International Journal of Agriculture, Environment & Biotechnology
Citation: IJAEB: 7(2): 191-204 June 2014
DOI: 10.5958/2230-732X.2014.00234.4

Biotechnology

Morphological, Cytological and Biochemical Characterization of wheat *Aegilops Longissima* Derivatives BC_1F_6 and BC_2F_4 with High Grain Micronutrient

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Paper No. 197 Received: February 03, 2014 Accepted: April 28, 2014 Published: May 13, 2014

Abstract

Micronutrient deficiency (Iron and Zinc) is the major problem worldwide mostly in the developing countries with high dependence on staple foods. Biofortification of staple cereal and tuber crops has been considered and taken up as the most effective, feasible and economic approach for alleviating micronutrient deficiency. The present study was the initiative towards biofortification of wheat where the previous work of wide hybridization between HD2687 and *Aegilops longissima* accession 3506 and subsequent backcrossing with *Triticum aestivum* cultivar WL711 has been continued. In this study the alien chromosome introgression, chromosomal stability of BC_1F_6 and BC_2F_4 wheat-*Ae. longissima* derivatives has been investigated for their potential as germplasm for future breeding and biofortification program through morphological, cytological and biochemical analysis. We found that the selected derivatives showed stable 42, 44 and 46 chromosomes for most of the plants where single plant reported for each 41, 43 and 45 chromosomes with 19-22 bivalents and few trivalent. The GISH analysis of derivative 79-1-4-8-1-2-2 revealed introgression of two univalent S^l chromosomes. Finally four derivatives were selected from BC_1F_6 (79-1-4-8-10-2-2, 79-1-4-8-10-2-5, 79-2-4-4-1-1-3 and 79-2-4-4-1-1-5) and three from BC_2F_4 (HD2687/L3506//WL711-3///WL711-1-2-7-1, HD2687/L3506//WL711-3///WL711-1-2-7-3, HD2687/L3506//WL711-3///WL711-1-2-7-5) as stable biofortified lines for future breeding to alleviate hidden hunger.

Highlights

- Biofortification of staple crops is the most effective approach against hidden hunger
- *Aegilops* sp. known for robustness against different stresses, therefore deployed for transfer of variability
- High Fe and Zn containing stable wheat-*Ae. longissima* derivatives BC_1F_6 were selected as future breeding material

Keywords: Wide hybridization, biofortification, genome *in situ* hybridization, hidden hunger, *Ae. longissima*

Malnutrition caused by deficiencies of minerals and vitamins is also known as “hidden hunger”, since physical symptoms associated with hunger and malnutrition are not distinguished in most of the affected people. According to the United Nation’s Standing Committee on Nutrition (UN/SCN), over three billion people worldwide are afflicted due

to micronutrient malnutrition, particularly deficiencies in Fe and Zn which results in significant decrease in health development, cognitive and motor function impairment, complications during pregnancy and anemia that leads to increased morbidity and mortality rates as well as reduction in productivity (Bouis, 2007; Peleg *et al.*, 2008; Salim-Ur-Rehman *et al.*, 2010; Welch and Graham, 2004).

Biofortification refers to the development of nutritionally enriched new and existing crops via traditional breeding approach or genetic modification. The initiatives were started since late 2002 to till now by different international agencies such as CGIAR (Consultative Group for International Agricultural Research) and Harvest Plus funded by The Bill and Melinda Gates Foundation, GAIN (Global Alliance for Improved Nutrition), Golden Rice Project, Zero Hunger, WHO (World Health Organization) and World Food Program. Nutrient supplementation and food-based approaches has been used for alleviation of micronutrient deficiency. Nutrient supplementation or fortification approach has been mostly neglected due to overburden on individuals budget and as add-on to regular dietary habits. Food-based approach has been effectively utilized with availability of diverse crop varieties, plant and animal species under varying environment and geographic region with diversified food culture.

Among cereals wheat is the second-most produced crop, occupying 17% (one sixth) of crop acreage worldwide, feeding about 40% of the world population and providing 20% (one fifth) of total food calories and protein to human nutrition. In 2013, the total wheat production in India was about 92.46 million tons (mt) from 29.65 million hectare (mha) area however the target was 95 mt (http://faostat.fao.org). Further the task of increasing wheat production faces challenges of terminal heat stress, dwindling resources and growing threat from diseases such as wheat rusts. Wild relatives of wheat have been used as source of variability and robustness against climate change and different biotic-abiotic stresses. In 2006-2007 *Aegilops longissima* (S^lS^l) accession 3506 derivatives were prepared after crossing with HD2687, subsequent backcrossing with WL711 and selfing upto BC_1F_3 population (Neelam et al., 2013). The prerequisites for using these derivatives as breeding material were chromosome constitution, meiotic stability and micronutrient concentrations. This article deals with the cytological and biochemical characterization of advanced generation BC_1F_6 of wheat-*Ae. longissima* derivatives with higher grain micronutrients.

Materials and Methods

Plant material and generation of mapping population

Aegilops longissima (S^1S^1) accession 3506 with high grain Fe and Zn concentration was obtained from the wheat germplasm collection of Punjab Agriculture University, Ludhiana, India, used as donor male plant whereas bread wheat cultivars WL711 and HD2687 were used as recipient female plants. *Ae. longissima* (S^1S^1) accession 3506 was crossed with elite *T. aestivum* cultivar HD2687, producing F_1 hybrids in 2006-2007. The F_1 generation so obtained was partially fertile due to unreduced gamete formation. The amphiploid was backcrossed with another wheat cultivar WL 711, generating two viable BC_1 seeds in 2007-08. BC_1 plants ($AABBDDS^1$) were allowed to self, generating BC_1F_3 plants. BC_1 plants were further backcrossed with WL711, producing BC_2 backcross derivatives, grown at The Indian Institute of Technology, Roorkee in 2009-2010 (Neelam *et al.*, 2013). BC_1F_3 and BC_2 derivatives were selfed subsequently to get BC_1F_6 and BC_2F_4 generation of backcross derivatives respectively after morphological and genetic screening. BC_1F_6 derivatives along with their parents were grown at research field, Eternal University, Baru Sahib (2012-2013). Development of wheat-*Ae. longissima* derivatives upto BC_1F_6 generation is shown in Fig. 1 and the details of the plant materials used are given in Table 1. Five plants from each derivative were used for analysis.

Table 1: Plant materials used for the study (2012-2013)

S.No.	Plant Materials
1.	*Triticum aestivum* WL711
2.	*Triticum aestivum* HD2687
3.	*Aegilops longissima* 3506 (L3506)
4.	BC_1F_6 HD2687/L3506//WL711-1-4-6-2(79-1-4-6-2)
5.	BC_1F_6 HD2687/L3506//WL711-1-4-6-4-1(79-1-4-6-4-1)
6.	BC_1F_6 HD2687/L3506//WL711-1-4-8-5-2(79-1-4-8-5-2)
7.	BC_1F_6 HD2687/L3506//WL711-1-5-5-1(79-1-5-5-1)
8.	BC_1F_6 HD2687/L3506//WL711-1-5-7-6(79-1-5-7-6)
9.	BC_1F_6 HD2687/L3506//WL711-1-4-8-10-2(79-1-4-8-10-2)
10.	BC_1F_6 HD2687/L3506//WL711-1-6-5-6-6(79-1-6-5-6-6)
11.	BC_1F_6 HD2687/L3506//WL711-2-4-4-1-1(79-2-4-4-1-1)
12.	BC_1F_6 HD2687/L3506//WL711-2-4-5-2-6(79-2-4-5-2-6)
13.	BC_1F_6 HD2687/L3506//WL711-2-1-14-1-6(79-2-1-14-1-6)
14.	BC_2F_4 HD2687/L3506//WL711-3///WL711-1-2-7

