INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 10, No. 1, p. 100-108, 2017

RESEARCH PAPER OPEN ACCESS

Exploring novel diversity for biofortification in Elite D-genome synthetic hexaploid wheat (AABBDD)

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Key words: Micronutrients, Hidden hunger, SHWs, Elite D-genome, Bio-fortification

http://dx.doi.org/10.12692/ijb/10.1.100-108 Article published on January 15, 2017

Abstract

Micronutrient deficiencies otherwise termed as hidden hunger, are serious health concern for more than 2 billion people worldwide including developing and under developed countries. Wheat is an important staple crop because it is a major source of dietary energy and protein for more than one third of world population. Thus, biofortification of wheat can play a vital role to overcome hidden hunger in the countries where wheat is the foremost source of protein and nutrients. We examined 128 Elite D-genome synthetic hexaploid wheats (SHWs) to explore new genetic and phenotypic variability that may be exploited for biofortification of wheat. Grain iron (Fe) ranged between 7.45-70.33 mg kg-1 with an average of 29.56 mg kg-1and grain zinc (Zn) ranged between 5.32-171.38 mg kg-1 with an average of 43.87 mg kg-1. Some accessions (68.111/RGB-U//WARD/3/*Ae. tauschii* (326), DVERD_2/*Ae. tauschii* (221), GAN/*Ae. tauschii* (897) showed exceptionally high iron and zinc contents while maintaining thousand grain weight and number of spikelet per spike. In the study, the effect of the tauschii parent as well as the durum parent of the alleles was observed on the phenotypic traits and on Fe and Zn. These varieties can be used in future for wheat bio-fortification breeding program.

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Introduction

Micronutrient deficiencies, especially zinc (Zn) and iron (Fe), lead to serious health problems for more than 3 billion people worldwide (Velu *et al.,* 2011). Almost 160 million children under the age of 5 are lacking essential proteins, which lead to an economic burden for society (Uauy C. *et al.,* 2006a). Current estimates show that malnutrition results in 3 million deaths per year, among children in developing countries. Wheat is the major staple food crop in many parts of the world, contributing 28% of the world edible dry matter production and up to 60% of daily energy intake in several developing countries and a good source of micronutrients(Cakmak Ismail 2008). Wheat is a good source of micronutrients (including Iron, Zinc, and vitamin A). Increasing world population and deteriorating land resources have made International wheat breeders to intensify wheat yield, but these efforts have seriously hampered its quality including micronutrients.For that reason, developing genetically micronutrient enriched cereals and refining their bioavailability (biofortification) by using under-utilized primary gene poolconsidered promising and cost-effective approaches for lessening malnutrition (Xu *et al.,* 2012). Micronutrient enrichment traits are present in the genomes of major staple crops that could allow substantial increases in Iron, Zinc, and pro-vitamin A carotenoids without negatively impacting the yield (Welch and Graham 2002).

Genetic changeability is enough for the progress of wheat varieties along with much higher Zn, Fe concentration in cereals. The most sustainable approach, cheaper and more feasible means is biofortification through the blend of molecular and conventional assortment. The significant positive correlation among Fe and Zn concentrations shows that both these traits can be bred simultaneously (Monasterio and Graham 2000). Inbread wheat, Fe and Zn concentration ranges between 26-41 mg kg-1 and 19-60 mg kg-1, respectively (Paltridge *et al.,* 2012). Synthetic hexaploid wheats (AABBDD *T. turgidum* x *Ae. tauschii*) had 25 to 30% more Fe, and Zn concentrations in their grains than common wheat cultivars (Calderini and Ortiz-Monasterio 2003) and significantly higher phytase contents which assist bioavailability of micronutrients (Ram *et al.,* 2010). Narrow genetic background due to continous selection in bread wheat has deteriorated the genetic diversity in bread wheat. Synthetic Hexaploid wheat is a dynamic tool to enhance genetic diversity in primary gene pool, ensuring easy use to allelic variations in new breeding programs. Very rare information is available on biofortification potential of a wide range of available SHWs and a complete lack of information about genomic regions prevailing micronutrients in SHWs.

