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Morphological and molecular genetic variation in wheat for salinity tolerance at germination and early seedling stage

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Abstract

Salinity is one of the major constraints to wheat production. Salt affected soils can be better utilized by developing and growing salt tolerant wheat varieties. Genetic diversity for salt tolerance is a prerequisite for developing salt tolerant wheat varieties. Therefore, the present study was conducted to evaluate the level of genetic diversity among 172 (123 Pakistani and 49 exotic) wheat genotypes for salinity tolerance at germination and early seedling stage. All the genotypes were first tested at 200 mM NaCl stress. Based on the results, 34 genotypes were selected and subsequently tested at 250 mM and 300 mM NaCl stress. Genetic variation for salt tolerance existed in the studied wheat genotypes. Plumule growth was affected more than radicle growth at higher salinity levels. Based on salt tolerance index, 18 accessions were identified as salt tolerant at 200 mM NaCl stress. Egyptian accession 11466 was the most salt tolerant at 250 mM NaCl stress, whereas Pakistani accession 11299 and Egyptian accession 11466 were the most salt tolerant at 300 mM NaCl stress. Genetic similarity coefficients based on RAPD marker data ranged from 0.38 to 0.95. RAPD primer OPA 2 produced a unique fragment of 1000 bp, whereas OPF 13 generated two fragments of 1200 bp and 1400 bp only in some tolerant genotypes. Genetic similarity coefficients for SSR markers ranged from 0.45 to 0.95. Both RAPD and SSR markers revealed genetic variation in the studied genotypes. The salt tolerant landraces identified in this study could be used as parents to incorporate salt tolerance in future wheat cultivars. The unique DNA fragments observed in this study should be further investigated in segregating populations to determine their usefulness in Marker assisted selection for salt tolerance in wheat.

Keywords: Genetic diversity; germination stage; RAPD; Salinity tolerance; SSR; *Triticum aestivum* L.
Abbreviations RAPD; Random amplified polymorphic DNA; SSR; simple sequence repeats or microsatellite DNA markers: STI; Salt tolerance index: STTI; salt tolerance trait index.

Introduction

Over 800 million ha of land worldwide (Munns, 2005) and about 6 million ha in Pakistan (Chatrath et al., 2007) is salt affected. Salinity is a major constraint to food production as it limits crop yields and restricts the use of previously cultivated lands. The canal irrigated areas of Pakistan, especially that in Punjab province are the main contributors to crop production in the country. Salinity problem is becoming more severe in the canal irrigated areas of Pakistan (Evans et al., 2012). Soil reclamation and proper drainage may considerably ameliorate salinity problems. However, the area affected by salt is so large that these solutions appear to be unrealistic. One of the possible ways to bring salt affected land under cultivation is by growing salt tolerant crop cultivars. Salt tolerant cultivars are capable of maintaining active water uptake by root cells at high salt levels in the soil solution (Ashraf, 2004). Bread wheat (*Triticum aestivum* L.*)* provides more than half of the caloric and protein requirements to one-third of the world's population (Dhanda et al., 2004). Although wheat is one of the most salt tolerant cereal crops (Badridze et al., 2009), its yield substantially decreases as the soil salinity level rises to 100 mM NaCl (Munns et al., 2006). To date, Kharchia 65 is the only available donor variety of salt tolerance in wheat and has been extensively used in breeding salt tolerant wheat cultivars globally (Chatrath et al., 2007). Therefore, new sources of salt tolerance in wheat need to be identified to broaden the gene pool and to provide donor parents in locally adapted genetic backgrounds. Due to soil heterogeneity, field screening for salt tolerance is difficult. Therefore, most of the studies on salt tolerance of wheat have been carried out in controlled environments. Identification of salt tolerant genotypes at both the germination and seedling stages is particularly useful (Mano and Takeda, 1997). Germination is a crucial stage for plant establishment (Song et al., 2008) and poor germination may lead to poor stand establishment, resulting in lower grain yields. The seedling stage is generally the most sensitive phase of plant development, and studies on salt tolerance in different crop species has mostly included plant assessment at this stage (Song et al., 2008; Tlig et al., 2008; Badridze et al., 2009). Development of salt tolerant wheat varieties requires an efficient system for assaying genetic variation in adapted as well as exotic germplasm. Due to the complex nature of salt tolerance, DNA markers may provide a valuable tool for assessing genetic diversity. Among different marker systems, random amplified polymorphic DNA (RAPD) is a simple technique for diversity analysis (Sun et al., 1997; Fu et al., 2002). However, RAPD analysis is sensitive to reaction conditions

and may not be reproducible (Devos and Gale, 1992). On the other hand, simple sequence repeats (SSRs) are genome specific, highly accurate and repeatable across environments (Roder et al., 1995). Therefore, these have been successfully used for genetic diversity analysis (Huang et al., 2002; Hao et al., 2006). Kurup et al. (2009) reported that results obtained from RAPD markers supported the morphological and physiological results and could, therefore, be used for screening salinity tolerant genotypes before planting the crop. Rahman et al. (2004) studied RAPD markers linked to genomic regions controlling salinity tolerance in wheat and concluded that efficiency of plant breeding programs can be increased by marker-assisted selection. The present study was conducted to assess the extent of genetic variation in landraces/cultivars of Pakistani and exotic wheat based on morphological data at germination and early seedling stage along with RAPD and SSR marker data.

