-1-5-5 (186.04 %) whereas the highest concentrations for grain zinc, copper, calcium, manganese, magnesium and potassium were observed in the derivatives 79-1-6-10 (241%), 79-2-4-5 (151%, 223%), 79-1-6-5 (162%), 79-1-8-3 (43.9%) and 79-1-5-6 (35.0%), respectively.

Characterization of introgression lines by in situ hybridization

Introgression of a pair of *Ae. longissima* chromosomes was observed in the BC_1F_3 derivative 79-2-1-4 (2n=44, Fig. 2a). In 79-2-1-25 derivative (2n=46), the *Ae. longissima* probe strongly hybridized with two pairs of chromosomes. The presence of a satellite in the short arm of a pair of chromosomes and its comparison with the standard karyotype indicated that it could be $1S^l$ of *Ae. longissima* (Fig. 2b). In 79-1-5-5, hybridization with S genome probe showed the introgression of a pair of S^l chromosomes and two other S^l chromosomes (Fig. 2c).

Molecular characterization

On the basis of molecular marker analysis, it was found that chromosome $2S^l$ of *Ae. longissima* 3506 was present in all of the selected derivatives whereas the introgression of $7S^l$ chromosome was found in 11 out of 16 selected derivatives (Fig. 3). The introgression of $4S^l$ chromosome was observed only in BC_1F_379-1-6-5 whereas two of the selected derivatives 79-2-1-4 and 79-2-1-19 showed the presence of $5S^l$ chromosome of *Ae. longissima* (Fig. 3). There was no introgression of $3S^l$ and $6S^l$ chromosomes in any of the selected derivatives. Only the complete introgression of $2S^l$ and $7S^l$ chromosomes not involving any translocation was found in the derivatives as the markers of both short and long arms were present in them. This was also confirmed by *in situ* hybridization analysis (Fig. 2). It was observed that the derivatives with only introgression of group 2 and 7 chromosomes of *Ae. longissima* (79-2-4-5, 79-1-4-2, 79-1-5-5, 79-1-5-6, 79-1-6-1, 79-1-6-5, 79-1-6-10, 79-1-8-3) had higher grain micronutrient concentrations than the derivatives with introgression of other chromosomes and their combinations (Table 3, and Fig. 3). The derivatives with $1S^l$, $2S^l$ or only $2S^l$ chromosome also showed comparable grain zinc concentration to that of the derivatives with $2S^l$ and $7S^l$ chromosomes (Fig. 3).

High molecular weight glutenin subunit (HMW-GS) profile

High molecular weight glutenin subunit (HMW-GS) profiles of some of the selected BC_1F_3 derivatives along with both parents are shown in Fig. 4. The HMW glutenin subunits of *Ae. longissma* ($1S^l$) had lower electrophoretic mobility than the wheat HMW subunits controlled by *Glu A1*, *Glu B1* and *Glu D1* loci. Therefore comparative analysis of HMW-GS could provide useful information regarding the introgression of group 1 chromosome of *Ae. longissima*. The HMW-GS of the *Ae. longissima* showed two migrating zones. The one with slower electrophoretic mobility was located above the *Glu D1* subunit 2 of *T. aestivum* HD2687 and WL711 whereas the other band with faster electrophoretic mobility was located between the *Glu D1* subunit 2 and *Glu B1* subunit 7 of the wheat parents. The group 1 of *Ae. longissima* was found to be present in five of the selected derivatives (Table 3, Fig. 4). The BC_1F_3 79-2-1-4 and 79-2-1-14 showed the addition of *Ae. longissima* subunits in the wheat background whereas the selected derivatives 79-2-1-19, 79-2-1-24 and 79-2-1-25 showed the loss of *Glu D1* subunit 12 of wheat parents (Fig. 4) suggesting the substitution of $1S^l$ for

Table 3. Chromosome number, introgressed *Ae*. *longissima* chromosomes and chromosome pairing at metaphase I of BC_1F_3 derivatives.

BC_1F_3 progenies	Introgressed chromosome	2n	Univalent (I) Mean ± S.D	Bivalent (II) Mean ± S.D	Trivalent (III) Mean ± S.D
79-2-1-4	1,2,5	44	1.6 ± 0.5	21.2 ± 0.4	-
79-2-1-14	1,2	46	6.0 ± 1.2	20.0 ± 1.2	-
79-2-1-19	1,2,7,5	43	3.0 ± 0.4	20.0 ± 0.9	-
79-2-1-24	1,2	43	4.4 ± 0.7	19.3 ± 1.3	-
79-2-1-25	1, 2,7	46	2.1 ± 0.8	21.5 ± 1.4	0.3 ± 0.1
79-2-4-5	2,7	45	3.0 ± 0.9	21.0 ± 1.2	-
79-1-4-2	2, 7	42	3.8 ± 1.0	19.0 ± 0.9	0.4 ± 0.0
79-1-4-6	2	41	2.6 ± 0.6	18.6 ± 1.4	0.3 ± 0.1
79-1-4-8	2	43	2.6 ± 0.8	20.2 ± 2.3	-
79-1-5-5	2,7	46	3.3 ± 1.0	21.3 ± 0.8	-
79-1-5-6	2,7	43	2.4 ± 0.9	19.2 ± 1.3	-
79-1-5-7	2,4,7	43	3.1 ± 0.6	19.9 ± 1.7	-
79-1-6-1	2, 7	45	3.7 ± 1.2	20.6 ± 2.1	-
79-1-6-5	2, 7	41	1.0 ± 0.2	20.0 ± 2.0	-
79-1-6-10	2, 7	43	2.5 ± 0.8	20.2 ± 1.5	-
79-1-8-3	2,7	47	2.7 ± 0.6	21.7 ± 0.9	0.2 ± 0.0

