Identification of Salinity Resistance Wheat (*Triticum Aestivum* L.) Genotypes by SSR Markers

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ABSTRACT

Wheat is an important staple food and cash crop of the world. To obtain better yield from saline soils and saline waters, it is imperative that salt tolerant wheat varieties should be developed. Soil salinity is one of the greatest problems faced by agriculture today. There are many mechanisms by which plants tolerant the problems associated with salinity all of which are genetically controlled. Salt tolerance is a mixture of different morpho-physiological processes which are controlled by many genes across the wheat genome. DNA markers enhance the recovery rate of isogenic recurrent genome after hybridization and facilitate the introgression of multiple genes with additive effects necessary to increase stress tolerance. Molecular markers are used to transfer alleles of interest from wild relatives into cultivated genotypes. SSR primer pairs were used to find DNA marker linked with salinity resistance. A total 200 SSR primer pairs used. Out of which four SSR primers were polymorphic with the parents. These four polymorphic primers applied on F2 population. Development of microsarellite markers provided a new approach to MAS in breeding practice. Based on these criteria microsatellite markers are preferable to RFLP markers. Once developed microsatellite markers are easy to use and less expensive to assay because the procedure for microsatellite analysis virtually involves only amplification and electrophoresis.

Introduction

The salt concentration in the soil, when reached a level that is harmful for growth, salinity is said to be developed. The accumulated salts are mostly the soluble salts of calcium, magnesium and potassium. The anions found are mostly chlorides and sulfates with some carbonates and bicarbonates [1]. Although plants growing in salty soils (halophytes) do not normally required high salinity, some like Halogeton glomerata, will not survive in non-saline culture solution, while other like salicornia, and grow only poorly [2]. One solution is to make the soil environment more suitable for the healthy growth of a wide range of plants. This can be achieved through a hydrological agricultural engineering approach in conjunction with the chemical/biological reclamation of salt-affected soils. However, certain limitations especially insufficient supply of canal water, saline underground irrigation water, the increasing price of energy-intensive inputs, poor soil drainage and mismanagement of water and soil resources mean that the technological approach is unlikely to achieve the desired success.

Since many of salt affected soils are beyond the reach of conventional reclamation techniques, a major scientific thrust has been aimed at developing suitable salt tolerant cultivars to bring these lands into agricultural productivity. Nevertheless, it must be emphasized that both approaches are complementary and not mutually exclusive. In Pakistan, wheat is grown on about 8.46 million hectares with total production of over 21.07 million tones, but a significant part of the wheat-growing area suffers from salinity and sodicity which adversely affects crop fields. If varieties of wheat capable of giving high yields on slight to moderately salt-affected soils could be developed, the productivity of such lands would be increased manifold and it might also permit expansion of agriculture into more marginal lands.

Salinity tolerance in wheat has been and is being extensively researched in Pakistan and elsewhere in the world but still 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. Wheat tolerance to salinity varies with the stage of plant growth, nature and level of salinity, duration of stress and is affected by soil moisture, climate, nutrition and management practices. 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 [3]. The demand for wheat is expected to grow faster than any other major agricultural crop. Due to land limitations, the enhancement of wheat production must come from higher absolute yields, which can only be met by the concerted action of scientists involved in diverse agricultural disciplines and in particular by increased efforts in plant breeding [4].

In addition to continuous investments in conventional breeding methods, biotechnological tools and the better understanding of the current and expanded genetic diversity should be considered for raising the yield frontier in wheat. Genetic diversity is pre-requisite for any successful breeding program. Cultivation or germplasm with a narrow genetic base entails a risk due to genetic vulnerability. Many wheat cultivars conserved in seed banks are not sufficiently characterized to inspire breeders 'interest for their efficient exploitation. Patterns of genetic variation within and among wheat land cultivars are usually unknown [5]. Little information about the genetic variation within and among land cultivar accessions is available.

Molecular markers can support a more detailed characterization of genetic resources. Avast potential lies in their ability to identify the structure of genetic diversity within and among accessions, which can be of great importance for the optimization of collections, the planning of seed regeneration, and the successful implementation of pre breeding approaches. Molecular markers provide a direct measure of genetic diversity and go beyond the indirect diversity measures based on agronomic traits or geographic origin. Simple sequence repeats (SSRs) are highly polymorphic in wheat and, therefore, suitable for the discrimination of genotypes. They are generally genome specific, abundant, co dominant, and cover all 21wheat chromosomes. They have been successfully employed to characterize genetic diversity in seed bank collections of improved wheat germplasm [6] and wild relatives [7,8].

Materials and Methods

The Plant Material

In the present studies, F_2 segregating populations and their parents, which obtain by crossing

(S-24 x DN-27).