Results

Morphological screening

The effects of genotype, treatment and genotype × treatment interaction were highly significant for all traits studied (Supplementary Table 1). Based on STI values, the 172 wheat genotypes were classified into four groups (Table 1), namely salt tolerant (STI= 81 to 100%), moderately salt tolerant (STI= 67 to 80%), moderately salt susceptible (STI= 54 to 66%) and salt susceptible (STI= below 54%). Only 18 accessions fell in the tolerant class, including 8 accessions from Pakistan, 6 from Iran, 3 from Syria and 1 from Egypt. The check cultivars, SARC VII and Shorawaki were also among the tolerant genotypes. Forty seven accessions were moderately salt tolerant; 82 accessions were moderately salt susceptible and 25 accessions were salt susceptible. Most of the salt susceptible wheat accessions were from Pakistan. STTI for germination rate at 250 mM NaCl concentration ranged from 4.6% to 92.6%. At 300 mM NaCl concentration, STTI for germination rate ranged from 4.0% to 32.6%. Plant growth at seedling stage was determined by plumule's and radicle's fresh and dry weights at 250 and 300 mM NaCl stress levels. The increase in NaCl concentration decreased the plumule fresh weight. At 250 mM NaCl stress, STTI for plumule fresh weight ranged from 2.6% to 56.9%. At 300 mM NaCl stress, accession 11466 had the maximum STTI (21.7%) for plumule fresh weight, followed by 21% for 11299. Only seven accessions including 11299, 11383, 11454, 11460, 11466, Karaj II and Maroon survived at 300 mM NaCl stress (Table 3). At 250 mM NaCl stress, STTI for radicle fresh weight ranged from 47% to 99.9% (Table 2). At 300 mM NaCl stress, STTI for radicle fresh weight ranged from 45% to 90% (Table 3). Fresh biomass of all genotypes significantly reduced with increasing NaCl stress. Considerable reduction in plumule and radicle dry weights was observed at 250 and 300 mM salt stress levels (Tables 2 and 3). At 250 mM salt stress, STTI for plumule dry weight ranged from 5.9% to 81.1% (Table 2). At 300 mM salt stress, accession 11460 acquired the maximum STTI (27.6%) for plumule dry weight. At 250 mM NaCl stress, STTI for radicle dry weight ranged from 67% to 97%. At 300 mM NaCl salt stress, accession 11383 acquired the maximum STTI (85.7%) for radicle dry weight followed by 11466 (77.4%).

RAPD analysis

A total of 26 RAPD markers were tested to investigate genetic diversity in selected 41 wheat genotypes. Of these, only 15 produced polymorphic bands and were further used for genetic diversity analysis. These 15 markers generated 142 polymorphic DNA bands of different sizes. The number of bands ranged from 7 (for OPC 6) to 12 (for OPB 8) with an average of 9.5 bands per marker. The salt tolerant cultivar SARC IV produced the maximum number of bands (105) with 15 markers, followed by accession 11545 (92 bands). Genetic similarity coefficients based on RAPD data ranged from 38 to 95%, indicating the diverse origin of genotypes studied (Fig. 1). Average genetic similarity was 69%. Cluster analysis grouped the studied genotypes into two major clusters A and B. Cluster A consisted of two sub clusters. Cluster A1 consisted of five salt susceptible and three salt tolerant genotypes. Cluster A2 included three salt tolerant genotypes. Cluster B was more diverse as it included 18 salt tolerant and 12 salt susceptible genotypes (Fig. 1). The salt tolerant cultivar SARC IV and accession 11545 were grouped together in cluster B1. Of the seven surviving genotypes at 300 mM NaCl stress, five grouped in cluster B and two in cluster A. All the international genotypes from Iran, Syria and Egypt fell in cluster B. Cluster A included all the genotypes from Pakistan. This indicated the diverse nature of genotypes in different ecological zones of the world. Cluster B1 consisted of seven salt tolerant genotypes. Cluster B2 consisted of three salt tolerant and 10 susceptible genotypes. Cluster B3 included four salt tolerant and two susceptible genotypes. Cluster B4 included only two salt tolerant genotypes. Generally, salt tolerant genotypes tended to cluster together in small groups within the sub clusters. Accessions 11388 and 11399 clustered together in sub cluster 'B2', indicating that these are genetically similar (95% similarity). Accession 10818 from Pakistan was genetically the most distant genotype as it showed the least similarity with all genotypes tested and was placed separately in cluster B. The RAPD Primer OPA 2 produced a unique 1000 bp fragment in tolerant genotypes only. Likewise, RAPD primer OPF 13 generated two fragments of 1200 bp and 1400 bp in some tolerant genotypes but not in susceptible genotypes. These markers further need to be tested on populations segregating for salt tolerance to confirm linkage with genomic regions controlling salt tolerance.

