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Bulk Segregate Analysis Markers in Response to Salinity Tolerance Genes in Wheat (*Triticum aestivum*)

Ayman Amin

Department of Plant Physiology,
Faculty of Agriculture, Cairo University, Giza, Egypt

Abstract: Increasing salinity tolerance of wheat become an important issue for many wheat breeding program, as salinization severely affects plant productivity in many regions of the world, both in irrigated and dry-land agriculture. The aim of this study was to investigate natural allelic variants contributing to quantitative variation for salinity tolerance in bread wheat, a molecular marker-assisted breeding approach was used to analyse 154 F_6 composite population under saline hydroponics at a concentration of 150mM NaCl and their eight parents, to detect areas of the genome that may be linked to salinity resistance genes using molecular markers. Bulked segregate analysis (BSA) was employed on two bulks constituted by separately the 20 most-tolerant and the 20 most susceptible individuals. Both parents and individuals from each pool were screened with 175 Simple sequence repeats (SSR) markers and Chi square analysis was used to determine significant segregation events. Nine SSR markers were detected on chromosomes 7A, 2A, 3A, 4B, 7D, 1B, 2B and 4D that were significantly segregation events. Also five RFLP markers were significantly loci segregate on i.e.3B, 4B, 7D and 4A. This study demonstrates a new method of use BSA in plant breeding, which may help in detecting salt stress tolerance or other quantitative traits.

Key words: Bulked segregate analysis (BSA) · Salinity tolerance · Wheat · Molecular marker · SSR · *Triticum aestivum*

INTRODUCTION

Salinity is one of the most prevalent abiotic stresses affected on Earth's surface. As salt tolerance is a quantitative trait controlled by many genes, using molecular marker technologies give the physiologist the opportunity to enhance the efficiency of conventional plant breeding by tightness between DNA markers to the trait of interest. Molecular genetic mapping in wheat has been developed since 1960s when pioneering work on identification and chromosomal mapping of isozymes [1]. Detailed RFLP linkage and physical maps of wheat, comprising more than 2000 DNA markers have been published covering all seven homologous groups in wheat. Recent advances in genome mapping techniques based on the polymerase chain reaction (PCR) [2-7], have accelerated a novel DNA marker system, termed SSRs [8]. This is much easier to perform than RFLP analysis and show much higher levels of polymorphism and in formativeness in hexaploid wheat than any other marker system [9, 10]. Nowadays, the availability of detailed linkage maps of molecular markers makes it possible to dissect quantitative traits into discrete genetic factors, called Quantitative Trait Loci (QTL) [11]. The genetic analysis of quantitative traits under stressed environments and salt stress in particular has had little investigation until a few years ago. Stress response traits exhibit continuous variation and are caused by the segregation of many genes [12]. With this type of work it is difficult to distinguish between those traits that will actually improve yield under abiotic stresses and other stress response traits that have no effect on yield [13]. However, QTL analysis can make a considerable contribution to increasing the efficiency of breeding varieties with improved responses to abiotic stresses; e.g. drought and salinity [14, 15]. One of the most important

Corresponding Author: Ayman Amin, Department of Plant Physiology, Faculty of Agriculture, Cairo University, Giza, Egypt. E-mail: ayman_amin_3@hotmail.com.

goals of QTL mapping is the possibility of using the information obtained on gene number and their effects for plant improvement via marker-assisted selection [16]. Also, this is the area of molecular marker technology which is likely to help bridge the gap between detection and determination of the exact chromosomal location and cloning of a QTL [17]. However, the main factor contributing to the slow progress in mapping QTL for salinity tolerance relaying on the low heritability of yield itself under stress conditions and difficulty in distinguishing between those traits that will actually improve yield under stress and traits that have no effect on yield [13].

Development of wheat varieties having salt tolerance potential using conventional breeding methodologies is complicated as tolerance to salinity is controlled by many genes. The use of genetic and genomic analysis to help identify DNA regions tightly linked to quantitative traits in crops, called "molecular marker-assisted breeding", can facilitate breeding strategies for wheat improvement [18]. The use of molecular markers for the indirect selection of improved wheat speeds up the selection process by alleviating time-consuming approaches of direct screening under greenhouse and field conditions. Although the effect of salinity is also dependant on environmental factors, such as the strength and the time of salinisation, the characterisation of these genes would aid the development of salt tolerance cultivars [19]. This has been attempted with some success, for instance, in wheat; it has been shown that chromosome 4D holds genetic factors influencing sodium exclusion [15]. Nevertheless, the exact locations of most genes that control salt tolerance remain undefined.