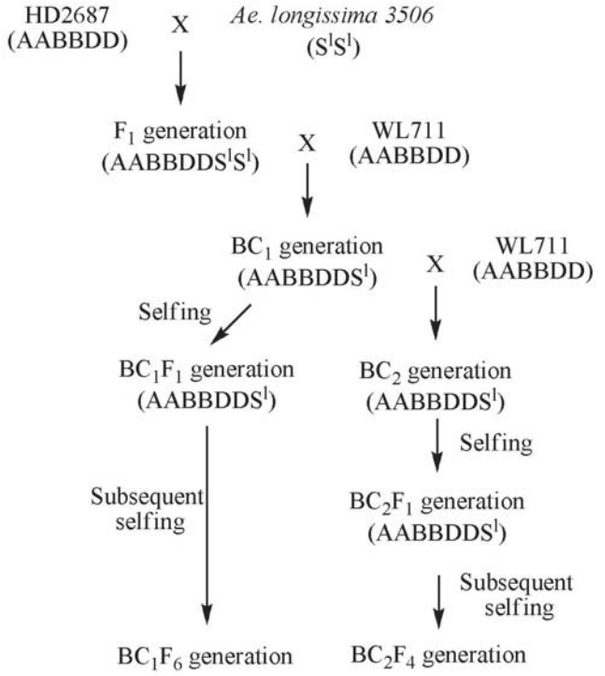

Fig. 1: Development of wheat-*Ae. longissima* derivatives

Morphological traits

The data on morphological traits on plants of different BC_1F_6 derivatives such as plant waxiness, grain color, number of seeds per spike and weight of 10 seeds was recorded in the field.

Micronutrient content analysis

Micronutrient analysis of grain was performed for BC_1F_6 and BC_2F_4 backcross derivatives, since derivatives with high micronutrient (Fe and Zn) were further selected for identification and characterization of alien gene introgression. To understand the distribution of micronutrients, mobilization and efficiency of overall system, micronutrient analysis of straw and leaves was performed for the selected BC_1F_6 backcross derivatives.

Digestion of sample

For chemical analysis whole grain samples from cultivated and wild accessions were washed with distilled water and dried in hot air oven at 80ÚC for 4 hours. A constant weight of 0.5 grams of sample (seed, straw, leaf) was used in present study. The samples were digested using 5ml of nitric acid (for seeds) and 6ml of nitric acid with 1ml of hydrofluoric acid (for straw and leaves) under the conditions of ramp-time of 15 min during which temperature rises to 180 ÚC and at this temperature digestion takes place for 20 min followed by cooling to room temperature in 15 min. Digested samples were diluted to make final volume upto 25ml and dilution factor was calculated by using formula: weight of sample/ final volume i.e. 25ml. Fe and Zn content was measured by using Agilent atomic absorption spectrometer (AAS) and the concentration was calculated in mg/kg.

Cytological studies

Selected BC_1F_6 and BC_2F_4 backcross derivatives plants were fixed in Carnoy's solution (6 ethanol: 3 chloroform: 1 acetic acid) for 24 hours and stored in 70% ethanol at 4ÚC. Cytological studies performed under microscope (Magnüs) taking anthers on the slide from the lowermost floret of the fixed ear. Anthers were crushed in acetocarmine dye to separate out pollen mother cells (PMCs), debris was removed and the slide was observed under 10X magnification of microscope. If appropriate stage (metaphase I) was observed, then slide was covered with coverslip, heated upto 65ÚC over spirit lamp, pressed hard to spread the chromosomes and observed under 100X oil immersion where number of chromosomes and pairing pattern was noted. If the appropriate stage was not observed, the upward floret was examined by repeating the same procedure.

Genomic *In Situ* Hybridization (GISH) Analysis

Comprehensive chromosomes analysis of selected BC_1F_6 plants for introgression of *Ae. longissima* chromosome was done according to the method described by Neelam *et al.*, (2013).

HMW glutenin SDS PAGE

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) with some modifications.

Results and Discussion

Morphological traits

The data of various morphological traits of parental lines and selected fertile plants of BC_1F_6 derivatives is given in Table 2. All the selected plants were non-waxy and had amber coloured grains except 79-1-5-5-1, which had red coloured grains.

Table 2: Morphological characterization of selected wheat-*Ae. longissima* derivatives (2012-2013)

Parents/Derivative	Weight of 10 seeds (in gm)	Average no. of seeds/spike	Seed Color	Waxiness
Ae. longissima 3506		35.5	Red	Non-waxy
T. aestivum cv. WL711		45.6	Amber	Waxy
T. aestivum cv. HD2687		43.2	Amber	Waxy
79-1-4-6-4-1-1	0.322	29.4	Amber	Non-Waxy
79-1-4-6-4-1-2	0.409	38.0	Amber	Non-Waxy
79-1-4-6-4-1-3	0.357	38.0	Amber	Non-Waxy
79-1-4-8-10-2-1	0.318	49.2	Amber	Non-Waxy
79-1-4-8-10-2-3	0.299	39.3	Amber	Non-Waxy
79-1-4-8-10-2-4	0.437	31.4	Amber	Non-Waxy
79-1-5-5-1	-	-	Red	Non-Waxy
79-1-5-7-6-1	0.308	36.7	Amber	Non-Waxy
79-1-5-7-6-3	0.354	49.9	Amber	Non-Waxy
79-1-5-7-6-4	0.315	35.0	Amber	Non-Waxy
79-1-6-5-6-6-5	0.310	06.1	Amber	Non-Waxy
79-2-1-14-1-6-2	0.327	30.6	Amber	Non-Waxy
79-2-1-14-1-6-3	0.301	29.2	Amber	Non-Waxy
79-2-1-14-1-6-4	0.297	22.3	Amber	Non-Waxy
79-2-4-4-1-1-2	0.390	34.9	Amber	Non-Waxy
79-2-4-5-2-6-2	0.260	44.8	Amber	Non-Waxy
79-2-4-5-2-6-3	0.282	34.2	Amber	Non-Waxy
79-2-4-5-2-6-4	0.277	33.6	Amber	Non-Waxy
79-2-4-5-2-6-5	0.348	33.0	Amber	Non-Waxy

Grain micronutrient concentration

The wheat cultivars *Triticum aestivum* cv. WL711 and HD2687 had Fe content of 37.6 and 32.7 mg/kg respectively and Zn content of 29.8 and 27.6 mg/kg respectively. *Ae. longissima* 3506 had 88.3 mg/kg of Fe and 46.7 mg/kg of Zn, which is about 2.0 fold higher in grain Fe and 1.5 fold higher in grain Zn concentration. All the selected wheat-*Ae. longissima* derivative plants showed significant difference in grain micronutrient concentration among themselves and when compared to parental wheat cultivars. The grain Fe concentration ranged from 25.7 to 76.9 mg/kg and grain Zn concentration ranges from 24.2 to 94.5 mg/kg among derivatives (Table 3). The highest increase in grain Fe concentration was observed in the plant 79-1-6-5-6-6-3 (104.43%) and the highest concentration for grain Zn was observed in the same plant (217.25%) but the average number of seeds per spike was very low in this plant (2.4 seeds/ spike).

Derivatives 79-1-4-8-10-2-2,79-1-4-8-10-2-5, 79-2-4-4-1-1-3 and 79-2-4-4-1-1-5 had high increase in Zn (94.13%, 58.9%, 64.7% and 51.7%, respectively) and moderate increase in concentration of Fe (6.14%, 44.6%, 24.3% and 32.1%, respectively) with threshold number of seeds/spike i.e. 39.3, 38.0, 32.6 and 39.9 seeds, respectively.

Similarly, BC_2F_4 derivative HD2687/L3506//WL711-3///WL711-1-2-7-1 had very high increase in Fe (149.7%) and Zn (362.0 %) concentration but had low harvest index whereas harvest index of other plants varied from 16.3 to 24.1 (unpublished data) with percentage increase in Fe concentration upto 35.9% and of Zn concentration 150.8%. The micronutrient data of BC_2F_4 derivatives is given in Table 4.

