



Screening and validation of salt-stress responsive cg-SSR markers in wheat (*Triticum aestivum* L.) germplasm of Pakistan

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Abstract

Background Soil salinity has been affecting wheat production worldwide over past few decades. Evaluation of wheat genotypes for salinity tolerance at germination and vegetative growth level is crucial. Marker assisted selection is a technique used extensively for choosing salt-tolerant genotypes from breeding populations to introduce novel genes.

Methods and materials The current study's main goal was to discover salt-stress resistant genes; genetic divergence and genome-wide connection by using recently designed candidate gene-based simple-sequence-repeat markers (cg-SSRs). The phenotypic connection of morphological features during the germination growth stage i.e., germination period, root length/weight and shoot length/weight, and vegetative growth stages i.e., root length/weight and shoot length/weight were tested in a group of 50 wheat genotypes. Significant difference was observed in germination rate, root length and weight among control and saline treatments.

Conclusion Total 30 SSR markers were utilized to test salinity resistance genes in wheat genotypes. Three (10%) of which were monomorphic, one (3.34%) showed no result, and the other 26 (86%) were polymorphic. Using 30 polymorphic markers discovered total 37 alleles. The polymorphic information content (PIC), quantifies each SSR locus capacity to discriminate between wheat, varied from 0.00 to 0.38 with an average of 0.19. Association analysis revealed that 26 primers were associated with morphological features, 03 with root length and the remaining 23 with germination. Utilizing morphological data, stress tolerance index (STI) was designed concluding that Auqab-2000, Margala-99 and Ufaq showed better resistance against salinity among other wheat genotypes. Cluster analysis demonstrated that wheat genotypes have vast genetic variability.

Keywords Marker-assisted selection · Salinity · SSRs · Wheat

Introduction

Wheat is an important cereal grain being harvested globally that is a dietary staple everywhere in the world also serving as key ingredient in a range of processed goods popular in urban life [9, 28]. The global amount of wheat produced was around 765.41 million metric tons in the crop year 2019–2020. The average annual wheat production of Pakistan is 45.6 million tons being one of the ten highest wheat-producing countries, compared to a target of 48 million tons, with 45 million tons consumed annually. Wheat production is being impacted by salt conditions all over the world resulted by global warming. High-yielding crop types have a high capacity for nutrient absorption, resulting in soil depletion of micro and macronutrients. As a result, nutrient supplementation by chemical or biological means is required to maintain and restore soil fertility. Soil salinity is posing a serious threat to global food productivity, with both

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developing and industrialized countries facing comparable basic issues in farming crops under saline circumstances [26, 32]. According to estimates, quarter of the world's land under irrigation is now saline. Soil salinity is a severe concern in Pakistani agriculture; more than 6.8 million hectares of land has salinity levels that exceed crop tolerance limits. The main cause of this scenario is ground and canal irrigation water, as more than 70% of tube wells pump salty water and canal irrigation adds salt to the land with each application. Due to increased salt content in soil, enormous area of fertile land could not be employed for conventional crop cultivation. These issues challenge the entire arid and semi-arid region's production system [11]. When roots grow in salty soils, they are subjected to osmotic stress, which causes a reduction in water potential and, as a result, a loss of cell turgor in the roots. Salinity stress has been shown to have an impact on photosynthesis, ion regulation, water relations, and yield [31]. Plant does not allow salt to rise up to the leaves. At the level of the endoderm, the root's inner layer, there is a first barrier. This barrier, however, can be broken, especially during the root's emerging stages. Other processes prevent salt from passing from the roots to the leaves, but the genes that control them are mostly unknown [4]. High genetic variation is found in wheat for salt tolerance, there have been few attempts to investigate the genetic and physiological complexity of multi-genic salinity-related characteristics [25]. Because of advancements in the fields of statistical genomics and molecular markers, quantitative trait loci (QTL) mapping in crop plants has become a most used technique. The concept behind QTL analysis is to find correlation between and marker genotype and phenotype [17]. The detection and selection of desirable genes in breeding populations can be done considerably more quickly than traditional breeding, using genomic technologies in combination with MAS. SSR markers, also known as microsatellites, are PCR-based markers found in abundance in animal and plant genomes. The number of copies of these repetitions creates difference in individuals that causes polymorphism in plants. Locus specific primers are designed for the use in PCR reactions as the DNA sequences bordering these regions are frequently conserved. Because of their high level of allelic variation, microsatellite loci are effective genetic markers, which is one of their most essential characteristics. Many SSR markers that are breeder friendly, have been produced and are now being used by breeders in variety of species [35]. Genome data sets have been produced due to Advances in genotyping techniques and DNA sequencing that are the sources to develop SNP markers and sequence-based simple sequence repeats (SSRs), which has hindered progress in MAS for wheat until recently [13]. Over last 4 decades, SSRs are the most extensively used markers for plant genotyping because of their characteristics: extremely informative, co-dominant,