SSR analysis

The 38 SSR markers generated 82 alleles of varying sizes in 41 wheat genotypes (Table 4). Number of alleles ranged from one to four with an average of 2.2 alleles per locus. The salt tolerant accession 11545 amplified maximum (56) bands. The SSR marker *Xwmc 773* amplified a 143 bp DNA fragment in seven salt susceptible genotypes only. Polymorphic Information Content (PIC) value provides information on allele diversity and frequency among different genotypes. PIC value of each SSR marker can be evaluated on the basis of its number of alleles amplified. PIC values varied greatly for all the SSR loci tested. Eight markers (*Xbarc128, Xbarc159, Xbarc273, Xcfd60, Xgwm18, Xgwm133, Xgwm371* and *Xgwm674*) generated single allele and their PIC value was, therefore, 0. PIC value of rest of the 30 markers ranged from 0.27 for *Xwmc367* to 0.73 for *Xwmc44* with an average of 0.39. PIC value of 14 markers was above 0.5 (Table 4). Genetic similarity coefficients for SSR markers ranged from 0.45 to 0.95 with an average of 0.69. Maximum genetic similarity (95%) was recorded between genotypes Maroon (Iran) and 11466 (Egypt), whereas the minimum genetic similarity (45%) was found between the Pakistani salt tolerant accession 11299 and Italian salt susceptible accession 11371. Minimum genetic similarity was generally observed between salt tolerant and

Table 1. Salt tolerance categories of wheat landraces/cultivars on the basis of salt tolerance index (STI).

Salt tolerance category	Range of salt tolerance index	No. of Accessions	Accession (Country of collection)
Tolerant	80-100%	18	11454 (Syria), Maroon (Iran), SARC VII (Pak.), Shorawaki (Pak.), 11299 (Pak.), Roushan (Iran), Chenab (Iran), 11453 (Syria), Chakwal 86 (Pak), Chods (Iran), 11467 (Syria), Sakha-92 (Egypt), 10851 (Pak.), 11401 (Pak.), 11383 (Pak.), Sardari (Iran), Local white (Pak.), 11186 (Pak.), Karaj II (Iran),
Moderately Tolerant	67-80%	47	SARC IV (Pak.), 11526 (Pak.), 11417 (Pak.), 11464 (Syria), Gulestan (Iran), Darab (Iran), 11465(Syria), Bayat (Iran), 11409 (Pak.), 11385 (Pak.), 10859 (Pak.), 10783 (Pak.), 11460 (Syria), 11458 (Syria), Pasban 90 (Pak.), Omid (Iran), 11466 (Egypt), Arvand (Iran), Pavon (Pak.), 11522 (Pak.), 10831 (Pak.), 11416 (Pak.), LU-26 (Pak.), 10850 (Pak.), 11546 (Pak.), 11407 (Pak.), Parula (Pak.), 10807 (Pak.), 11459 (Syria), 11545 (Syria), Rasool (Iran), Sabalan (Iran), Falat (Iran), 11384 (Pak.), 11287 (Pak.), Azadi (Iran), 10824 (Pak.), 11456 (Syria), 10833 (Pak.), 10792 (Pak.), 11478 (USA), 10854(Pak.), 11900 (Egypt), 10828 (Pak.), 11897 (Egypt), 11918 (Iran), 11214 (Pak.)
Moderately susceptible	54-67%	82	Karaj I (Iran), Bezostaya (Iran), 11406 (Pak.), 10810 (Pak.), Chakwal-97 (Pak.), Yecora 70 (Pak.), 11901 (Italy), 11461 (Syria), 11555 (Pak.), 11400 (Pak.), 11133 (Pak.), 11374 (Italy), 10843 (Pak.), 11372 (Italy), SARC V (Pak.), ERA (Pak.), Sakha-61 (Egypt), 11415 (Pak.), 11408 (Pak.), 10832 (Pak.), 11171 (Pak.), 11302 (Pak.), 11193 (Pak.), SARC I (Pak.), India (Iran), 11414 (Pak.), 10840 (Pak.), 11248 (Pak.), 11244 (Pak.), 10862 (Pak.), 10803 (Pak.), 11457 (Syria), 10829 (Pak.), 10791 (Pak.), 10790 (Pak.), 10809 (Pak.), 11386 (Pak.), 11403 (Pak.), 10767 (Pak.), Tabasi (Iran), 11462 (Syria), 10830 (Pak.), 10834 (Pak.), 10772 (Pak.), 11379 (Pak.), 11378 (Pak.), Kayeh (Iran), 11240 (Pak.), 10819(Pak.), Blue silver (Pak.), 10853 (Pak.), Morghan-I (Iran), 11463 (Syria), LU-26S (Pak.), 10815 (Pak.), 10777 (Pak.), 11221 (Pak.), 11382 (Pak.), 11369 (Italy), 10770 (Pak.), Tobari-66 (Pak),11195 (Pak.), 10798 (Pak.), 10771 (Pak.), 10839 (Pak.), 10841 (Pak.), 11242 (Pak.), 11418 (Pak.), 10806 (Pak.), 11380 (Pak.), 10800 (Pak.), 11335 (Pak.), 10813 (Pak.), 11370 (Italy), 10784 (Pak.), SARC III (Pak.), 10821 (Pak.), 10861 (Pak.), 10805 (Pak.), 10835 (Pak.), 10812 (Pak.), Frontana (Pak)
Susceptible	Below 54%	25	11388 (Pak.), 11381 (Pak.), 10788 (Pak.), Hirmad (Iran), 10793 (Pak.), S-24 (Pak.), 10789 (Pak.), 10823 (Pak.), 11387 (Pak.), 11371 (Italy), 10849 (Pak.), 10818 (Pak.), 11373 (Italy), 10775 (Pak.), 10811 (Pak.), 11289 (Pak.), 10801 (Pak.), 10826 (Pak.), 11290 (Pak.), 11399 (Pak.), 10795 (Pak.), Kohistan-97 (Pak.), 11402 (Pak.), 10786 (Pak.)