Grain protein content (GPC) is an important factor in the production of quality bread and pasta and in the human diet. It is also an important feature for wheat producers because higher prices are often paid for wheat having high GPC. A promising source of alleles increased GPC was identified on chromosome 6B *Triticum turgidum* var. *dicoccoides* accession FA-15-3 (DIC). Two quantitative trait loci (QTL) stated above, that the positive effect of DIC-6B was connected to a single locus between the centromere and NorB2 locus on the short arm of chromosome 6B (Olmos *et al.,* 2003). The high-yielding varieties from CIMMYT breeding program derived from SHWs contains preferred processing quality traits and 10–90% higher grain micronutrient content than the famous commercial varieties(Guzmán *et al.,* 2014). Therefore, the objectives of the present study were to (1) identify the morphological diversity of micronutrients among Elite varieties of SHWs. (2) increase in production of the wheat varieties with improved micronutrient content so the formation can be used by the breeders all over the world for the biofortification of wheat, and (3) Biochemical and Molecular characterization of Fe, Zn trait in synthetic Hexaploid wheat.

Materials and methods

Plant material and phenotyping

The present study was conducted to calculate Iron (Fe) and Zinc (Zn) concentrations in Synthetic Hexaploid wheat grains. Total 95 lines of Elite-I (consists of 95 primary synthetic Hexaploid Wheat varieties derivatives of cross combinations of 34

durum wheat and 74 *Ae. tauschii* accessions) and 33 varieties of Elite-II (consist of 33 primary synthetic hexaploid wheat varieties. The synthetic hexaploid are derived from a combination of 14 durum wheat and 32 *Aegilops tauschii* accessions) synthetic hexaploid wheat grains cultivated in Pakistan were found from National Agriculture Research Centre (NARC), Islamabad (Wheat Wide Crosses and Cytogenetic Lab). Elite 1 was amongst earlier subsets having 95 lines derived from first 450 synthetics produced in CIMMYT (Mujeeb-Kaz,i 2003).

The Fe and Zn concentrations of grain were calculated according to the standard set protocols of (Zarcinas *et al.,* 1987). There were no particular permits required for the described field trails. The field trials were performed by applying alpha lattice design consisting of five replications with plot size of 1 x 2 m2. Soil was collected from four random sites of field and soil parameters were recorded before planting site with Mean value EC (dS m-1) 0.30, pH 7.8, Organic matter (%) 0.70, Available-P (mg kg-1) 4.5, Available-K (mg kg-1) 130, textural class loam 13, Zinc concentration 0.32mg/kg.

The concentration of Fe and Zn were calculated from digested sample of grains with spectrophotometer in 2014 and the phenotypic data for agronomic traits were calculated from cropping season in 2013-2014 at National Agriculture Research Centre, Islamabad, Pakistan which is situated between 33.7167° N, 73.0667° E in arid zone of Pakistan and majorly depends on rainfall. The total rainfall during the planting season 2013-14 was recorded 490 mm. The phenotypic traits evaluated in this study included yield parameters which were Plant height, tiller number, spike length, number of spikelet/spike, and number of spike, 1000 grain weight, and heading days.

Molecular analysis

The plant material was used for molecular evaluations for evaluating their DNA based diversity. This was done by using two primers (Table 1) according to the given protocol.

Genomic DNA extraction

Genomic DNA was extracted from young leaf tissues where 3 to 4 fresh leaves were collected, frozen in liquid nitrogen and grinded in that liquid nitrogen to obtain an approximately 0.5g powder. This powder was then transferred to 1.5ml Eppendorf tubes. 600 µl pre-warmed DNA extraction buffer was added to each Eppendorf tube that contained the crushed leaf material and was mixed well. Samples were incubated at 65°C in the water bath for 30 minutes. 600µl of chloroform: Isoamyl alcohol (24:1 v/v) mixture was added and to form homogenous mixture tubes were vortex. Samples were centrifuged for 15 min at 5000 rpm. The supernatant was transferred to a new Eppendorf tube. For the precipitation of DNA, 1000 µl cold isopropanol was added to the tube and tubes were gently inverted for mixing to suspend the DNA. The incubation of one hour was given to the samples at -20°Cfor good suspension. Samples were centrifuged at 10,000 rpm for 5 minutes to make the DNA pellet. The supernatant was decanted and the pellet was washed with 70% ethanol. Pellets were dried at room temperature for an hour. To remove RNA, DNA was treated with 40µg RNAse-A (20 µl of commercially supplied RNAse-A) at 37°Cfor 1 hour.