Generation of Bluks

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 15 F_2 plants showing resistance to salinity, and the other was similarly formed from DNA of 15 F_2 plants susceptible to salinity. These two DNA were then used in polymerase Chain Reaction (PCR) to find polymorphism.

DNA Extraction

DNA was extracted following (Plaschke et al. 1995) with some modifications. Fresh leaf tissue (0.1-0.2 g) was ground with a pestle and mortar in liquid nitrogen and transferred into two Eppendrof tubes and added to them 0.75 ml of heated 2% CTAB buffer (Cetyltrimethyl ammonium bromide) containing 6μl mercapto-ethanol. This was incubated for 30 minutes at 65°C, then 0.75ml of chloroform: isoamyl-alcohol (24:1) was added and the tube was inverted gently for its proper mixing. Spinning it at 10,000 rpm for 5 minutes separated the plant debris. Avoiding the interface, the supernatant containing nuclear material was removed to a new tube and an equal volume of ice-cold isopropanol was added to it. Gentle mixing for 5 minutes allowed the DNA to precipitate and further centrifugation for 5 minutes at 10,000 rpm caused DNA to accumulate as a pellet. Supernatant was poured off and DNA was allowed to dry by inverting the tube over a tissue paper. DNA was resuspended in 1 ml 70% ethanol.

Ethanol was discarded after spinning for 5 minutes at 13,000 rpm in a microcentifuge. To further purify the DNA, it was resuspended in 400 μl TE (0.01M Tris HCl and 0.001 M EDTA (ethylene diamine tetra acetic acid disodium salt), pH 8.0), 40 μl 3M sodium acetate and 800 μl 95% ethanol. After 5-7 minutes this DNA was again centrifuged at 13,000 rpm for five minutes and supernatant was discarded leaving the DNA as pellet in the tube. This DNA was re-suspended in 1 ml 70% ethanol and spun at 13,000 rpm for 5 minutes. The supernatant was discarded, and DNA pellet was dried under vacuum by inverting the tube upside down for 30 minutes. The DNA pellet obtained was suspended in 100 μl sterile deionised water (SDW) at room temperature to dissolve it properly and stored in an Eppendorf tube at –20°C until required for further use.

DNA Quantification

Solutions of nucleic acids strongly absorb ultraviolet light with λmax 260 nm. The intense absorption is due to presence of aromatic purine and pyramidine bases. A solution of a double stranded DNA at a concentration of 50 μg/mL in a 1-cm quartz cuvette will give an A260 reading of 1.0. Therefore, the concentration (X) of double stranded DNA solution with A260 of 0.15 is,

50/1.00 μg/mL = X/0.15 μg/mL then   X = 7.5 μg/m

SSR Analysis

The amplification reaction was performed in a total volume of 20 μl following Roder et al. (1998) protocol SSR (PCR) reaction. Polymerase Chain Reaction (PCR) mixture was prepared using the

following composition for each DNA sample.

Sterilized Deionized Water (SDW)=		8.0µl
Primer Forward	=	0.5µl
Primer Reverse	=	0.5µl
DNA template	=	1.0µl
ReadyMixTM PCR Master Mix	=	10.0µl
Total volume	=	20.0µl

Out of the total PCR volume, 10 µl of each sample was taken and loaded in the high resolution (Metaphor) 2% agarose gel for visualization of PCR products

SSR Profile

A total of 61 Polymorphic SSR primers were applied to fingerprint the accessions. Roder et al. [10] protocol was used for SSR (PCR) profile:

Lid temperature		110 °C	
Initial Denaturation	94 °C	4 minutes	
Denaturation	94 °C	1 minute	
Annealing	58-60 °C	1 minute	45 Cycles
Extension	72 °C	1 minute	
Last extension	72 °C	10 minutes	
Hold at	22 °C		

Preparation of 2% Agarose Gel

The PCR products were run on 2% fine Agarose (EL grade) and bands were resolved by staining with ethidium bromide solution. Gel tray and combs were rinsed in distilled water. Appropriate combs were placed in gel tray. Prepared 100mL of 0.5 X TBE electrophoresis buffer was measured with graduated cylinder and poured into 250 mL conical flask. Added 2 gm of Agarose powder was to make 2 % gel and placed in microwave until it boils (1-2 minutes). Add 4 µL ethidium Bromide was added and gently swirled. The Agarose solution was gently added to gel tray taking care that no bubble was formed and removed if any formed. The gel was allowed to settle at room temperature for about 20-30 minutes then combs and end pieces of paper tape were removed.

Running a Gel

Gel box was filled by 0.5X TBE buffer and gel was placed in gel box. Added 3 µL migration dye was added to PCR products. Using the pipette 10 µl of each sample is loaded into appropriate lanes of the gel. 5 µl of ladder DNA was loaded on each end of wells. Run at 80 volts for 1hour. When dye is reached to opposite ends, the gel tray was removed, and gel was placed on UV Transiluminator to see the bands.

