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APPLICATION OF RANDOM AMPLIFIED POLYMORPHIC DNA (RAPD) TECHNIQUE FOR THE IDENTIFICATION OF MARKERS LINKED TO SALINITY TOLERANCE IN WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

DNA marker for a low Na uptake trait in hexaploid wheat (*Triticum aestivum* L.) was identified. The individual plants from F_3 population segregating for salinity tolerance and the parents (LU-26S & Rohtas-90) were grown in polyethylene tubes under saline conditions (EC 25 dS m^{-1}) and screened for K:Na ratio, chloride ions and net photosynthesis at the fourth leaf stage. The plants were then transplanted into pots filled with 7 Kg of fertile soil and supplied with optimum water and nutrients until maturity. Correlations of K:Na and net photosynthesis with yield components were calculated. Genomic DNA from 15 tolerant and 15 sensitive F_3 plants was extracted. The bulked segregant analysis was used in the random amplified polymorphic DNA (RAPD) technique. DNA polymorphisms were observed using 148 primers. The primer OPZ-10 amplified a 680 bp polymorphic DNA fragment which linked to K:Na ratio trait. This DNA fragment can be used for marker-assisted selection to breed for salinity tolerant wheat. The K:Na ratio and net photosynthesis were not correlated with yield components.

Introduction

Abiotic stresses such as salinity, heat, cold and drought are major threats to crop productivity worldwide. Approximately, 20% of the world's agricultural land is affected by salinity (UNEP 1992; Flowers & Yeo, 1995). However, in the Indo-Pakistan subcontinent the extent of salt affected lands in irrigated areas ranges between 0.8-38% (Singh, 1992). In Pakistan, about 6.3 million hectares of land are salt affected (Khan, 1993).

Physiological basis of salinity tolerance in wheat and other crop plants has been widely studied and it is generally accepted that high net photosynthesis, high K:Na ratio and low chloride ions accumulation may be used as general screening criteria for salinity tolerance (Shah & Wyn Jones, 1988; Gorham & Wyn Jones, 1990; Wu *et al*., 1996; Jain & Selvaray, 1997; Noctor & Foyer, 1998; Munns *et al*., 2000). A lot of variation for K and Na ions accumulation at low and high salinity has been reported in the tribe Triticeae. It has been reported that variation in accumulation of K and Na ions is controlled by a gene on long arm of chromosome 4D, designated as *Kna* (Gorham *et al*., 1991).

Reclaiming the saline soil or developing salinity tolerant varieties could solve the salinity problem. Since, soil reclamation is not always economical or practical, the development of salinity tolerant cultivars is a good alternative. Efforts have been made to evolve salinity tolerant varieties by pyramiding salinity traits in a single genotype.

However, very few lines/varieties with good salt tolerance under field conditions have been reported (Flowers & Yeo, 1995). The constraints with the conventional breeding approaches are complexity of salt tolerance trait (Zhang & Blumwald, 2001), low genetic variance of yield components under stress conditions, lack of proper screening procedure (Ribaut *et al*., 1996; Frova & Devos, 1999) and absence of suitable genetic model systems (Zhu, 2001). These constraints can be overcome by using DNA marker-assisted selection for the traits of interest, because these markers provide genotypic basis of a plant (Tahir, 2001). Among the DNA-based markers; restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), microsatellites (SSRs) and amplified fragment length polymorphism (AFLP) have been used in field crops (Voss *et al*., 1995).

RAPD analysis is relatively easy and less expensive, so it has been extensively used to document genetic variation in *Triticum* spp., (Cao *et al*., 1998; Sun *et al*., 1998; Yuejin & Lin, 2000; Mukhtar *et al*., 2002). It has been used to tag genes for disease resistance (Rafalaski *et al*., 1991; Laucou *et al*., 1998), and for fingerprinting of genomes (Welsh & McClleland, 1990; Rahman & Zafar, 2001; Rahman *et al*., 2002). The identification of RAPD markers needs a pairs of near-isogenic lines for the trait. The development of near isogenic lines is costly, laborious and time consuming. Bulked segregant analysis (BSA) proposed by Michelmore *et al*., (1991) can over come this problem. BSA makes use of F_2 or F_3 population and it has been extensively used for the identification of RAPD markers linked to various genes (Poulsen *et al*., 1995; Mackay *et al.,* 2000; Ni *et al.,* 2001).

The objective of the present work was to find DNA markers for low Na uptake using an F_3 population of a cross between salinity tolerant and salinity sensitive wheat genotypes. Furthermore, correlation studies were conducted to understand a relationship between physiological traits conferring salinity tolerance and yield components.