experimentally replicable multi-allele genetic markers and convertible among associated specie [23]. SSRs are proven to be particularly beneficial in the creation of plant species' integrated maps when linkage maps are generated with the use of full-sib families [15, 27] and for incorporating physical, genetic, and sequence-based maps [36] offering tool to correlate genotypic and phenotypic diversity for breeders and geneticists. Current investigation aimed to evaluate wheat germplasm against saline conditions phenotypically by growing them in sand culture providing artificial salt-stress and genotypic evaluation has been done using recently designed cg-SSR markers using collection of fifty wheat accessions including forty-five Pakistani and five Chinese cultivars provided by National Agriculture Research Centre, Islamabad. Salt responsive cg-SSR markers utilized in this research project were designed in 2018 and had not been validated before. The study proposes the validation of markers and their association with the phenotypic traits.

Materials and methods

Plant material and experimental design

A set of 50 *Triticum aestivum* L. accessions were used for two separate experimentations which included 45 local varieties and 5 Chinese wheat varieties, conducted at Nation Institute of Genomics and Biotechnology, NARC, Islamabad. Experiments followed complete randomized design with three replications.

Salt treatments

In present experiment, wheat genotypes were grown in sand culture where one set was kept control and one was provided with saline conditions, each with three replicates. Tap water including Hoagland's solution was added to sand every other day in both sets. Adding a calculated amount of NaCl developed the saline solution. The salt was dissolved in tap water and thoroughly mixed with sand for developing saline soil [37]. Plants were allowed to grow for a month. Seeds of wheat genotypes were germinated first in Petri dishes and rinsed thoroughly with sterilized water. In this experiment, wheat genotypes were germinated in a control and saline solution {50DeciSiemens per meter (dSm-1)} where 10 seeds of each genotype were placed in a Petri dish (diameter 9 cm), kept in the laboratory under normal growing conditions. In the case of salinity stress, the Petri dishes were irrigated daily with the required amount of saline solution in increments of 3 dSm-1 per day up to the final salinity (15 dSm-1). Seeds allowed to grow up to a week and then shifted to sand culture. Germination characters were counted at 48-h intervals.

Germination characters

Observations of Germination had been taken at 48-h intervals and continued up to 6th day. The germination of a seed was when the radicle came out and were > 2 mm long. Rate of germination, root length/weight and plumule length/weight were calculated, formula used for germination rate by Krishnasamy and Seshu [19] is as follows:

Rate of germination (%)

$$= (\text{Seeds germinated at 48 h} / \text{Seeds germinated at 144 h}) \times 100$$

Germination percentage was calculated using the following formula [22].

$$\text{Germination Percentage} = (\text{Seeds germinated} / \text{Total seeds}) \times 100$$

Seedling growth

Germinated seeds were transferred to sand culture. For a week, plants were grown normally with nutrient solution. A set of 50 wheat genotypes with three repeats was exposed to salt stress, while the other set was retained as control. Salt stress was gradually increased, the pH of the water was maintained, and EC (electrical conductivity) was measured before and after salt stress was applied [29].

Salt tolerance index

Salt tolerance index (STI) was calculated for germination characters and early seedling growth as described by Goudarzi and Pakniyat [14] by the following formula:

Salt tolerance index

$$= \left(\frac{\text{Variable measured under stress condition}}{\text{Variable measured under normal condition}} \right) \times 100$$

DNA extraction and PCR amplification

4–5 young grown leaves from all germplasm groups were gathered and stored at 80 °C for DNA extraction. The Cetyltrimethyl-ammonium bromide (CTAB) method was used to isolate genomic DNA from frozen leaf tissues as reported by Gale [24]. For molecular marker analysis, PCR was performed in a final volume of 10 µL containing: 2 µL genomic DNA (50 ng), 2 µL 5 × PCR6 buffer (Promega, Madison, USA), 1 µL dNTPs (0.2 mM for each dNTP), 1 µL of each primer (10 pM), 0.2 µL MgCl₂ (1.5 mM), and 0.05 µL of Go-Taq DNA polymerase (5 UµL⁻¹) (Promega); double distilled sterile water was added to a final volume of 10 µL. DNA amplification was carried out in a thermocycler (Eppendorf Master cycler Gradient, Eppendorf, Hamburg,

Germany). PCR amplification cycling profile used is as follows: 1 cycle (10 min) at 94 °C, 35 cycles (1 min) at 94 °C, 45 s at 50–60 °C, 1 min at 72 °C of 10 min at 72 °C, standard protocol provided by Zhang [37]. Ethidium Bromide-stained Agarose gel (1.5–3%) was used to resolve the PCR amplified products.