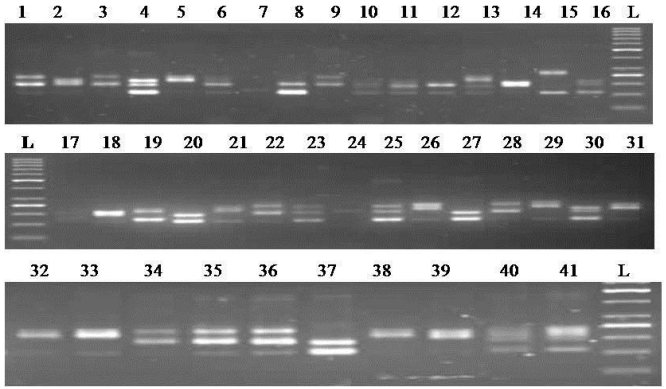

Fig 1. Agarose gel (2.5%) electrophorogram showing PCR products of primer Xwmc661 from DNA of 41 wheat genotypes. The lane L represents 50 bp DNA Ladder (Fermentas Life Sciences)

susceptible genotypes. Accessions collected from the same geographical area were genetically more similar than those collected from different areas. Accessions collected from different countries showed less genetic similarity. Cluster analysis grouped the 41 wheat genotypes into four clusters (Fig. 2). Cluster A consisted of three salt tolerant and five salt susceptible genotypes. The largest and phenotypically the most diverse group was cluster B that included 11 salt tolerant and 11 susceptible genotypes. Cluster C consisted of six salt tolerant and one salt susceptible genotype. Cluster D included two salt susceptible and cluster E included two salt tolerant genotypes only. Significant ($P<0.01$) positive correlation (0.22) was observed between similarity coefficients of RAPD and SSR markers data.

Discussion

Results of the present study indicated that morphological and molecular genetic variation for salt tolerance existed in the wheat genotypes tested. The genotypes responded differently to the different NaCl stress levels. STTI for germination rate under 250 and 300 mM NaCl stress showed that germination was significantly suppressed with increased NaCl concentration. The increase in salinity not only decreased percentage germination but also reduced germination rate. A high germination rate is required to avoid the adverse effect of salt stress at early growth stages.

Sabir and Ashraf (2007) also reported a reduction in germination rate at high salinity levels. Shahzad et al. (2012) reported a reduction in the vegetative growth of wheat landraces at 200 and 250 mM salt stress. They also observed morphological and molecular diversity in the wheat landraces they studied. The STTI values for fresh and dry radicle weights in the present study were always higher than STTI values for fresh and dry plumule weights under NaCl stress. The possible reason for the greater radicle growth compared to plumule may be the fact that under stress condition salt stress induces physiological drought, and plants tend to proliferate roots more at higher stress levels in order to absorb more water. Under stress conditions, root to shoot ratio shifts towards roots and food reserves in seed endosperm are utilized more in root development than in the shoot. As a result, plants having greater radicle weight index tend to show lesser values for plumule weight. Better growth at high salinity levels is required for salt tolerance. Ma et al. (2007) reported that under imposed stress, shoot growth was inhibited more than root growth. El-Hendawy et al. (2005) reported that salt stress decreased dry weight plant^{-1} at all growth stages. Our results confirmed that dry matter accumulation was significantly affected by NaCl stress beyond 200 mM concentration. Furthermore, the proportion of dry weight allocated to roots increased with increasing NaCl levels. Therefore, an increased root/shoot ratio seems to be an adaptation to salinity, resulting in more efficient water and nutrient uptake under salinity stress. Ali et al. (2009, 2011) reported the root/shoot ratio to be important selection criteria for drought tolerance in 16 sorghum accessions including landraces. Based on STI, wheat varieties Chenab, Local white, Maroon, Shorawaki, SARC VII and accessions 11453, 11299, 11417, 11454 and 11466 were relatively salt tolerant under 200 mM NaCl stress. Accession 11466 was the most salt tolerant at 250 mM NaCl stress, followed by 10841, 10824, 11383 and 11385. At 300 mM NaCl stress, accession 11299 ranked as the most salt tolerant, followed by 11466, Karaj II, 11460, Maroon, 11454 and 11383, indicating that these accessions have the potential to tolerate high salt stress at germination and early seedling stage. Maroon, 11383 and 11466 consistently performed well under all three salt stress levels. These genotypes can be used in hybridization programs to incorporate salt tolerance into future wheat varieties. Relatively large number of alleles (8.87 per primer) was detected by RAPD analysis than previously reported 5.5 alleles per primer (Rahman et al., 2004). However, Ojaghi and Akhundova (2010) reported 15.2 alleles per primer. The salt susceptible accession 10818 had the least similarity with salt tolerant accessions 10790 and 11299, indicating the reliability of RAPD markers for assaying genetic variation for salt tolerance in wheat. Results of RAPD analysis showed that maximum salt tolerant genotypes were concentrated in cluster B. Salt tolerant genotypes grouped into two clusters having maximum genetic divergence and could be used efficiently in breeding programs for salt tolerance. Our results are in disagreement with the findings of Cao et al. (1999), Mukhtar et al. (2002), Rahman et al. (2004) and Ahmed et al. (2010) who reported less genetic variation in the wheat material tested.

