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Full Length Research Paper

Identification of RAPD markers linked to salinity tolerance in wheat

Waqas Manzoor Bhutta* and Muhammad Hanif

Department of Botany, Government College University Faisalabad, 38040-Pakistan.

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Genetic diversity can be measured by a number of ways, including pedigree, phenotype and allelic diversity at loci controlling phenotypes of interest. A DNA marker for root length in wheat (*Triticum aestivum* L.) was identified. The individual plants from F_2 population segregation for salinity tolerance and the parents (S-24 and DN-27) were grown in polyethylene tubes under saline conditions (EC 16 dS m^{-1}) for root length at the four leaf stage. The plants were then transplanted into pots supplied with optimum water and nutrients until maturity. Genomic DNA from 50 tolerant and 50 sensitive F_2 plants was extracted. The bulked segregants analysis was used in the random amplified polymorphic DNA (RAPD) technique. DNA polymorphisms were observed using 240 primers. The primer, GLE-14 amplified a 970 bp polymorphic DNA fragment and this DNA fragment can be used for marker-assisted selection to breed for salt tolerant wheat. This marker should be further used while selecting different crops under salt stress.

Key words: Marker, RAPD, root length, salinity, wheat.

INTRODUCTION

Salinity is a serious constraint to crop yield and is one of the big problems of irrigated agriculture in the world. The adverse effects of soil salinity on crop growth and yields are of multifarious nature. In salt-affected soils, excessive concentration of neutral soluble salts adversely affects the growth and yield of most plants. The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution causing physiological drought, nutritional imbalances and specific ion toxicity or combination of all these factors (Gorham and Wyn Jones, 2002). Because of their frequent potential for multiple cropping, the arid and semi-arid regions of the earth offer considerable promise for development as major food producing regions. Many of the inhospitable deserts of such regions require only a source of water for conversion to prime agricultural land. A frequent problem with developing such lands is the accumulation of soluble salts, which imposes a stress on growing crops that can lead to decrease yield and, in severe cases, complete crop failure (Zhang and Blumwald, 2001).

The extent, distribution and nature of the salt-affected lands of the world are not well known in most countries due to lack of standardization of characterization criteria. According to Dulal and Purnell (1986) the percentages of the salt-affected land in different regions are as follows: Asia, 2.1%; Australia, 42.3%; Asia, 21%; South America, 7.6%; Europe, 4.6%; Africa, 3.5%; North America, 0.9%; Central America, 0.7%. Estimates of the extent of the world's saline soil range up to 955×10^6 ha (Szabolcs, 1991), a significant number considering that total world crop land is only 1450 × 106 ha (UNEP, 1992). It corresponds to approximately 25% of the world's irrigated land and 60% of the cultivated land (Suarez and Rhoades, 1991).

Pakistan occupies a total geographical area of 80.5×10^6 ha, out of which 32.95×10^6 ha is considered suitable for cultivation while only 20.36×10^6 ha is actually under cultivation. An area of 16.23×10^6 ha is irrigated through canals and tube well and the remaining 4.13×10^6 ha is dependent on rain. Salt affected lands in Pakistan are estimated about 6.3×10^6 ha (Khan, 1998). So, salinity is one of the major constraints responsible for low crop productivity in Pakistan. Consequently, there is a pressing need to develop wheat varieties with a better ability to grow varieties and produce grain in places where wheat is grown inefficiently or not at all today (Flowers and Yeo,

*Corresponding author. E -mail: wmbhutta4055@yahoo.com.

1995). Salinity tolerance in wheat has been and is being extensively researched in Pakistan and elsewhere in the world, yet efforts to improve salt tolerance have been hampered by a number of factors, particularly the lack of understanding of the mechanisms of salt tolerance and interaction of salinity with various environmental factors with regards to plant growth (Ribaut et al., 1996; Frova and Devos, 1999).