PCR conditions and gel electrophoresis

PCR reactions were carried out in 25 µl reaction. The primers were extended by PCR with *Taq*. polymerase using the following amplification conditions: 95°C for 5 min; 95° C for 1 min; 63-58°C for 1 min; 72°C for 45 seconds and final extension for 5 min at 72°C. 0.8% agarose/TBE. Gels were used for the electrophoresis of the products, and visualized with ethidium bromide under UV light chamber and observed by means of the computer program UVI Photo MW.

Statistical analysis

All the statistical investigations were done using XLSTAT 2014 statistical software. The Analysis of Variance (ANOVA) was used to analyze the variations in group means and their related procedures. Pearson's correlation was used to analyze the association between all the parameters that were studied.

Results

Phenotypic Variations

Significant variations were observed for all the traits studied including number of spike, grains per spike, plant height, Fe and Zn concentration in SHWs (Table 2).Grain iron (Fe) ranged between 7.45-70.33 mg kg-1 with an average of 29.56 mg kg-1and grain zinc (Zn) ranged between 5.32-171.38 mg kg-1 with an average of 43.87 mg kg-1.Highest Fe content was found in Elite-I DVERD_2/*Ae. tauschii* (221) which was 42.2 mg/kg. Highest Zn content was found in Elite-I 68.111/RGB-U//WARD/3/*Ae. tauschii* (326) which was 102.83 mg/kg.Some accessions (68.111/RGB-U//WARD/3/*Ae. tauschii* (326), DVERD_2/*Ae. tauschii* (221), GAN/*Ae. tauschii* (897) showed exceptionally high iron and zinc contents while maintaining thousand grain weight and number of spikelet per spike.

Table 1. Primers designed for Nac gene along with their sequence, fragment size, position at allele and chromosome.

Marker	Primer sequence (5'-3')	Allele	Expected Fragment Size	Chromosome	Reference
Xucw108	Forward: AGCCAGGGATAGAGGAGGAA Reverse: AGCTGTGAGCTGGTGTCCTT	*Gpc-B1* and *Yr36*	217	6BS	Uauy *et al.*, 2006
Xuhw89	UHW89-BF: TCTCCAAGAGGGGAGAGACA UHW89-R: TTCCTCTACCCATGAATCTAGCA	*Gpc-B1* and *Yr36*	126	6BS	Distelfeld *et al.*, 2006

Table 2. Descriptive statistical analysis of the data regarding phenotypic traits of elite D-genome synthetic hexaploid wheat with respect to durum parents and tauschii parents.

Variables	No. of observations	Minimum	Maximum	Mean	Std. deviation
Fe (mg/kg)	128	7.45	70.33	29.56	14.906
Zn (mg/kg)	128	5.32	171.38	43.87	39.712
Spike length (cm)	128	7.800	17.60	11.58	1.498
No. of spikelet/spike	128	10.40	21.00	17.53	1.780
Plant height (cm)	128	50.00	123.0	85.09	13.431
No. of tillers	128	2.000	42.00	17.30	8.847
Days to 50% heading	128	134.0	171.0	153.1	6.972
Thousand Kernel Weight (TKW)	128	16.60	51.10	32.91	7.706

According to the Correlation matrix (Fig. 1), the Fe has significantly positive correlation with Zn (r=0.20). Fe has a significant correlation with thousand kernel weight (TKW) (r=0.19) having no correlation with spike length (r=-0.094). Zn has a positive correlation with No. of Spikelet/spike (SP/S) (r=0.275) and has a significant correlation with spike length (SL) (r=0.06) and have no correlation with TKW (r=-0.04).