The possible reason for this disagreement is probably that we used landraces that have not gone through artificial selection pressure and are, therefore, more diverse. Secondly, the wheat material used was from different geographical regions, thus having relatively large genetic variation. However, our results are in agreement with the findings of Bibi et al. (2009) and Ojaghi and Akhundova (2010) who reported high molecular genetic variation in wheat genotypes. Simple sequence repeats analysis revealed that number of alleles ranged from one to four with an average of 2.2 alleles per marker. Almanza-Pinzon et al. (2003) reported 2 to 4 alleles per locus in 70 wheat genotypes with 37 SSR markers. Ahmad (2002) reported 3.6 alleles per locus in 13 wheat genotypes with 43 SSR markers, whereas Singh et al. (2006) reported that number of alleles ranged from 2 to 6 with an average of 2.9 alleles per SSR locus. Genetic similarity coefficients for SSR markers ranged from 0.45 to 0.95, indicating the presence of genetic variation in the genotypes tested. Singh et al. (2006) reported an average PIC value of 0.47 using 21 SSR markers. Similarly, Almanza-Pinzon et al. (2003) reported an average PIC value of 0.45 with 37 SSR markers. Cluster analysis revealed that some accessions from the same geographical region were grouped together while others showed more deviation. This may be due to the reason that genotypes in the same geographical region have greater chances of being descended from similar ancestors. The relative ranking of some genotypes also varied between different treatments, which may be due to the large genetic variability often present in landraces and the complex nature of salt tolerance mechanisms.

However, the salt tolerance response of some of the genotypes was stable across different NaCl concentrations. These genotypes showed the potential to have good plant stand establishment and biomass production at early seedling stage, which contribute towards high production under salt stress. Dry matter production is a useful criterion to evaluate salt tolerance because it permits direct estimation of economic return under saline conditions (Mass, 1986). RAPD analysis was found useful to evaluate salt tolerance in wheat but efficiency of RAPD depends upon right choice of the RAPD primers. Salt tolerant genotypes having maximum genetic variation could be efficiently used to broaden the genetic base for salt tolerance breeding.

Table 2. Salt tolerance trait indices (STTIs) of 5 traits studied on 34 wheat landraces/cultivars at 250 mM NaCl stress.

Sr. No.	Accession No	Area of Collection	Germination rate	Plumule Fresh Weight	Radicle Fresh Weight	Plumule Dry Weight	Radicle Dry Weight	STI	Rank
1	10812	Faisalabad (Pak)	15.2	10.0	93.3	9.2	96.0	44.7	25
2	10824	Faisalabad (Pak)	24.8	33.6	99.9	51.2	94.0	60.7	3
3	10828	Faisalabad (Pak)	20.1	26.8	86.1	8.5	85.0	45.3	24
4	10831	Faisalabad (Pak)	12.8	25.9	87.1	28.6	94.0	49.7	21
5	10833	Faisalabad (Pak)	57.9	38.8	47.4	53.8	96.0	58.8	6
6	10841	Faisalabad (Pak)	56.3	30.9	84.3	54.2	83.0	61.7	2
7	10850	Faisalabad (Pak)	14.9	9.3	47.0	7.2	84.0	32.5	32
8	10851	Faisalabad (Pak)	22.2	29.5	81.2	34.7	89.0	51.3	17
9	11186	Kharan (Pak)	32.5	12.1	94.6	31.3	94.0	52.9	12
10	11214	Loralai (Pak)	34.4	9.8	76.9	14.3	96.0	46.3	22
11	11287	Pishin (Pak)	38.2	11.1	98.1	18.8	86.0	50.4	19
12	11299	Chagi (Pak)	28.3	22.8	95.4	22.6	89.0	51.6	15
13	11383	Hyderabad (Pak)	33.5	22.4	85.5	64.3	96.0	60.3	4
14	11385	Nausharo Feroz (Pak)	36.5	33.5	87.3	53.7	89.0	60.0	5
15	11401	Larkana (Pak)	7.4	2.6	84.7	0.0	77.0	34.3	31
16	11409	Jacobabad (Pak)	31.4	4.0	78.9	63.1	87.0	52.9	13
17	11417	Bhakkar (Pak)	27.6	0.0[x]	0.0 [x]	0.0 [x]	0.0 [x]	0.0 [x]	0 [x]
18	11453	Syria	19.0	21.4	90.8	27.0	95.0	50.6	18
19	11454	Syria	25.8	13.7	82.8	5.9	75.0	40.6	27
20	11460	Syria	53.1	10.9	80.1	32.9	80.0	51.4	16
21	11466	Egypt	33.3	56.9	80.7	81.1	87.0	67.8	1
22	11478	Italy	40.0	37.5	84.0	41.1	87.0	57.9	8
23	11545	Syria	7.8	13.3	78.0	21.8	79.0	39.9	29
24	Sakha-92	Egypt	15.9	15.7	98.2	41.0	94.0	53.0	11
25	Karaj II	IRAN	31.4	13.8	79.1	11.6	81.0	43.4	26
26	Chenab	IRAN	21.7	0.0	0.0	0.0	0.0	0.0	0
27	Maroon	IRAN	43.4	25.9	81.8	33.6	97.0	56.3	10
28	Roushan	IRAN	92.6	15.8	77.7	23.2	82.0	58.3	7
29	4098775	Faisalabad (Pak)	26.1	0.0	77.1	0.0	80.0	36.6	30
30	4098805	Faisalabad (Pak)	20.1	30.6	85.7	19.6	72.0	45.6	23
31	Pasban 90	Pakistan	18.1	52.2	76.8	54.3	81.0	56.5	9
32	SARC IV	Pakistan	45.4	15.3	66.7	0.0	67.0	38.9	28
33	SARC VII	Pakistan	26.2	52.0	74.6	31.1	77.0	52.2	14
34	Shorawaki	Balochistan (Pak)	46.5	20.7	87.4	9.3	86.0	50.0	20
SE_{diff} [x]			2.74	2.5	5.40	3.59	5.48	3.15	