Cytological studies

The BC_1F_5 plants (2011-2012) with high concentration of Fe (54-108 mg/kg) and Zn (44-131 mg/kg) were selected and selfed to produce BC_1F_6 derivatives in 2012-2013 with 41-46 chromosomes having varied frequency of bivalents ranged from 19 (in 79-1-4-6-4-1-1 plant) to 23 (in 79-25-4-5-2-6-3 plant) whereas univalent frequency varied from one (79-1-4-8-5-2-2, 79-1-4-8-10-2-3, 79-1-4-8-10-2-5, 79-1-5-5-1-2, 79-2-4-5-2-6-3) to three (79-1-4-6-2-1-1, 79-1-4-6-4-1-4, 79-1-4-8-10-2-2, 79-2-4-5-2-6-1) with occasionally trivalent and quadrivalent. The cytological data of selected plants are summarized in Table 3 and in Table 5 for rest of the plants.

BC_2F_4 derivatives had 43-44 chromosomes with 22 bivalents (HD2687/L3506//WL711-3///WL711-1-2-7-4 and

Table 3: Micronutrient and cytological data of morphologically selected BC_1F_6 progeny of cross HD2687/L3506//WL711

Derivative	Average no. of seeds/ spike	Fe(mg/kg) ± RSD	% inc. in Fe*	Zn(mg/kg) ± RSD	% inc. in Zn*	Chromosome No.	Bivalent (II)Mean	Univalent (I)Mean	Trivalent (III)Mean
79-1-4-6-2-1-1	18.4	56.74 ± 0.9	50.91	85.17 ± 0.9	186	44	20.4	2.6	0.1
79-1-4-6-2-1-2	13.3	60.86 ± 1.0	61.85	37.41 ± >100	25.62	-	-	-	-
79-1-4-6-2-1-3	21.3	34.85 ± 6.0	-7.33	51.96 ± 0.5	74.48	-	-	-	-
79-1-4-6-2-1-4	20.6	62.35 ± 2.0	65.83	93.47 ± 0.5	213.86	45	20.6	3.7	-
79-1-4-6-4-1-4	15.4	43.65 ± 0.4	16.09	62.11 ± 0.4	108.56	43	19.8	2.8	0.2
79-1-4-6-4-1-5	27.5	40.20 ± 0.6	6.91	60.74 ± 0.1	103.96	-	-	-	-
79-1-4-8-10-2-2	39.3	39.92 ± 1.7	6.17	57.81 ± 0.5	94.13	42	20.4	1.4	-
79-1-4-8-10-2-5	38	54.36 ± 2.8	44.56	47.33 ± 0.8	58.92	42	20.7	1.2	0.3
79-1-5-7-6-2	21.8	42.57 ± 1.9	13.22	55.81 ± 0.4	87.4	-	-	-	-
79-1-5-7-6-5	39.4	35.09 ± 3.0	-6.67	49.41 ± 0.3	65.91	43	20.3	2.4	-
79-1-6-5-6-6-1	2.6	59.34 ± 1.5	57.82	84.99 ± 0.7	185.39	-	-	-	-
79-1-6-5-6-6-3	2.4	76.87 ± 3.4	104.43	94.48 ± 0.3	217.25	-	-	-	-
79-1-6-5-6-6-4	3	65.06 ± 2.5	73.02	75.67 ± 0.5	154.09	-	-	-	-
79-2-1-14-1-6-1	26.5	33.67 ± 2.2	-10.46	45.36 ± 0.4	52.32	-	-	-	-
79-2-1-14-1-6-5	27.5	37.48 ± 0.3	-0.33	45.06 ± 0.9	51.31	-	-	-	-
79-2-4-4-1-1-3	32.6	46.73 ± 1.9	24.28	49.05 ± 0.5	64.7	42	21	0.0	-
79-2-4-4-1-1-5	39.9	49.68 ± 2.4	32.12	45.18 ± 0.3	51.7	-	-	-	-
Ae. longissima 3506	31.6	88.31 ± 0.6	134.87	46.7 ± 0.6	56.83	-	-	-	-
T. aestivum cv. WL711	-	37.60 ± 3.2	0.01	29.78 ± 0.5	0	-	-	-	-
T. aestivum cv. HD2687	-	32.71 ± 2.7	-13.01	27.58 ± 0.4	-7.38	-	-	-	-

*compared with WL711

Table 4: Micronutrient data of BC_2F_4 derivatives

Derivative	Fe(mg/kg) ± RSD	% inc. in Fe*	Zn(mg/kg)± RSD	% inc. in Zn*
HD2687/L3506//WL711-3///WL711-1-2-7(Bulk)	48.17 ± 0.1	67.53	81.53 ± 0.4	173.78
HD2687/L3506//WL711-3///WL711-1-2-7-1	71.76 ± 0.1	149.59	137.59 ± 0.2	362.02
HD2687/L3506//WL711-3///WL711-1-2-7-2	27.71 ± 0.1	-3.61	43.87 ± 0.2	47.31
HD2687/L3506//WL711-3///WL711-1-2-7-3	36.81 ± 0.1	28.04	60.52 ± 0.3	103.22
HD2687/L3506//WL711-3///WL711-1-2-7-4	37.13 ± 0.1	29.14	74.68 ± 0.3	150.76
HD2687/L3506//WL711-3///WL711-1-2-7-5	39.07 ± 0.1	35.89	73.67 ± 0.3	147.39

*compared with WL711

Table 5: Chromosome number and pairing of wheat-*Ae. longissima* derivatives (2012-2013)

Derivative	Chromosome No.	Bivalent (II)Mean	Univalent (I)Mean	Trivalent (III)Mean	Quadrivalent (IV)Mean
79-1-4-6-4-1-1	42	19.4	1.7	0.1	0.3
79-1-4-8-5-2-2	42	20.9	0.1	-	-
79-1-4-8-5-2-4	42	21.0	0.0	-	-
79-1-4-8-10-2-3	42	20.0	1.0	0.3	-
79-1-5-5-1-2	42	20.0	0.7	-	0.3
79-1-5-5-1-3	43	20.7	1.7	-	-
79-1-5-5-1-4	43	20.1	2.0	0.3	-
79-1-6-5-6-6-2	42	20.8	0.4	-	-
79-2-1-14-1-6-2	42	20.6	0.8	-	-
79-2-1-14-1-6-3	42	21.0	0.0	-	-
79-2-4-5-2-6-1	43	20.2	2.6	-	-
79-2-4-5-2-6-3	46	22.7	0.7	-	-
79-2-4-5-2-6-4	44	20.8	1.8	0.2	-
79-2-4-5-2-6-5	41	19.8	1.5	-	-

HD2687/L3506//WL711-3///WL711-1-2-7-5), three univalents (HD2687/L3506//WL711-3///WL711-1-2-7-1) however, HD2687/L3506//WL711-3///WL711-1-2-7-3 had one trivalent with no univalent. The results of cytological studies for BC_1F_6 and BC_2F_4 backcross derivatives are shown in Fig. 2.

GISH analysis

GISH was performed for selected wheat-*Ae. longissima* derivatives where 79-1-4-8-10-2-2 derivative showed clear signal of *Ae. longissima* univalent chromosome (S^l) transfer in selected background of *T. aestivum* cv.WL711 (Fig. 3)