Microsatellite loci analysis

Microsatellites were selected based on available information with respect to proximity to known quantitative trait loci that control wheat quality traits to directly validate the reliability of any associations. Thirty SSR markers were chosen from the publicly available set published by Singh et al. BMC Plant Biology [33]. The presence (1) or absence (0) of an allele was used to establish a binary matrix composed of 0 s and 1 s. From this matrix, 26/30 loci resulted in polymorphic alleles that could be scored as either 0 or 1. Local white and Pasban-90 wheat were used as a standard marker.

Statistical analysis

Statistical Analysis System (SAS, 2008) was used to perform ANOVA (analysis of variance) using GLM procedure for evaluation of effects of salt stress on germination and seedling of 50 genotypes. The data collected from DNA analysis has been used to create a similarity matrix utilizing the Nei (1983) method with the data obtained from 30 DNA markers. A Mixed Linear Model (MLM) was constructed in PowerMarker v3.25 software to investigate associations between quality-related traits and SSR markers.

Results

Germination characters in sand culture

The characters under study i.e., germination rate, plume weight/length and root weight/length, showed significant observations overall. ANOVA represents the significance with *P* values ranging from 0.0000 to 0.0002 (< 0.005). The difference in minimum and maximum observations show that salinity has weakens the growth into half. The most affected trait is root length.

Pearson correlation coefficient (Table 1) shows the proximity/correlation coefficient matrix of four agronomic traits. RW had a negative relationship with GP2D and a positive relationship with GP4D, GP6D, and RL. RW was adversely connected with PL, while GP2D, GP6D, GP6D, and RL were positively correlated. RW and PL were linked, significantly. PW was also linked to RW in a substantial way.

Table 1 Pearson's correlation among all morphological traits at germination stage with significance value

	GP2D	GP4D	GP6D	RL	RW	PL	PW
GP2D	1						
GP4D	0.642338	1					
GP6D	0.556695	0.800381	1				
RL	0.626144	0.351929	0.353254	1			
RW	-0.046 ^a	0.124846	0.090235	0.0031 ^b	1		
PL	0.628333	0.400911	0.359225	0.739113	-0.01 ^a	1	
PW	0.486656	0.214065	0.209467	0.641002	0.0097 ^b	0.777522	1

^aSymbol representing highly significant level

^bSignificance level, GP2D, germination percentage after 2 days; GP4D, germination percentage after 4 days; GP6D, germination percentage after 6 days; RL, root length; RW, root weight; SL, shoot length; SW, shoot weight

Table 2 Pearson's correlation coefficients between vegetative traits in wheat accessions

	RL	RW	SL	SW
RL	1			
RW	-0.00358 ^a	1		
SL	0.280987	0.04693 ^b	1	
SW	0.093353	0.138212	0.406624	1

^aSymbol representing highly significant level

^bSignificance level, RL, root length; RW, root weight; SL, shoot length; SW, shoot weight

Vegetative characters in sand culture

The characters under study i.e., shoot weight/length and root weight/length, showed significant observations overall. ANOVA represents the significance with *P* values ranging from 0.0000 (<0.005). The difference in minimum and maximum observations shows that salinity has weakened the growth almost four times. The most affected trait is root weight.

Pearson correlation coefficient for pair wise alignment was used to investigate correlation among all agronomical traits. It demonstrated the relationship between two variables, exposing the attributes correlation more effectively. Table 2 shows the proximity/correlation coefficient matrix of four agronomic traits. RW and RL had a weak relationship. SL had a positive relationship with RL and RW. RW and SL had a strong relationship.

Interrelationship among germination stage agronomical traits

PCA analysis, which is based on correlation, is used to classify data patterns based on trait relationships. Based on eigenvalue and variability, data is currently classified into seven principal components (PCs) or factors (Fs).