Spike length has significantly positive correlation with No. of the spikelet (r=0.19) and has a significant correlation with Zn (r=0.06) and negative correlation with Days to 50% heading (r=-0.30). No. of Spikelet has a significant correlation with No. of Tillers (T No.) (r=0.17) and has no correlation with Days to 50% heading (DH) (r=-0.011). Plant height (PH) has a positive correlation with No. of tillers (r=0.43) having no correlation with Days to 50% heading and TKW (r=-0.34) (r=-0.084) respectively. No. of Tillers has a significant correlation with TKW (r=0.16) and no correlation with Days to 50% heading (r=-0.41).

Days to 50% heading has a negative correlation with TKW (r=-0.11).

Molecular Validation

We selected molecular markers Xuhw89 and Xucw108 and amplified at different gradients.

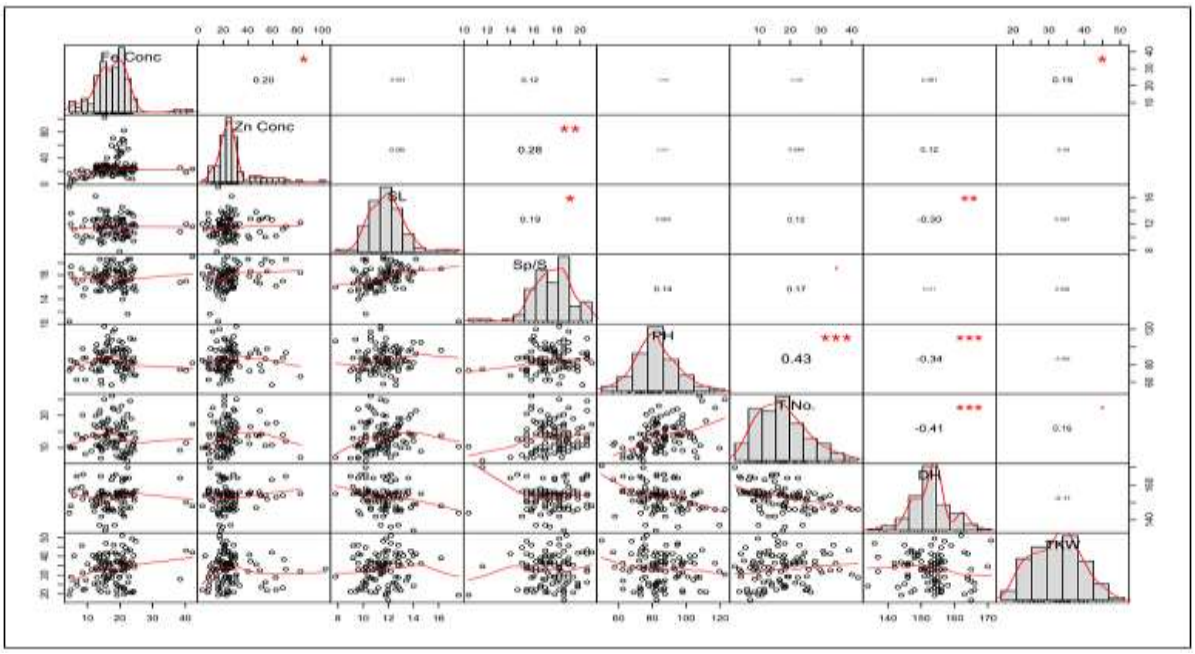

Fig. 1. Correlation Matrix representing correlation among Iron and zinc concentration, spike length, No. of spikelet per spike, Plant height, No. of tillers, Days to heading and Thousand kernel weight.

The ancestral wild wheat allele encodes a NAC transcription factor (NAM-B1) that accelerates senescence and increases nutrient remobilization from leaves to developing grains, whereas modern wheat varieties carry a nonfunctional NAM B1 allele.

The primers Xucw108 and Xuhw89 in Elite 1 and Elite 2 showed no results that revealed the lack of NAC domain (TtNAM-B1 allele) as the primers were genome specific and designed on TtNAM-B1 sequence. Whereas, it showed results in other bread wheat varieties showing 2000-2500bp bands (Fig 2 & 3).