The use of BSA assisted with molecular markers may offer a good opportunity to provide a rapid method with an efficient strategy for identifying DNA markers linked to regions of interest using as MAS. To perform BSA, DNA samples from individuals that are phenotypically similar or identical are bulked together. For instance, tall and short plants are bulked separately. The theory states that the two bulks should be genetically dissimilar in the region controlling the trait examined and are seemingly heterozygous at all other points [20]. Differences are detected by examining the marker patterns of the two bulks. If the marker appears only in one bulk and not in the other on statistical basis, then the position of the marker indicates a part of the genome that may be linked to the trait studied.

Two types of application of the BSA technique are possible depending on whether the plants are derived from a cross between two parental lines using Quantitative traits loci (QTLs) analysis, or from a population of plants with diverse genetic backgrounds such as composite populations using Chi squared statistical analysis [21]. In both applications, when using co-dominant markers (e.g. RFLPs or SSR) with bulks of genetically diverse individuals, where several marker alleles may be present, more than 15 individuals would need to be combined to ensure that each allele frequency represented that in the population as a whole [22].

MATERIALS AND METHODS

This research experiment was carried out at John Innes Center, UK. The F6 seeds for 154 lines were supplied from the germplasm store at John Innes Centre Norwich Research Park, England. The eight parents used for the crossing research were selected either because of their known salt tolerance or because of their good agronomic adaptation. Sources for Salt tolerance are KTDH 19 (UK, double haploid line), KTDH 59 (UK, double haploid line), KRL3-4 (Indian), SARCI (Pakistan) and Meteor (UK, old variety, tall). The KTDH lines are derived from the parents Kharchia and TW161, Kharchia has tolerance against high salt concentration in the tissues, whereas TW161 excludes Na^+ ions from the leaves. Sources for adaptation and agronomy are Blue Silver (CIMMYT selection), Punjab 85 (Pakistan, semi-dwarf), WH157 (Indian, semi-dwarf). In previous work, Each KTDH line was crossed with every other line. The Fl generation from each KTDH containing line was then crossed with the F1 generation from the non-KTDH lines, producing a total of 54 F2 lines. The 154 lines represent the highest salt tolerant lines selected until the F5 generation. This took place in glasshouse under saline hydroponics at a concentration of 150mM NaCl. F6 seeds were collected from self pollinated plants under normal condition to increase the seed number from each line.

Hydroponics Medium Culture: Seeds were placed on filter paper soaked in sterile water and left to germinate in a 20°C incubator for 3 days. For each line, 5 germinated seeds with similar hypocotyl lengths were chosen and planted in horticultural grade sand in pots (7 cm x 7 cm). Pots were placed in the glasshouse and irrigated with tap water everyday for one week, with a day/night

Fig. 1: Hydroponics culture medium before (a) and after (b) addition of salt addition

temperatures of 20°C/12°C in a hydroponic system following the protocol established by Amin [23] (Figure 1). One seedling was removed, leaving four young plants per pot. Once leaf 2 had emerged in the majority of the population Hoagland's basal salt mixture was added to the irrigation water at half strength. The irrigation solution was changed every 3 days. After leaf 3 was fully measured in most cases, NaCI of 25mM was applied daily until the target concentration of 150 mM NaCl was reached. Measurements of leaf 2 length was made from sand surface to tip. Similarly leaf 5 was measured. Flag leaves were removed after emergence and leaf length was measured from the base of the lamina to leaf tip. Flag leaf samples were collected as they emerged and freeze-dried (-20 °C) for 48 hr. All flag leaf samples were then placed in 5 ml hinged-cap tubes with 3 Tungsten Carbide beads 3mm (QIAGEN) and milled for 5 minutes. The dry weight of the flag leaves was recorded.

Agronomic data i.e. ears emerged, spikelet number and grain number were collected before whole plant were harvested (above ground material was used) and left to dry overnight in a 60°C incubator and then milled in 75x25 mm milling tubes, with 8mm beads again for 5 minutes and between 0.01-0.04 g of dry matter was weighed out.

All samples were placed in porcelain crucibles and incinerated at 550°C for 5 hours. After cooling, the ash was dissolved using nitric acid and diluted to 15 ml using ddH_2O. Sodium and potassium ions were measured using a flame photometer. In each case readings were compared to a predetermined calibration curve to obtain the ion concentration in mmols. NaCI and KCI standards used to create the calibration curve were prepared at 0.2 mM intervals ranging from 0 to 2 mM.

RFLP Markers: DNA was cut using the following 6-base cutting enzymes – *EcoRI, EcoRV, DraI, HindIII, BamHI*

and *Bgl II*. Restriction reactions were set up in a final volume of 25 µl as follows. This mixture was incubated overnight at 37 °C and the reaction terminated by the addition of 5 µl of loading buffer (40% sucrose in 1 x TE, 0.25% bromophenol blue). The digestion was checked by running out 5 µl of the product on a 0.8% agarose gel containing 5 µl ethidium bromide (10mg/ml) per 100 ml of gel solution.

