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QTL MAPPING OF WHEAT (*TRITICUM AESTIVUM* L.) IN RESPONSE TO SALT STRESS

AYMAN Y. AMIN[1] & AYMAN A. DIAB[2]

[1]Department of Plant Physiology, Faculty of Agriculture, Cairo University, Giza, Egypt

[2]Agricultural Genetic Engineering Research Institute, Agricultural Research Center, Giza, Egypt

Faculty of Biotechnology, October University of Modern Sciences and Art (MSA), Egypt

ABSTRACT

Molecular markers provide a rapid approach to breeding for desired traits. To use them, it is necessary to determine the linkage between quantitative trait loci (QTL) and such markers. This study was conducted to investigate the genetic basis of salinity responses in Egyptian bread wheat (*Triticum aestivum* L.). In this context, a doubled haploid (DH) population (SGDH) of 139 individuals was produced from the cross between two Egyptian breeding cultivars (Sakha 93, salt tolerant) and (Gemmeza 7, salt susceptible). The DH population was tested under saline hydroponics culture and various plant responses were measured. A molecular genetic map of the SGDH population covering 3645.3 cM, was constructed using Restriction Fragment Length Polymorphism (RFLP), Microsatellite or Simple Sequence Repeats (SSR), and Amplified Fragment Length Polymorphism (AFLP) markers. In total, 325 loci along the 21 wheat chromosomes were mapped. The B genome showed the highest number of mapped markers followed by the A and the D genomes respectively. Interval and composite interval mapping (using QTL cartographer) were used to identify the genomic regions controlling traits related to salt tolerance with a threshold of LOD 3. Analysis of QTLs has revealed the approximate location of the significant markers associated with 12 traits related to salt tolerance traits across the A, B and D genomes. Fifty five significant QTL were detected on 15 of the 21 chromosomes mapped in this study, for some of these more than one QTL was identified. In many cases QTL were mapped very close to each other, indicating possible gene clustering or pleiotropy. For some traits, the total percentage of phenotypic variation explained by all QTL exceeded 50-60% but on average it was in the region of 15%. Chromosomes of homologues groups 2 and 5 exerted the biggest effect on most phenotypic traits, especially chromosomes 2B and 5B. The results indicated that there is considerable potential for improving salt tolerance of hexaploid wheat by using marker-assisted selection.

KEYWORDS: Genetic Map, Molecular Marker, Quantitative Trait Loci (QTL), Salt Stress, Wheat *Triticum aestivum*

INTRODUCTION

Salt tolerance is a quantitative trait controlled by many genes; therefore, using molecular marker technology gives the physiologist and the breeders 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 Gary Hart worked on identification and chromosomal mapping of isozymes. Detailed RFLP linkage and physical maps of wheat, comprising more than 2000 DNA markers (Chao *et al.*, 1989; Devos and Gale 1993; Xie *et al.*, 1993; Van Deynze *et al.*, 1995; Marino *et al.*, 1996; Hohman *et al.*, 1994; Gill *et al.*, 1996) have been published covering all seven homologous groups in wheat. Recent advances in genome mapping techniques based on the polymerase chain reaction (PCR), have accelerated a novel DNA marker system, termed SSRs (Tautz *et al.*, 1986). This is much easier to perform than RFLP analysis and show much higher levels of polymorphism in hexaploid wheat than any other marker system (Röder *et al.*, 1995; Bryan *et al.*, 1997). In

addition, AFLP is more powerful in detecting a large number of loci, producing high complexity DNA fingerprints which can be used for high resolution mapping and marker-assisted cloning. 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) (Gelderman 1975). The genetic analysis of quantitative traits under stressed environments and salt stress in particular has had little investigation until a few years ago. With this type of work it is difficult to distinguish between those traits that will actually improve yields under abiotic stresses and other stress response traits that have no effect on yield (Quarrie 1996). However, QTL analysis can make a considerable contribution to increasing the efficiency of breeding varieties with improved responses to abiotic stresses; e.g. drought (Quarrie *et al.,* 1997), and salinity (Dubcovsky *et al.,* 1996). One of the most important 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 (Lande and Thompson 1990). 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 (Paterson 1995). However, the main factor contributing to the slow progress in mapping QTL for salinity tolerance relaying on the low heritability of yield traits under stress conditions (Quarrie 1996). To map QTL one has to set up a cross between tolerant and susceptible parents, measure the traits of interest and screen the experimental population for genetic markers.

Dubcovsky *et al*., 1996, have mapped one major QTL for salinity tolerance in inbread wheat. They identified the position of a QTL affecting K/Na ratio which, most likely, is a single gene *Knal* controlling Na/K discrimination, and which was located on the long arm of chromosome 4D. The effect of the QTL was very strong in a greenhouse experiment (under 50 mM NaCl). Nevertheless, a specific molecular-marker map with more than 250 markers covering 20 of the 21 wheat chromosomes was developed by Semikhodskii *et al.,* (1996) for the detection of genomic regions influencing salt tolerance in wheat. Quarrie *et al.* (2005) showed that the major QTL involved in accumulation of Na and K was detected on the long arm of chromosome 5A. An additional QTL was identified on chromosome 4A and found to be the major region corresponding to chlorine accumulation. (Quarrie *et al.,* 2005). Thus, QTL analysis with molecular markers can be a powerful tool to analyse the genetic basis of complex traits such as salt tolerance. In this study several DNA-based markers (RFLP, SSR and AFLP) were used to construct a genetic linkage map and to identify chromosomal regions associated with salt tolerance in hexaploid wheat (*Triticum aestivum*).

This work provides a molecular tool for breeders and physiologists to facilitate the selection of wheat varieties under salt stress in a strategic improvement program for Egyptian wheat using marker assisted selection.