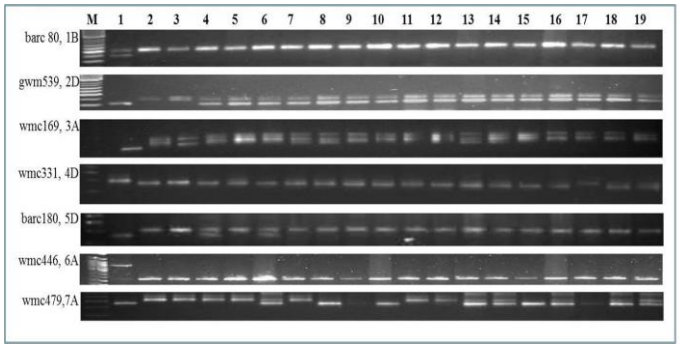

Fig 3. SSR marker analysis of group 1 to 7 chromosomes of wheat on selected wheat- *Ae*. *longissima* derivatives: Lane M- Ladder (50 bp), 1. *Ae*. *longissima*, 2. *T*. *aestivum* cv. WL711, 3. *T*. *aestivum* cv. HD2687, 4. $BC_1F_3$79-2-1-4, 5. $BC_1F_3$79-2-1-14, 6. $BC_1F_3$79-2-1-19, 7. BC_1F_3 79-2-1-24, 8. $BC_1F_3$79-2-1-25, 9. $BC_1F_3$79-2-4-5, 10. $BC_1F_3$79-1-4-2, 11. $BC_1F_3$79-1-4-6, 12. $BC_1F_3$79-1-4-8, 13. $BC_1F_3$79-1-5-5, 14. $BC_1F_3$79-1-5-6, 15. $BC_1F_3$79-1-5-7, 16. $BC_1F_3$79-1-6-1, 17. $BC_1F_3$79-1-6-5, 18. $BC_1F_3$79-1-6-10, 19. $BC_1F_3$79-1-8-3.

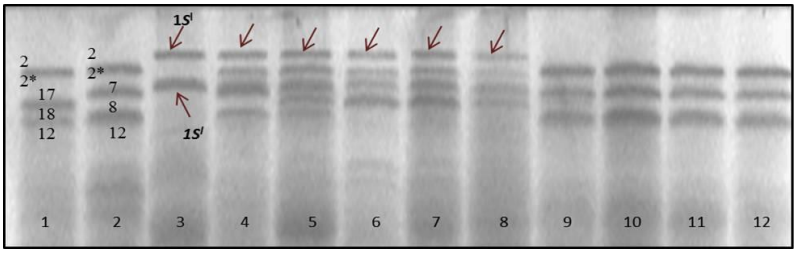

Fig 4. High Molecular Weight Glutenin Subunit profile of some selected BC_1F_3 derivatives 1. *T*. *aestivum* cv. WL 711, 2. *T*. *aestivum* cv. HD2687, 3. *Ae*. *longissima* 3506, 4. $BC_1F_3$79-2-1-4, 5. $BC_1F_3$79-2-1-14, 6. $BC_1F_3$79-2-1-19, 7. $BC_1F_3$79-2-1-24, 8. $BC_1F_3$79-2-1-25, 9. $BC_1F_3$79-2-4-5, 10. $BC_1F_3$79-1-4-2, 11. $BC_1F_3$79-1-4-6, 12. $BC_1F_3$79-1-4-8, Numerals: HMW Glutenin subunits in wheat parents, Solid arrows: Introgressed $1S^l$ *Ae*. *longissima* specific HMW Glutenin subunits.

1D. No introgression of group 1 chromosome of *Ae*. *longissima* was found in the other selected derivatives (data not shown).