[x] Standard error of the difference between STTI/STI.

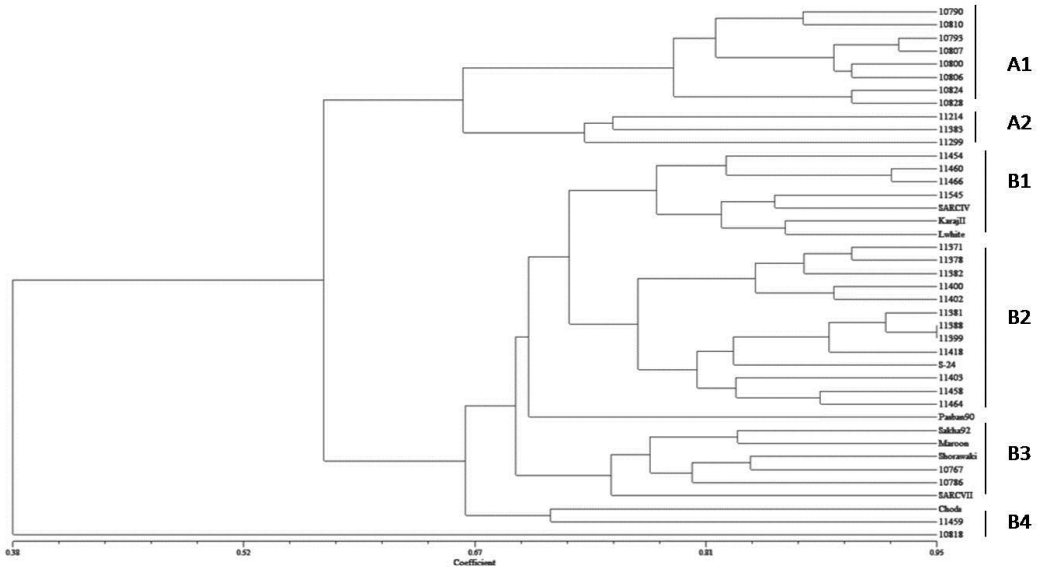

Fig 2. Genetic relationship determined by UPGMA cluster analysis of Dice Similarity Coefficient based on RAPD data for 41 wheat genotypes

The salt tolerant landraces identified in this study need to be further tested at seedling stage in hydroponics and at maturity in the field to make them a valuable genetic resource for incorporating salt tolerance traits in future wheat cultivars of Pakistan and elsewhere. The present study revealed genetic variation for salt tolerance in wheat genotypes based on morphological and RAPD as well as SSR marker data.

Although, significant positive correlation was observed between the similarity coefficients of both marker types but the correlation coefficient was small. However, the ranges of similarity coefficients for the two marker systems were almost similar, indicating that both types are equally good for genetic diversity analysis. A relatively high polymorphism was detected in the landraces than in the cultivars as revealed by average allelic richness. This indicated that modern plant breeding has reduced genetic variability in the commercial wheat cultivars. Salt tolerant genotypes produced more alleles than sensitive ones. Generally, salt tolerant cultivars tended to cluster together, indicating less genetic variation among them. Moreover, landraces generally clustered far from modern cultivars, indicating that these two are genetically more divergent. Therefore, the landraces still provide a largely unexplored gene pool with great potential for broadening the genetic base of modern wheat cultivars. A salt tolerant cultivar 'Pasban 90'(Mujeeb-Kazi and Diaz de Leon, 2002) from Pakistan was found genetically different from all other cultivars studied. This cultivar, along with other salt tolerant genotypes should be used in breeding programs for improving salt tolerance in future wheat cultivars. Four RAPD primers OPA 2, OPB 7, OPB 11 and OPF 13 generated specific fragments in salt tolerant genotypes only. These unique DNA fragments should be further studied in segregating populations to identify genomic regions controlling salt tolerance in wheat. Once the linkage of these fragments with genomic regions is confirmed, these can prove useful in Marker assisted selection of salt tolerance in wheat.

