A rapid method for identifying markers associated with salinity tolerance genes in hexaploid wheat (*Triticum aestivum*)

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ABSTRACT

Increasing salinity tolerance of wheat become an important issue for many wheat breeding programs, as salinisation 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 lines and their eight parents under saline hydroponics at a concentration of 150mM NaCl, to detect areas of the genome that may be linked to salinity resistance genes using molecular markers. Bulked segregant analysis (BSA) was employed on two bulks constituted by separation of the 20 most-tolerant and the 20 most susceptible individuals. Both parents and individuals from each pool were screened with 175 simple sequence repeat (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. 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 segregant analysis (BSA), salinity tolerance, wheat, molecular marker, SSR, Triticum aestivum.*

INTRODUCTION

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 (Munns and Tester, 2008). 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 (Maas and Grieve 1990), the characterisation of these genes would aid the development of salt tolerant cultivars. This has been attempted with some success, for instance, in wheat; it has been shown that chromosome 4D holds genetic factors influencing sodium exclusion (Dubcovsky *et al.*, 1996). Nevertheless, the exact locations of most genes that control salt tolerance remain undefined. The use of bulked segregant analysis (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 separetly. 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 (Michelmore *et al.*, 1991). 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 (Babu *et al.*, 2004), or from a population of plants with diverse genetic backgrounds such as composite populations using Chi square statistical analysis. 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 (Quarrie *et al.*, 1999). The aim of this study was to investigate natural allelic variants contributing to quantitative variation for salinity tolerance in bread wheat.

MATERIALS AND METHODS

This research experiment was carried out in 2005-2006 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 F_1 generation from each KTDH containing line was then crossed with the F_1 generation from the non-KTDH lines, producing a total of 54 F_2 lines. The 154 lines represent the highest salt tolerant lines selected until the F_5 generation. This took place in glasshouse under saline hydroponics at a concentration of 150 mM NaCl. F_6 seeds were collected from self pollinated plants under normal conditions to increase the seed number from each line.

Hydroponic 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 temperatures of 20°C/12°C in a hydroponic system following the protocol established by Amin (2002). 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

emerged 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 was 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.

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 F_6 lines each was selected from putative salt tolerant and intolerant extremes of the populations and designated as salt resistant (R) 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 F_6 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 used for the microsatellite screening, were developed in John Innes Centre by Mike Gale's group the rest of SSR markers were available in the public domain. 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 follows: 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 and 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 (2002).

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 aneuploid 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 response before and after salt stress amongst the F_6 progenies

Plant growth responses are reflected in the leaf length measurements. Leaf 2 was measured before salt stress was applied, so that the plant natural vigour could be ascertained before selection was imposed. The measurements of leaf 5 and flag leaf reflect the plant response to the level of salt. With respect to the progenies 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 Table 1, the results are all significant, indicating a significant amount of genetic variation produced by the crosses.

Ion distribution in the parents and progenies

The concentration of Na and K ions in the whole plant were normally distributed between the lines (Fig. 1a,b). However, when the ions in the flag leaf were examined, there was a skewed curve toward the lower data (Fig. 2a,b). This was possible 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 progenies showed a wider range than the parents.

Table (1): Analysis of variance for growth response before and after salt stress of F_6 composite population.

	Source of variation	Degrees of freedom	Mean Squares	F value	Probability
Leaf 2	Between progenies	153	57.29	5.56	0.001
	Within progenies	459	10.3		
	Total	612			
Leaf 4	Between progenies	153	151.75	5.63	0.001
	Within progenies	459	26.96		
	Total	612			
Flag-leaf	Between progenies	108	39.26	1.742	0.002
	Within progenies	312	22.54		
	Total	420			

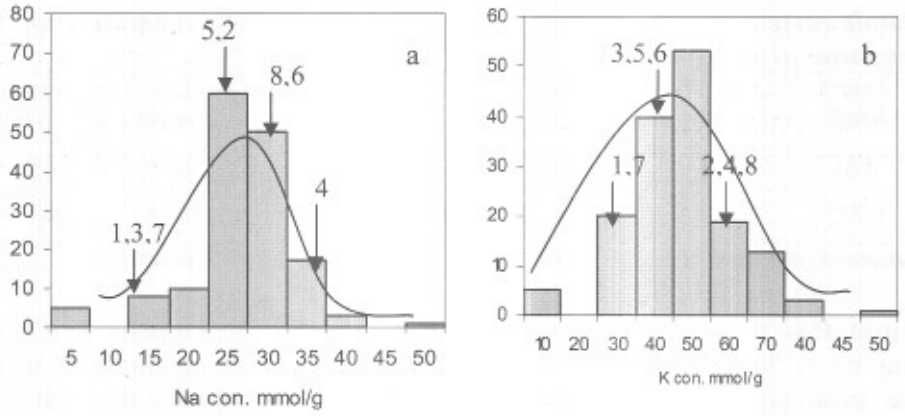

Fig. (1): Distribution of concentrations for Na (a) and K (b) ions in the whole plant. The arrows indicate the parental means, with 1- (KTDH 19), 2- (Blue Silver), 3- (KTDH59), 4- (WH157), 5- (Punjab 85), 6- (Sarc-1), 7- (Meteor), and 8- (KRL3-4). The black lines show the curves for the distributions.