Wheat tolerance to salinity varies with the stage of plant growth, nature and level of salinity, duration of stress, effect of soil moisture, climate, nutrition and management practices (Gorham and Wyn Jones, 1990; Wu et al., 1996; Jain and Selvaray, 1997; Bhutta et al, 2005). Different physiological traits such as selectivity for potassium, exclusion and compartmentation of sodium and chloride ions, an osmotic adjustment by the accumulation of organic solutes have all been related to salt tolerance of wheat crop plants (Watson et al., 2001). Soil and water salinity directly affects wheat production (Zhu, 2001). The reduction in growth of crop plants by salinity may result from its effect on dry matter production, ionic relations, water status, physiological disorders, biochemical reaction or a combination of all these factors. Various varieties of wheat do not equally respond to soil salinity. Some varieties have been observed to be very sensitive to salinity, while others have shown high salt tolerance (Akhtar et al., 2003). To obtain better yield from saline soils and saline irrigation waters on sustained basis, it is imperative that along with improved agronomic practices the genetic resources should be exploited with the help of modern plant molecular techniques, such as marker assisted breeding, gene transformation and tissue culture to develop high yielding salt tolerant rice varieties (Voss et al., 1995). One of the main uses of DNA markers in agricultural research has been in the construction of linkage maps for a range of crop species (Cao et al., 1998; Bhutta et al., 2006). Linkage maps have been utilized for identifying chromosomal regions that contain genes controlling simple traits and quantitative traits, using QTL analysis. DNA markers that are tightly linked to agronomically important genes may be used as molecular tools for marker-assisted selection in plant breeding. Several mapping studies have identified QTLs associated with salinity tolerance in wheat (Rafalaski et al., 1991).

In contrast, RAPD markers pre- identified in a bulked segregant analysis were found to be useful in discriminating fusarium head blight sensitive and tolerant wheat genotypes (Sun et al., 2003). The plants from such segregating populations can be grouped (bulked) according to phenotypic expression of a trait and tested for differences in allele frequency between the population bulks: bulk segregant analysis (BSA) (Quarrie et al., 1999). The other variant of BSA is that the plants of the same species from different genetic backgrounds are bulked according to phenotypic expression and genotyped with DNA markers. The BSA was first used for simple trait like disease resistance mapping using RAPDs and RFLPs (Michelmore et al., 1991) and was used to find DNA markers linked to a complex trait like salt tolerance in wheat (Welsh and McClelland, 1990). The identification of RAPD markers needs a pairs of near isogenic lines for the traits. Bulked segregant analysis proposed by Michelmore et al. (1991) can overcome this problem. BSA makes use of F_2 F_3 or F_4 population and it has been extensively used for the identification of RAPD markers linked to various genes (Poulsen et al., 1995; Mackay and Caligari, 2000; Ni et al., 2001; Bhutta, 2007). Keeping this background in view, the present research work has been designed to find DNA markers linked to salt tolerance traits in common wheat, using RAPD analysis and bulk segregant analysis.

MATERIALS AND METHODS

Experiment 1

Screening of different wheat genotypes for salinity tolerance in hydroponics culture

Seeds of each 25 genotypes were sown on iron trays, randomized within trays. One seed per plug and five plants per family per tray were germinated, with a total of ten trays being used. The trays were placed over vermiculite moistened with a solution containing 2.0 mol m^{-3} of aerated, phostrogen-based nutrient solution containing 2.26 mol m^{-3} K, 0.1 mol m^{-3} Na, 0.24 mol m^{-3} Ca, 0.31 mol m^{-3} Mg, 2.8 mol m^{-3} NO_3, 0.57 mol m^{-3} PO_4 and micronutrient (Gorham, 1994).

Transplanting

At two-leaf stage (almost one week after emergence of seedling) the seedling were transplanted from iron trays to the 200 L capacity tubs. Two plants per hole of each genotype were transplanted into foam-plugged holes in thermo pole sheets.

Treatments

After 3 days of transplanting, salt was added to the nutrient solution starting on the 15 day after germination, in increment of 25 mol m^{-3} NaCl day^{-1} to a final level of 120 and 240 mol m^{-3} NaCl. $CaCl_2$ was also added to maintain a Na:Ca ratio of 20:1. The pH was adjusted at 6.0 to 6.5 daily by adding HCl (I N) or NaOH (I N). Solutions were changed after 8 days during the entire experimental period.

