Morphological analysis and screening of wheat generations derived from HD2967 × Kharchia65 for salt tolerance

Varsha¹, S. Yashveer^{1*}, V. Singh² and P. Kumar²

¹Department of Molecular Biology, Biotechnology and Bioinformatics, CCS Haryana Agricultural University, Hisar-125 004, India

²Department of Genetics and Plant Breeding, COA, CCS Haryana Agricultural University, Hisar-125 004, India

*Corresponding Author Email : shikhayashveer@gmail.com

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Abstract

Aim: To screen wheat generations derived from cross HD2967 × Kharchia65 for two *Nax* loci imparting salt tolerance and background selection of the plants using polymorphic SSRs.

Methodology: The study for salt tolerance was carried out on three generations, i.e., BC_1F_3 , BC_2F_2 and F_4 , derived from the cross HD2967 × Kharchia65 in net house. Salt stress was provided at germination stage and the plants were grown to maturity. Data was recorded for various agro-morphological traits which contributed to yield. DNA isolated from young leaves of morphologically superior plants were checked for the presence of *Nax1* and *Nax2* genes using gene specific primers. Plants having either or both *Nax* loci were then subjected to polymorphic SSR markers screening for background selection of foreground selected plants.

Results: On the basis of agro-morphological performance and presence of either or both *Nax* genes, 68 high yielding plants were selected. Out of total 178 SSR markers screened covering the whole genome uniformly (A, B and D), 31 markers were polymorphic for the parents HD2967 and Kharchia65. These polymorphic SSR markers were used to produce molecular diversity among the selected progeny plants. Cluster analysis of parents and all the three generations, showed that all the selected plants were inclined towards recurrent parent.

Interpretation: This study showed that a linked marker like *Nax1* and *Nax2* could be a promising tool for breeding wheat with enhanced tolerance to salinity conditions. However, growth rates and biomass production provide reliable criteria for assessing the degree of salt stress and the ability of a plant to withstand it. Therefore, initial screening of seeds in the presence of salt stress provides additional advantage in directional selection. Plants selected with *Nax* loci, better mean performances, high heritability, and high genetic advance as 5% of mean for the studied traits could be further backcrossed with the recurrent parent to develop salt tolerant wheat lines.

Key words: Na⁺ exclusion, *Nax* genes, Salinity, Wheat

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Introduction

Globally, wheat (*Triticum aestivum* L. em. Thell) is an important staple food crop and has been cultivated in major civilizations of Europe, West Asia, and North Africa. Today, wheat is grown on more land area than any other commercial crop, covering about 219 million ha with annual production of 760.3 million tons in 2017-18 (FAOSTAT, 2018). It continues to be the most important food grain source contributing about a fifth of total calories consumed by humans. Crop production has been threatened by changes in climate for the last few decades (Ahmad et al., 2010; Ahmad, 2012). The production of wheat has unveiled a significant reduction due to soil salinization year by year (Oyiga et al., 2016). Application of poor quality irrigation water and shallow saline groundwater contributes to the salinization of upper soil. These particular processes contributing to salinization, combined with the influence of other climatic and landscape features and the effects of human activities determines the accumulation of salt in the landscape (Rengasamy, 2010). Salinity adversely affects the cultivated land area and the salinized area is increasing because of unreasonable irrigation, unsustainable cultivation and climate change (Qadir et al., 2014; Jayakannan et al., 2015). Response of wheat plant to soil salinity is complex and depends on several factors like growth stage, concentration of salts in soil and type of salts (Khan et al., 2010).

Currently, two methods are being followed for managing the problem of salinity; first is transforming soil with chemical or physical methods and secondly cultivation of salt-tolerant varieties developed through biotechnology. The erstwhile is costly and raise the secondary salinization of soil and also adds up chemical substances. Therefore, cultivating salt-tolerant crop varieties is recommended. Attempts to improve salt tolerance of crops through traditional breeding programs have achieved little success due to genetic and physiological complexity of the trait. DNA markers are highly effective in identifying genes and selecting multi-genic traits and genes which are mainly influenced by environmental conditions. SSRs are being used for investigation, genetic divergence, genome and QTL mapping for salt tolerance in different crops (Liu et al., 2001; Munns, 2002; Ma et al., 2007; Kurup et al., 2009).