Southern Blotting: The products of digestion were run overnight at 25 V. When the blue dye had migrated 10 cm the electrophoresis was stopped. The gels were then cut and depurinated by incubation in 0.25 M HCl until the blue tracking dye turned yellow.DNA was transferred onto nylon "Hybond N^+" membrane (Amersham) by the alkaline method [24].

Pre-Hybridization of Filters: Filters were placed in boxes and covered with pre-hybridization solution containing 20% (v/v) 5xHSB (3M NaCl, 100 mM PIPES, 20 mM EDTA, pH 6.8), 10% (v/v) Denhardt's III reagent (2% gelatin, 2% Ficoll-400, 2% PVP-360, 10% SDS, 5% $Na_3PO_4.10H_2O$) and 0.0005% (v/v) salmon testes DNA. The box was incubated for 2-3 h or overnight at 65°C with gentle shaking.

Probe Labelling and Hybridization: Radiolabelling of probes was performed as described in Feinberg and Vogelstein [25]. 25 ng of the insert DNA (for 1-3 filters) in a volume of 15 µl was denatured at 100°C for 5 min and quickly chilled on ice to avoid reannealing of DNA strands. The following components were then added: 2 µl bovine serum albumin (10 mg/ml), 5 µl oligolabelling buffer, 2µl of Klenow polymerase (6u/µl) and 1 µl of ^{32}P-αCTP. Labelling was carried out for 2 h at 37°C.

The labelled probe was denatured for 5 min by adding 1/10 volume of 3 M NaOH and then added to the box with prehybridized filters. Hybridization was carried out overnight at 65°C with gentle agitation. After hybridization, the radioactive solution was discarded and filters were washed twice in 2 x SSC/0.5% SDS followed by two washes in 0.2 SSC/0.5% SDS. All washes were performed at 65°C for 15 minutes each. The filters were placed in a sandwich of 3MM Whatman paper, wrapped in Saran wrap and exposed to Kodak BioMax film between two intensifying screens (Fuji High speed X) inside a cassette at -70°C. The time of exposure depended on the radioactivity on the filters. For example, filters of 1-2 counts per second (cps) were exposed for 7-10 days, 7-10 cps – 3 days, 20-50 cps 5 -7 h, etc. Autoradiographic films were developed in an RGII Fuji X-ray developer using the manufacturer's recommended reagents. Filters were stripped for further use by washing four times (5min each) in boiling 0.1 x SSC/0.5% SDS, blotted dry with 3MM Whatman paper, wrapped in Saran Wrap and stored at –20°C. For RFLP analysis, only wheat genomic PSR probes were employed in this study. All of them are available in the Crop Genetics Department of the John Innes Centre.

SSR Markers Screening and Bulk Segregant Analysis: Once the 5th leaf had emerged for most of the plants, a side shoot was collected from each individual plant and stored at –70°C for DNA extraction. Based on the results from salinity screening of 154 F6 lines each were selected from putative salt tolerant and intolerant extremes of the populations and designated salt resistant or susceptible (S) lines. Then, DNA was extracted from the individual plants of the two bulk materials using a Mini-kit (QIAGEN), following the manufacturer's instructions. Concentrations of DNA were assessed by viewing 2 µl of DNA solution on a 0.8% agarose gel with lambda DNA, i.e., 25, 50, 100 and 150ng. DNA was then diluted with 1x TE buffer to a final concentration of 20 ng/µl. The eight parents were screened with 175 microsatellite primers. One hundred and ten SSR markers that were polymorphic between the 8 parents were used to screen the bulks from the F6 generation. Bulks were made according to the grain number. Trait was ranked from highest to lowest and the top 20 and bottom 20 lines were analysed. Therefore, for both tolerant and susceptible plants 40 DNA individuals were subjected to PCR. The PSP primers were used for the microsatellite screening, were developed in John Innes Centre by Mike Gale's group the rest of SSR markers available in the public domain (http://wheat.pw.usda.gov/GG2/index.shtml).

DNA was plated on a 96 well microtitre plate in 3 µl aliquots (25 ng/ µl). To each aliquot 17 µl of the PCR master mix was added. The master mix consisted of: 1x PCR buffer supplied with 1.5 mM $MgCl_2$, 2.5 mM of dNTP, 0.1 µM of forward and reverse primers and 1 unit of Taq DNA polymerase. The PCR was carried out as following: For PSP primers: 1 cycle of 94°C for 3 min, followed by 45 cycles of 94°C for 1 min, -33°C decreasing 0.5°C/s, 61°C for 30 sec. +11°C increasing 0.5°C/s to a

final extension of 72°C for 5 min was performed. For Xgwm primers, PCR was carried out as: 1 cycle of 94°C for 3 min, followed by 45 cycles of 94°C for 1 min, 50-65 °C for 1 min. final extension of 72°C for 5 min was performed before samples were placed at 4°C. Amplification products (DNA fragments) were resolved by polyacrylamide gel electrophoresis using vertical unit and silver staining following the protocol established by Amin [23].