Harvesting

Plants were harvested after 5 weeks of salinity development. Plants were washed with distilled water and dried with blotting paper. At the time of harvesting, data were recorded, containing root length (cm). Relative salt tolerance = (Value of a character in NaCl / Value of a character in control) X 100

Experiment 2

The plant material

The total DNA was extracted from young leaves of F_2 population,

which were obtained by crossing S-24 x DN-27.

DNA extraction

After screening of plants for the physiological traits, 50 plants giving highest root length and fifty plants giving lowest root length were selected from the F_2 population for DNA extraction. The wheat populations were grown in plastic containers (250 ml) and 0.2 - 0.3 g leaf tissues were obtained from the 8 day old seedlings of the wheat genotypes. After cutting into small pieces, the leaf tissues were transferred immediately into zipper plastic bags containing 1.5 ml CTAB. DNA was isolated from the leaves using the method proposed by Rogers and Bendich (1988). Leaf materials were completely homogenized with a hand roller. After incubation at 65°C for 30 min, the homogenized leaf tissues were transferred into two 1.5 ml Eppendorf tubes. Equal volume (0.75 ml) of chloroform-isoamylalcohol was added and the tubes were inverted vertically 5 - 10 times, followed by spinning at 13,000 rpm for 10 min in centrifuge (MSB010 CX1.5, MSE, UK). After centrifugation supernatant was transferred from both tubes into another 1.5 ml Eppendorf tube. Then approximately 700 µl (0.9 volumes) of isopropanol was added into the supernatant and mixed by inverting the tube about 10 times. The DNA was pelleted and washed and resuspended in 150 µl of 0.1X TE and treated with RNase for one hour at 37°C to digest RNA. Finally, the concentration of DNA was measured at 260 nm in a spectrophotometer (Cecil CE 2021 2000 Series, Cambridge, UK). The quality of DNA was checked by running 5 µl DNA on 0.8% agarose gel, prepared in 0.5X TBE buffer. The DNA samples giving smear in the gel were rejected.

Amplification reaction

RAPD amplification was performed in volumes of 20 µl containing 1×PCR buffer, 3 mM MgCI2, 100 µM each of dATP, dCTP, dGTP, dTTP (Promega), 0.4 µM 10-mer primers, 20 ng of template DNA and 1 unit of Taq DNA polymerase. A total of 240 random primers with 10-base oligonucleotide obtained from Gene Link Co. (USA) were used for the amplification of the genomic DNA. Taq polymerase together with buffer, MgCl2, dNTPs and gelatin were purchased from Fermentas. The RAPD amplification was carried out in 24-well automated thermocycler (Eppendrof). The program consisted of denaturation at 94°C for 3 min, followed by 40 cycles (denaturation at 94°C, annealing at 36°C for 1 min, extension at 72°C for 1 min), finishing at 72°C for 9 min. The amplified products were separated by electrophoresis in 1.2% agarose gel and visualized by ethidium bromide staining (Sambrook et al., 1989). The fragment size was determined by 100 bp DNA ladder. Reactions were repeated from 2 to 3 times to check the consistency of the amplified products and only easily resolved and bright DNA bands were counted.

Random amplified polymorphic DNA (RAPD) analysis

The DNA of 100 F_2 plants (50 salinity resistant and 50 salinity susceptible) were selected on the basis of root length to generate bulks. Concentration of the DNA was measured by spectrophotometer. DNA samples were diluted to 1.25 ng/µl by adding deionized distilled water.

Generation of bulks

Two DNA pools contrasting for salinity resistance were formed by mixing the same amount of DNA from individual, plants. One pool or bulk consisted of equal amounts of DNA of 50 F_2 plants showing resistance to salinity with highest root length and the other was similarly formed from DNA of 50 F_2 plants susceptible to salinity with low root length. These two DNA were then used in polymerase Chain Reaction (PCR) to find polymorphism.