Materials and Methods

The research work was conducted in the Department of Plant Breeding and Genetics (PBG), University of Agriculture, Faisalabad (UAF) and Plant Biotechnology Division, National Institute for Biotechnology & Genetic Engineering (NIBGE), Faisalabad, Pakistan. The F_3 population and parents (LU-26S & Rohtas-90) were grown in polyethylene tubes filled with 400 grams of air-dried sand. There were 20 plants of each parent and 200 plants of the F_3 population. The plants were supplied with Hoagland nutrient solution. Moisture level was kept uniform in all the tubes by simple weighing.

The electric conductivity (EC) of the sand was adjusted to 10 dSm^{-1} using Jenway Meter Model 4070 when the plants were at the 3rd leaf stage and was increased gradually to 25 dSm^{-1} over two weeks using NaCl. Potassium: sodium ratio (K:Na), net photosynthetic rate (Pn, μmol CO_2 m^{-2} s^{-1}) and chloride ion concentration (mg L^{-1}) were determined at the 4th leaf stage.

Leaf samples were collected at 8 A.M. and washed with distilled water to determine ion uptake. The samples were blotted with tissue paper, placed in 1.5 ml eppendorf tubes, labelled and stored at -10°C for cell sap extraction. Frozen leaves were crushed in the eppendorf tubes using stainless steel rod with tapering end. The cell extract was centrifuged at 6500 rpm for 10-15 minutes. The supernatant was transferred into a clean eppendorf tube. The cell extract was diluted 5-10 times with distilled water. Potassium and sodium ratio was calculated by determining the concentration of potassium and

sodium ions using flame photometer. The chloride ion concentration was estimated by titration of the leaf extract with silver nitrate using potassium chromate as an indicator.

Net CO_2 assimilation rate (Pn) of fully expanded 4th leaf was measured with a portable, open infra-red gas analyser (IRGA, Model LCA3) and Parkinson Leaf Chamber after attaining steady reading within 30-60 s. Net photosynthetic rate calculated on IRGA was based on the formula Pn = (f/s) . c (Long & Hallgren, 1987) where c, f and s were the CO_2 differential between the reference and analysis air stream (mol mol^{-1}), leaf area (m^2) and flow rate (cm^3 s^{-1}), respectively.

Salinity stress was alleviated after measuring the physiological traits and the plants were transplanted into pots filled with 7 kg rich field soil. The plants were grown to maturity under optimum nutrition and moisture conditions. At maturity, plant height, spike length, number of spikelets per spike, number of grains per spike, grain weight per spike and 100 grain weight of the plants was measured. Correlation and analysis of variance of the data were performed using Minitab computer program

Molecular analysis

A total of 15 plants exhibiting highest K:Na ratio and 15 plants showing the lowest K:Na ratio were selected for DNA extraction. DNA was extracted from the leaves by a method proposed by Rogers & Bendich (1988). The DNA concentration was measured using spectrophotometer. The quality of the DNA was checked by observing ratio at 260 and 280 nm wavelength, the quality was further checked by running a sample of 25 ng DNA on an agarose gel. Fifteen tolerant and 15 sensitive plants were selected to formulate the respective DNA bulks.

For RAPD analysis, 10-base oligonucleotide primers were obtained from Operon Technologies, Inc. (USA). PCR was performed in volumes of 50 µl containing 5 µl of 10 X buffer [100 mM Tris-HCl (pH 8.8), 500 mM KCl and 0.8% Nonidet P40], 6 µl $MgCl_2$, 2 µl each of dATP, dCTP, dGTP, dTTP (2.5 mM), 4 µl of primer (15 ng/µl), .001% gelatin, 5 µl of genomic DNA (5 ng/ µl), 2 unit of *Taq polymerase*, and dH_2O. A total of 148 primers were used for RAPD analysis. *Taq polymerase*, together 10 X buffer, $MgCl_2$, dNTPs and gelatin were obtained from Perkin Elmer (Norwalk, Conn. USA). Amplification was performed in Perkin Elmer DNA thermal cycler 480 programmed for a first denaturation step of 5 min., at 94°C followed by 40 cycles of 1 min at 94°C, 1 min., at 36 °C and 2 min., at 72°C. The PCR tubes were kept at 72°C for 10 min., and then held at 4°C until the tubes were removed.

Amplification products were analyzed by electrophoresis on 1.2% agarose gels and detected by staining with Ethidium bromide. All visible and unambiguously scorable fragments amplified by the primers were scored under the heading of total scorable fragments. The fragments that were repeatedly present in one bulk and absent in the other were scored as polymorphic fragments.

When a primer was found to give rise to a polymorphism between the bulks, it was used to amplify DNA from the individual F_3 plants of the population to verify the linkage of marker with the trait.