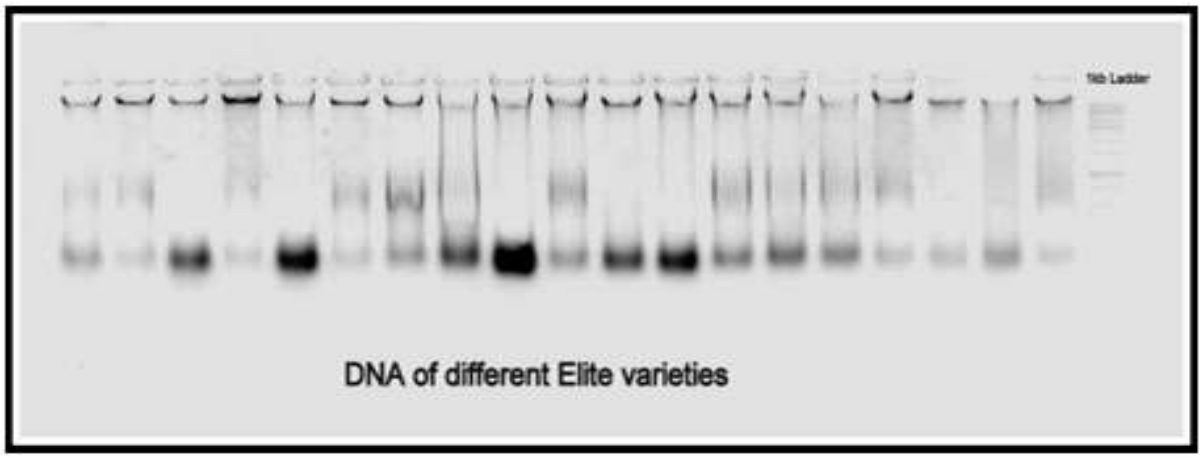

Fig. 2. DNA extracted from young leaf tissues.

Discussion

Iron and Zinc Concentration

Average micronutrient concentration reported by (Zhang Huakun *et al.,* 2014) in bread wheat (Fe=48.80±11.26 and Zn=30.4± 21 mg/kg) were three-fold more than wild relative and their synthetics (Fe=91.59±37 and Zn=70.88±29.40 mg/kg).

This showed high variability for Fe and Zn trait. (Velu *et al.,* 2011) reported the concentrations of Fe and Zn as 23-52mg/kg and 19-52 mg/kg respectively examined in the year 2008-09. While in the investigation of 2010-11 Fe and Zn concentrations were reported as 27-43 mg/kg and 15-51 mg/kg respectively.

According to (Guzmán *et al.,* 2014) the mean grain Fe and Zn concentration in the varieties was 29.4 mg/kg and 21.7 mg/kg, respectively. According to (Zhang Yong *et al.,* 2010), the genotypes showed almost the same mean value and range of Fe and Zn concentration, compared with the report of (Yong *et al.,* 2007), ranging from 28.0 to 60.2 mg/kg for Fe with mean of 39.2 mg/kg, and from 21.4 to 58.2 mg/kg for Zn with mean of 32.3 mg/kg.