PCR material

The 10-base oligonucleotide primers used in the PCR were from operon Technologies, Inc. USA. Taq DNA polymerase, together with 10 X PCR buffer, $MgCl_2$, dNTP's and gelatin were of perkin Elmer.

Preparation of 1% mini agarose gel for DNA samples quantification

To prepare the gel, 0.5 g of agarose was added to 50 ml 1 × TBE in flask. Then it was heated in a microwave oven for 1.5 min at medium setting and allowed to cool to 50 - 60°C and 2.5 µl ethidium bromide (10 µg/µl) was added. This mixture was poured into a gel tray. After the gel became solidified, it was placed in a gel tank containing 1× TBE buffer to cover the gel. 5 µl of each DNA sample along with λ DNA standards (supplied by BDH chemicals, UK) of 5, 10, 20, 50, 100 and 500 ng/ µl was loaded in the wells and gel was run at 50 V for 15 min. Then gel was taken out and photographed in the dark room using UV light. The intensity of bands was compared with DNA standards in the gel and quantified visually.

Analysis of RAPD data

Data were scored from good quality photographs of each amplification reaction. The left of the gel was considered as lane-1. Amplified fragments were scored by starting from the top of the lane to its bottom. All visible and unambiguously scorable fragments amplified by primers were scored under the heading of total scorable fragments. Bands of less than 400 bp were in some cases difficult to score and were not considered. The fragments that were repeatedly present in one bulk and absent in the other were scored as polymorphic fragments.

RESULTS AND DISCUSSION

Experiment 1

Relative root length

Relative salt tolerance for relative root length provided further estimates of the salinity tolerance of genotypes (Table 1). Comparison of genotypes based on relative root lengths showed that some of the genotypes were more tolerant than others at 12 dS m^{-1} NaCl. Relative Root lengths of CIM-3 and SQ-26 were 76.05 and 57.30%, respectively in 12 dS m^{-1}. With increased salinity level (24 dS m^{-1}), relative root length of all the genotypes were affected but to varying degrees. Under 24 dS m^{-1} genotype, S-24 with salt tolerance index of 75.19% appeared to be less affected. From the comparison of overall performance, the salt tolerance of different genotypes could be clearly assessed (Tahira et al., 2006). Genotypes, S-24 and DN-34 with tolerance indices of 84.10 and 81.98% respectively for root length in

Table 1. Relative root lengths (%) of 25 genotypes grown in control and at two salinity levels.

Genotype	12 dS m^{-1}	24 dS m^{-1}	Mean of two salinities
CIM-31	48.9	38.0	43.5
CIM-3	76.0	46.0	61.0
SQ-26	57.3	48.6	52.9
SQ-77	56.6	42.6	49.6
SS-17	56.3	40.1	48.2
Y02-2	47.3	41.8	44.5
Y28	57.7	45.5	51.6
WC-65	58.9	43.6	51.2
KRL-24	52.8	35.6	44.2
S-24	84.1	75.1	79.6
KRL—20	65.1	53.1	59.1
SQ-78	42.8	34.1	38.4
WC-78	45.1	35.5	40.3
DN-25	51.2	34.2	42.7
DN-26	52.6	44.6	48.6
DN-27	42.5	25.2	33.9
K-65	65.0	55.1	60.1
LU-26S	48.3	38.8	43.5
DN-30	74.6	42.8	58.7
DN-33	72.6	37.1	54.9
DN-34	81.9	40.9	61.4
DN-35	81.1	42.3	61.7
DN-36	70.9	31.4	51.2
DN-37	62.4	28.0	45.2
DN-38	69.0	36.1	52.5

salinized solution, under 12 dS m^{-1} appeared to be the most tolerant genotypes. By contrast, DN-27 and DN-37 were 25.27 and 28.02% seems to be the most sensitive to salinity compared to DN-24 under 24 dS m^{-1}. Other genotypes produced shoot lengths ranging between 42.712 to 61.45% at both salinity levels.