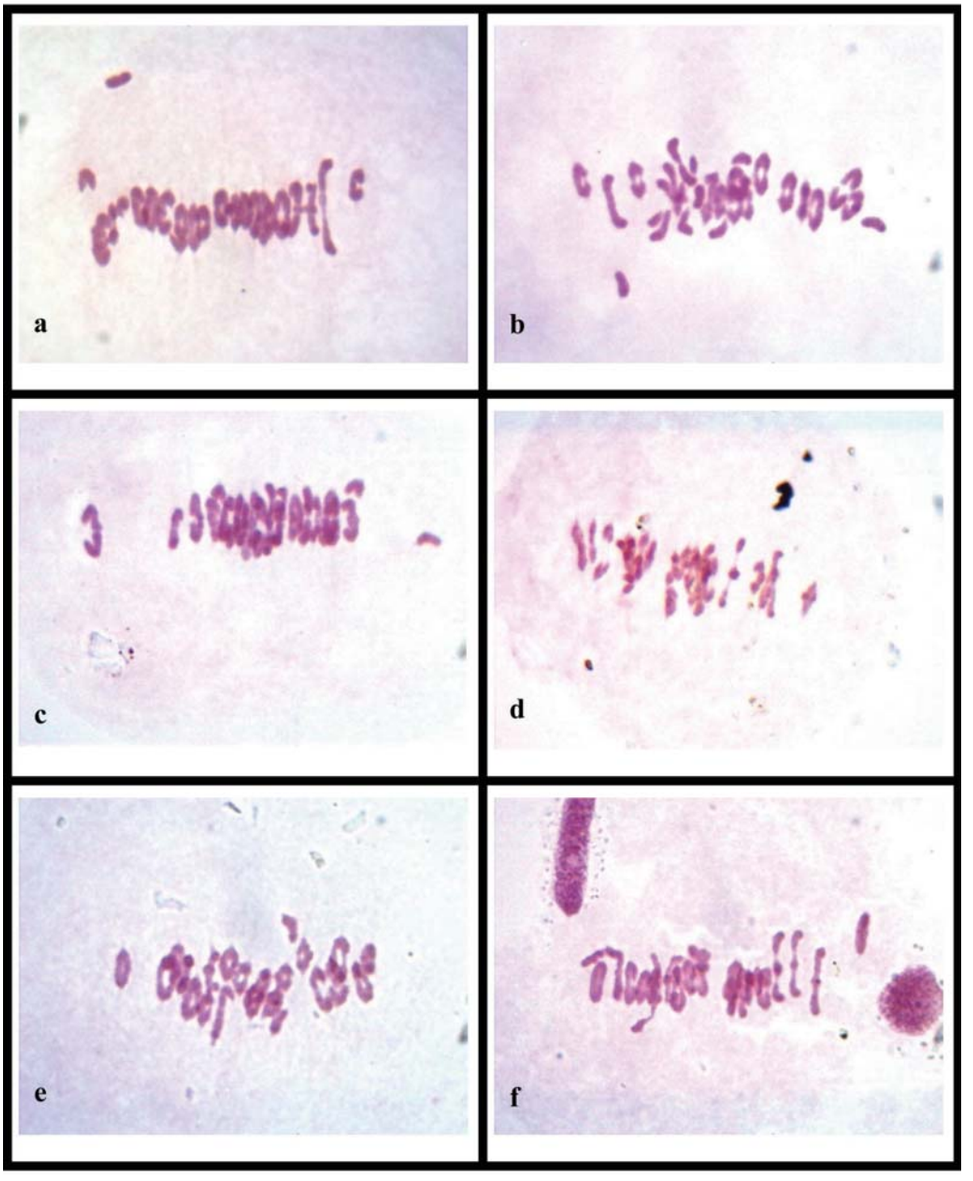

Fig. 2: Chromosome number and pairing in BC_1F_6 HD2687/L3506//WL711 (a) 79-1-4-6-2-1-1 (44 chromosomes; 21II+2I) (b) 79-1-4-6-2-1-4 (43 chromosomes; 20II+3I) (c) 79-1-4-8-5-2-2 (43 chromosomes; 21II+1I) (d) 79-1-4-8-5-2-4 (42 chromosomes; 21II) (e) 79-1-4-8-10-2-3 (42 chromosomes; 21II) (f) 79-1-4-8-10-2-5 (42 chromosomes; 21II) cont…

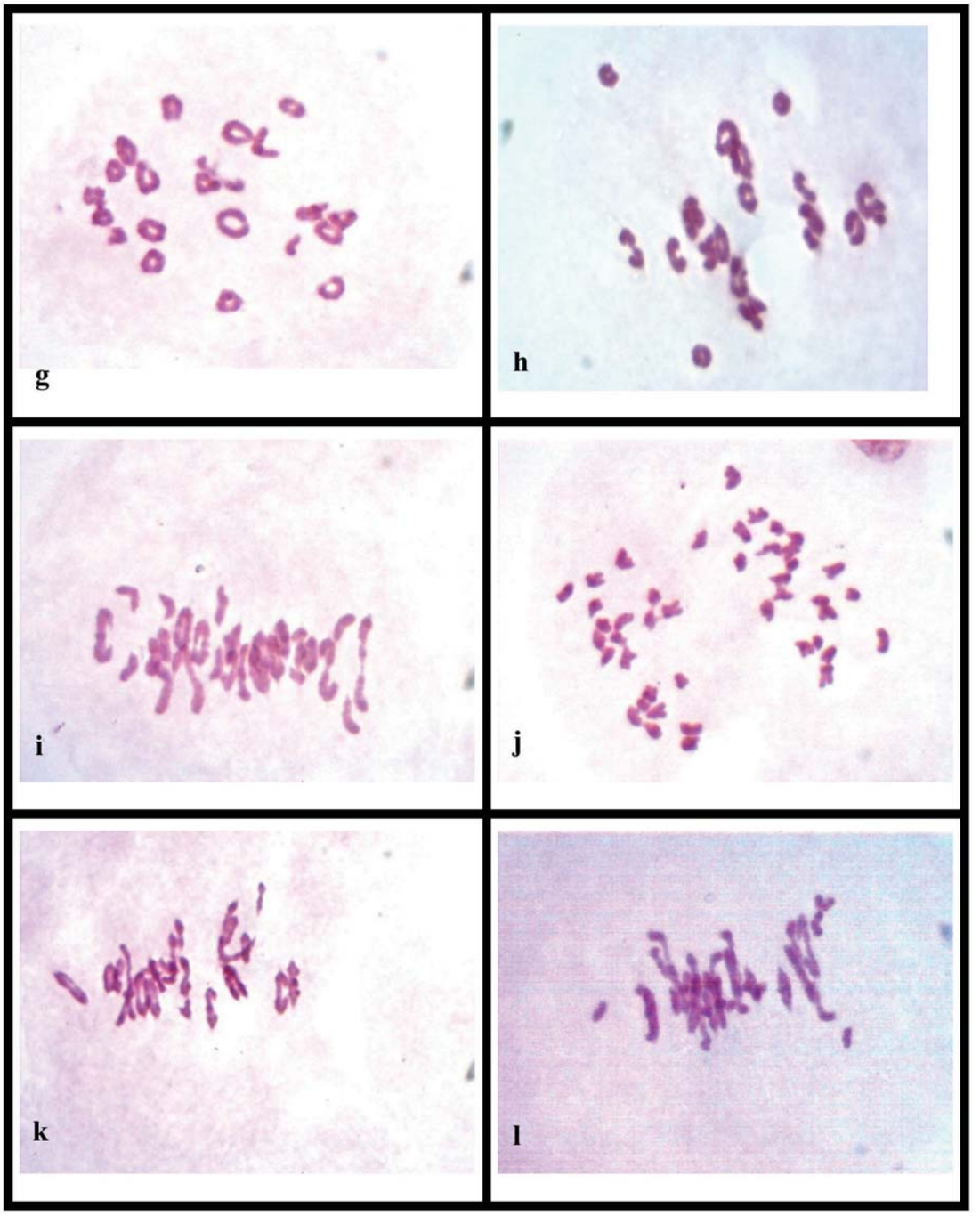

Fig. 2: Chromosome number and pairing in BC_1F_6 HD2687/L3506//WL711 (a) 79-1-4-6-2-1-1 (44 chromosomes; 21II+2I) (b) 79-1-4-6-2-1-4 (43 chromosomes; 20II+3I) (c) 79-1-4-8-5-2-2 (43 chromosomes; 21II+1I) (d) 79-1-4-8-5-2-4 (42 chromosomes; 21II) (e) 79-1-4-8-10-2-3 (42 chromosomes; 21II) (f) 79-1-4-8-10-2-5 (42 chromosomes; 21II) cont…
Fig. 2 Chromosome number and pairing in BC_1F_6 HD2687/L3506//WL711 (g) 79-1-5-5-1-2 (42 chromosomes; 21II) (h) 79-1-5-5-1-3 (43 chromosomes; 21II+1I) (i) 79-1-5-5-1-4 (42 chromosomes; 21II) (j) 79-1-5-7-6-5 (43 chromosomes) (k) 79-1-6-5-6-6-2 (42 chromosomes; 21II) (l) 79-2-1-14-1-6-2 (43 chromosomes; 19II+5I) cont…