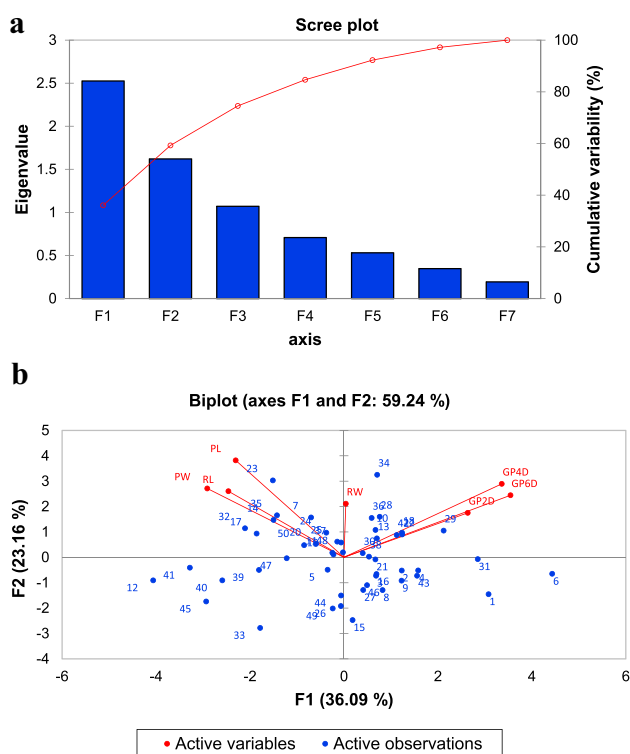


Fig. 1 a Scree Plot showing principle factors based on cumulative variability and eigenvalue of wheat agronomical traits. b Biplot representation of correlation among 7 agronomical traits in 50 wheat varieties

Seven factors under observation are GP2D, GP4D, GP6D, RL, RW, PL, PW, respectively. The first three PCs, which have eigenvalue greater than one, provide more variability. These three variables account for 74.5 percent of the variance. The cutoff value for selecting PCs for further investigation was eigenvalue one. The first 2 factors, F1 (36%), and F2 (23%), contributed more variability, indicating their relevance in limiting the Scree plot and biplot (Fig. 1a, b). The importance of traits and their implications

in future breeding programs are revealed by their contribution to factor variability.

Interrelationship among vegetative stage agronomical traits

PCA analysis, which is based on correlation, is used to classify data patterns based on trait relationships. Based on eigenvalue and variability, data is currently categorized into four primary components (PCs) or factors (Fs). Components under observation are RL, RW, SL, SW, respectively. With an eigenvalue of > 1, the first PC contributes higher variability. This factor accounts for 56.28 percent of overall variability. The first factor, F1, contributes the most variability (56.28 percent), indicating its relevance in limiting biplot.

The importance of traits and their implications in future breeding programs are revealed by their contribution to factor variability.

Scree plot based on cumulative variability and eigenvalue, reveal four principal components and their contribution (Fig. 2a, b). The first principal component to touch the curve Line had the most impact on genotype distribution based on agronomic traits. The remained three components with eigenvalue of 1 contributed a small amount of variability, accounting for 43.72 percent of the total variability.

Salt tolerance index

Salt tolerance evaluation of wheat genotypes under study has been done using formula: salt tolerance index = (Variable measured under stress condition/Variable measured under normal condition) × 100

Wheat genotypes have been classified as ST: salt tolerant, HST: Highly salt tolerant, MST: moderate salt tolerant, HSS: highly salt sensitive, SS: salt sensitive (Table 3).

Genetic diversity assessment and SSR variation

Genetic diversity research was conducted on all wheat lines using 30 widely used DNA marker reported by Singh et. al [33]. Three of the thirty markers were monomorphic, one had no result, and the other 26 were polymorphic. Using 30 polymorphic markers discovered total 37 alleles. Other than six primers, each marker yields a single allele. SSR13TaNAC47 had three alleles, SSR25TaPP2C1, SSR109TaGAPC1, SSR111TaSOS1, SSR115TaMYB72, and SSR98TaSST had two alleles. The alleles ranged in size from 50 to 400 base pairs. The frequency of the main allele ranged from 52 percent (SSR13TaNAC47) to 100 percent (SSR13TaNAC47, SSR25TaPP2C1, SSR75F3H1, SSR39BI-85). On the other hand, gene diversity ranged from 0.00 (SSR25TaPP2C1,