Nullisomic Tetrasomic (NT) Analysis: In some cases, markers showed more than one band (locus) on the gel and it was difficult to identify which chromosome each band was on. In such case, the DNA from Chinese Spring nullitetrasomic aneuploids lines of the 21 chromosomes (John Innes Centre), as well as controls of disomic Chinese Spring, were used to ascertain the identification of the chromosome position for each allele when the band is absent.

RESULTS

Plant Growth Responses Before and after Salt Stress Amongst the F6 Progeny: Plant growth responses are reflected in the leaf length measurements. Leaf 2 was measured before salt stress was applied, so that the plants natural vigour could be ascertained before selection was imposed. The measurements of leaf 5 and flag leaf reflect the plants response to level of salt. With respect to the progeny population, the range of response was more extreme which is reflected in the diversity created by the cross. To test whether the crosses created a large amount of variation, ANOVA was performed (Table 1). As shown in Table1, the results are all significant, indicating a significant amount of genetic variation produced by the crosses.

Ion Distribution in the Parents and Progeny: The concentration of Na and K ions in the whole plant were normally distributed between the lines (Figure 2a, b). However, when the ions in the flag leaf were examined, there w as a skewered curve toward the lower data (Figure 3a, b). This was possibly because plants that have a low Na content are more likely to produce flag leaves and therefore the population sampled would have a higher proportion of plants with low Na concentration. Nevertheless, in all cases the progeny shows a wider range than the parents. This phenomenon is due to transgressive segregation and indicates that ion accumulation is a quantitative trait. When the parents are compared, ion accumulation is not less in salt tolerant parents. Although ion accumulation has been postulated as an important influence on salt tolerance, it is shown in the correlation data that ion accumulation is not a direct measure of salt tolerance.

In the whole plant, sodium level (Na-WP) is negatively correlated with length of leaf 5 (Table 2), as plants with high sodium content are under a higher degree of salt stress and therefore less likely to be capable of producing large leaves later in development.

The reason the lower leaves are unaffected may be as Na^+ accumulates in the plant and the treatment had not taken full effect while these leaves were emerging. Flag leaf Na^+ concentrations (Na-FL) correlate with length of leaves 2 and 5 and with K^+ ion concentration in the flag leaf (K-FL) (Figure 4). The correlations with leaf length could be a consequence of these leaves acting as a reservoir for Na^+ ions. The Na^+/K^+ ratio for the majority of the progeny was 1:1, which is reflected in the median. The range is large, with a minimum Na^+/K^+ ratio of 0.02 and a maximum of 5.3.

Table 1: Analysis of variance for growth response before and after salt stress of F6 composite population.

		Sum of Squares	Degree of freedom	Mean Square	F value	Sig.
Leaf 2	Between Groups	8764.65	153	57.29	5.56	0.
	Within groups	4731.98	459	10.3		
	Total	13496.63	612			
Leaf 4	Between Groups	23215.5	153	151.75	5.63	0.
	Within groups	12376.63	459	26.96		
	Total	35592.13	612			
Flag-leaf	Between Groups	4239.76	108	39.26	1.742	0.002
	Within groups	7237.1	312	22.54		
	Total	11476.86	420			

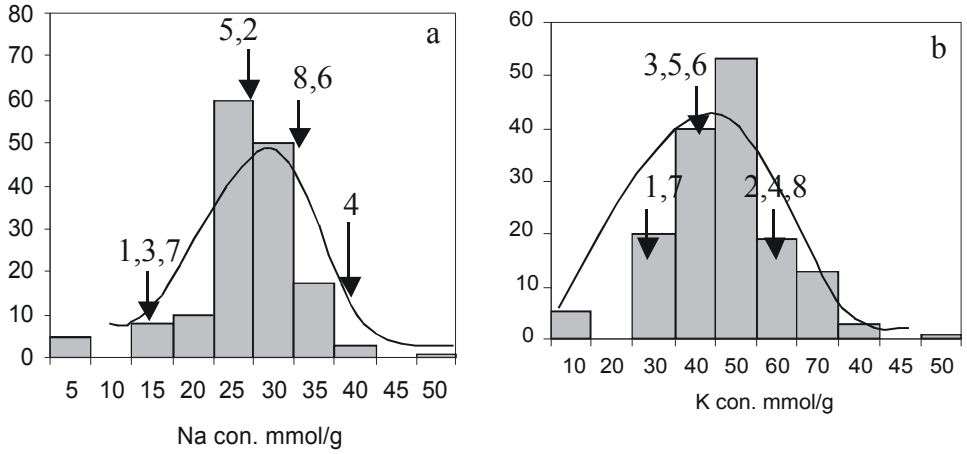

Fig. 2: Distribution of concentrations for Na (a) and K (b) ions in the whole plant. The arrows indicate the parental means, with 1- (KTDH 19), 2- (Blue Silver), 3- (KTDH59), 4- (WH157), 5- (Punjab 85), 6- (Sarc-1), 7- (Meteor), and 8- (KRL3-4). The black lines show the curves for the distributions