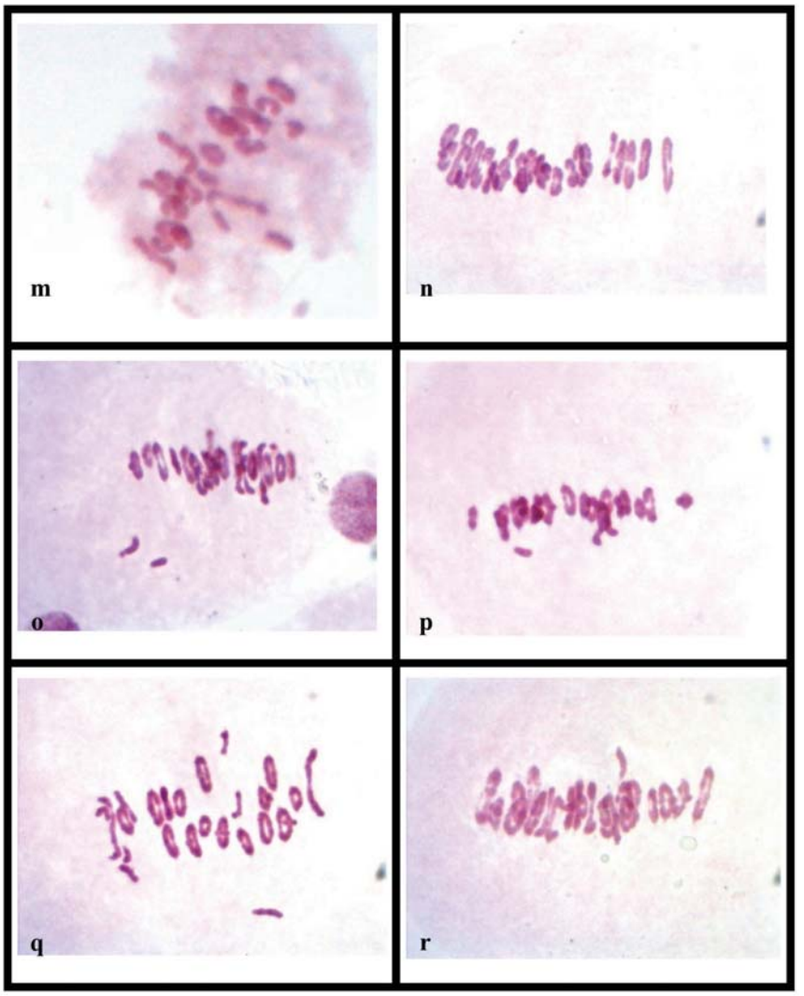

Fig. 2 : Chromosome number and pairing in BC_1F_6 HD2687/L3506//WL711 (m) 79-2-4-5-2-6-1 (43 chromosomes; 19II+1III+2I) (n) 79-2-4-5-2-6-3 (46 chromosomes; 23II) (o) 79-2-4-5-2-6-4 (44 chromosomes; 21II+2I) (p) 79-2-4-5-2-6-5 (41 chromosomes; 20II+1I) (q) BC_2F_5 HD2687/L3506//WL711-3//WL711-1-2-7-1 (43 chromosomes; 21II+1I) (r) BC_2F_5 HD2687/L3506//WL711-3//WL711-1-2-7-3 (42 chromosomes; 21II) cont…

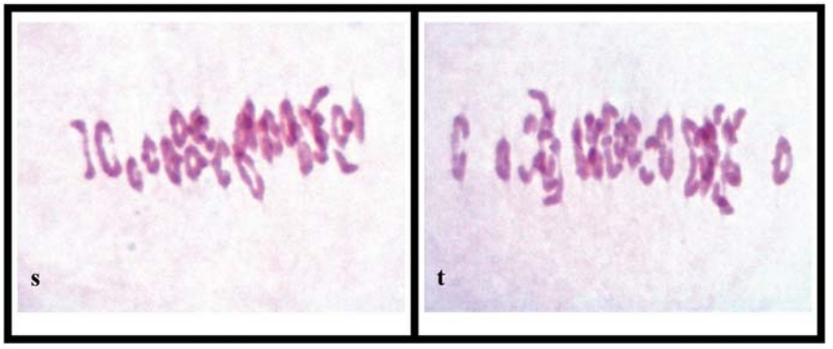

Fig. 2: Chromosome number and pairing in (s) and (t) BC_2F_5 HD2687/L3506//WL711-3//WL711-1-2-7-5 (44 chromosomes; 22II)

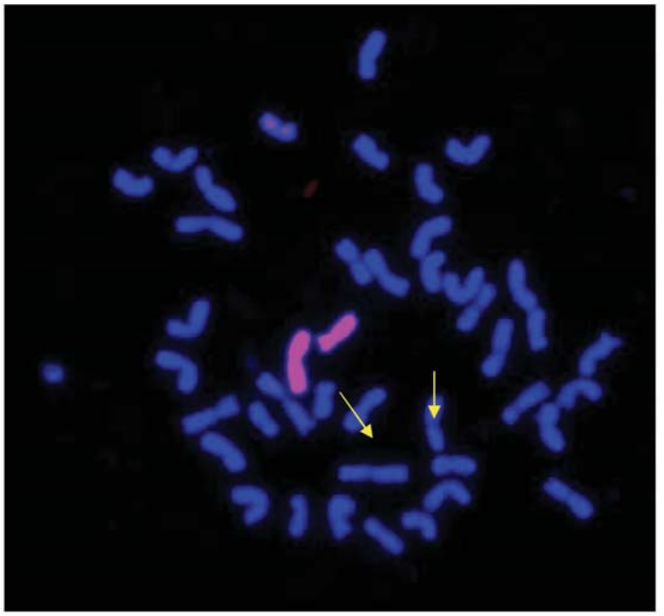

Fig. 3: GISH study of *Ae. longissima* derivative 79-1-4-8-10-2-2. *Ae. longissima* chromosomes are in pink colour.

High molecular weight glutenin subunit (HMW-GS) profile

The high molecular weight glutenin subunit (HMW-GS) profiles of selected BC_1F_5, BC_1F_6 and BC_2F_4 derivatives along with parents are shown in Fig. 4, Fig.5 and Fig. 6 respectively. The HMW glutenin subunits of Ae. *longissima* ($1S^l$) had distinct protein band than that of wheat HMW glutenin subunits. After SDS-PAGE of proteins from single seed, introgression of group 1S from *Ae. longissima* was observed in 79-1-5-7-6, 79-1-6-5-6-6 and 79-2-4-5-2-6 of BC_1F_5 sown in 2011-2012 (Fig. 4) however in BC_1F_6 generation sown in 2012-2013 only 79-1-6-5-6-6-1 and 79-1-6-5-6-6-3 showed presence of chromosome 1 (Fig. 5). No introgression was found in 79-1-5-7-6-2, 79-1-5-7-6-5, 79-1-6-5-6-6-2, 79-1-6-5-6-6-4 and other derivatives of BC_1F_5 and BC_1F_6 generation. No introgression was found in BC_2F_4 derivatives (Fig. 6).