Table 1. Generation means and generation mean squares for net photosynthesis (Pn, µmol CO_2 m^{-2} S^{-1}), K:Na ratio, Chloride ions (Cl^-, mg L^{-1}), plant height (Pl.Ht., cm), Spike length (Sp.leng, cm), Grains/spike (Gr./Sp.), Spikelet per spike (Splt/sp.) and 100 grain weight (100 Gr.wt., gm) of the parents and the F_3 generation in wheat.

	ROHTAS-90	LU-26S	F_3	Gen. MS
Pn	1.28	1.09	1.04	0.057^{**}
K:Na	1.24	1.01	1.10	0.04^{**}
Cl^-	368.75	363.47	351.78	226.1^{NS}
Pl.Ht.	44.93	53.44	54.06	78.02^{*}
Sp.Leng.	8.077	8.51	7.90	0.29^{*}
Gr./Sp.	51.58	25.78	23.91	717.23^{*}
Splt/Sp.	14.27	8.89	9.28	26.95^{**}
100 Gr.Wt.	3.54	4.48	4.39	1.98^{**}

*=(P < 0.05 or lower); **=(P < 0.01 or lower); NS= Non-significant.

Table 2. Correlation of physiological traits with yield components in wheat.

	Net Photosynthesis	K:Na ratio
K: Na ratio	0.147	
Plant height	-0.11	-0.107
Spike length	-0.143	- 0.034
Number of spikelets	-0.143	-0.079
Number of grains	-0.114	- 0.022
Grain weight/spike	0.118	- 0.095

All values are Non-significant

Results and Discussion

In the present studies, generation means of the parents and the F_3 population were significant (p< 0.05 or less) for net photosynthesis, K:Na ratio, Na^+ concentration, K^+ concentration and for yield components in wheat. However, the means were non-significant for Cl^- concentration (Table 1). The data from individual F_3 plants was used to determine the correlation of net photosynthesis and K:Na ratio with yield components. The results indicated that genetic factors controlling K:Na, net photosynthetic rate and yield components segregated independently (Table 2). These results are not in line with some earlier studies (Rana, 1985; Salam *et al*., 1992; Frova & Devos, 1999).

It has been reported that salt tolerant plants maintain high net photosynthetic rate under saline stress (Gorham & Wyn Jones, 1990). The excessive salt accumulation in wheat (*Triticum aestivum*) and other crop plants, first inhibits photosynthesis by decreasing stomatal conductance and mesophyll conductance to CO_2 diffusion and then impairs ribulose-1,5-bisphosphate carboxylase/oxygenase (Shah & Wyn Jones, 1988; Gorham & Wyn Jones, 1990; Delfine *et al*., 1998). The correlation of different traits conferring salt tolerance in wheat has been reported in studies where plants were grown until maturity under stress conditions (Salam *et al*., 1992; Frova & Devos, 1999). However, in the present findings, salt stress was alleviated after recording the data of physiological parameters. Similar studies were conducted on spinach (Delfine *et al*.,

1999). Moreover, the salt stress imposed for short period of growth (3^{rd} to 4^{th} leaf stage) did not impair the biochemical and photochemical characteristics of the plants, and the plants reverted to normal growth (Delfine *et al*., 1999). In another study, reported by Delfine *et al*., (1998) different physiological parameters were measured on the same leaves under a mild salt stress and after alleviating the stress. The net photosynthesis and stomatal and mesophyll contents were partially recovered on alleviating the stress, however, the plants were not able to recover when exposed to stress for long time. It may be suggested from the present studies that the gene(s) conferring K and Na ion uptake and net photosynthetic rate are not linked with the yield genes and may be present on different chromosomes.

DNA marker studies

A total of 148 primers of 10-mer oligonucleotides were used in PCR, 128 (86.5%) primers amplified the genomic DNA in both the bulks. The size of the amplified DNA bands ranged from 285 bp to 3.5 kb base pairs with an average of 5.5 bands per primer. The range in number of DNA fragments amplified was 1 to 13. However, Malik (1995) has reported an average of 5.2 bands per primer in wheat (*Triticum aestivum* L.), while 8.7 bands per primer in sugarcane (Harvey *et al*., 1995) and 9 bands per primer in lettuce (Michelmore *et al*., 1991) has been reported. Different number of bands in each crop plant reflects that annealing sites of the primer vary not only among the plants belonging to different families but also in plants of the same family. However, the difference in the total number of RAPD bands among the cultivars or strains of the same species is non-significant. The results indicated that the varieties evolved in this region have a common gene pool. In the light of these findings it may be suggested that breeders are working with very narrow genetic base to tailor wheat cultivars (Cao *et al.,* 1998; Sun *et al*., 1998; Mukhtar *et al*., 2002; Rahman *et al*., 2002).