Materials and methods

Plant material

Seeds of 172 wheat landraces/varieties (Table 1) including 7 check cultivars/lines, namely, Shorawaki, Pasban 90, SARC III, SARC IV, SARC VII, LU 26S and LU 26, were acquired from Plant Genetic Resources Program, National Agricultural Research Centre, Islamabad; Saline Agriculture Research Center (SARC), University of Agriculture, Faisalabad; and Ayub Agricultural Research Institute, Faisalabad. The landraces/cultivars included 123 accessions from Pakistan, 23 from Iran, 13 from Syria, 5 from Egypt, 7 from Italy and 1 from the USA.

Morphological screening

Ten seeds of each accession were surface sterilized and sown following the method described by Tlig et al. (2008). Seeds of all genotypes were surface sterilized in 0.58% sodium hypochlorite solution for 1 minute; subsequently washed 4-5 times with distilled water and air dried for germination experiments. In the first phase of the study, all accessions were tested at two treatments: control (distilled water) and 200 mM salt (NaCl) solution.

Seeds of each genotype were germinated in filter paper lined petri dishes at room temperature. The experimental design was a factorial combination of treatments and genotypes arranged in a completely randomized design with three replications. A seed was considered germinated when plumule was longer than half of the length of the seed, and the radicle was equal to or longer than the seed length. Data were recorded on germination rate, plumule fresh and dry weights and radicle fresh and dry weights. Germinating seeds were daily counted till a constant number. Germination rate (GR) was calculated as:

$$GR = X_i / Y_i + X_{ii} / Y_{ii} + \cdots\cdots\cdots + X_n / Y_n$$

(Maguire, 1962)

where X is the number of seeds germinated for the day, Y is number of days from the first seed germination and i, ii…n are number of days. Eight days old plumules and radicles, emerged under control and 200 mM NaCl stress, were weighed and then oven-dried at 70°C, following which dry weights were recorded. Salt tolerance trait index (STTI) at germination was calculated according to the formula of Ali et al. (2007):

$$STTI = \frac{Value \cdot of \cdot trait \cdot under \cdot salt \cdot stress}{Value \cdot of \cdot trait \cdot under \cdot control} \times 100$$

Salt tolerance index (STI) was calculated as the mean of salt tolerance trait indices (STTIs). STIs were used to group the 172 wheat genotypes into four classes (Table 2).

In the second experiment, 50 genotypes were selected from the four previously defined classes (salt tolerant, moderately salt tolerant, moderately susceptible and susceptible), and were evaluated at 250 and 300 mM salt stress. Of these, 34 genotypes survived at 250 and 7 at 300 mM salt stress. Data for 250 and 300 mM salt stress were collected and analyzed as previously described. STTI values were separated by standard error of mean. The STI data for 34 surviving genotypes were used to rank these genotypes at 250 mM NaCl stress.

RAPD and SSR analysis

Based on the relative performance at 200, 250 and 300 mM NaCl stress, 41 genotypes including 24 salt tolerant and 17 salt susceptible were selected for molecular diversity analyses. Genomic DNA of these genotypes was isolated from fresh leaves. DNA isolation, purification, and quantification were carried out following Shah et al. (2009). PCR reactions were carried out according to the method described by Shah et al. (2009) with minor modification. PCR products were separated on 1.5% agarose gel. PCR products were visualized under UV pro transilluminator to capture gel image. A total of 26 RAPD and 53 SSR markers were used to study the genetic diversity in 41 wheat genotypes. Only 15 RAPD markers including OPA 1, OPA 2, OPA 3, OPA 5, OPA 10, OPA 13, OPA 18, OPB 1, OPB 7, OPB 8, OPB 11, OPC 6, OPE 1, OPF 13 and OPJ 13 produced polymorphic bands and were used for diversity analysis. Primer sequences and genomic locations of SSR markers were obtained from http://wheat.pw.usda.gov. Thirty eight SSR markers were polymorphic among the 53 tested.

Table 3. Salt tolerance trait indices (STTIs) of five traits studied on seven wheat landraces/cultivars at 300 mM NaCl stress

S. No.	Accession No	Area of Collection	Germination rate	Plumule Fresh Weight	Radicle Fresh Weight	Plumule Dry Weight	Radicle Dry Weight	STI	Rank
1	11299	Chagi (Pak)	32.6	21.0	90.0	10.3	57.4	42.3	1
2	11383	Hyderabad (Pak)	4.0	4.8	45.0	9.3	85.7	29.8	7
3	11454	Syria	12.6	11.5	72.5	9.5	52.5	31.7	6
4	11460	Syria	17.2	8.2	68.6	27.6	58.5	36.0	4
5	11466	Egypt	18.3	21.7	66.7	17.5	77.4	40.3	2
6	Karaj II	Iran	31.4	10.5	70.6	8.3	64.9	37.1	3
7	Maroon	Iran	17.8	15.5	68.2	5.3	57.4	32.8	5
SE_{diff}[x]			3.81	2.41	4.97	2.87	4.62	1.73	

[x]Standard error of the difference between STTI/STI

Table 4. SSR markers along with their annealing temperatures (T_A), allele size, chromosome number, number of polymorphic alleles detected (P. Allele), and polymorphism information content (PIC)