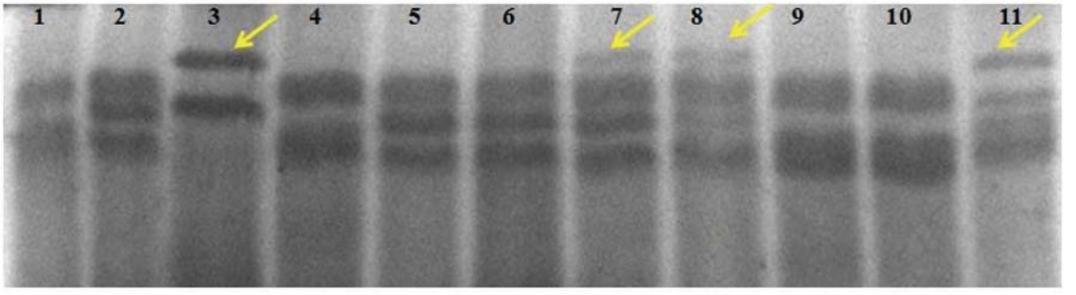

Fig. 4: HMW glutenin subunit profile of BC_1F_5 Generation sown in 2011-2012
1. *T. aestivum* cv. WL711, 2. *T. aestivum* cv. HD2687, 3. *Ae. longissima* 3506, 4. 79-1-4-6-4-1, 5. 79-1-4-8-5(bulk), 6. 79-1-5-5(bulk), 7. 79-1-5-7-6, 8. 79-1-6-5-6-6, 9. 79-2-1-14-1-6, 10.79-2-4-4-1-1, 11. 79-2-4-5-2-6

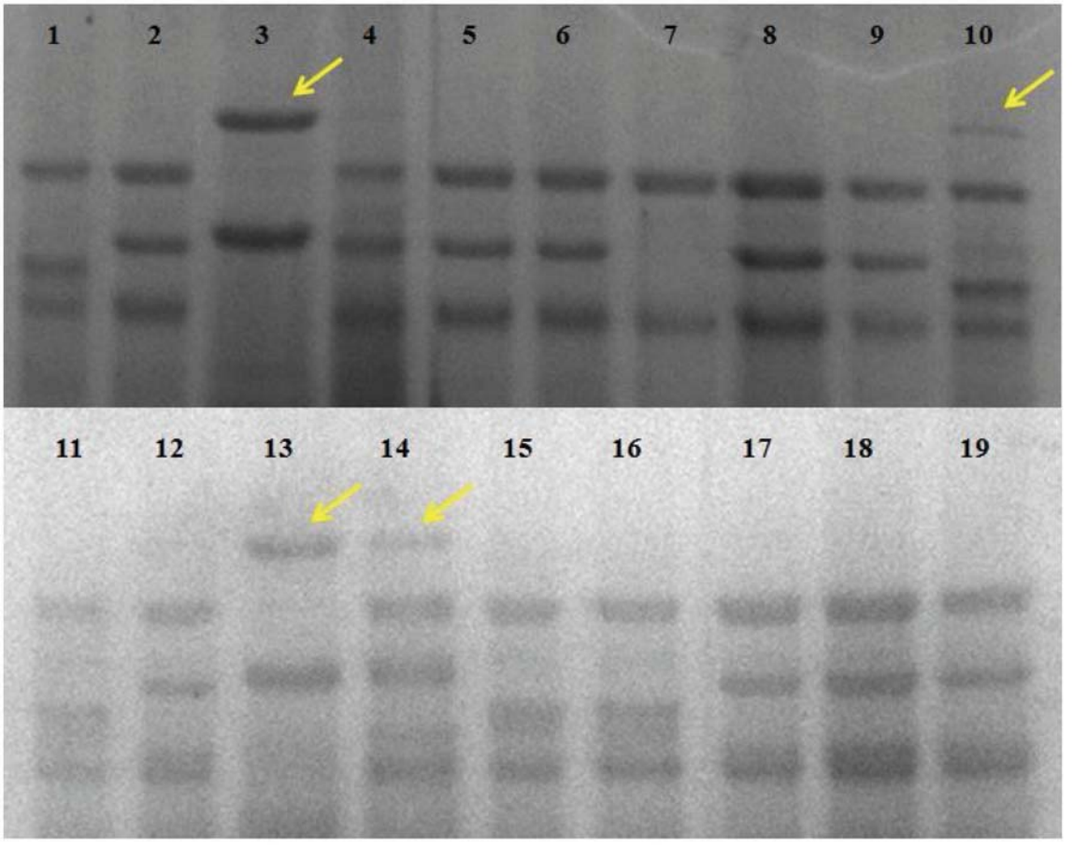

Fig. 5: HMW glutenin subunit profile of BC_1F_6 Generation
1. *T. aestivum* cv. WL711, 2. *T. aestivum* cv. HD2687, 3. *Ae. longissima* 3506, 4. 79-1-4-6-2-1-1, 5. 79-1-4-6-2-1-2, 6. 79-1-4-6-2-1-4, 7. 79-1-5-7-6-2, 8. 79-1-5-7-6-5, 9. 79-1-4-8-10-2-5, 10. 79-1-6-5-6-6-1, 11. *T. aestivum* cv. WL711, 12. *T. aestivum* cv. HD2687, 13 *Ae. longissima* 3506, 14. 79-1-6-5-6-6-3, 15. 79-2-4-5-2-6-2, 16. 79-2-4-5-2-6-4, 17. 79-2-4-8-5-2(bulk), 18. 79-1-5-5-1(bulk), 19. 79-1-5-5-1

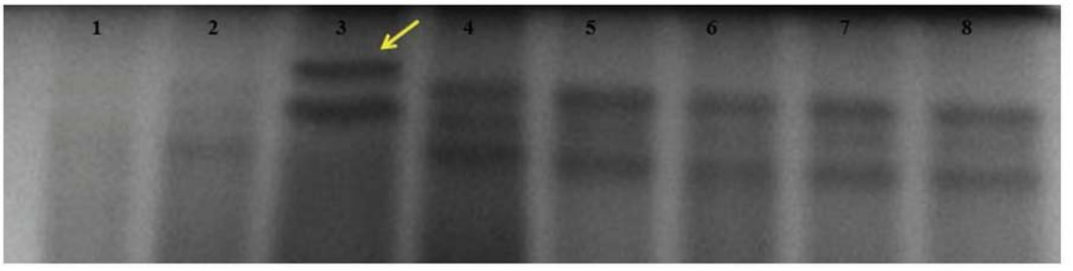

Fig. 6: HMW glutenin subunit profile of BC_2F_4 Generation
1. *T. aestivum* cv. WL711, 2. *T. aestivum* cv. HD2687, 3. *Ae. longissima* 3506, 4. HD2687/L3506//WL711-3///WL711-1-2-7-1, 5. HD2687/L3506//WL711-3///WL711-1-2-7-2, 6. HD2687/L3506//WL711-3///WL711-1-2-7-3, 7. HD2687/L3506//WL711-3///WL711-1-2-7-4, 8. HD2687/L3506//WL711-3///WL711-1-2-7-5

Micronutrient analysis

Comparative micronutrient analysis of Fe and Zn was done in straw, lower leaves and flag leaf of selected derivatives with wheat-Ae. *longissima* 3506 at maturity stage. Concentration of Fe in straw was found to be almost constant in all the derivatives with the variation in lower leaves and flag leaf. The concentration was found to be highest in lower leaves followed by flag leaf and straw in

11 selected derivatives whereas 8 derivatives showed higher Fe in flag leaf followed by lower leaf and straw. Concentration in straw, lower leaves and flag leaf ranged from 22-88 mg/kg, 104-563.0 mg/kg and 91-281 mg/kg respectively (Table 6 and Fig. 7).

Concentration of Zn in straw was found to be highest in most of the selected derivatives. The pattern revealed highest Zn concentration in straw followed by lower leaves and flag leaf in almost all the derivatives. Concentration in straw, lower leaves and flag leaf ranged from 3-38 mg/kg, 4.0-14 mg/kg and 4.0-17 mg/kg respectively (Table 7 and Fig. 8).