Data Analysis

The fingerprints were examined under ultraviolet Transilluminator and photographed using SyneGene Gel Documentation System. The SSR bands were counted, and the presence and absence of bands were scored by 1 (present) and 0 (absent). The data was analyzed for estimation of genetic diversity and similarities among accessions by using Popgen software Version 1.32.

Result and Discussion

They arise from different classes of DNA mutations such as substitution mutations, rearrangements or errors in replication of tandemly repeated DNA [11]. A total of Sixty one SSR primer pairs were used to find DNA markers linked with salinity tolerance. The conditions for polymerase chain reaction (PCR) were optimized for each of the SSR primers. Microsatellites are simple, tandemly repeated sequence motifs flanked by unique sequences. SSR polymorphism (SSRP) reflects polymorphism based on the number of repeat units in a defined region of the genome being investigated [12]. The genomic DNA of S-24 and DN-27 wheat parents were used to optimize the PCR condition for the SSRs. A concentration of 32.6 ng was found optimum for good amplification in final volume of 20 µl. Similarly, 2mM of $MgCl_2$ was found optimum in 20 µl final volume. In PCR, 2-2.5 units of Taq Polymerase are normally used in 100 µl final volume. However, in the present study, 1 unit/20µl reaction was used to amplify the loci without non-specific products [13-20].

Annealing temperature is one of the most important parameters that need adjustment in the PCR. The normal range of annealing temperature is 36-75µC.

Screening of Wheat Genotypes Involved in Crossing

The S-24 and DN-27 were screened with the SSR primer pairs. Polymorphisms were observed among the parents with Xgwm52-3D, Xgwm98-3D, Xgwm 115-4B and Xgwm137-2A primer pairs. The other was found monomorphic. The primer pair Xgwm52-3D amplified two alleles. The estimated size of alleles at one locus was 190 bp (a1) in S-24 and 185 bp (a2) in DN-27 but at the second locus the parents were monomorphic. Similarly, two alleles were amplified with Xgwm98-3D. The approximate size of polymorphic allele at one allele was 205 bp in the salinity resistant parent S-24, while it was absent in salinity sensitive parent (DN-27). Thus, the behaviour of this allele was dominant. The second allele was found monomorphic (Figure 1). These markers are selectively neutral because they are usually located in non-coding regions of DNA. With the use of molecular marker techniques, it would now be possible to hasten the transfer of desirable gene among varieties and to introgression of novel genes from related wild species. Polygenic traits which were previously very difficult to analyze using traditional plant breeding methods, would now be easily tagged using molecular markers [21-23].
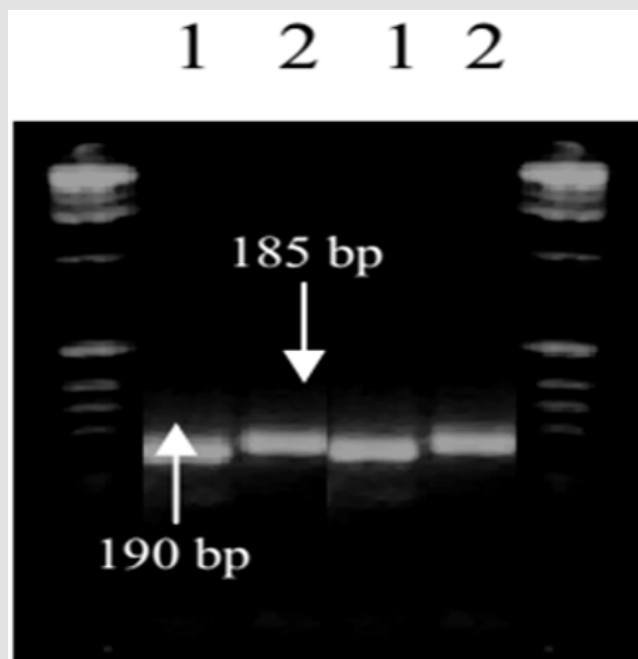
Figure 1: Screening of wheat genotypes involved in crossing with Xgwm52-3D. 1= S-24, 2= DN-27.
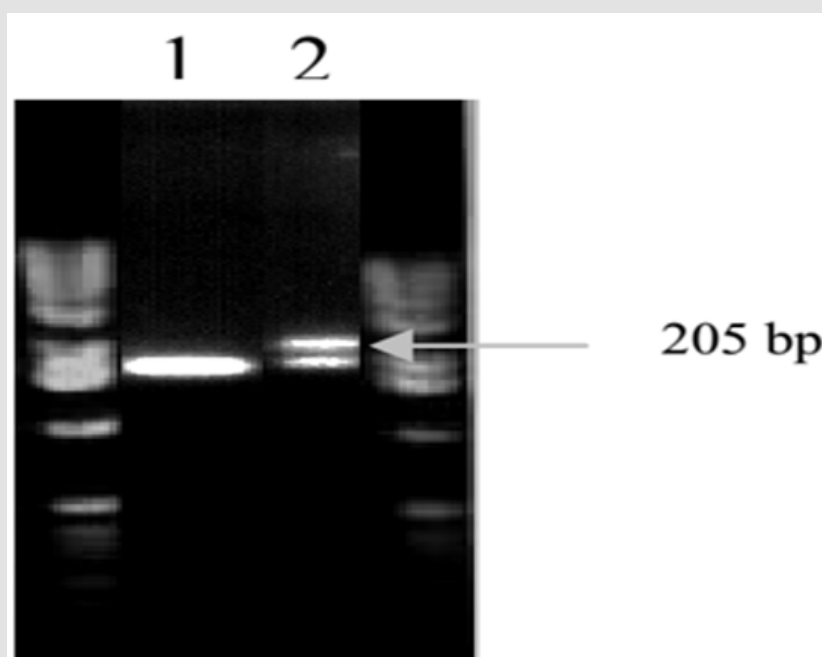