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.

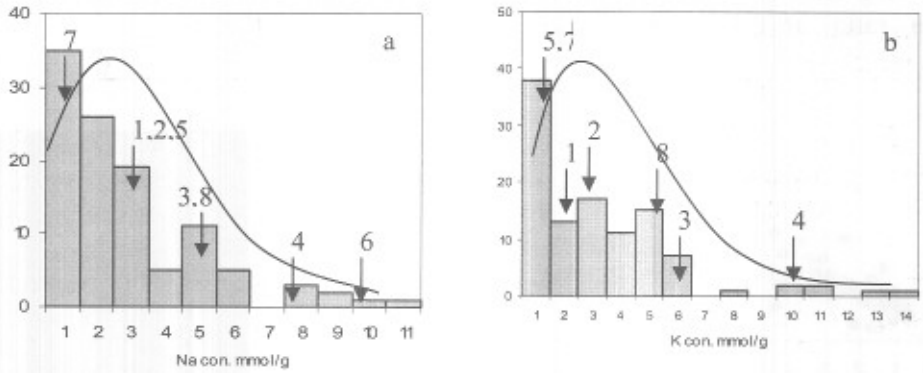

Fig. (2): Distribution of concentrations for Na (a) and K (b) in the flag-leaf. The arrows indicate the parental means, with 1- (KTDH 19), 2- (Blue Silver), 3- (KTDH59), 4- (WH157), 5- (Punjab 85), 6- (Sarc-1), 7- (Meteor), and 8- (KRL3-4). The black lines show the curves for the distributions.

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.

Table (2): Simple correlation coefficients between Na and different characters for 154 F6 lines, in response to salt conditions.

	Leaf 2 length	Leaf 5 length	Flag-leaf length	No. ears	No. Spikelets 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**

* and ** indicate significant correlation at 0.05 and 0.01 level of probability, respectively

The reason that 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^+ concentration (Na-FL) correlated with length of leaves 2 and 5 and with K^+ ion concentration in the flag leaf (K-FL) (Figure 3). The correlation 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 progenies 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. 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 were 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 (1990) noted that in barley there was a low discrimination between the transport of Na^+ and K^+ to the shoot.

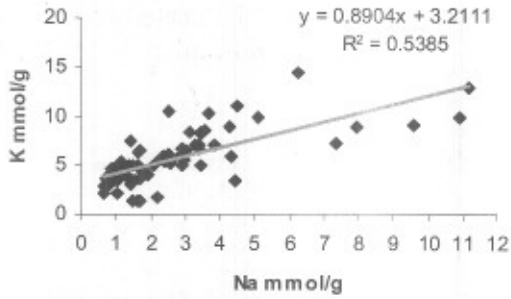

Fig. (3): Relationships between flag-leaf Na content and flag-leaf K content.

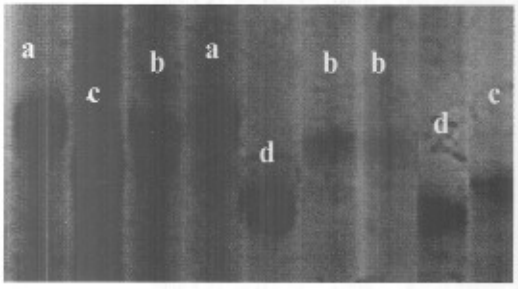

Fig. (4): The polymorphism amongst the 8 parents using SSR marker (PSP 3001).

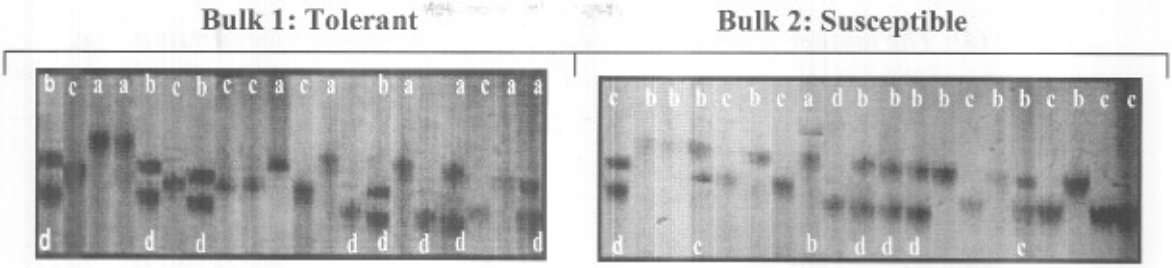

Fig. (5): Segregation of the microsatellite PSP3001 among the individuals from tolerant and susceptable bulks.