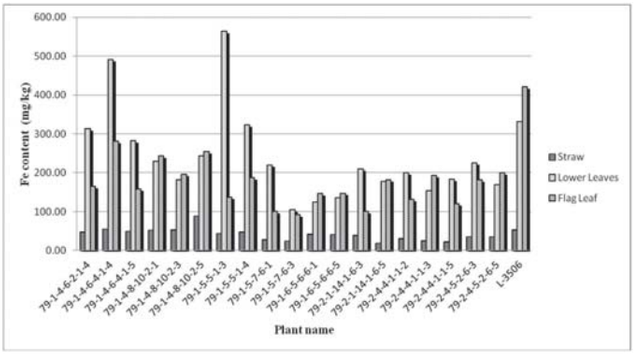

Fig. 7: Iron content in mg/kg in straw, flag leaf and lower leaves of wheat-Ae. *longissima* derivatives/ wild relatives

Table 6: Comparative analysis of Fe content in mg/kg in straw, lower leaves and flag leaf of wheat-*Ae. longissima* by AAS reading at maturity

Derivative	Straw Fe(mg/kg) ± RSD	Lower leaves Fe(mg/kg) ± RSD	Flag leaf Fe(mg/kg) ± RSD
79-1-4-6-2-1-1	49.10 ± 0.8	404.11 ± 0.4	155.47 ± 0.9
79-1-4-6-2-1-4	47.61 ± 1.6	312.62 ± 3.0	164.79 ± 0.3
79-1-4-6-4-1-4	54.40 ± 2.3	490.91 ± 1.4	280.51 ± 0.7
79-1-4-6-4-1-5	47.92 ± 2.5	281.93 ± 1.8	157.95 ± 1.4
79-1-4-8-10-2-1	50.90 ± 0.9	228.57 ± 0.6	242.86 ± 1. 6
79-1-4-8-10-2-3	53.28 ± 1.4	181.88 ± 1.2	196.10 ± 0.8
79-1-4-8-10-2-5	87.63 ± 0.12	242.57 ±0.15	254.05 ± 0.16
79-1-5-5-1-3	43.45 ± 0.3	562.98 ± 1.5	137.13 ± 1.3
79-1-5-5-1-4	47.10 ± 4.2	322.74 ± 1.9	186.78 ± 1.4
79-1-5-7-6-1	27.53 ± 1.1	219.90 ± 0.7	99.80 ± 2.1
79-1-5-7-6-3	23.18 ± 0.7	104.64 ± 0.8	91.33 ± 2.8
79-1-6-5-6-6-1	41.94 ± 2.6	123.49 ± 0.4	146.02 ± 1.1
79-1-6-5-6-6-5	40.24 ± 1.9	135.46 ± 1.4	147.21 ± 1.6
79-2-1-14-1-6-3	38.15 ± 1.3	209.45 ± 1.4	100.00 ± 1.0
79-2-1-14-1-6-5	17.40 ± 1.5	177.07 ± 0.9	181.63 ± 1.9
79-2-4-4-1-1-2	30.14 ± 1.0	199.70 ± 2.3	131.45 ± 1.1
79-2-4-4-1-1-3	24.85 ± 0.6	153.63 ± 1.0	192.86 ± 0.7
79-2-4-4-1-1-5	22.49 ± 0.07	183.26 ± 0.20	120.24 ± 0.16
79-2-4-5-2-6-5	34.29 ± 0.9	168.69 ± 0.7	199.50 ± 1.5
79-2-4-5-2-6-3	34.43 ± 0.7	225.30 ± 0.4	181.15 ± 1.5
Ae. longissima 3506	53.02 ± 0.6	331.00 ± 0.4	420.42 ± 0.3

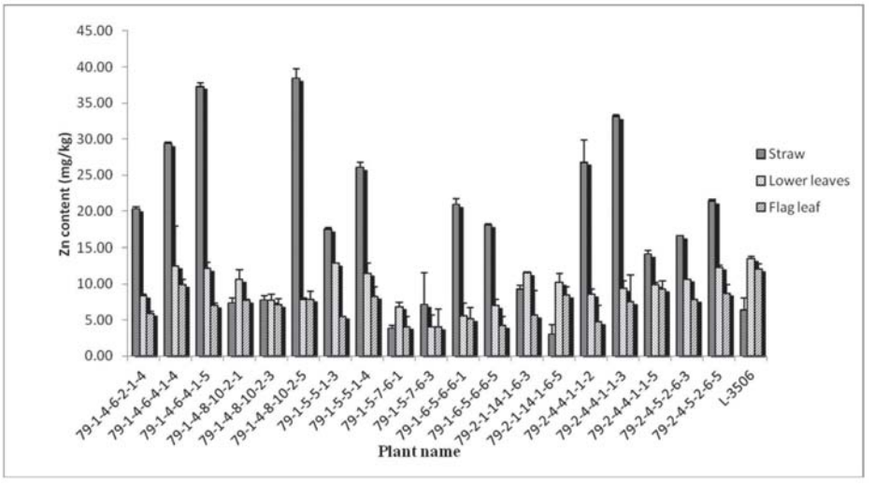

Fig. 8: Zinc content in mg/kg in straw, flag leaf and lower leaves of wheat-Ae. *longissima* derivatives/wild relatives

Table 7: Comparative analysis of zinc content in mg/kg in straw, lower leaves and flag leaf of wheat-*Ae. longissima* by AAS reading at maturity

Derivative	Straw Fe(mg/kg) ± RSD	Lower leaves Fe(mg/kg) ± RSD	Flag leaf Fe(mg/kg) ± RSD
79-1-4-6-2-1-1	21.17 ±0.3	9.15 ± 0.1	16.96 ± 0.3
79-1-4-6-2-1-4	20.31 ± 0.2	8.42 ± 5.4	5.93 ± 0.7
79-1-4-6-4-1-4	29.35 ± 0.4	12.52 ± 0.9	9.93 ± 0.3
79-1-4-6-4-1-5	37.33 ± 0.7	12.13 ± 1.3	7.05 ± 0.1
79-1-4-8-10-2-1	7.33 ± 0.6	10.62 ±0.8	7.74 ± 0.8
79-1-4-8-10-2-3	7.79 ± 1.3	7.76 ± 0.2	7.13 ± 1.1
79-1-4-8-10-2-5	38.41 ± 0.21	7.85 ± 0.01	7.90 ± 0.01
79-1-5-5-1-3	17.52 ± 0.7	12.86 ± 1.4	5.53 ± 1.3
79-1-5-5-1-4	26.06 ± 0.4	11.49 ± 0.6	8.28 ± 1.4
79-1-5-7-6-1	3.83 ± 4.4	6.86 ± 1.7	4.10 ± 2.5
79-1-5-7-6-3	7.13 ± 0.8	4.01 ± 1.7	4.02 ± 1.5
79-1-6-5-6-6-1	20.92 ± 0.1	5.64 ± 0.9	5.19 ± 1.3
79-1-6-5-6-6-5	18.12 ± 0.5	6.99 ± 0.1	4.23 ± 3.4
79-2-1-14-1-6-3	9.28 ± 1.3	11.53 ± 1.2	5.66 ± 1.1
79-2-1-14-1-6-5	3.03 ± 3.0	10.20 ± 0.7	8.45 ± 2.3
79-2-4-4-1-1-2	26.81 ± 0.2	8.58 ± 1.1	4.75 ± 3.7
79-2-4-4-1-1-3	33.12 ± 0.5	9.35 ± 0.3	7.53 ± 1.1
79-2-4-4-1-1-5	14.09 ± 0.06	9.93 ± 0.04	9.29 ± 0.04
79-2-4-5-2-6-3	16.56 ± 1.6	10.68 ± 0.3	7.85 ± 0.7
79-2-4-5-2-6-5	21.40 ± 0.2	12.33 ± 0.3	8.73 ± 1.2
Ae. longissima 3506	6.44 ± 0.1	13.53 ± 0.9	12.07 ± 2.0

Ae. longissima 3506 micronutrient content was found to be 2.3 fold higher in Fe and 1.6 fold higher in Zn concentration than the recipient wheat cultivars. High variability for grain micronutrient in wild *Triticum* and *Aegilops* species was reported in recent studies (Rawat *et al*., 2009). In an another study Tiwari *et al*., (2008) analysed the grain ash for Fe and Zn content in the putative amphiploids between *Triticum turgidum* and *Ae. longissima* accessions having bolder seeds and found higher Fe and Zn content in grain ash, confirming the superior genetic system of *Ae. longissima* for micronutrients uptakes, transportation and sequestration. *Ae. longissima* (S^lS^l) having higher similarity with the B genome donor of hexaploid wheat, *Ae. speltoides* (SS) could be effectively utilized for transfer of QTL/ genes responsible for high grain micronutrient concentration to elite wheat cultivars (Dvörak and Zhang, 1990; Friebe *et al.*, 1996). In present study most of the selected wheat- *Ae. longissima* derivatives had higher grain Fe and Zn concentration than both the wheat and *Ae. longissima* parents. These enhanced micronutrient contents in derivatives might be due to introgression of group 2 and group 7 chromosomes of *Ae. longissima* which have been reported to carry the genes for enhanced grain micronutrients (Rawat *et al.*, 2009; Neelam *et al.*, 2013).