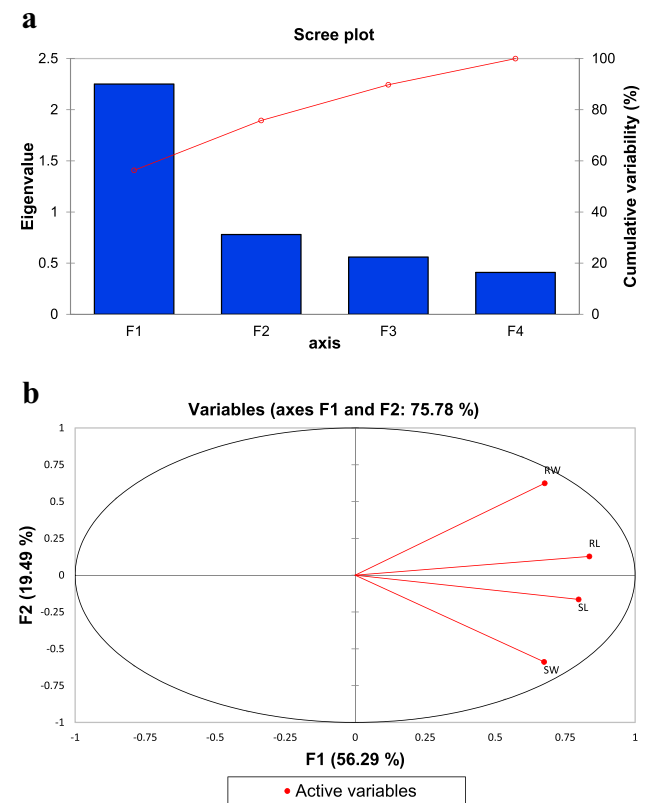


Fig. 2 a Scree Plot showing principle factors based on cumulative variability and eigenvalue of wheat agronomical traits. b Biplot representation of correlation of 4 agronomical traits with 50 wheat genotypes

SSR75F3H1, SSR39BI-85) to 0.50 (SSR75F3H1, SSR39BI-85, SSR13TaNAC47). The polymorphism information content (PIC), which measures how well each SSR locus can distinguish different wheat varieties, ranged between 0.00 and 0.38, with average value of 0.19.

Genetic relationship and cluster analysis

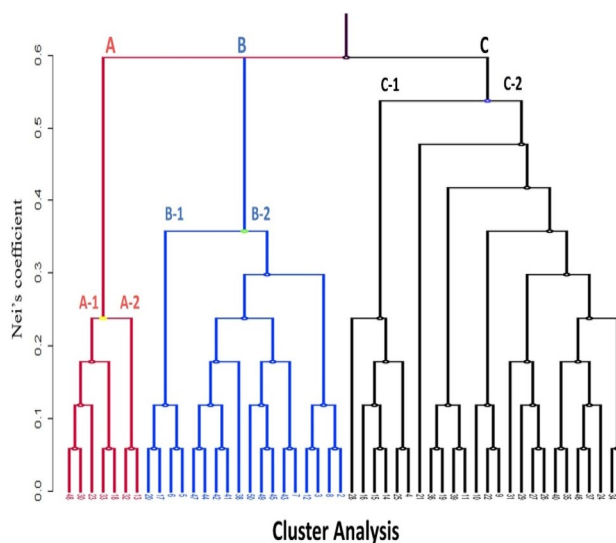
The data collected from DNA analysis has been used to create a similarity matrix utilizing the Nei (1983) method with the data obtained from 30 DNA markers. The resulted matrix revealed significant amount of genetic relatedness among the 50 genotypes, demonstrating a mean genetic similarity. The genetic similarities between the genotypes in the paired combinations ranged from 0.02 to 0.57.

The dendrogram was created for 50 genotypes with 30 SSR markers using clustering based on UPGMA analysis, as shown in (Fig. 3). Dendrogram analysis separated wheat genotypes in 3 major groups: Group A, B, C, and D. Each