Discussion

All of the selected derivatives showed non- waxy leaf sheaths as that of *Ae*. *longissima* parent. The plant non- waxiness in wheat is controlled by a dominant gene which is epispastic to the waxiness and is controlled by homoeologous group 2 chromosomes (Liu et al., 2006). Therefore, the presence of non-waxiness in all of the derivatives indicated the introgression of group 2 chromosome of *Ae*. *longissima*. The four BC_1F_3 derivatives 79-2-1-24, 79-2-1-25, 79-1-5-5 and 79-1-5-6 had brittle rachis and red grain color as that of *Ae*. *longissima* parent. A dominant gene for red grain color and brittleness are known to be present on homoeologous group 3 of wheat (Metzger and Silbaugh, 1970; Watanabe et al., 2002, Himi et al. 2011). Thus, these derivatives might have had the introgression of chromosome $3S^l$ from *Ae*. *longissima*. The reasons for the failure of polymorphic SSR markers to detect the introgression of $3S^l$ was however, not clear. *Ae*. *longissima* acc. 3506 had nearly 1.4 to 3.4 fold higher grain micronutrient concentrations than the wheat

cultivars. The wild *Triticum* and *Aegilops* species were reported to have high variability for grain micronutrient (Gregorio GB, 2002; Rawat et al., 2011). Ozkan et al. (2007); observed a wide range of variation for grain iron, zinc, manganese and copper among 54 accessions of *Triticum monococcum*. Tiwari et al. (2008) also reported higher grain ash iron and grain ash zinc content in the putative amphiploids between *T. durum* and *Ae. longissima* accessions having bolder grains, confirming the superior genetic system of *Ae. longissima* for uptake and sequestration of micronutrients. *Ae. longissima* being a S^l genome species, could effectively be utilized for transfer of high grain micronutrient concentration to elite wheat cultivars as it has high similarity with the B genome of hexaploid wheat (Dvörak and Zhang, 1990; Friebe et al., 1996). The diploid S-genome *Aegilops* species have been effectively used for the transfer of disease resistance by various workers (McIntosh, 1991; Ceoloni et al., 1992).

Most of the selected derivatives had higher grain micronutrients Fe, Zn, Cu and Mn concentrations than both the wheat as well as *Ae. longissima* parents whereas the concentrations of Mg, Ca and K was as high as that of the better parent *Ae. longissima*. The isolation of transgressive derivatives for the micronutrient concentrations indicated additive action of the genes of the parental species for these nutrients. These derivatives also had bolder grains further supporting the proof of the concept that *Ae. longissima* possesses superior genetic system for micronutrient biofortification in the wheat background which may not be due to the usual concentration effect. On the basis of GISH analysis, molecular markers and HMW GS profiling, most of the derivatives with high grain micronutrients concentrations showed the introgression of $2S^l$ followed by $7S^l$ and $1S^l$ chromosomes. One or two species of the *Sitopsis* section had contributed the S genomes to some tetraploid *Aegilops* species including *Ae. kotschyi* and *Ae. peregrina* (Kimber and Feldman, 1987; Badaeva et al., 2004). Tiwari et al. (2010) found that the substitution of the group 2S and 7U chromosomes of *Ae. kotschyi* led to the enhancement in grain iron and zinc concentration as well as content in the backcross derivatives. The introgression of $7U^p/7S^p$ and $4S^p$ chromosomes from *Ae. peregrina* were reported to confer 100-200% higher grain iron and zinc density in the backcrossed derivatives over the elite wheat cultivars (Neelam et al., 2010). Two major QTL explaining 12.6 % and 11.7% of the variance for grain iron concentration were mapped on chromosomes 2A and 7A and one major QTL explaining 18.8% of the variance for grain zinc concentration on 7A by Tiwari et al. (2008); in *T. boeoticum* x *T. monococum* RIL population. Shi et al. (2008) identified QTL for grain zinc concentration (mg/kg) on wheat chromosomes 4 and 5 contributing 11.9% and 10.9% to the variance whereas for grain zinc content (µg/seed) major QTL were found on chromosomes 2 and 7 in a double haploid wheat population. Genc et al. (2009) also reported major QTL for grain zinc concentration on chromosomes 4 and 7 in wheat. Peleg et al. (2009) in a durum wheat x wild emmer wheat RIL population, reported significantly contributing QTL on on chromosome 2 and 7 for grain iron, zinc, manganese and magnesium whereas for copper and calcium the QTL were on chromosomes 2, 4 and 7. Norton et al. (2010); observed QTL for grain zinc on rice chromosomes 6 and 10, for magnesium on chromosome 4 and for potassium chromosome 7. The rice chromosomes 6 and 10 are colinear with wheat chromosomes 7 and 1, respectively whereas rice chromosomes 4 and 7 correspond to wheat group 2 chromosomes (Gale and Devos, 1998; Sorrells et al., 2003). The presence of two co-located QTL for grain zinc concentration and content was observed on barley (*Hordeum vulgare L.*) chromosome 2H (Lonergan et al., 2009) which is syntenic to its wheat homoeologue (Deynze et al., 1995; Cho et al., 2006). All of these findings strongly support our results that the *Ae. longissima* $2S^l$ and $7S^l$ chromosomes possess orthologs for grain micronutrient concentrations. Addition of more than one chromosome has been observed in most of the backcross derivatives which led to extensive linkage drag and reduced fertility and harvest index. For commercial exploitation of biofortified wheat, recombinants of these addition/ substitution lines of group 1, 2 or 7 chromosomes with fine transfers in elite wheat cultivars will be required. The work on homoeologous chromosome paring and irradiation induced fine compensating transfers from the derivatives in elite wheat cultivar background without linkage drag is in progress.

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