Ae. longissima had non-waxy leaves and leaf sheaths. Non-waxy trait is contributed by a dominant gene of group 2 chromosomes. All the wheat- *Ae. longissima* derivatives had non-waxy, leaves, leaf-sheaths and spikes trait indicating the introgression of $2S^l$ chromosome Group 2 chromosomes also contain genes involved in effective uptake, translocation and grain sequestration of the micronutrients as a consequence *Ae. longissima* with $2S^l$ exhibits higher micronutrient than wheat cultivar.Since earlier studies revealed the presence of two QTL exhibiting 12.6% and 11.7% of increase in grain Fe in diploid wheat RIL population (Tiwari *et al.*, 2008). Another study reported major QTL for grain Zn content (ìg/seeds) was found on chromosome 2 and 7 in a double haploid wheat population (Shi *et al.*, 2008). Major QTL was grain Zn concentration was also mapped on chromosome 4 and 7 (Genc *et al.*, 2009).

Peleg *et al.*, (2009) reported QTL on chromosomes 2 and 7 for grain Fe, Zn, Mn and Mg whereas for Cu and Ca, QTL were found on chromosome 2, 4 and 7 in a durum wheat x wild emmer wheat RIL population. Chromosomes 2H of barley (*Hordeum vulgare* L.) possess two co-located QTL for grain zinc (Lonergan *et al.*, 2009) which is syntenic to its wheat homoeologues (Cho *et al.*, 2006). All of the above findings strongly favour and support our results that the *Ae. longissima* $2S^l$ and $7S^l$ chromosomes possess orthologs for grain micronutrient concentrations.

The BC_1F_5 derivative 79-1-5-5-1 had brittle rachis and red grain colour as that of the *Ae. longissima* donor parent. Dominant gene on homoeologous group 3 of wheat are known to control red grain colour and rachis brittleness (Watanabe *et al.*, 2002), which may indicate the introgression of $3S^l$ from *Ae. longissima* in this derivative. The rachis brittleness of this derivative was the disarticulation of the entire spike above the basal spikelet as that of *Ae. longissima* indicating that the gene(s) for that type of rachis brittleness may be orthologous to the wedge type disarticulation of spikelet in wild *Triticum* species (Li and Gill, 2006; Endo and Gill, 1996). This derivative also had much higher grain micronutrient content the gene(s) for which may also be present on chromosome $3S^l$. The progeny of the derivative is true breeding for non-waxy leaves and brittle rachis controlled by $2S^l$ and $3S^l$,

respectively suggesting their disomic substitution/ addition. The presence of 43 chromosomes in this derivative indicates that there might be introgression of some other chromosome along with $2S^l$ and $3S^l$ which may be responsible for the high grain micronutrient content (Fig. 2h).

Additive action and differential expression of transporters and regulator genes of parental species in derivatives might lead to transgressive isolation of derivatives for the micronutrient concentration. We observed few derivatives had high micronutrient content but possess less number of seeds that might be due to concentration effect and chromosomal instability. Derivatives with 42 or 43 chromosomes were much similar to wheat morphologically and had bolder grains, high Zn and moderate Fe content with acceptable number of seeds/ spike (>30). These derivatives can be used for precise transfer of gene controlling HMW glutenin from *Ae. longissima* for further investigation and utilization in bread making quality (Garg *et al.*, 2007).

In the present study SDS-PAGE analysis of seed proteins of derivatives 79-1-6-5-6-6-1 and 79-1-6-5-6-6-3 exhibited distinct bands of HMW glutenin specific to the *Ae. longissima* 3506 donor indicating the introgression of chromosome $1S^l$ of *Ae. longissima* (Fig. 5). The genes for HMW glutenin subunits have been mapped on group 1 chromosomes of *Triticeae* (Stangoulis *et al.*, 2007).

Ae. longissima possess highest Fe content in flag leaf followed by lower leaves and straw indicating efficient working of transporters of Fe such as *IRT-1* and *-2, YSL-1*and *NRAMP 1-4*. Similarly the Zn content was highest in lower leaves followed by flag leaf and straw indicating proper functioning of Zn transporter such as *HMA-1, -4* and *-8, MTP-1, YSL-1* and *ZIP-4* (Colangelo and Guerinot, 2006). However, in the derivative plants distribution pattern revealed high Fe content in lower leaves and high Zn content in straw, thus indicated the restricted transportation of Fe and Zn from roots to seeds. The factors responsible for such regulation were under study.

The derivative 79-1-5-5 had high grain and lower leaves Fe as compared to straw and flag leaf. This might be further justified through differential expression analysis of Fe transporters spatially and temporally.

Considering the stability of chromosome, percentage increase in grain Fe and Zn and average number of seeds/ spike, four derivatives were selected from BC_1F_6 and three from BC_2F_4. Derivatives 79-1-4-8-10-2-5 and 79-2-4-4-1-1-3 had stable 42 chromosomes and stable chromosome pairing and high percentage increase in Zn (58.92% and 64.7%) and moderate percentage increase in Fe content (44.56% and 24.28%) respectively as compared to the parental wheat cultivars. The number of seeds/ spike was 38 and 32.6 respectively suggesting that the high grain micronutrient content in those derivatives was due to the superior genetic system of *Ae. longissima* instead of the concentration effect. In the derivative 79-1-4-8-10-2-2 with 42 chromosomes (20II, 2I) the GISH analysis revealed introgression of two *Ae. longissima* chromosomes. The grain micronutrient increase of Fe was 6.17% whereas of Zn it was 94.13% compared to the wheat parent and had 39.3 seeds/ spike. The derivative 79-2-4-4-1-1-5 also had high percentage increase in Zn (51.7%) with moderate increase in Fe (32.12%) with 39.9 seeds/ spike.

The BC_2F_4 derivative HD2687/L3506//WL711-3///WL711-1-2-7 had high Fe and Zn with high harvest index and stable 44 chromosome. Most of the transporters present in these derivatives were derived from introgression of *Ae. longissima* chromosome 7, since transporters play significant role in uptake, distribution and accumulation of micronutrients.

Conclusion

In the present study ten BC_1F_6 along with BC_2F_4 derivatives were selected and sown in the fields of Eternal University, Baru Sahib and were analyzed for their potential as biofortified breeding material having stable chromosomes with wild *Ae. longissima* chromosome introgression. After morphological, cytological and micronutrient data analysis most of the derivatives showed the stable 42, 44 and 46 chromosomes where single plant reported for each 41, 43 and 45 chromosomes with 19-22 bivalents and few trivalent. GISH analysis of the derivative 79-1-4-8-1-2-2 revealed introgression of two univalent S^l chromosome.All the selected derivative plants had non-waxy leaves and leaf sheaths indicating introgression of $2S^l$ chromosome of *Ae. longissima.* All the plants showed amber coloured seeds except *Ae. longissima* and BC_1F_5 derivative 79-1-5-5-1 exhibiting brittle rachis and red coloured seeds. Plants 79-1-6-5-6-6-1, 79-1-6-5-6-6-3 showed the two distinct *Ae. longissima* specific bands 1*Ax* and 1*Ay* of HMW-GS which were absent in wheat cultivars HD2687 and WL711. Micronutrient analysis of seed, straw, lower leaves and flag leaf of all the derivatives and parents revealed moderate

to high grain Zn content and low to high Fe content. Finally four derivatives were selected from BC_1F_6 generation 79-1-4-8-10-2-2, 79-1-4-8-10-2-5, 79-2-4-4-1-1-3 and 79-2-4-4-1-1-5 and three from BC_2F_4 generation(HD2687/L3506//WL711-3///WL711-1-2-7-1, HD2687/L3506//WL711-3///WL711-1-2-7-3 and HD2687/L3506//WL711-3///WL711-1-2-7-5 based on high micronutrient content, more wheat like morphological feature, stable chromosomes with *Ae. longissima* introgression. These selected derivatives will be used as high Fe and Zn containing germplasm for future breeding program and biofortification.

Acknowledgement

The authors would like to acknowledge the DBT, Government of India for providing research grant under DBT project. We would also like to acknowledge ASB, Eternal University for providing instrumentation facility and infrastructure.

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