A locus named, *Kna1*, for controlling K^+/Na^+ discrimination in wheat has been identified on chromosome 4DL (Gorham et al., 1987; Shah et al., 1987; Dubcovsky et al., 1996; Gao et al., 2001). *Nax1* and *Nax2* are two genetic loci that control the removal of Na^+ from the xylem and thereby help to exclude Na^+ from leaves of plants in saline soil. *Nax1* removes Na^+ from the xylem in roots and lower parts of leaves, leaf sheaths, and is found to be located on chromosome 2A (Lindsay et al., 2004), while *Nax2* is located on chromosome 5A (Byrt et al., 2007) and removes Na^+ from the xylem, only in the roots (James et al., 2006). Since *Nax* genes are not present in modern wheat (Huang et al., 2006), molecular markers linked with *Nax1* and *Nax2* genes can be employed as an effective screening tool to overcome the salinity problem which limits the wheat production. Yadav et al. (2017) confirmed and

validated the presence of *Nax1* and *Nax2* loci in Kharchia65 by using linked SSR markers. The present study therefore aimed at introgression of the genes for salt tolerance in HD2967 from Kharchia65 using conventional breeding methods and then screening the plants with linked markers for confirming the presence of introgressed genes. BC_1F_3 , BC_2F_2 and F_4 generations were selected using a combination of morphological parameters and molecular markers.

Materials and Methods

Raising of cross generations: A cross between well-known cultivar, HD2967 with good agronomic traits but susceptible to salinity and low yielding, otherwise tolerant to salinity and drought, Kharchia 65 was during 2012-13 and F_1 seeds were evaluated in the next season for phenotypic and molecular traits. Plants from F_1 progeny screened for *Nax* loci, using linked markers available plants with either both or any of *Nax* loci in combination with good yield under salt-stress conditions, were progressed to the next generation. Backcrossing of F_1 and elite parent (HD2967) was performed to obtain the BC_1F_1 and continuous selfing of F_1 and BC_1F_1 led to F_4 and BC_1F_3 generations, respectively. Second backcross of BC_1F_1 with the recurrent parent (2014-15) was made to obtain BC_2F_1 generation and then further selfing gave BC_2F_2 generation. Every year phenotypically superior plants were subjected to marker-assisted selection to obtain the next generations. The present study comprised of BC_1F_3 , BC_2F_2 and F_4 seeds of the cross.

The parental genotypes, backcrosses and F_4 generations were raised during Rabi 2016-17 in trays in the net house of the Department of Molecular Biology, Biotechnology and Bioinformatics, CCS Haryana Agricultural University, Hisar, India. The seeds were subjected to chloride dominated salt stress ($EC \sim 8 \text{ ds m}^{-1}$) at the time of germination. The seeds which germinated under salt stress were transplanted to pots with normal soil, i.e., deprived of salinity stress. Before transplanting, the original soil of the seedlings was neutralized by washing them in water and the plants growing in pots were given Hoagland nutrient solution every 10 days from the sowing date. The seedlings were grown to maturity and data was collected on various morphological traits like plant height, number of tillers per plant, spike length, number of grains per spike, number of spikelets per spike, 100-grain weight, grain yield per plant and biological yield per plant.

Isolation of Genomic DNA and SSRs screening: Whole genomic DNA was isolated from young leaf tissues of the selected morphologically superior progeny plants and parents using CTAB method (Saghai-Maroo et al., 1984). Parental DNA was amplified using 178 SSRs to study polymorphism. Phenotypically superior plants of all the generations were selected and checked for the presence of *Nax* loci using gene-specific SSR primers. Those having *Nax* loci were subjected to amplification using polymorphic SSRs for genotyping. PCR amplifications were performed using My-CYCLER (programmable thermal cycler

from BIORAD™ INTERNATIONAL). PCR reaction was conducted in a reaction volume of 20 µl containing 10X PCR buffer, 10 mM dNTPs, 0.4 µM of each primer, 1 unit Taq DNA polymerase and 50–80 ng template DNA. Amplified DNA products were resolved by submerged horizontal electrophoresis in 2.5% agarose gels, viewed under UV light fluorescence using Labnet Ultra violet trans-illuminator and image was taken by GenoSens Gel Doc system. The presence of DNA band on agarose gel was taken as one and the absence was read as zero.