MATERIALS AND METHODS

Two spring wheat were chosen as parents on the basis of contrasting response to salinity measured in previous work (Salah *et al.* 2005). The parent varieties were Sakha 93 (relatively salt tolerant) and Gemmeza 7 (salt susceptible). A cross between these two genotypes was made and the resulting F_1 plants were pollinated to produce a double haploid population in wheat using maize (*Zea mays L.*) pollen as the male parent to produce haploids, which were doubled by following the procedure described by Laurie and Reymondie (1991). However, here Dicamba is used as a synthetic growth regulator instead of 2,4-D.

RFLP Analysis

Total genomic DNA was extracted from young leaves of 20 days old plants using the CTAB method. DNA was extracted from leaf material of the 139 DH lines and the two parents Sakha 93 and Gemmeza 7 Fresh leaves (3-5 g), were ground in liquid nitrogen with a pestle and mortar and transferred to 50 ml tubes and extracted with 20 ml of CTAB buffer

pH 8.0 (5% NaCl, 0.8% Na_2EDTA, 0.2M Tris-HCl pH 8.0, 1mM DTT [2,3-Dihydroxybutane-1,4dithiol])) at 65oC for 2 h. After incubation, the tubes were left to reach room temperature, and 1.5 volumes of chloroform were added to them and mixed gently for 5 min, followed by centrifugation for 10 min at 3000 rpm. The supernatant was transferred into fresh tubes and 0.6 volume of cold isopropanol added for precipitating DNA at –20 ^oC for 30 min. After this, DNA was spooled using glass hooks, and washed with absolute ethanol for a few seconds. After evaporating, DNA was resuspended in 1 ml of sterile 1x TE buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 8.0). For measuring DNA concentration, 4 µl of resuspended DNA was mixed with 394 µl of 1x TE buffer and the optical density measured at λ260 nm. DNA concentrations were estimated in 1% agarose gels and adjusted to 50 ng/µl. 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: DNA 10 µg, Enzyme 20u, Buffer 1x, and ddH_2O µl (to a total of 25 µl). This mixture was incubated overnight at 37 ^oC 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. 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 (Southern, 1975). For labelling, only wheat genomic PSR probes were employed in this study. All of them are designed at Department of Crop Genetics, John Innes Centre. The PSP probes were labelled with digoxigenin (DIG)-dUTP using the PCR DIG Probe Synthesis kit (Roch). The membrane was pre-hybridized with DIG Easy Hybridization solution (Roche) for 1 to 2 hours and hybridized overnight at 42^oC with DIG-labeled probe. The membrane was washed twice for 5 min in 2x SSC, 0.1% SDS at room temperature and then twice for 15 min in 0.5x SSC, 0.1 % SDS at 68^oC with shaking. Detection was carried out according to the manufacturer's instructions using the DIG Luminescent Detection Kit (Roche). After washing, the membrane was incubated with CSPD(R) Chemiluminescent Substrate (Roche) and subsequently exposed to X-ray film (Lumi-Film, for Chemiluminescent Detection Film) for 30 min at 37^oC.

Microsatellites Analysis

DNA was extracted as indicated above and diluted with 1 x TE buffer to a final concentration of 20 ng/µl. The amplification mixture contained 20 ng of template DNA, 1 x PCR buffer supplied with 1.5 mM $MgCl_2$, 2.5 mM of dNTP, 0.1 µM of forward and reverse primers, and 0.2 unit of Taq DNA polymerase. The total volume of the reaction was 10 µl. The thermal conditions of the reaction were dependent on the set of *Xgwm* primers used as described by Röder *et al.*, (1995). For gel preparation the two gel plates were dried using 100% ethanol. The large plate was coated with a 2% solution of dimethyldichorosilane in octamethylcyclotetrasiloxane (Repelcoate (v)), left to dry and wiped with 100% ethanol. The small (bind) plate was coated with 30 µl of y-methacryloxypropyl-trimethoxysilane, left to dry and washed first with sterile water and then 100% ethanol. 300µl of ammonium persulphate (APS) and 30µl of N'N',N'N'-tetramethyl-ethylene diamine (TEMED) was added to 60ml of 6% polyacrylamide gel mix polyacrylamide gel [15% (v/v) Bis-acrylamide (19:1) 40%, 48% (w/v) urea, and 10% (v/v) 10x TBE buffer (0.09 M Tris borate, 0.002 M EDTA) was made up to 1 litre with distilled water]. The mix was then poured between the plates and a comb added. The gel was allowed to set for approximately 1 hour. 10 µl of the loading buffer (10 ml formamide, 10 mg xylene cyanol FF, 10 mg bromophenol blue, and 200 µl of 0.5mM EDTA pH 8.0) were added and mixed with the PCR products. The mixture was denatured for 5 minutes at 95^oC and 5 µl were loaded in to slots. Electrophoresis was performed in vertical slabs in 1x TBE for 75 min at 90 V. After electrophoresis, gels were fixed in 10% acetic acid for 30 min and washed in ddH_2O for 15 minutes on a shaker. Washed gels were stained for 30 minutes in silver-staining solution (0.1% $AgNO_3$ and 0.055%

formaldehyde) with gentle shaking. The gels were then rinsed with ddH_2O for 10 seconds and bands appeared after washing in developer solution (3% Na_2CO_3, 0.055% formaldehyde, and 0.0002% $Na_2S_2O_3$). Once the patterns of amplification fragments became visible the reaction was stopped with 10% acetic acid, the gel rinsed with water and left to dry.