In the present studies, four primers produced polymorphic DNA bands. Of these, 3 primers (OPA-16, OPR-14 and OPZ-10) produced polymorphic DNA bands in the salt sensitive bulk and one primer, OPM-14, produced a polymorphic DNA fragment in the tolerant bulk. The primer OPA-16 did not reproduce the polymorphic DNA band. The lack of reproducibility might be due to the formation of artificial heteroduplexes between multiple amplified fragments or due to a partial match of primer-target sequences (He *et al*., 1992). To avoid the problem of reproducibility can be avoided; only intensely stained bands are scored as polymorphic DNA marker (Laucou *et al*., 1998).

The polymorphisms reproduced with the other three primers (OPM-14, OPR-14 and OPZ-10) (Figs. 1-2) were further confirmed by running PCR with individual DNA samples from which the bulks were constituted. The polymorphic DNA produced by the primer OPM-14 and OPR-14 did not confirm the polymorphism. The primer OPZ-10 reproduced the polymorphic band of around 680 bp in 11 out of 15 salt sensitive plants DNA, and the fragment was also amplified in 2 tolerant plants DNA. Hence, the polymorphic DNA fragment is linked in trans phase with the K:Na ratio. The use of BSA has been reported to tag gene(s) for a number of traits in different crop plants (Poulsen *et al.,* 1995; Mackay *et al.,* 2000; Ni *et al*., 2001). It is suggested that the combination of RAPD and BSA, is a useful technique in finding DNA markers (Oh *et al*., 1994). Efficiency and speed of plant breeding programs can be accelerated by marker-assisted selection (MAS) and permit persistent progress in the advancement of selected material. Further studies are needed to map the DNA marker on the chromosome.

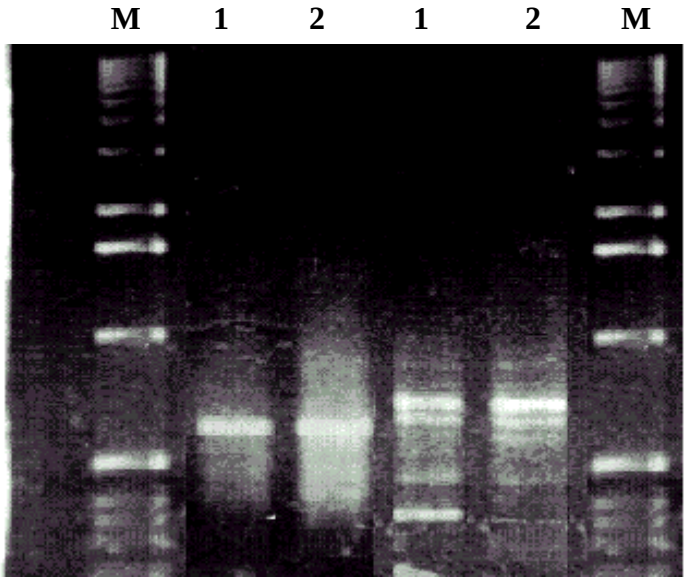

Fig. 1 Results of RAPD reactions involving two bulks (lane 1, salinity tolerant and lane 2 salinity sensitive) using 10-mer primers OPM-07 and OPM-14

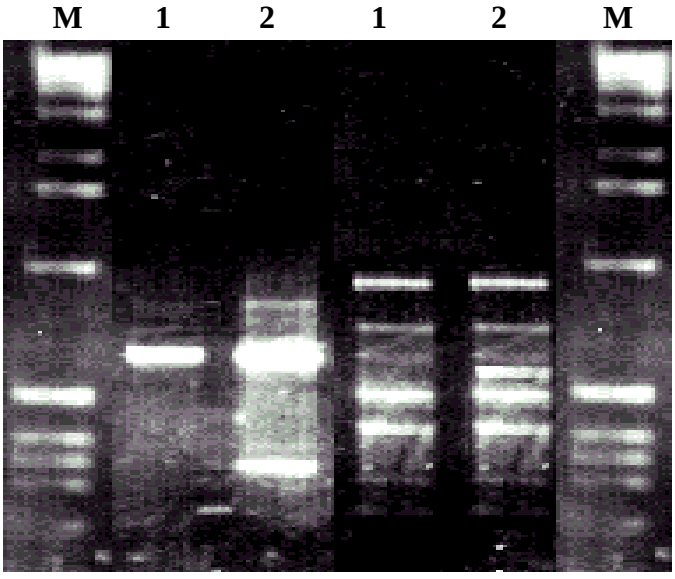

Fig. 2 Results of RAPD reactions involving two bulks (lane 1, salinity tolerant and lane 2 salinity sensitive) using 10-mer primers OPR-14 and OPZ-10.

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