Table 3 Salt tolerance evaluation of wheat genotypes under study

Genotypes	STTI (VS)	STTI (GS)	STI	Salt tolerance level
Zincol-2016	0.51	0.71	61	ST
Local white	0.74	0.68	71	ST
Ahsan-2016	0.53	0.67	60	ST
Gold-2016	0.56	0.78	67	ST
Johar-2016	0.67	0.57	62	ST
Pakhtunkhwa-2015	0.63	0.53	58	MST
Pakistan-2013	0.59	0.79	69	ST
Pirsabak-2013	0.49	0.83	66	ST
Shahkar-2013	0.53	0.83	68	ST
Galaxy-2013	0.67	0.81	74	ST
NARC-2011	0.67	0.89	78	ST
AAS-2011	0.53	0.74	63	ST
DHARAB-2011	0.51	0.5	50	MST
PUNJAB-2011	0.72	0.6	66	ST
Millet-2011	0.4	0.61	50	MST
NARC-2009	0.53	0.67	60	ST
Pirsabak-2008	0.59	0.79	69	ST
MEIRAJ-2008	0.67	0.61	64	ST
F-ABAD-2008	0.57	0.59	58	MST
FAREED-2006	0.62	0.81	71	ST
SEHER-2006	0.65	0.81	73	ST
CHAKWAL-50	0.66	0.97	81	HST
ATTA HABIB	0.79	1	89	HST
BARSAT	0.73	1.01	87	HST
WAFaq-2001	0.61	0.9	75	ST
PIRSABAK-2005	0.47	0.92	69	ST
AS-2002	0.44	0.96	70	ST
AUQAB-2000	0.67	1.13	90	HST
MARGALA-99	0.61	1.2	90	HST
SULEMAN-96	0.64	1.08	86	HST
M.H-97	0.55	1.12	83	HST
BAKHTAWAR-94	0.6	1.09	84	HST
INQILAB-91	0.5	0.32	41	MST
KHYBER-87	0.69	0.93	81	HST
UFAQ	0.74	1.11	92	HST
PASBAN-90	0.66	1.01	83	HST
PAROULA	0.64	1.03	83	HST
PASTOR	0.61	0.62	61	ST
WL-711	0.62	0.64	63	ST
PAK-81	0.65	1.02	83	HST
UJALA	0.53	1.03	78	ST
TATARA	0.59	0.67	63	ST
BAHTOOR	0.59	0.77	68	ST
PIRSABAK-2004	0.58	0.51	54	MST
BHAKKAR	0.57	0.49	53	MST
K4565-F5	0.46	0.85	65	ST
K4565-F4	0.51	0.77	64	ST
K4565-18Y4-5	0.5	0.35	42	MST
K4565-18Y4-51	0.35	–	35	SS
K4565-18Y2-3954	0.54	–	54	MST

ST, salt tolerant; HST, highly salt tolerant; MST, moderate salt-tolerant; HSS, highly salt sensitive; SS, salt sensitive

**Fig. 3** Diversion of whole wheat population shown in cluster analysis

group has further two subgroups, group A contains A-1 and A-2, group B has B-1 and B-2, and group C has C-1 and C-2. On the base of DNA markers, the maximum genetic distance observed in the group C-2. This group contained 19 genotypes. It is further divided in two sub-clusters. Genotype 21, 28 and 38 were the most diverged genotypes in whole experiment. Group B contained 18 genotypes with Nei value of 0.37. Cluster A with lowest diversity contained rest of 7 genotypes.

Marker-trait association for morpho-physiological traits

TASSEL software was used to estimate marker-trait relationships using information about lineage of wheat cultivars used and phenotypic data. Based on relatedness relationship and population structure (Q-matrix), the association analysis and identification of SSR markers linked with the salinity related attributes in wheat population under study (K-matrix), was conducted by utilizing a general linear model (GLM). The findings revealed that 26 primers are associated with morphological features, three of which are associated with root length and the remaining 23 with germination (Table 4).

Discussion

The development of quantitative and qualitative genetic features has always been the main interest of breeders using traditional breeding methods based on phenotypic evaluation and selection until recent advances in molecular technology

Table 4 Marker trait association of wheat genotypes

Sr. no.	Trait ~ Marker	P-value
1	GP2D ~ SSR85TaAFP-A	2.38E-18
2	GP2D ~ SSR13TaNAC47 200 bp	3.50E-13
3	GP4D ~ SSR85TaAFP-A	1.45E-10
4	GP2D ~ SSR121TaSrg6 140 bp	3.51E-09
5	GP4D ~ SSR1TaSRG	1.32E-07
6	GP2D ~ SSR1TaSRG	3.17E-07
7	GP2D ~ SSR126TaClpB2	2.91E-06
8	GP2D ~ SSR127TaClpB2	4.97E-06
9	GP2D ~ SSR40TaAOC1	2.70E-05
10	GP2D ~ SSR98TaSST 175 bp	4.09E-05
11	GP6D ~ SSR1TaSRG	0.000105
12	GP2D ~ SSR111TaSOS1 200 bp	0.00017
13	GP4D ~ SSR13TaNAC47 185 bp	0.000247
14	GP4D ~ SSR39BI-85	0.000391
15	GP2D ~ SSR101TaACO1	0.000916
16	GP2D ~ SSR136TaSP	0.001286
17	GP4D ~ SSR95TaABL1	0.004081
18	GP2D ~ SSR130TaClpB5	0.005205
19	GP4D ~ SSR101TaACO1	0.00521
20	GP4D ~ SSR13TaNAC47 200 bp	0.007723
21	Root length ~ SSR12TaNAC47	0.009137
22	GP6D ~ SSR39BI-85	0.01153
23	GP4D ~ SSR132TaCRY1a	0.014796
24	GP4D ~ SSR111TaSOS1 100 bp	0.015419
25	GP4D ~ SSR121TaSrg6 140 bp	0.015419
26	Root length ~ SSR79Tagpd1	0.020351
27	GP2D ~ SSR115TaMYB72 150 bp	0.02259
28	Root length ~ SSR109TaGAPC1 300 bp	0.025136
29	GP6D ~ SSR115TaMYB72 50 bp	0.030888
30	GP4D ~ SSR139TaWD40D	0.031545
31	Root length ~ SSR13TaNAC47 185 bp	0.031614
32	GP2D ~ SSR13TaNAC47 400 bp	0.033652
33	GP2D ~ SSR139TaWD40D	0.034141
34	GP4D ~ SSR115TaMYB72 50 bp	0.036456
35	GP4D ~ SSR44TaSOS1	0.048914
36	GP4D ~ SSR89TaOBF1b	0.048914