AFLP Analysis

DNA was extracted and diluted as above and AFLP was performed using primers obtained from KeyGene Ltd. For restriction and ligation 0.25 µg DNA was mixed in a volume of 25 µl with 2.5 units of rare cutter-*PstI*, 2.5 units of frequent cutter-*MseI*, 5 µl of 5 x Restriction-ligation (RL) buffer, 0.5 µl of *PstI*-adapter, 0.5 µl of *MseI*-adapter, 10 ul of ATP, and 1 unit T4 DNA ligase and incubated overnight at 37^{o}C. The same restriction and ligation was also prepared using *SseI* as rare cutter and *Sse1*-adapter. For Amplification of Restricted Fragments, 2.5 µl of the diluted restriction and ligation product (1:10) was mixed in a volume of 25 µl with 0.7 µl of *MseI*-primer M00, same amount for *PstI*-primer P00 (6 pb cutter), 1 µl of 5 mM dNTPs, 2.5 µl 10 x PCR buffer, 0.2 u of TAQ-polymerase. The cycle profile used was: 94ºC 30 sec, 65ºC 30 sec, 72ºC 60 sec (cycle 1), then annealing temperature is lowered 0.7ºC each cycle during next 12 cycles and 94ºC 30 sec, 56ºC 30 sec and 72ºC 60 sec from cycles 14 to 35. After pre-amplification, the product was diluted 1:20 with ddH_2O, and then used as a template for selective 23 primer combinations amplification to generate AFLPs. The selective amplification reaction was conducted in a final volume of 20 µl containing 5 µL of diluted pre-amplification reaction, 30 ng of each combination primers, 200 µ*M* dNTPs, 10 m*M* Tris-HCl (pH 8.3), 1,5 m*M* MgCl2, 50 m*M* KCl, and 0.2 unit Taq polymerase. The PCR was programmed with the same cycle profile above mentioned. Immediately before electrophoresis, 10 µl of loading buffer (98% formamide, 10mM EDTA pH 8.0, bromophenol blue and xylene cyanol as tracking dyes), was added and the mixture was denatured for 5 min at 94^{o}C and place immediately on ice. Gel preparation and electrophoresis were performed as mentioned above with minor change. After electrophoresis, the gel was silver-stained using the silver sequence DNA staining kit (Promega cat # Q4132) following the instructions provided to reduce gel noise. Only bands showing unambiguous polymorphism were entered into a data matrix.

Map Construction and Linkage Analysis

MAPMAKER program (Lander *et al.*, 1987) was used for constructing linkage groups and the distances between markers were calculated using the Haldane mapping function. The MAPMAKER program allows data to be analysed and used two-, three- and multipoint analysis for finding realistic genetic linkage groups, ordering loci within them and finding the maximum likelihood genetic map for any given order of loci.

Hydroponics Culture Medium

Four seedlings of each DH line were planted one per pot, with three replicates; Positions of the plastic pots were frequently rotated and random complete block design implemented. The hydroponics was set up as described by Amin (2002). For the germination, no salt addition was observed during the growth of seedlings, when leaf 2 was fully emerged salt-stressed conditions were established by mixing the half strength Hoagland nutrient solution with the salt containing a determined NaCl and $CaCl_2$ (4:1) gradually by 50mM a day to give a final concentration of 150mM. The experiment was performed in a greenhouse conditions, at about 26/20^{o}C with 14/10 day/night natural daylight. Before leaf 4 was collected, extension rate was measured day by day from the moment it protruded from the subtending leaf sheath, until no increase in length could be detected. After this Na^+, K^+ ions and K/Na ratio were analysed as described by Amin (2002), As`well as, fresh weight (lfw), dry weight (ldw) and percentage of water (W%) were measured. At the end of the experiment, some

yield parameters were measured such as spike number per plant (SNPP), number of spikelets per spike (NSPS), Number of Spikelets per Spike (NSPP), total grain number (TGN), grain weight per plant (TGW) and Total Dry Weight (TDW).

QTL Analysis

Both molecular mapping and phenotypic data were analysed for QTL using QTL Cartographer with Windows QTL Cartographer ver. 2.5 (Wang *et al*., 2006). Two methods for QTL analysis were employed interval mapping (Lander and Botstein, 1989) and composite interval mapping (Zeng, 1993). After preliminary investigation of a series of data the number of background parameters for Model 6 was selected to be 14 thus gaining a balance between the number of QTL detected and the reliability of their detection. On this set of data I found several chromosomal regions which are likely to be significant on the basis of significance with the linear regression model, when the likelihood ratio (LR) were also above thresholds of 5 for Model 3 and 15 for Model 6. The output data from QTL Cartographer were transferred to Excel 97 for visualisation. Additive effects are described in relation to Sakha 93 (+) the AA parent, and Gmaza (-) the BB parent in the analysis. The only QTLs identified using model 3 or model 6 had a significant effect with marker-by-marker linear regression analysis, are considered.

RESULTS

RFLP

Out of 35 DNA clones screened to identify polymorphisms between the two parental genotypes Sakha 93 and Gemmeza 7, twenty two probes revealed polymorphisms for at least one of the six (six-base) cutting restriction endonucleases (EcoRI, EcoRV, BamHI, HindIII, DraI, and Bgl II). 25 loci were mapped on the wheat genome. In general, the most likely positions for the RFLPs within chromosomes corresponded with published locations for the markers (Chao *et al*., 1989; Devos *et al*., 1992; and Graingenes database). The great majority of probes were low copy ones giving a simple hybridization pattern with 3 to 6 bands. With some probes more than one polymorphism per enzyme was observed (Figure 1). With regard to the segregation ratio between the two parents l alleles, most of the RFLP loci mapped followed the expected 1:1 segregation ratio. On the B genome all the 7 chromosomes were covered with at least one marker except chromosome 6B. The B genome was much better covered with RFLP markers than the A and D genomes with at least one marker in each chromosome.