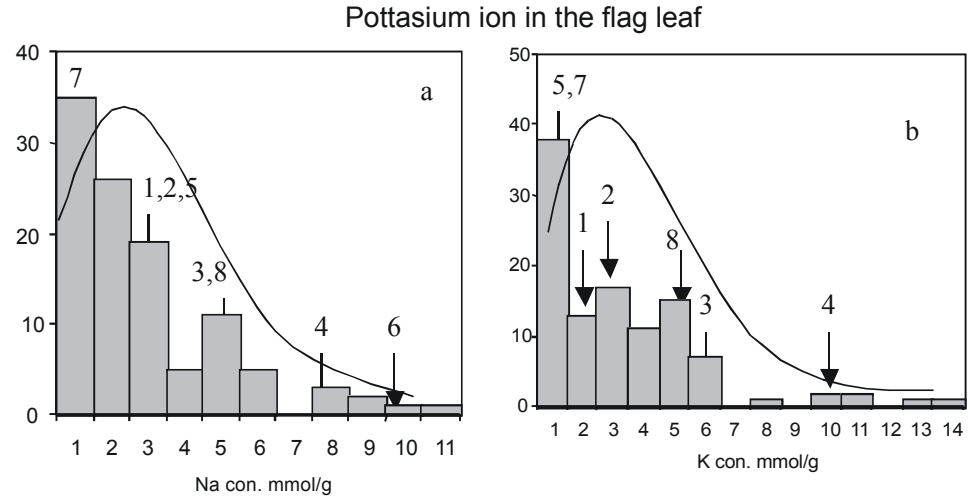

Fig. 3: Distribution of concentrations for Na (a) and K (b) in the flag-leaf. The arrows indicate the parental means, with 1- (KTDH 19), 2- (Blue Silver), 3- (KTDH59), 4- (WH157), 5- (Punjab 85), 6- (Sarc-1), 7- (Meteor), and 8- (KRL3-4). The black lines show the curves for the distributions

Table 2: Correlation coefficients between Na and different characters for 154 F6 lines, in response to salt conditions

	Leaf length 2	Leaf Length 5	Flag-leaf Length	No. ear	No. Spikelet Per Spike	Dry weight	K-WP	K-FL
Na-WP	0.01	-0.45**	0.11	0.06	0.04	-0.07	0.12	0.03
Na-FL	0.21*	-0.26*	0.13	0.04	0.08	-0.06	0.09	0.31**

** Correlation is significant at the 0.01 level

* Correlation is significant at the 0.05 level

Table 3: The RFLP markers that segregated amongst the two bulks for salt response

Marker	Chi square	d.f	*P*	Chr location	Allele MW
Dra1 Psr 567	6.88	2	0.03*	3B	210
Dra1 Psr 574	8.26	2	0.02*	4B	208
BamH1 Psr 115	5.33	1	0.02*	7D	165
HindIII Psr 194	15.39	3	0.00**	4A	210
EcoR1 Psr 967	21.01	3	0.00**	3B	200

* Significant at 0.05 * Salt tolerance parents

** Significant at 0.01 ** Agronomic parents

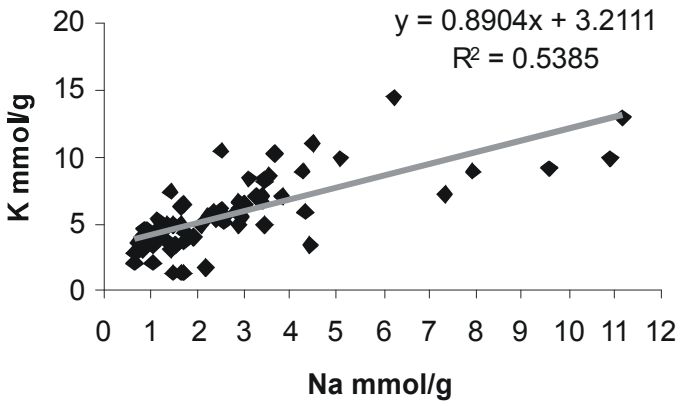

Fig. 4: Relationships between flag-leaf Na content and flag-leaf K content

This 1:1 relationship is not seen in the flag leaf data, the ratio is much higher. The ratio in the flag leaf is 4:1 (Na^+/K^+). This suggests that there is some selectivity over ion transport in the flag leaf. To determine whether the high Na^+/K^+ ratio is due to an increase in Na ion transport or to a decrease in K ion transport, the differences between whole plant and flag leaf data was examined. On average, K seems to experience a larger decrease in concentration than Na. This indicates that it is a decrease in K transport that increases the ratio. The positive correlation between Na^+ and K^+ ions in the flag leaf has been seen in other species, for instance, Gorham *et al.* [26] noted that in barley there was low discrimination between the transport of Na^+ and K^+ to the shoot.