and genetics [7]. Association mapping, on the other hand, is promising methodology to identify and dissect complicated features [8]. In comparison with conventional QTL mapping, the key advantages include reduced research time, improved mapping resolution, and increased allele count [37].

The development of molecular markers technology during the last century has made it possible to analyze direct statistical connections between phenotypic features in populations and genetic markers, a field called “association genetics” [20]. Relevant genetic markers’ availability has improved and made it possible to link variation in complex traits to genomic regions containing these markers. The association

mapping method was initially effectively used to dissect alleles at loci involved in human disease susceptibility [34].

The technique is being used to specify markers and inherited genes that are relevant to variety of traits in variety of plant species, including potato [21], barley [18], rice [1] and wheat [6]. By accounting for population structure and kinship, new computational algorithms provide more accuracy in recognition of marker–trait relationships in populations of complex-pedigree. Additionally, marker resolution can be determined by linkage disequilibrium (LD) in given population. Gene cloning of required genes will be made easier with these methods [5].

Because of high level polymorphism at marker loci, using SSRs to analyze diversity and population structure yields better results than using other marker types [10].

The first step in revealing the genetic diversity favored by genotypes is to morphologically characterize germplasm [3]. The degree of genetic diversity in a crop's breeding program is largely dictated by it [2].

The current study's primary goal was to investigate wheat germplasm for phenotypic alterations under saline stress conditions, as well as genetic diversity and similarity.

A total of 50 wheat genotypes were gathered and 7 morphological traits at the germination growth stage and 4 morphological traits at the vegetative growth stage were examined.

The vast variance shown by genotyped for Germination data, root/shoot length, root/shoot weight can be explained by the mean value of morphological features.

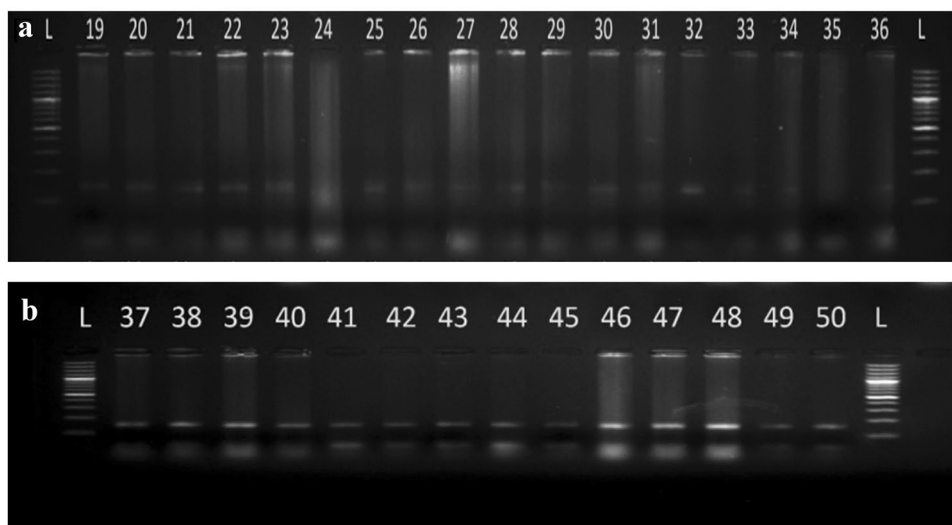
Analysis of variance for days to germination data, root/shoot length, root/shoot weight revealed a highly significant variation at (P 0.01) significance level. Results indicate difference in phenotype and growth pattern of stress and control genotypes. An effective screening method and reliable trait for salt tolerance at germination growth stage and vegetative growth stage, STI, is defined as ratio of values for salt (NaCl) affected plant to values for control. The equation for the calculation of STI is:

$$STI = \frac{\text{Mean value under salt stress}}{\text{Mean value under control}} \times 100$$

Five levels of salt tolerance are described as: salt sensitive (SS), highly salt sensitive (HSS), salt tolerant (ST), highly salt tolerant (HST), and moderately salt tolerant (MST). Results show that 13 out of 50 genotypes under study are highly tolerant while one genotype is salt sensitive remaining all are moderately or less tolerant.