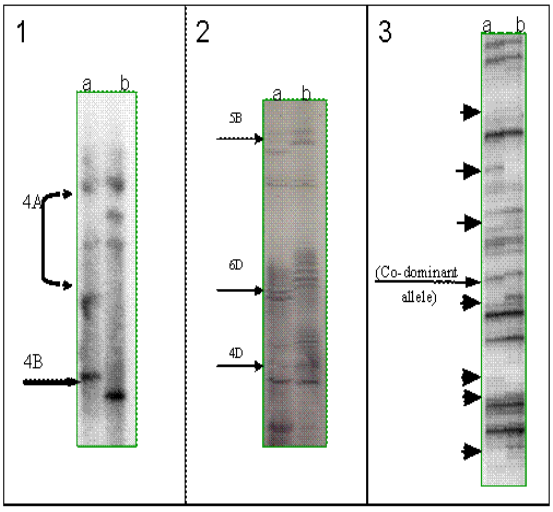

Figure 1: Segregation of the Three Different Markers with the DNA of Two Parents (a) Sakha 93 and (b) Gemmeza 7, 1- Southern Hybridization of psr914 to Filters with Hind III 2- the Microsatellites GWM133 among the Doubled Haploids and 3- AFLP Markers Amplified with Primer Combination (P68M78).

Microsatellites

This work was carried out using two types of primers: GWM (Röder *et al.*, 1998) and PSP (Bryan *et al.*, 1997). Using 122 wheat microsatellite primer pairs, 72 were shown to be polymorphic between Sakha 93 and Gimaza, thus making the level of polymorphic loci 60%. Seventeen markers scored with more than one locus (Figure 1) to allow 90 loci to be mapped. In some cases nullisomic-tetrasomic (NT) analysis was used for assigning chromosomes to the bands (Table 1), that corresponded to Chinese Spring. This analysis helped to identify 40 new polymorphic loci. The microsatellites showed uniform distribution over all the chromosomes. This uniformity was also observed among wheat genomes, with the B, D and A genomes respectively 40%, 27%, and 33% of the total number of SSR loci mapped.

Table 1: Nullisomic Tetrasomic Analysis with Some SSR Primers According to Chinese Spring (CS)

Primer	Chromosome locus	Band size (bp)
GWM11	1B*	190
GWM18	1B*	180
GWM55	2B/4D/1D*/5D/5B	154/134/110/100/85
GWM111	7D*/2D/2B*	201/128/110
GWM129	3B*/2B*/5B	235/225/215
GWM132	2D*/6B*/7D	138/122/95
GWM133	5B*/6D*/3A/5B/4D*	134/120/110/95/80
GWM159	5D*/5B	192/160
GWM160	4A*	180
GWM174	5D*	170
GWM191	6B/3D*/2B/5B/1D	140/125/120/110/100
GWM192	4B*/4D*/4A	190/142/130
GWM205	5A*/5D*	150/140
GWM261	2D*	175
GWM272	5D*/7B	140/128
GWM335	5B*/5D/5A*	220/100/140
GWM382	2D/2A*	125/120
GWM429	2B*	205
GWM645	3D*	145
PSP3001	3B*/3A*/7A/1A	210/175/104/100
PSP3029	6A*/2A*/5A	170/160/135
PSP3030	3B*	170
PSP3047	3A*	170
PSP3071	6A*/6B*	158/135
PSP3088	2A*	165
PSP3100	1B*	165
PSP3118	3B/5B*	201/160
PSP3148	5D*	150
PSP3159	4A*	175

* =loci were showed polymorphism between the two parants and mapp

AFLP

AFLP analysis was performed using two rare-cutter enzymes, SseI and PstI (SseI is an 8-base cutter while PstI a 6-base cutter), and a frequent cutter, MseI. For amplification, primers with 2 or 3-base selection were chosen. Twenty three primer combinations were screened with the doubled haploid population, and overall 368 AFLP loci were scored. Only 205 could be mapped reliably on the wheat genomes. On average 10 polymorphic loci were scored for each primer combination. Usually one parent had a null allele when a band was present in the other parent (dominant marker). However, we detected DNA polymorphism for some loci, which segregated as co-dominant markers having one allele for

each parent (Figure 1). AFLP loci were located to chromosomes by linkage to the SSR and RFLP anchor markers previously mapped. These covered all wheat chromosomes except 4D and 6B. In some cases, clusters of AFLP loci were observed (chromosome 2B, 3B, 4B, and 6D). The average distance between the markers within a "cluster" was usually *ca* 5 cM, and the length of a cluster often exceeded 30 cM.

Molecular Genetic Map of the SGDH Population

A molecular map was constructed with 139 DHs and 325 loci derived from 123 molecular markers, representing 21 linkage groups and constituting 3645.3 cM in a total distance (Figure 2). The mean distance between two markers was 11.3 cM. A LOD score of 3 was adopted as the threshold. The scoring was clear with little segregation distortion. Only a few polymorphisms were relatively difficult to score. All scoring was performed twice, and a missing value was assigned when clear identification of the allele was difficult. The markers were randomly distributed along the linkage groups, with clustering around 14 centromeric regions and all of the 21 wheat chromosomes were covered. Almost 48% of the markers (156 loci) were mapped into the B genome, 29% (94 loci) mapped to the A genome and the D genome was the least mapped by only 23% of the markers (75 loci).