Statistical Analyses: Morphological data was analyzed using INDOSTAT software. Molecular weights for SSR amplified products in base pairs were estimated and the summary statistical analysis of the genotypic data was performed using POWERMARKER software version 3.25 (Liu and Muse, 2005). Gene diversity, heterozygosity and polymorphism information content (PIC) values were calculated for each marker. Dendrogram for various generations were constructed using distance matrix by the unweighted pair-group method with arithmetic average (UPGMA) by POWERMARKER software version 3.25 and resultant files were visualized and edited using MEGA7.

Results and Discussion

In total, 1190 seeds were sown in the trays filled with soil and vermiculite mixture, out of which 220 plants of BC₁F₃, 44 of BC₂F₂ and 120 of F₄ survived up to maturity and were harvested separately. Statistical analyses of recorded data revealed that the selection criterion was in the right direction as plants having *Nax* loci showed better performance over HD2967 in each generation under saline condition. The perusal of data (Tables 1, 2 and 3) revealed that under salinity various genetic parameters, i.e., GCV, PCV, heritability, and genetic advance as 5% of mean reported more influence of plant's genetic background and less of the environment. The highest GCV and PCV was reported for the grain yield per plant in BC₁F₃ and F₄ generations, while in BC₂F₂, the number of tillers per plant showed the highest GCV and PCV. Among all the three generations, higher to moderate heritability

was reported for all the traits, except for number of tillers per plant in BC₁F₃.

Genetics advance estimates were highest for grain yield per plant in each generation during the study period, and higher to moderate genetic advance were reported for biological yield per plant, harvest index and number of tillers per plant. Ijaz and Shahzad (2015) evaluated important quantitative traits and found a similar pattern of GCV, PCV, and heritability for plant height, spike length and number of spikelets per spike, grain yield per plant and biological yield. Ahmed *et al.* (2016) also reported higher heritability estimates for most of the traits in entire cross combinations of his study. Yadav *et al.* (2017) reported confirmatory results in the backcross generation of the cross HD2851 x Kharchia 65. Direct selection can be done for improving the traits showing higher heritability values with high genetic advance as traits with higher heritability are less affected by environment and there would be greater correspondence between phenotypic and breeding values (Panse, 1957).

Selection of plants based on the phenotypic trait is limited and can be misleading due to variation in soil salinity and sodicity, hence marker-assisted selection approach is more effective (Ma *et al.*, 2007). Molecular markers are indispensable tools for improving plant breeding methods by facilitating early selection through linked genes of interest and also they have been profusely used for investigation of molecular breeding, genome mapping, and QTL mapping for biotic and abiotic tolerance in various crops (Liu *et al.*, 2001; Munns *et al.*, 2002; Ma *et al.*, 2007; Kurup *et al.*, 2009). These markers are not influenced by the environment and can be scored at all stages of plant growth, which saves time, resources and energy. SSRs linked to *Nax1* (5'-ATCGCATGATGCACG-3') and *Nax2* (5'-TCTCCATCATTCATC-3') loci gave amplification in Kharchia 65 only (Fig. 1) when used to amplify genomic DNA of both parents. One hundred seventy-eight SSR primers other than *Nax1* and *Nax2* were then used to study polymorphism between parents, out of which 31 SSRs were found to be polymorphic. Out of 384

Fig. 1: *Nax1* banding pattern in Kharchia 65 (KH), HD 2967(HD) and F₄ plants (1-15).

Fig. 2: Dendrogram showing relationship among (a) parents and BC₁F₃ plants; (b) parents and BC₂F₂ plants and (c) F₄ plants at 31 polymorphic loci.

plants, 167 phenotypically superior plants were selected from three generations to be screened for *Nax* loci. Sixty-eight plants confirmed the presence of either *Nax1* at a band size of 210 base pairs or *Nax2* at a band size of 225 base pairs or both *Nax* loci.