RFLP Results and Chi Squared Analysis: Parents were screened with 66 markers (represents 50 RFLPs and 16 SSRs). All were found to be polymorphic. Because of the limited amount of DNA available from each F2 individual plant only a few Psr markers were screened with the bulked DNA (bulking outlined in Methodology). In each case, the 40 tolerant and susceptible plants for salt stress were individually screened for that marker. Chi squared analysis was used to detect significant segregation events. For each significant marker, the frequency between high and low trait values was significantly different in at least one allele. The alleles giving rise to the significant effect were identified by examining the parental screen (Table 3).

Microsatellite Results and Chi Squared Analysis: Parents was screened with 175 markers and polymorphism were determined (Figure 5). 110 markers were found to be polymorphic and then screened with the bulked DNA (bulking outlined in Methodology). The parental alleles were identified in each line (Figure 6). The values were inputted into a table and Chi squared was used to determine if the segregation pattern was significant (Table 4).

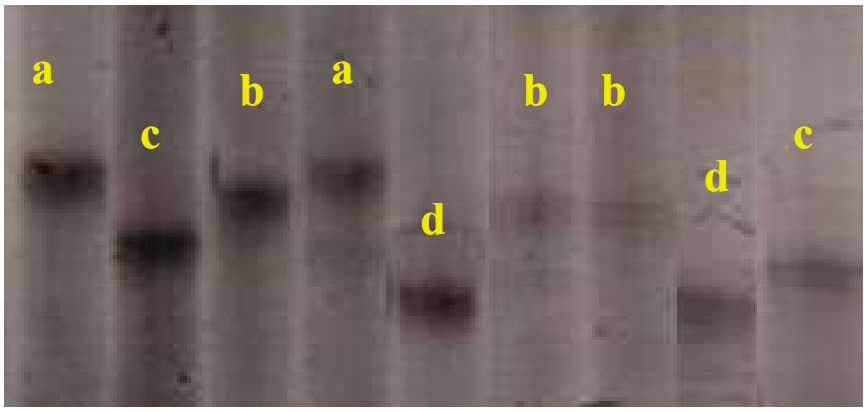

Fig. 5: The polymorphism amongst the 8 parents using SSR markers (PSP 3001)

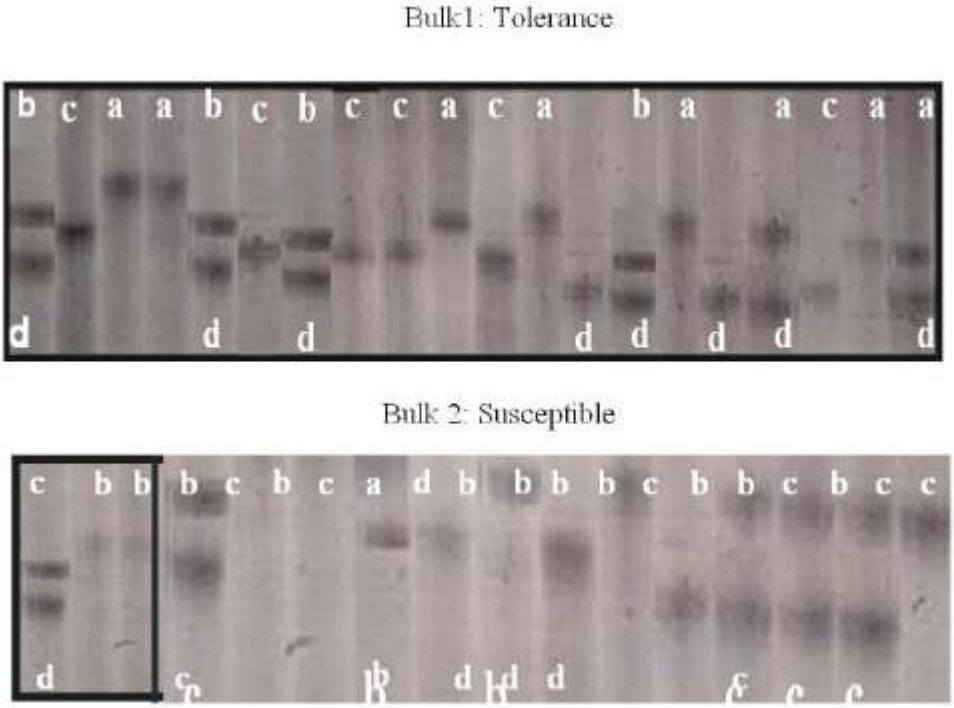

Fig. 6: Segregation of the microsatellites PSP3001 among the individuals from number of grains bulks