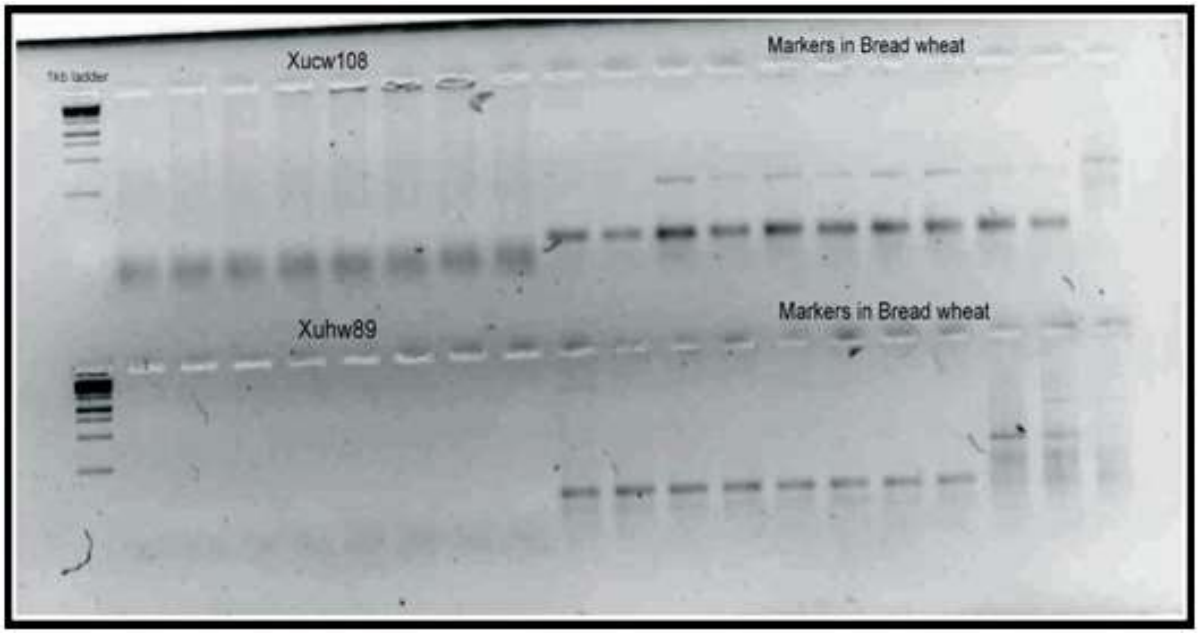

Fig. 3. The primers Xucw108 and Xuhw89 showing no results in Elite 1 and Elite 2 SHW that reveal the lack of NAC domain (TtNAM-B1 allele).

According to the statistical analysis of different morphological and biochemical parameters, positive correlation was found between Fe and Zn (r-0.416 p<0.01) (Velu *et al.,* 2011) while in our findings the correlation between Fe and Zn was significant (r=0.231).

The positive significant correlation was found between Fe and Zn across the groups (r = 0.37; P < 0.01) reported by (Guzmán *et al.,* 2014). Fe has a significant correlation with TKW (r=0.170) while Zn has a negative correlation with TKW (r=-0.057). The difference between all the Fe varieties was found significant (p=0.464) according to ANOVA with respect to tauschii parent and was found least significant (p=0.794) according to ANOVA with respect to durum parent. The difference between all the varieties of Zn was found highly significant (p=0.0000) according to ANOVA with respect to tauschii parents and was found also highly significant (p=0.038) with respect to durum parents.

We have found the effect of both tauschii parents as well as durum parents on these lines. Micronutrients concentrations have been reported to have significant negative impact on yield (Zhang Yong *et al.,* 2010); (Karami *et al.,* 2009) of bread wheat cultivars, whereas in synthetic bread wheat iron had significant positive correlation with thousand kernel weight as compare to Zn concentration, which had negative correlation with thousand kernel weight. It was concluded that higher grain Zn and Fe concentrations are not necessarily related to small grain size.

Plant Height

Synthetics are usually tall and the range is almost around 85 cm to a limit of 140 cm (anonymous). In our findings, the minimum plant height for all the lines was 50cm and the maximum was 123cm and mean plant height was 85cm. the data regarding days to 50% heading can vary because of the variations in the dates of planting.