These plants were then screened using 31 polymorphic SSRs. Abbasov *et al.* (2011) reported similar banding pattern for the *Nax* loci while studying Turkish wheat accessions. Yadav *et al.* (2017) obtained affirmative results in F₂ generation of cross HD2852 ×

Table 1: Mean, Range, GCV, PCV, H^2 and Genetic advance (% of mean) for various traits in BC_1F_3 population

Traits	BC_1F_3					
	Phenotypic parameters			Genetic parameters		
	Mean $\pm$ SE	Range	GCV (%)	PCV (%)	Heritability (%)	Genetic advance as 5% of mean
Plant height (cm)	80.57 $\pm$ 0.51	55.00-119.50	9.92	29.48	67.00	12.14
No. of tillers /plant	2.71 $\pm$ 0.05	1.00-6.00	27.01	36.64	27.10	59.11
Spike length (cm)	10.02 $\pm$ 0.10	6.50-15.00	17.31	21.48	66.00	38.72
Number of spikelets/spike	14.85 $\pm$ 0.16	7.00-25.00	18.21	27.98	60.20	31.03
Number of grains /spike	41.14 $\pm$ 0.68	8.00-70.00	27.11	28.38	78.10	37.32
100 grain weight (g)	3.99 $\pm$ 0.04	2.30-9.55	17.11	22.43	63.60	38.28
Grain yield/ plant (g)	3.36 $\pm$ 0.07	0.35-6.85	36.85	47.92	72.10	57.70
Biological yield/ plant (g)	9.15 $\pm$ 0.18	2.01-18.90	31.78	41.09	73.20	51.63
Harvest index (%)	36.69 $\pm$ 0.46	17.41-49.52	20.08	21.57	50.30	34.39

Table 2: Mean, Range, GCV, PCV, H^2 and Genetic advance (% of mean) for various traits in BC_2F_2 population

Traits	BC_2F_2					
	Phenotypic parameters			Genetic parameters		
	Mean $\pm$ SE	Range	GCV (%)	PCV (%)	Heritability (%)	Genetic advance as 5% of mean
Plant height (cm)	81.89 $\pm$ 0.96	70.50-96.50	7.78	17.93	68.80	11.95
No. of tillers /plant	2.75 $\pm$ 0.23	1.00-8.00	54.40	61.32	64.70	44.97
Spike length (cm)	10.60 $\pm$ 0.36	6.20-16.60	22.77	24.64	55.30	23.33
Number of spikelets/spike	16.54 $\pm$ 0.56	9.00-27.00	22.40	24.15	78.00	32.81
Number of grains /spike	41.15 $\pm$ 1.47	22.00-66.00	23.73	24.15	66.50	48.03
100 grain weight(g)	3.84 $\pm$ 0.09	2.91-4.92	15.18	15.99	70.20	29.71
Grain yield/ plant(g)	3.23 $\pm$ 0.19	1.25-5.68	38.88	45.21	64.00	52.49
Biological yield/ plant(g)	9.36 $\pm$ 0.52	2.99-17.80	36.76	38.40	71.60	48.90
Harvest index (%)	35.03 $\pm$ 1.18	21.26-48.27	22.33	22.93	54.90	44.82

Kharchia 65. Munns *et al.* (2012) showed that the presence of *Nax2* could increase the grain yield of wheat on salt-affected farmers' fields by up to 25%. Xu *et al.* (2018) proposed that the underlying transport properties of *TmHKT1;5-A* and *TaHKT1;5-D* contribute to their unique ability to improve Na^+ exclusion in wheat that leads to improved salinity tolerance in the field. Fine mapping of the *Nax1* locus indicated that the candidate gene was *TmHKT1;4-A2* (Huang *et al.*, 2006), which likely encodes for a Na^+ -specific transporter located on the plasma membrane of cells surrounding xylem vessels (Munns *et al.*, 2012). Polymorphic SSRs, providing uniform coverage of all three wheat genomes (12, 8 and 11 markers on A, B, and D chromosome, respectively) were used for background selection of 68 selected plants from all three generations. Molecular marker analysis of selected plants using polymorphic SSRs revealed that these plants either had alleles in homozygous state or heterozygous state at varying frequencies.

The major allele frequency ranged from 0.500 to 0.926 in BC_1F_3 , 0.458 to 0.916 for BC_2F_2 and 0.500 to 0.900 for F_4 generation.