With data from 30 DNA markers, data from DNA study has been used to construct a similarity index using Nei (1983) approach. The resulting matrix revealed a significant level of genetic relatedness among the 50 genotypes, demonstrating a mean genetic similarity. The genetic similarities

Fig. 4 a Polymorphism revealed by SSR109TaGAPC1 maker in wheat varieties; L: ladder (100 bp), lane 19–36 *Triticum aestivum* L. cultivars. **b** Monomorphism revealed by SSR76TaRab7 maker in wheat varieties; L: ladder (100 bp), DNA Marker; lanes 37–50 *Triticum aestivum* L. cultivars



between the genotypes in the paired combinations ranged from 0.02 to 0.57.

Cluster analysis is a useful tool for identifying homogeneous plant groups. The current study split whole Lines into four groups based on their Nei values, which ranged from 0.02 to 0.57. The genetic connection of different agricultural characteristics offers the possibility of improving economic qualities [30]. Understanding the genetic linkage between various characteristics is critical for the creation of varieties with a genetic attribute that ultimately enhance crop acceptance.

UPGMA analysis-based clustering was used to construct a dendrogram for 50 genotypes along with 30 SSR primers, as shown in (Fig. 3). Dendrogram analysis separated wheat Lines in 3 major groups: Group A, B, C, D. Each group has further two subgroups, group A contains subgroups A-1 and A-2, group B has B-1 and B-2, and group C has C-1 and C-2. On the base of DNA markers, the maximum genetic distance observed in the group C-2. This group contained 19 Lines. It is further divided in 2 sub-clusters. Line 21, 28 and 38 were most diverged Lines in whole experiment. Group B contained 18 lines with Nei value of 0.37. Cluster A with lowest diversity contained rest 7 lines.

The difference and similarity between two variances can be measured by mean of correlation coefficient [12]. The study of relationships between diverse morphological traits is essential for cultivar development in plant breeding. Without a thorough grasp of character relationships in complex biological organisms, genetic improvement could not be carried out accurately. Significant ($P=0.01$) correlation coefficient was also found between relative values of most of the attributes. Because of the link between shoot length and root weight, shoot length should be used as a selection criterion for increasing salt stress tolerance in crop.

The SSR markers identification, associated to phenotypic features of wheat implicated in salt stress resistance was the study's second goal. Based on relatedness relationship, the association analysis has been conducted using software “TASSEL” to find the microsatellite markers linked with salinity related attributes in structured wheat population. The findings revealed that 26 primers are associated with morphological features, three of which are associated with root length and the remaining 23 with germination (Table 4).

Conclusion

The current study's overall aim was to validate cg-SSR markers created from salt responsive genes for their potential to identify salt tolerant wheat genotypes. A genetic diversity research was conducted on all wheat lines using 30 widely used DNA markers. Three of the thirty markers were monomorphic, one showed no result, and the other 26 were polymorphic (Fig. 4a, b). As a result, 26 of 30 markers provided results that were mentioned in the literature [33]. Total 37 alleles have been discovered utilizing 30 polymorphic primers. Other than six primers, each marker yields a single allele. Three alleles were found in SSR-13TaNAC47, two alleles were found in SSR25TaPP2C1, SSR109TaGAPC1, SSR111TaSOS1, SSR115TaMYB72 and SSR98TaSST. The size of the alleles ranged from 50 to 400 bp. The major allele frequency varied from 52 (SSR-13TaNAC47) to 100% (SSR25TaPP2C1, SSR75F3H1, SSR39B1-85). Gene diversity on the other end, varied from 0.00 (SSR25TaPP2C1, SSR75F3H1, SSR39B1-85) to 0.50 (SSR13TaNAC47). The polymorphic information content (PIC), which quantifies each SSR locus's capacity to discriminate between wheat, varied from 0.00 to 0.38 with

an average of 0.19. The findings of this study would further promote testing of these techniques in diverse genetic organizations, like how they were employed for wheat.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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