Figure 2: Screening of wheat genotypes involved in crossing with Xgwm98-3D. 1= S-24, 2= DN-27.

A total of two loci were detected with the primer pair Xgwm 115-4B. The estimated size of polymorphic allele at one locus was 240 bp in S-24 and was dominant in nature. At the second allele the parents were monomorphic. However, the polymorphism was only at first allele. Typically, they may be di-nucleotides (AC)n, (AG)n, (AT)n; trinucleotides (TCT)n, (TTG)n; or tetra-nucleotides (TATG)n, where 'n' is the number of repeating units. The (AT)n di-nucleotides are the most abundant type of SSR in plants, and AG, TC and AC motifs are less frequent (Powell et al., 1996). Similarly, the amplified product size with the primer pair Xgwm137-2A was in the range of 175-200 bp. The polymorphic product of Xgwm137-2A locus was easily resolved on 2.5% agarose gel. A total of two alleles were detected and designated as `a1' (200bp) and `a2' (175 bp). The polymorphic allele (a1.200bp) was present in S-24 but it was absent in DN-27. Thus, the expression of this locus was dominant (Figure 2). The second locus was found monomorphic. They are

valuable genetic markers because they are co-dominant, detect high levels of allelic diversity, and are easily and economically assayed by the polymerase chain reaction (PCR). Results from screening a wheat genomic library suggest that there are about 5700-10,000 microsatellite in wheat, with different repeats and their relative frequency decreases with increasing size of the repeat motif [24-28].

Microsatellites are highly mutable lici which may be present at many sites in a genome. As the flanking sequences at each of these sites may be unique, once SSR loci are cloned and sequenced. Primers can be designed to the flanking sequences. The resultant sequence tagged microsatellite usually identifies a single locus which, because of the high mutation rate of SSR, is often multi-allelic. Alleles which differ in many base pairs of length can be resolved on agarose gels but often SSR,s are visualized on sequencing gels where wingle repeat differences can be resolved and thus, all possible alleles can be detected. SSR are high informative markers because they are co-dominant (unlike RAPDs and AFLPs) and generally highly polymorphic. The nature of the PCR-based assay used in their amplification and detection implies that they are highly reproducible between laboratories.

Linkage of Microsatellite Markers with the Salinity Resistance Gene

The primer pairs Xgwm52-3D, Xgwm98-3D, Xgwm 115-4B and Xgwm137-2A that amplified polymorphic products among the parents were selected to amplify the genomic DNA of 30 each F_2 extreme salinity resistant and salinity sensitive plants derived from the crosses S-24 x DN-27. Firstly, the linkage analysis was conducted on F_2 resistant plants. The primer pair Xgwm52-3D amplified two alleles. At the first locus, 26 out of 30 homozygous resistant F_2 plants amplified `a1' allele. While, `a2' allele was amplified by 24 out of 30 homozygous susceptible F_2 plants. Thus, at this locus, `a1' allele was linked with the salinity resistant locus. The recombination frequency at this locus was 18.7%. Similiarly at the second locus two alleles were amplified (b1and b2). The recombination frequency at this locus was 20.1%. Two alleles were found with primer Xgwm137-2A in F_2 population of S-24 x DN-27 CROSS.A total of 30each salinity resistance plants were surveyed with the primer pair. Out of 30 salinity resistance plants amplified `a2' allele and the rest were recombinants. But seven of the salinity sensitive plants yielded amplification profile like parent (a2) indicating recombinant plants. The frequency of the recombination at this locus was 19%.

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