Gene diversity for the screened generations varied from 0.135 to 0.618 for BC_1F_3 , 0.152 to 0.611 for BC_2F_2 and 0.180 to 0.605 for F_4 generation. Out of 31 polymorphic SSRs, four SSRs (Barc 344, *Wmc11*, *Wmc88* and *Xcfd7*) were heterozygous for all the three generations, while *Xgwm577* was heterozygous only for BC_1F_3 plants. The PIC values ranged from 0.118 (*wmc11*) to 0.500 (*wmc577*) with an average value of 0.381 (Table 4). De Bustos *et al.* (2001) developed gene-specific markers to assist selection in backcross progenies in Spanish wheat, and for recovering the genetic background of recurrent parent, selection was also done by other polymorphic markers (AFLPs) located across the complete genome of wheat. James *et al.* (2012) reported similar results after introgression of salinity tolerance genes from durum to bread wheat.

The range of major allele, PIC and gene diversity in all three generations of the present study showed that selected plants were significantly variable at genetic level too. Homozygosity of most of the markers in both backcrossed generations confirmed the recovery of recurrent parent's genome.

Table 3: Mean, Range, GCV, PCV, H² and Genetic advance (% of mean) for various traits in F₄ population

Traits	F ₄					
	Phenotypic parameters			Genetic parameters		
	Mean ± SE	Range	GCV (%)	PCV (%)	Heritability (%)	Genetic advance as 5% of mean
Plant height (cm)	80.21±1.01	50.80-106.50	12.09	22.46	63.10	12.79
No. of tillers /plant	3.06±0.11	1.00-7.00	32.91	39.33	52.90	38.53
Spike length (cm)	10.36±0.24	4.30-18.00	23.97	25.05	75.30	25.22
Number of spikelets/ spike	14.61±0.29	7.00-23.00	20.56	23.09	69.23	37.70
Number of grains /spike	37.10±1.31	3.00-66.00	36.04	36.12	68.50	36.70
100 Grain Weight (g)	3.71±0.05	2.59-4.84	14.31	16.67	73.60	25.29
Grain yield/ plant(g)	3.07±0.16	0.42-10.08	55.57	59.77	66.50	63.46
Biological yield/ plant(g)	9.02±0.33	2.60-21.90	37.37	39.89	74.80	52.12
Harvest index (%)	32.90±0.90	12.87-51.64	27.88	28.40	76.40	46.38