Table 2: Significant QTL Detected in all Experiments Performed

Trait Name	Chromosome location	Marker	% of variance explained	LR statistic	Additive effect	*P* LR analysis
Na concentreation	2B	P68M78-2	4.716	4.50	-0.042	0.036*
	4B	Gwm368	17.930	18.86	-5.321	0.007**
	5D	Gwm174	8.754	4.49	0.057	0.043*
K concentration	2B	psr126	29.045	54.22	-0.491	0.008**
	2D	Gwm132	6.691	4.46	0.147	0.049*
	3D	Gwm191	5.660	5.51	0.135	0.011*
	5D	Gwm174	22.306	27.59	-0.288	0.026*
K/Na ratio	2B	P68M78-2	5.369	5.62	0.007	0.034*
	2D	Gwm261	6.277	5.68	-0.007	0.008**
	3B	P73M91-5	4.873	4.68	-0.006	0.030*
	4A	P73M78-5	6.423	12.78	0.007	0.007**
	7A	S15M47-6	8.534	15.66	-0.009	0.022*
	5B	S15M47-3	8.828	16.72	0.009	0.046*
Growth Rate (under salt stress)	1A	P20M68-3	5.920	4.95	0.105	0.013*
	2A	P68M81-15	8.741	8.64	0.125	0.005**
	2B	S15M17-1	5.914	5.78	0.105	0.020**
	4A	P75M71-11	4.414	4.74	0.092	0.007**
	3A	P73M91-6	4.535	5.00	0.097	0.038*
Leaf Fresh Weight	3A	psp3001	9.768	18.48	-1.108	0.041*
	3B	P78M50-11	12.215	15.00	1.133	0.046*
	4B	P75M71-12	6.832	6.39	-0.826	0.035*
	5D	Gwm174	10.547	5.18	1.024	0.038*
	5B	P65M17-11	8.265	15.47	0.976	0.011*
Leaf Dry Weight	1D	P73M91-22	13.751	18.32	0.033	0.004**
	4B	P65M21-6a	9.642	7.45	-0.027	0.011*
	5D	Gwm159	13.734	10.85	0.033	0.005**
% of water content	3D	Gwm383	10.570	14.47	4.613	0.001***
	5B	psr162	6.811	6.42	3.698	0.019*
Number of spikes per plant	2B	P65M83-3	6.056	5.94	0.052	0.015*
	4B	P65M21-6a	10.184	19.26	-0.073	0.011*
	5B	Gwm499	20.699	36.49	-0.108	0.038*
	7D	P75M81-4	6.554	6.36	-0.054	0.019*
	5B	psr162	7.004	6.26	0.058	0.024*
Number of spikelets per spike	1D	Gwm642	9.453	21.07	0.446	0.009**
	2B	P65M83-3	6.929	6.82	0.340	0.008**
	3B	P77M21-14	21.353	20.17	-0.638	0.013*
	4B	P65M21-6a	10.664	22.41	-0.493	0.017*
	5B	Gwm499	8.546	8.36	-0.382	0.014*
	7D	P75M81-4	5.754	5.56	-0.309	0.032*
Total grain Number per plant	1B	psr325-2	10.948	21.77	0.219	0.037*
	1D	Gwm642	9.907	21.43	0.194	0.005*
	2B	P65M83-3	8.169	17.93	0.261	0.006**
	3B	P78M69-1	8.424	6.90	-0.170	0.008**
	5B	Gwm499	6.841	6.63	-0.156	0.035*
Total grain weight per plant	1D	Gwm642	5.111	4.96	0.658	0.015*
	2B	P65M83-3	15.143	23.34	1.761	0.013*
	3B	P78M69-1	16.339	22.16	-1.235	0.012*
	3D	Gwm645	9.342	14.91	0.930	0.019*
	5B	psr162	8.514	7.74	0.864	0.015*
Total DryWeight	1D	P73M91-22	10.209	8.10	25.684	0.008**
	2A	P77M21-10	10.125	15.36	27.374	0.002**
	3A	Gwm674	5.707	5.58	-19.168	0.021*
	4B	P77M25-6	7.520	8.51	-23.632	0.007**
	5D	psp3148	12.263	10.40	28.094	0.001***
	5D	P77M21-4	9.394	17.25	-25.165	0.003**

With linear regression model the significance at the 5%, 1%, 0.1% and 0.01% levels are indicated by *,**,*** and ****, respectively

QTL Analysis

QTL for the 12 measured traits were detected and mapped. Fifty five significant QTLs were detected on 15 of the 21 chromosomes mapped in this study. However, the distribution of QTL was not uniform (Table 2 and Figure 2). Three markers located on chromosomes 2B, 4B and 5D significantly affected sodium concentration (Table 2). The QTL on 2B and 4B were identified with Model 3 in the regions P68M78-2 (4.7% of variance) and GWM368 (17.9% variance), respectively.