Experiment 2

Random amplified polymorphic DNA (RAPD) analysis

Recent development in DNA marker technology together with the concept of marker assistance selection provides new solutions for selecting and maintaining desirable genotypes. Once markers closely linked to desirable traits are identified markers assisted selection can be performed in early segregating populations and early stage of plant development. MAS can be used to pyramid the major genes including resistance genes, with the ultimate goal of producing varieties with more desirable characters (Frova and Devos, 1999).

Availability of tightly linked genetic markers for resistance genes will help in identifying plants carrying these genes simultaneously without subjecting them to the pathogen in early generations. The breeder would require little amount of DNA from each of the individual plants to be tested without destroying the plants. Using the known set of primers for PCR, the products of the reaction would have to be run on agarose gels and the genotypes of the individual plant for resistance or susceptibility could then be directly ascertained by the presence or absence of the marker band on the gel. Salinity resistance DNA marker studies were not conducted on wheat. Two bulks contrasting for the traits were constructed by pooling an equal amount of DNA from the 50 salt tolerance and 50 salt sensitive homozygous plants to find the DNA marker linked with the trait of interest salinity. The results from individual F_2 populations were used to determine the correlation of osmotic pressure and absolute root length with yield. The results indicated that genetic factors controlling relative root length (Table 1) segregated independently (Rana, 1985; Salam et al., 1992). The adverse effects of soil salinity on the crop growth and yields are of multifarious nature. In salt-affected soils, excessive concentration of soluble salts adversely affects the growth and yield of most plants.

The deleterious effects of salinity on plant growth are

associated with low osmotic potential of soil solution causing physiological drought, nutritional imbalance and specific ion toxicity or combination of all these factors (Gorham and Wyn Jones, 1990). The growth of plants under saline environment depends upon a number of morphological, physiological, biochemical and anatomical adaptations, which enable the plant to grow at high salt concentrations. Generally, the mechanism of salt tolerance involves ion exclusion, compartmentation, high K^+/ NA^+ ratio, K^+ selectivity and ion discrimination of leaves. The decreasing stomatal conduction inhibits osmotic pressure and then impairs ribulose-1,5-bisphosphate carboxylase/oxygenase (Gorham and Wyn Jones, 1990; Delfine et al., 1998; Pakniyat et al., 1997). The ability of plants to regulate influx of salt is obviously one of the major factors determining salt tolerance. As cytoplasmic Na^+ is toxic above threshold level, it is extruded by the plasma membrane Na^+/H^+ antiporters that are energized by proton gradients generated by the plasma membrane ATPase. The cytoplasmic Na^+ may also be removed at tonoplast by vascular Na^+/ H^+ antiporters into the cell vacuoles (Salam et al., 1992; Frova and Devos, 1999). To obtain crop production on salt affected soils, a genetic approach has been proposed in conjunction with normal reclamation and management practices. This approach emphasizes breeding and cross breeding of cultivars, followed by selection (Delfine et al., 1999), which is an important tool for improving plant salt tolerance. Plant salt tolerance is a complex mixture of different morpho-physiological traits, which are controlled by many genes across the wheat genome and are known as polygenic or quantitative traits. The different physiological traits were observed on the leaves under a mild salt stress. The plants were not to recover when salinity period is long (Delfine et al., 1998). The osmotic pressure and stomatal and mesophyll contents were partially recovered on alleviating the stress. The gene conferring absolute root length and osmotic pressure are not linked with the yield genes and may be present on different chromosomes.

DNA marker studies

A total of 240 available decamer random primers were surveyed with bulked segregant analysis (BSA) used. Two polymorphic DNA bands ranging from 265 to 1320 base pairs with an average of 5.4 bands per primer were amplified in the resistance bulk. However, the polymorphic bands could not be confirmed in individual plant analysis for which the bulk was constituted. Thus, from the BSA none of the detected polymorphic DNA fragments were linked to the salinity resistance. Then, we reduced the bulk size to 5 resistance and sensitive plants each and conducted RAPD analysis with the above 240 primers, while two polymorphic fragments were detected between the reduced bulks.