Table 4: Summary statistics for all three generations of the cross

Marker	BC1F3				BC2F2				F4			
	Major allele frequency	Gene diversity	Heterozygosity	PIC	Major allele frequency	Gene diversity	Heterozygosity	PIC	Major allele frequency	Gene diversity	Heterozygosity	PIC
Barc26	0.7317	0.3926	0.0000	0.315	0.8333	0.2778	0.0000	0.239	0.8000	0.3200	0.0000	0.268
Barc310	0.6098	0.4759	0.0000	0.362	0.5833	0.4861	0.0000	0.368	0.5000	0.5000	0.0000	0.375
Barc344	0.6341	0.4640	0.2439	0.356	0.8333	0.2778	0.1667	0.239	0.6250	0.4688	0.1500	0.358
Barc263	0.5122	0.4997	0.0000	0.374	0.7500	0.3750	0.0000	0.304	0.7500	0.3750	0.0000	0.304
Wmc3	0.8049	0.3141	0.0000	0.264	0.5833	0.4861	0.0000	0.368	0.8000	0.3200	0.0000	0.268
Wmc382	0.7073	0.4140	0.0000	0.328	0.8333	0.2778	0.0000	0.239	0.6500	0.4550	0.0000	0.351
Wmc11	0.5000	0.5922	1.0000	0.509	0.4583	0.6111	1.0000	0.534	0.5000	0.6050	1.0000	0.527
Wmc46	0.7317	0.3926	0.0000	0.315	0.6667	0.4444	0.0000	0.345	0.7000	0.4200	0.0000	0.331
Wmc134	0.5122	0.4997	0.0000	0.374	0.5000	0.5000	0.0000	0.375	0.7000	0.4200	0.0000	0.331
Wmc145	0.5610	0.4926	0.0000	0.371	0.5833	0.4861	0.0000	0.368	0.7000	0.4200	0.0000	0.331
Wmc304	0.6341	0.4640	0.0000	0.356	0.7500	0.3750	0.0000	0.304	0.7000	0.4200	0.0000	0.331
Wmc41	0.7561	0.3688	0.0000	0.300	0.5000	0.5000	0.0000	0.375	0.6000	0.4800	0.0000	0.364
Wmc47	0.6341	0.4640	0.0000	0.356	0.6667	0.4444	0.0000	0.345	0.6000	0.4800	0.0000	0.364
Wmc758	0.6341	0.4640	0.0000	0.356	0.9167	0.1528	0.0000	0.141	0.6500	0.4550	0.0000	0.351
Wmc437	0.8049	0.3141	0.0000	0.264	0.5000	0.5000	0.0000	0.375	0.6000	0.4800	0.0000	0.364
Wmc719	0.5122	0.6187	0.0000	0.549	0.5833	0.4861	0.0000	0.368	0.6000	0.4800	0.0000	0.364
Wmc67	0.6341	0.4640	0.0000	0.356	0.5833	0.4861	0.0000	0.368	0.6500	0.4550	0.0000	0.351
Wmc577	0.6341	0.4640	0.0000	0.356	0.8333	0.2778	0.0000	0.239	0.6500	0.4550	0.0000	0.351
Wmc88	0.7439	0.3810	0.5122	0.308	0.7917	0.3299	0.4167	0.275	0.7000	0.4200	0.6000	0.331
Wmc104	0.6341	0.4640	0.0000	0.356	0.6667	0.4444	0.0000	0.345	0.6000	0.4800	0.0000	0.364
Wmc161	0.8537	0.2499	0.0000	0.218	0.6667	0.4444	0.0000	0.345	0.9000	0.1800	0.0000	0.163
Wmc428	0.9268	0.1356	0.0000	0.126	0.6667	0.4444	0.0000	0.345	0.9000	0.1800	0.0000	0.163
Wmc93	0.8537	0.2499	0.0000	0.218	0.5833	0.4861	0.0000	0.368	0.8500	0.2550	0.0000	0.222
Wmc48	0.9024	0.1761	0.0000	0.160	0.9167	0.1528	0.0000	0.141	0.8500	0.2550	0.0000	0.222
Xcfd7	0.7561	0.3688	0.4878	0.300	0.7083	0.4132	0.5833	0.327	0.6000	0.4800	0.8000	0.364
Xgwm46	0.5610	0.4926	0.0000	0.371	0.9167	0.1528	0.0000	0.141	0.8500	0.2550	0.0000	0.222
Xgwm577	0.7317	0.3989	0.0244	0.328	0.5000	0.5000	0.0000	0.375	0.7500	0.3750	0.0000	0.304
Xgwm480	0.7805	0.3427	0.0000	0.283	0.8333	0.2778	0.0000	0.239	0.6000	0.4800	0.0000	0.364
Xgwm443	0.6829	0.4331	0.0000	0.339	0.5833	0.4861	0.0000	0.368	0.6500	0.4550	0.0000	0.351
Xgwm455	0.6829	0.4331	0.0000	0.339	0.6667	0.4444	0.0000	0.345	0.6500	0.4550	0.0000	0.351
Xpsp3000	0.6585	0.4497	0.0000	0.348	0.7500	0.3750	0.0000	0.304	0.7000	0.4200	0.0000	0.331
Mean	0.6876	0.4108	0.0732	0.328	0.6841	0.3999	0.0699	0.316	0.6895	0.4096	0.0823	0.324

Separate dendrograms were prepared for all three generations by UPGMA method. Each of the three generations was grouped into two clusters. Major cluster I consisted of Kharchia 65 while cluster II contained all plants of respective progeny with the parent genotype HD2967 (Fig. 2). The finest breeding strategy is to select high yield under non-saline conditions followed by backcross of the selected plants. The genotype with higher productivity at lowest salinity should be used as recurrent parent. Though, in this study, plants were selected under saline conditions they showed that linked markers, i.e., *Nax1* and *Nax2* could present a valuable tool for breeding wheat with improved tolerance to salinity.

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