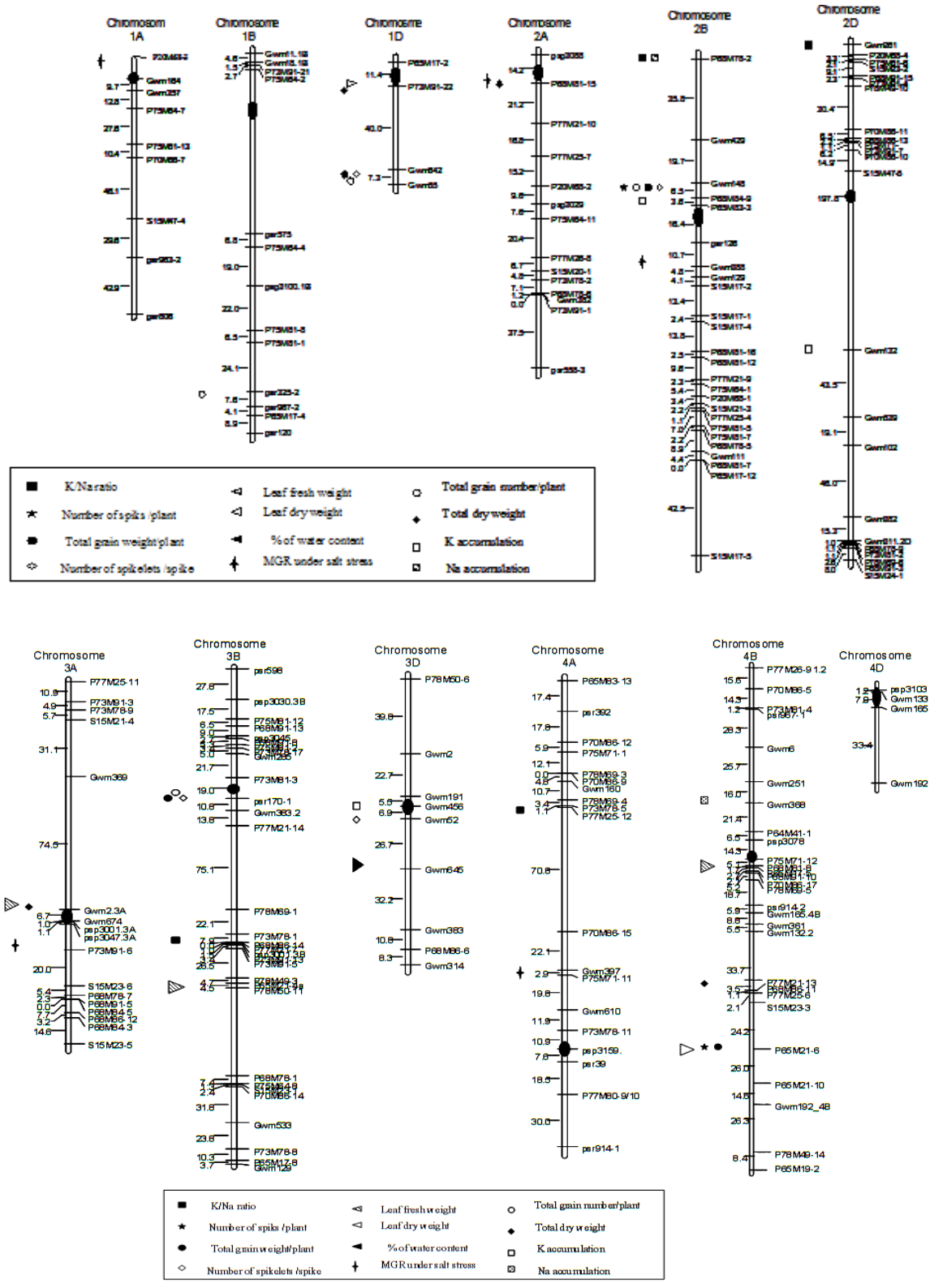

Figure 2: Continued.,

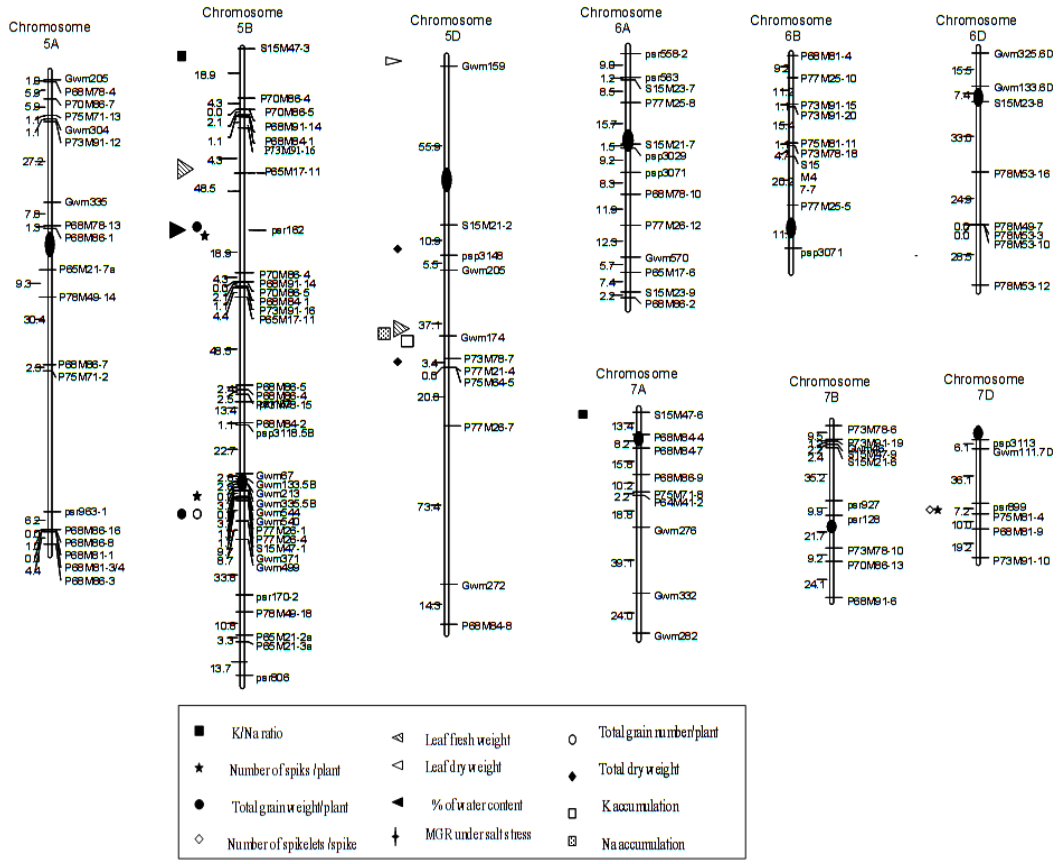

Figure 2: Genetic Map of Sakha 93 x Gemmeza Using 139 DH Lines. Ovals Mark Approximate Position of Centromeres. Distances in cM Using the Haldane Function. Location of Significant QTL for Salt-Related Traits Indicates by Symbols