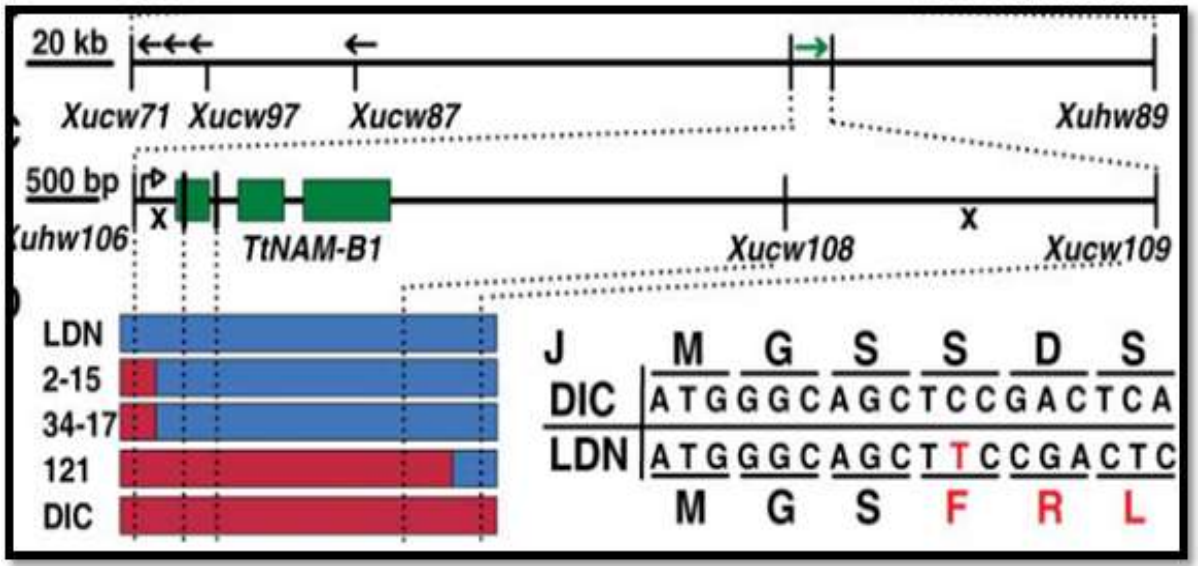

Fig. 4. First 18 nucleotides of DIC and LDN Tt NAM-B1 alleles and their corresponding amino acid translation.

Thousand Kernel Weight

Thousand kernel weights of all varieties ranged from 16.6 to 51.1g with the mean of 32.9 g. According to (Zhang Yong *et al.,* 2010), the TKW ranged from 28.0 to 48.7 g. The entries that were having 1000 grain weight of more than 45 g are considered to be healthy and performing well i.e. SCA/*Ae. tauschii* (518), YAV_2/TEZ//*Ae. tauschii* (895), STY-US/CELTA//PALS/3/SRN_5/4/*Ae. tauschii* (431) and GREEN/*Ae. tauschii* (458).

Nac Transcription Factor

A QTL for GPC was mapped on chromosome arm 6B. Olmos *et al.*, 2003 mapped this QTL as a simple Mendelian locus, Gpc-B1. Molecular markers within this region flank a 245-kb physical contig, including Gpc-B1. We selected molecular markers Xuhw89 and Xucw108 and amplified at different gradients. The ancestral wild wheat allele encodes a NAC transcription factor (NAM-B1) that accelerates senescence and increases nutrient remobilization from leaves to developing grains, whereas modern wheat varieties carry a nonfunctional NAM B1 allele. Figure 4 is representing first 18 nucleotides of DIC and LDN TtNAM-B1 alleles and their corresponding amino acid translation. The LDN allele carries a 1-bp insertion (red T) that disrupts the reading frame (indicated by red amino acid residues) (Uauy C. *et al.,* 2006a).

Elite 1 and Elite 2 show no results that reveal the lack of NAC domain (TtNAM-B1 allele). As Primers were genome specific and designed on TtNAM-B1 sequence. The sequence has one bp substitution within first intron at position 11. Mutation at this position will result in the lack of amplification. TtNAM-B1 allele was found in 42 wild emmer and 17 domesticated emmer lines and no results in 57 cultivated durum lines which suggest frame shift insertion of 1bp during the domestication of durum wheat (Uauy Cristobal *et al.,* 2006b).

This study strongly supports that viable iron and zinc bio fortified varieties can be developed and this is need of an hour. Improved Fe and Zinc contents of wheat could make an important contribution towards the reduction of malnutrition and health issues which effects 25% world population (Borrill *et al.,* 2014).

Conclusion

In the study, the effect of the tauschii parents as well as the durum parents of the alleles was observed on the phenotypic traits and on Iron (Fe) and Zinc (Zn). These varieties can be used in future for wheat biofortification breeding program to increase the yield of Elite synthetic Hexaploid wheat. The competitive Zn and Fe biofortified varieties can be developed. If it becomes successful, then this would affectedly contribute to improve the health and livelihood of several micronutrient-deficient people in many developing countries.

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