Microsatellite results and Chi Square analysis

Parents were screened with 175 markers and polymorphism was determined (Fig. 4). 110 markers were found to be polymorphic, and then screened with the bulked DNA. The parental alleles were identified in each line (Fig. 5). The values were tabulated and Chi square was used to determine if the segregation pattern was significant (Table 3). With PSP3001 marker the segregation was significant. Table (3) 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 grains under salt conditions. This analysis was carried out for all markers screened. Nine markers that significantly segregated with traits are listed in Table 4. 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) analysis represents a perfect way for assigning chromosomes to the bands. Thirty markers were examined and many unknown loci were identified. For instance, in comparison with the previously published SSR wheat genetic map (Röder *et al.,* 1998) one change was made. GWM192 was previously mapped on chromosome 5D, but according to the NT analysis (Fig. 6), it is located on chromosome 4B with the same molecular weight (190 bp).

Table (3): Segregation pattern for parental alleles with PSP 3001 marker.

PSP3001	Observed grain number		Total	Expected
allele	High	Low		
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 (4): 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 level of probability
** Significant at 0.01 level of probability

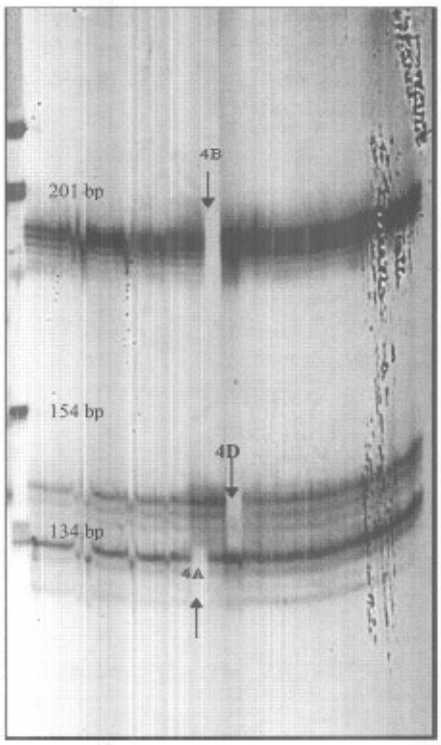

Fig. (6): NT analysis among the 21 chromosomes of Chinese Spring with SSR marker xgwm192.

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 (are not presented). 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 that 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 conclusion can be made, more harvests must be analysed. Correlations that were significant between Na and K ions (Figure 3) 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 (Maas *et al.*, 1996). 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 tolerance to saline conditions.

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الملخص العربي

طريقة سريعة لتحديد المعلمات الوراثية المرتبطة بجينات تحمل الملوحة فى قمح الخبز

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زيادة تحمل الملوحة أصبح يمثل أهمية كبرى لدى الباحثين المهتمين ببرامج تربية القمح وذلك لتأثيرها الواضح على إنتاجية المحصول على المستوى العالمي سواء في الأرض الخصبة أو المستصلحة حديثاً. ويعتبر الهدف الرئيسي في هذه الدراسة البحثية هو التعرف على المواقع الجينيه الطبيعية والتي تساهم في زيادة تحمل الملوحة لقمح الخبز. وعلى ذلك فقد تم استخدام المعلمات الوراثية (الواسمات الجزيئية) كعامل مساعد فعال في انتخاب السلالات المرغوبة في برامج التربية. وقد اختبرت هذه المعلمات الوراثية على 154 سلاله من جيل الاخصاب الذاتى السادس لهجين بين ثمانية أباء بعد نموها فى بيئة ملحية تركيزها 150 ميكرومول من كلوريد الصوديوم بالأضافه إلى الإباء (لتحديد) مواقع الجينات المقاومة لظروف الملوحة داخل المادة الوراثية للقمح .استخدمت طريقة التحليل بنظام المجموعات المنتخبة (Bulk Segregant Analysis BSA) وقسمت النباتات الى مجموعتين بداخل كل مجموعه 20 سلالات نباتية، المجموعة الأولى تمثل السلالات الأكثر تحملاً للملوحة والثانية للسلالات الأكثر حساسية للملوحة وقد تم اختبار أفراد كل مجموعه والإباء بصفه مستقلة مع 175 من المعلمات الوراثية من نوع (Simple Sequence Repeats (SSR وتحليل النتائج احصائياً باستخدام مربع كاى (Chi square) لتعيين الفروق المعنوية للتوزيعات والاختلافات الوراثية لهذه السلالات موضع الدراسة. تسعة من المعلمات الوراثية تم تحديدها على الكر وموسومات (7A, 2A, 3A, 4B, 4B, 7D, 1B, 2B & 4D) حيث وجد أن لها تأثير معنوي واضح تحت ظروف الملوحة لمحصول القمح. وتوضح هذه الدراسة أسلوب جديد لاستخدام التحليل بنظام المجموعات المنتخبة BSA والذي يساهم بشكل مناسب في انتخاب السلالات النباتية والاقلمه تحت ظروف الملوحه او الظروف المعاكسة الأخرى.