For both QTL, the Gemmeza 7 allele had an increasing effect on the tissue concentration of sodium as expected. The 2B marker is coincident with other QTL that had significant effect for K/Na ratio in opposite direction. Another QTL on chromosome 5D was identified in the SSR locus GWM174. It explained 8.7% of the trait variability, and coincided with 5D QTL controlled the K accumulation and leaf fresh weight (Figure 2). Four chromosomes were implicated in controlling the concentration of K in leaves. The first QTL, on 2B, explained 29% of the variation in potassium content. At this QTL region on chromosome 2B, the increase in potassium content came from Sakha 93 parent. QTL for some other traits (spike number per plant, number of spikelets per spike, total grain number per plant and total grain weight per plant) were also present in the same region. The second QTL was identified near GWM174 on chromosome 5D. The percentage of the phenotypic variation explained was 22% (Table 2). Using model 3, two further QTLs on chromosomes 2D and 3D were found to harbour QTL with additive effects opposite to the QTL on 5D for potassium accumulation in the regions GWM132 and GWM191 respectively, explaining in total 13.3% of variability for this trait (Table 2). Significant QTL for K/Na ratio were also located on four more chromosomes 3B, 4A, 7A, and 5B (S15M47-3–Psr162). The QTL on chromosome 2B explained only about 5.4% of the total phenotypic variance, The Gemmeza 7 allele at this locus (P68M78-2) was associated with high sodium content and consequently with a low K/Na ratio. In contrast, the QTL for K/Na identified on regions (P73M91-5 and S15M47-6) of chromosomes 3B and 7A respectively, showed Gemmeza 7 to have the increasing effect and together they explained more than 13% of the variance. The significant QTL on 5B detected by composite interval mapping and located in interval S15M47-3–P70M86-4 (Figure 2) accounted for 8.3% of the K/Na ratio variation.

In general, the Sakha 93 alleles increased the growth rate under salt stress for all the QTL identified (Table 2). The two QTLs for leaf growth rate under salinity were detected on 4A and 3A. Another QTL accounting for 8.7% of the variance was detected by interval mapping on chromosome 2A (PSP3088 – P68M81-15) at the same interval as the 2A QTL for total dry weight. These results may indicate that some genes induced under salt stress, e.g. on chromosome 2A, controlled the subsequent growth rate of the plant.

Under salt stress conditions, strongest QTL was detected by composite interval mapping on the long arm of chromosome 3A. It was placed in the interval GWM674-PSP3001, and accounted for 9.8% of the total leaf fresh weight variation. This region was coincident with other QTL for total dry weight (Figure 2). The QTL on chromosomes 5D (GWM272-P68M84-8) explained 10.5% of the leaf fresh weight. Significantly, this QTL was located at the same place as a QTL having a similar significant effect for Na accumulation (Figure 2) with the Sakha 93 allele increasing Na accumulation. Also a second QTL on 5B mapped at almost the same position as the QTL for spike number per plant and the total grain weight, both of which were increased by the Sakha 93 allele. QTL mapping identified a region on the long arm of chromosome 1D which had a large effect on leaf dry weight, accounting for 13.7% of the variance (Table 2). The same marker on the 1D QTL region significantly affected the total dry weight under salt stress (Figure 2). Moreover, a QTL for leaf dry weight (9.4% of the total phenotypic variance) was detected on the long arm of chromosome 4B in the region of P65M21-6a, its effect came from Sakha 93 allele decreased leaf dry weight and associated with reductions in some yield components i.e. SNPP, and NSPS (Figure 2).

Only two QTL controlling % water content were identified. The first was located on the long arm of chromosome 3D in the interval GWM383–P68M86-6 where the QTL for total grain weight was identified. This QTL was detected accounted for 10.6% of the total phenotypic variation. The second QTL was found on 5B (S15M47-3–Psr162), and explained 6.8% of the variation of percent water content. At the same region, QTL for spike number per plant, and total grain weight were also associated (Figure 2).For yield characters under salt conditions, all five significant QTL for SNPP were detected on chromosome 2B, 4B, 5B, 7D, and 5B (Table 2), were also associated with those found for NSPS (Table 2). Moreover, composite interval mapping identified another two QTLs On the long arm of chromosome 1D in the interval P73M91-22-GWM642 and long arm of chromosome 3B in the region of P77M21-14. These QTL explained 9.4% and 21.4% of the total NSPS variability, respectively.

Certain genomic regions were found on chromosome 1B in the interval Psr325.2-Psr967.2 had a strong effect on TGN. It was identified by Model 6; the phenotypic variation explained by this QTL was almost 11%. Plants which carried the Gemmeza 7 allele of this QTL, produced fewer grains in total under salt stress. Markers on chromosomes 1D, 2B, 3B, and 5D linked to QTL for TGN also showed significant coincidence with QTL for NSPP and NSPS (Table 2 and Figure 2). The strongest LR statistic effect on TGW was associated with a QTL located on the short arm of chromosome 2B in the region of QTL for other traits including SPNP, NSPP, TGN, and K concentration. In addition, chromosome 3D contained a QTL detected by Model 6. It was located in the interval P78M69.1–P73M78.1 on the long arm and accounted for 9.3% of the phenotypic variability. Another QTL was found on 5B (S15M47.3–Psr162). It was located in the region Psr162 by Model 3 and explained 8.7% of grain weight variability.

The Sakha 93 allele had an increasing effect was observed for the other QTL identified on chromosome 3D (GWM645) using model 6. It explained 9.3% of the total variance of this trait. It was close to a QTL described above controlling percentage of leaf water content (Figure 2). In general, the QTL for leaf dry weight were also linked to the QTL for total dry weight on chromosomes 1D, 4B, and 5D (Table 2). These QTL accounted for about 30% of the total trait variability. Three more QTLs were detected for TDW, one of these being identified by interval mapping, located on

chromosomes 2A (P68M81-15-P77M21-10) was coincident with the QTL for maximum growth rate under salt stress (Figure 2).