Table 4: Segregation pattern for parental alleles with PSP 3001 marker

PSP3001	Observed grain number			
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Allele	High	Low	Total	Expected
A	8	1	9	4.5
B	4	11	15	7.5
C	5	9	14	7
D	8	4	12	6
Total	25	25	50	25
Chi Sq.	d.f.	Prob.		
7.2	2	0.027		

Table 5: The markers that segregated amongst the two bulks for salt response

Marker	Chi square	d.f	*P*	Chr location	Allele MW (Pb)
PSP3001	7.2	2	0.027*	7A	104
PSP3029	5.33	3	0.02*	2A	160
PSP3047	21.01	3	0.00**	3A	170
PSP3159	2.41	2	0.002**	4B	175
Xgwm 192	3.37	2	0.04*	4B	190
Xgwm 276	6.88	2	0.03*	7D	165
Xgwm 18	8.26	2	0.02*	1B	180
Xgwm 55	15.39	3	0.00**	2B	154
Xgwm 192	1.34	2	0.031*	4D	142

* Significant at 0.05

** Significant at 0.01

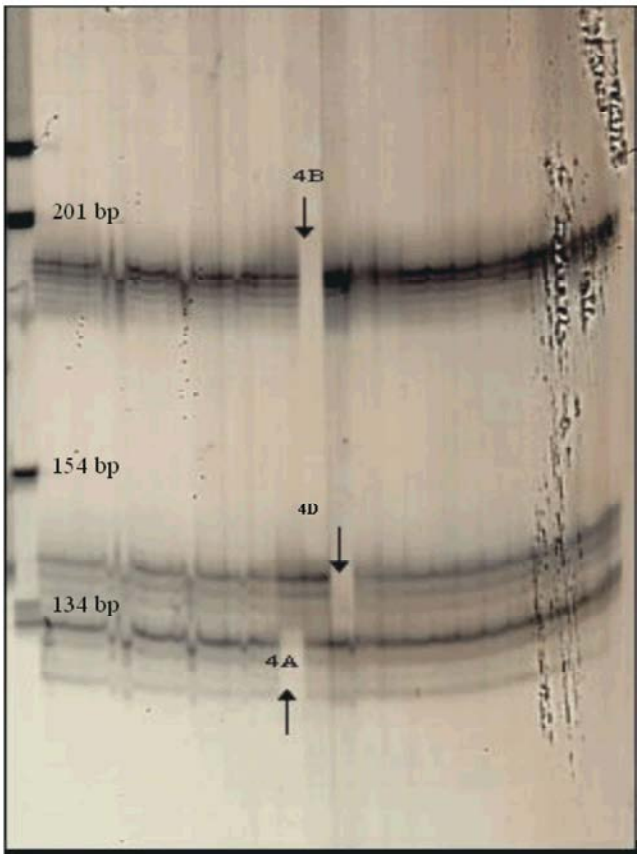

Fig. 7: NT analysis among the 21 chromosome of Chinese Spring with SSR marker xgwm192

With PSP3001 marker the segregation was significant. Table 4 also shows which parental alleles influenced the trait at this genomic position. In this case, allele A mostly occurs in lines that have high grain number. Whereas allele B occurs mainly in lines that have low grain number.

By examining the parental screen, it can be concluded that allele A occurs in KTDH 19 and KTDH 59, therefore, either one or both may hold factor(s) that increase number of grain under salt condition. This analysis was carried out for all markers screened. Nine markers that significantly segregated with traits are listed in Table 5. These markers could be linked to genes affecting these traits under saline conditions.

To determine the exact locations of these factors, more markers must be screened in the relevant areas for confirmation.

A Nullisomic-tetrasomic (NT) results represent a perfect way for assigning chromosomes to the bands. Thirty markers were examined and many unknown loci were identified.

For instant, in comparison with the previously published SSR wheat genetic map [27] one change was made. GWM192 was previously mapped on chromosome 5D, but according to the NT results (Figure 7), it is located on chromosome 4B with the same molecular weight (190 bp).

DISCUSSION

The hydroponics experiment gave an indication about which lines perform well under saline conditions. This can be reflected in the data for dry weight and ear emergence (did not present), the lop 20 of each trait are shown below. Alongside this is the genetic contribution of each parent to these top 10 lines.