Sr. No.	Locus/Marker	T_A	Size	Location	P. Allele	PIC Value
1	*Xbarc12*	52	150-200	3A	2	0.47
2	*Xbarc60*	58	200-250	1B/4B	2	0.41
3	*Xbarc124*	52	220-280	2D/2A	2	0.47
4	*Xbarc128*	52	300	2B/5BL/7D	1	0.21
5	*Xbarc151*	55	200-250	5A/7A	2	0.50
6	*Xbarc159*	52	175-240	2B/2D	1	0.23
7	*Xbarc273*	52	225	6D	1	0.22
8	*Xcfd13*	60	254-322	6B/6D	2	0.50
9	*Xcfd19*	60	267-294	1D/5D/6D	2	0.49
10	*Xcfd49*	60	214-324	6D	3	0.61
11	*Xcfd60*	60	218	6D	1	0.19
12	*Xcfd66*	60	202-276	7D	3	0.62
13	*Xgwm02*	52	132-208	3A	3	0.62
14	*Xgwm18*	52	186	1BL	1	0.24
15	*Xgwm111*	55	150-200	7B/7D	2	0.49
16	*Xgwm133*	60	175	3AL	1	0.20
17	*Xgwm148*	60	150-200	2B	2	0.46
18	*Xgwm174*	55	150-200	5D	2	0.49
19	*Xgwm210*	55	150-220	2A	2	0.48
20	*Xgwm249*	55	130-180	2A	2	0.41
21	*Xgwm296*	55	130-250	2D/7D	4	0.64
22	*Xgwm304*	55	200-250	5A	2	0.46
23	*Xgwm314*	55	100-200	3D	2	0.52
24	*Xgwm371*	60	200	5B	1	0.26
25	*Xgwm413*	60	100-150	1A/1B	2	0.46
26	*Xgwm455*	55	120-180	2D	3	0.56
27	*Xgwm539*	60	100-150	2D	2	0.31
28	*Xgwm604*	50	100-180	5B	3	0.47
29	*Xgwm674*	60	130	3A	1	0.22
30	*Xwmc44*	61	242-432	1B	3	0.73
31	*Xwmc169*	61	167-245	3A	3	0.65
32	*Xwmc170*	61	230-450	2A/2D	3	0.52
33	*Xwmc367*	61	154-191	1B	2	0.27
34	*Xwmc407*	61	135-154	2A	2	0.50
35	*Xwmc419*	61	141-185	1B/4B/6B	2	0.41
36	*Xwmc661*	61	226-458	2B	4	0.64
37	*Xwmc764*	61	180-220	2B	2	0.46
38	*Xwmc773*	61	143-323	5B/6D	3	0.52

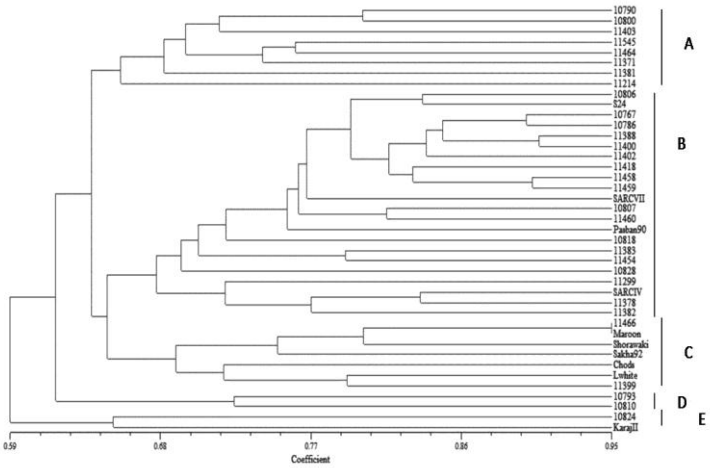

Fig 3. Genetic relationship determined by UPGMA cluster analysis of Dice Similarity Coefficient based on SSR data for 41 wheat genotypes

Statistical analysis

The morphological data from all experiments were analyzed using the GLM procedure in SAS (SAS Institute 2003). The means obtained were separated by standard error of the difference between means. For statistical analysis of RAPD markers, the score able bands were considered as a single locus. The loci were scored as present (1) or absent (0). RAPD analysis was done using the DICE coefficient (NTSYS-pc version 2.2) as described by Nei and Li's (1979). The similarity matrix obtained was then used to construct a dendogram. For SSR method, each fragment generated by a primer pair was considered to be an allele of the same marker locus. The diversity analysis based on SSR markers was done using Simqual sub program in similarity routine of NTSYS pc version 2.2 (Rohlf, 2005). Polymorphic Information Content (PIC) value of the SSR marker was calculated following Anderson et al. (1993). Pearson's correlation coefficient was calculated to determine the relationship between similarity coefficients of RAPD and SSR data.

Conclusion

The present study was conducted to assess genetic variation for salinity tolerance in 172 spring wheat genotypes based on morphological and DNA marker data. Morphological data

were obtained by screening the genotypes under control and NaCl stress. Morphological and molecular genetic variation was observed for salt tolerance in the wheat genotypes tested. Egyptian accession 11466 and Pakistani accession 11299 were the most salt tolerant wheat genotypes. Three unique DNA fragments were produced from the RAPD primers OPA 2 and OPF 13 in some tolerant wheat genotypes only. These fragments should be further studied to confirm their association with genes for salinity tolerance. The salt tolerant landraces identified in this study could be used as parents to incorporate salt tolerance in future wheat cultivars.

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