None of the fragment was linked with the salinity resistance based on the subsequent analysis of individual plants. Then, parents were screened with the available random primers. The DNA fragments amplified in PCR were in the range of 250 to 2100 bp. A total of 1552 DNA fragments were amplified with an average of 6.5. Out of these, 13.7% were polymorphic between the parents. While in barley 7.9 bands per primer (Harvey et al., 1995) and 8.3 bands per primer in tomato (Michelmore et al., 1991) had been studied. Then 30 each homozygous salinity resistant and salinity sensitive F_2 plants were screened with the polymorphic random primers. Only the polymorphic DNA fragments amplified with GLE-14 primer (approximately size 970 bp) was linked with the salinity resistance in repulsion phase (Figure 1). It was present in 27 out of 30 salinity sensitive plants. Similarly, the DNA fragment was present in 8 out of 30 salt tolerant plants. Thus, the recombination frequency between the marker and the resistance locus was around 12%. The polymorphic DNA fragment was designated as GLE-14 $_{970}$ using the same nomenclature as that of Michelmore et al. (1991). The annealing sites of different fragments of different crop plant may differ in plants belonging to different family as well as the same family.

Salt tolerance is a quantitative trait and it is controlled by more than one gene. But, there are reports that transfer of a single gene in a plant increased its tolerance by many folds (Ray et al., 2002). Traditional breeding strategies have been attempted to utilize genetic variation arising from varietals germplasm. Inter species or intra species hybridization, induce mutations or somaclonel variation of cell and tissue cultures have met with only limited success. Only few new plant introductions with improved stress resistance under field conditions have produced result (Tahira et al., 2006). Traditional approaches are limited by the complexity of stress tolerance traits, low genetic variation and yield components under stress and lack of efficient selection techniques. Furthermore, salinity traits that are linked to tolerance at one stage of development can differ from those that are linked to tolerance at other stages (Bhutta and Hanif, 2008). Once desired traits are identified, there are usually extensive breeding efforts to restore desirable trait along with retrogressed tolerance trait. Nonetheless, marker assisted selection (MAS) of specific secondary traits can be indirectly related to yield (Poulsen et al., 1995; Mackay and Caligari, 2000; Ni et al., 2001).

Osmotic adjustment or physiological tolerance indices might prove increasingly useful as the resolution of genetic and physical maps of chromosomes of different crop species improves (He et al., 1992). This strategy could be used in combination with pyramiding strategies or consecutive selection and accumulation of physiological yield components traits (Flowers and Yeo, 1995). The cultivars of the same species having different number of RAPD bands are non-significant and results observed that genotypes evolved in this region have the same gene pool (Cao et al., 1998).

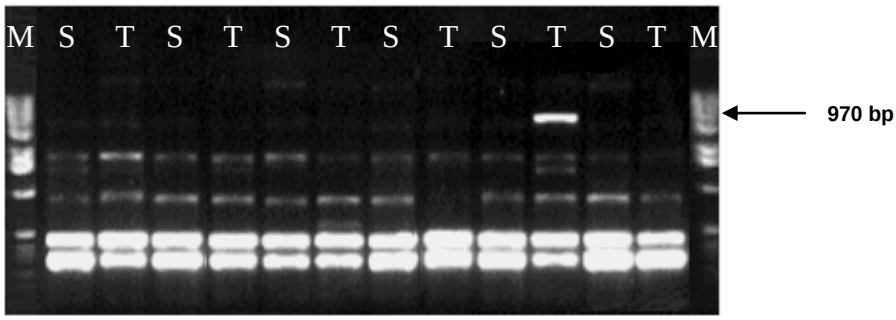

Figure 1. RAPD reactions involving two bulks using 10-mer primers GLE-14.

Genetic diversity is pre-requisite of narrow genetic base for any successful breeding program and breeder continuous use of narrow genetic base for breeding. Therefore, conventional breeding techniques can be more productive if integrated with new technologies of plant molecular biology. The genetic diversity presents in wheat germplasm has narrowed down; this could affect the development of improved salt tolerant wheat varieties.

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