KTDH 19 and Punjab 81 have the most influence in both traits, whereas KTDH 59 and Sarc 1 have little influence. Both agronomical and salt tolerant parents seem to contribute to high trail values. This is not surprising, as the microsatellite results also indicated that

these parents have genes that are important for salt tolerance.

Correlations observed between Na and other traits in Table 2 indicate where selection based on physiological traits is feasible. For instance, a long leaf 2 could indicate plants that are likely to have a high Na content in the flag leaf. Before such conclusions can be made, more harvests must be analysed.

Correlations that have been significant between Na and K ions (Figure 4) reflect more probable links between ion transportation and accumulation. It is more correct to base selection on MAS (marker-assisted selection). This is because selection based on physiological traits is restricted by variations in soil salinity and sodicity [28]. The work done in this experiment, points to areas of the genome that need to be studied in more detail. If these areas are truly linked to traits, the markers isolated could provide a valuable tool for breeding wheat with enhanced agronomy under saline conditions.

Molecular Marker Analyses: All parents were screened with 66 markers (represents 50 RFLPs and 16 SSRs). All were found to be polymorphic. Because of the limited amount of DNA available from each F2 individual plant only a few Psr Markers were screened with the bulked DNA (bulking outlined in Methodology). In each case, the 40 tolerant and susceptible plants for salt stress were individually screened for that marker. Chi squared analysis was used to detect significant segregation events.

Firstly, the parents were scored with the markers to check for polymorphisms. The marker was then used on the DNA bulks. The individual DNA samples that made these two bulks were screened, the parental alleles were identified in each lane and Chi squared analysis was used to determine if the segregation pattern was significant between the two groups (Table 4.5). For each significant marker, the frequency between high and low trait values was significantly different in at least one allele. The alleles giving rise to the significant effect were identified by examining the parental screen. Thus the parental origin of an allele having a significant influence on the trait at this genomic region can be determined. Sometimes (e.g. marker PSP3113) it was difficult to detect which parent had influenced the trait because only two alleles identified across all 8 parents. Salt tolerant parents (e.g. KTDH 19) seemed to carry most of the alleles close to genes that increase the resistance of plants under stress, with the exception of KRL3-4 near marker PSP3113 and Sarc-1 had two sensitive alleles closely linked to markers PSP3113 and PSP3119. On the other hand, the parents had good agronomic background seem sometimes to contain alleles that may also have a high effect on plant salt tolerance, i.e. Punjab 85 and Blue Silver with marker PSP3113 and PSP3144 respectively. However these alleles were not unique for those parents. When an allele has been connected to a trait it is impossible, from a group of parents with the same allele, to detect which parent influences the trait.

In fact, the agronomic cultivars may have some hidden genes that are advantageous to a plant under salt stress, which were reflected within each cultivar. These alleles seem to aid salt t olerance in the population studied. A contrasting effect can be seen with alleles resulting from salt tolerant cultivars, which have an adverse effect on plant vigour under salt stress.

The chromosome location for markers PSP 3001, PSP3071 and PSP3159 were identified from the NT analysis and mapping on chromosomes 3BL, 6AL and 4A (centromeric region) respectively. The results for QTL analysis in (23) showed a significant QTL for leaf fresh weight under salt stress and K/Na ratio closely linked to the region PSP3001. The same marker had a significant effect on salt response traits using BSA (Table 4). Previously, Kasinathan [29] identified a significant effect on length of flag leaf with the same marker (PSP3001) under salt stress using BSA. However, (29) did not indicate the chromosome location of the band studied.

Another QTL marker (PSP3030) had a significant effect in the region PSP3030 on plant under salt stress, with the allele from KTDH59 increasing the tolerance of the plant. The chromosome location of this marker has been mapped previously on the long arm of chromosome 4B [23]. Four significant QTL were observed by QTL mapping analysis controlling leaf dry weight and some yield characters to be associated with markers in the region of the PSP3030 locus.

In addition, two more significant affects were identified on homoeologous group 7 on the long arm of chromosomes 7B and 7D in the regions of PSP3119 and PSP3113 respectively (Table 4). PSP3113 appears to have an effect on salt tolerance using BSA as well as QTL analysis, which showed an effect on number of spikelets

per spike on 7D [23]. The last marker having a significant effect with BSA was PSP3144. This allele, with a molecular weight of 200 bp have been previously mapped on chromosome 3A (170 bp, according to the NT of CS) close to the centromeric position related to our map construction. However, if it was the correct chromosome location on 3A, then it could be coincident with the result in Amin [23] that indicated two QTL closely-linked to that chromosome position affecting leaf fresh weight under salt stress and total dry weight. More work is necessary to identify its position more precisely.

In conclusion, some significant markers were identified using BSA and these could give a quick guidance to genes which might affect the tolerance of plants under severe saline conditions.

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