DISCUSSIONS

The development of genetic linkage map is a first step towards the detection of factors controlling the expression of important traits. In this study, the RFLP level of polymorphism between the two parents was relatively high (65%). This result contrasts with a previous study (Chao *et al.* 1989) which found a low level of allelic variation (<10%) among cultivated varieties. In the present work, RFLP probes have been pre-selected on the basis of low copy number and high allele number. A further source of increased RFLP frequency may be due to the highly contrasting between the parents in response to salt stress.

Since microsatellite primers are regarded as being largely locus specific, only one locus was expected to be amplified by each primer. However, Röder *et al.,* (1998) detected more than 1 locus per microsatellite primer pair and mapped them onto bread wheat genetic maps. In this study, 55 primers detected only a single polymorphic locus and 17 of them detected more than one polymorphic locus (Figure 1). Salina *et al.*, 2000, noted that the microsatellites are abundant and evenly distributed throughout the genome. In agreement with this, the currant map showed 90 microsatellite loci gave a good coverage of the three genomes A, B, and D (28%, 33% and 39% respectively). Salina *et al.* (2000) observed that the D genome had less microsatellite loci in comparison with genomes A and B. Nevertheless, this result could not be confirmed, because in the present map the A genome had less microsatellite polymorphisms than the D genome (Figure 2). However, in agreement with them, the B genome contains the highest number of microsatellites.

Previous studies noted that AFLP are dominant markers (Zabeau and Vos, 1993). However, our study indicated, that in some cases the AFLP patterns showed co-dominance (Figure 2). The distribution of AFLP loci amongst the 7 homologues groups was more or less uniform. In fact, this marker system has been described as random markers and therefore the relationship between AFLP markers and particular linkage groups and chromosomes is unknown (Nandi *et al.*, 1997). In General, the D genome was least polymorphic with only 23% of the markers identified on it and the most polymorphic genomes were the B and A with 48% (156 loci out of 325 mapped) and 29% of the total number of loci mapped respectively. Similarly, Quarrie *et al.* (2005) observed in the genetic map of the hexaploid wheat population that is developed from the cross Chinese Spring x SQ1 that the D genome contained only 8% of markers. In contrast with the genetic map constructed in this study, the A genome had the highest mapped markers (48.5%) and about 43% for the B genome

In this work, the total phenotypic variation explained by QTL varied from 10-15% for the majority of the traits. However, in many cases of previous QTL mapping, the total phenotypic variation explained by all QTL averaged 20% or less (Xiao *et al*., 1996) and QTL detection depended on how contrasting were the parental genotypes that have been used to develop the mapping population. It is likely that some other QTL may be found in the poorly mapped regions of the genome. Nevertheless, on the well-mapped chromosomes, QTL were usually not randomly distributed. QTL for different traits were located to specific regions on these chromosomes. On chromosome 2B, QTL for several traits were found in the same interval P65M83.3–Psr126. Although, significant QTL were identified for many traits, none of these traits had a single major QTL, and overall they were controlled by more than one locus. In previous studies by Fitter and Hay (1987), they reported that the accumulation of Na as negative factor and K as a positive factor in plant tissues, can be used as the main criteria for salinity tolerance in wheat. Currant results were clearly indicated that the B and D genomes are mainly responsible for the significant QTL of K accumulation (Table 2), whereas Quarrie *et al*. (2005) found that the Na and K

accumulations were controlled largely by the A genome and in particular chromosome 5A. There have been many reports in the literature on association of the D genome with ion uptake in bread wheat. Under salt stress, Wyn Jones *et al*. (1984) observed greater discrimination (in favour of K and against Na accumulation) was shown by hexaploid bread wheat than by tetraploid durum wheat, so it was concluded that the character is resided in the D genome. Moreover, Dubcovsky *et al*., 1996 found that the marker psr375 co-segregated with the KnaI gene which is responsible for sodium/potassium discrimination in the Triticeae.

As yield parameters are importance in plant cultivation, the use of yield components as criteria is utterly justified. In previous work, Semikhodskii (1997) found that the most abundant QTL for these traits were close to the region on the long arm of the chromosome 5A which contains the vernalization response gene (Vrn1). In this study, the majority of the QTL affecting these traits were found on the B and D genomes (Figure 2), with no QTL for yield component were found on the A genome. In particular, the long arm of the chromosome 5A had a big gap, which needs further work. Nevertheless, Semikhodskii (1997) observed QTL for grain number per spike on chromosome 5B, which is similar to the results obtained in this study for grain number per plant.

In this work, Multiple QTLs were found for almost all traits at different regions; the number of QTL identified for each trait varied from 3 to 6, indicating that the genome contains multiple genes affecting each trait. The overlapping of these QTL indicates that either there are closely linked genes or the same gene is affecting different traits, our data tend to support this (Figure 5). Multiple traits can be correlated due to linkage, pleiotropy, or the correlated traits may be components of a more complex variable. The distinction between linkage and pleiotropy is important for breeding purposes as well as for scientific reasons. However, without fine resolution mapping or molecular cloning of QTLs, such distinction would be difficult and at best one can make inferences based on morphological and/or physiological relationships between traits under consideration.

The most practical application of the identified QTL is to perform marker-assisted selection aimed at efficient pyramiding of favourable QTL alleles to improve wheat yield and agronomic traits under salt stress. Additional QTL studies are useful for identifying the chromosomal regions that are more consistently associated with salt tolerance. Further studies involving fine mapping of genomic regions associated with salt tolerance across genetic backgrounds or species will be required for cloning of genes controlling tolerance to salt stress in plants.

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