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Review

USE OF MOLECULAR MARKER TECHNOLOGY: WHEAT (AN OVERVIEW)

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ABSTRACT:

Molecular markers technology has significant impact in identification; isolation of genes or genomic regions associated with any discrete trait and manipulated genomic regions through marker associated selection. Selection of elite material through marker-assisted selection (MAS) provides an efficient complementary breeding tool. Genotypes selected on the basis of desired traits can be differentiated through molecular markers. Molecular markers allow cultivars identification in early stages of plant development as being neutral to environmental effects. A large number of molecular markers associated with different traits have been identified in various plants. This paper will focus a brief overview on molecular markers their useful in plant breeding particularly for wheat

INTRODUCTION:

Markers are "characters" whose inheritance pattern can be followed by at the morphological (color size), biochemical (proteins or enzymes) or molecular (DNA markers) level. Morphological markers are easily influenced by environment, while the molecular markers are numerous, based on variations in genomic DNA sequences and have no phenotypic effect on the plant, and do not influence by the environment (Jones *et al.*, 1997; Jefferies *et al.*, 1999). Molecular genetics is the study of the agents that pass information from generation to generation. These molecules (genes) are long polymers of DNA, composed of four chemical building blocks: guanine (G), adenine (A), thymine (T) and cytosine (C). DNA markers can be based on restriction fragment length polymorphism (RFLPs) or on the polymerase chain reaction (PCR) technique. The determination of genetic polymorphism (variation) at the DNA levels made possible through the use of cellular restriction enzymes (endonucleases). These restriction enzymes are able to cut DNA only at their specific in restriction site, which are unique & specific sequences of usually 4-6 base pairs (bp) in length. Generally, restriction sites are occurring throughout the genome, and consequently, application of the enzyme to total genomic DNA results in its conversion into millions of fragments. Restriction enzymes that cut at sites that are of common occurrence (frequent cutters) in a given genome will result in very large numbers of small fragments, whereas, restriction with an enzyme that cuts sites which occur rarely (rare cutters) will result in fewer and larger fragments. The different fragments can be separated according to their length (and hence molecular weight) by gel electrophoresis. (agarose or polyacrylamide).

The smallest fragments of DNA move faster through the gel and will separate towards the lower end of the gel, whereas, the larger fragments will move more slowly and will separate at the above end (top). Several different samples can be run in parallel on the gel, each sample resulting in a track composed of different fragment lengths. Each track can be visualized by addition of a dye known as ethidium bromide to the loading sample or gel. The dye intercalates with the DNA and the bands can be examined under ultraviolet

light. The plant and animal genome containing no much amount of DNA that the fragment tracks visualized after electrophoretic separation appears as smear. Therefore, some specialized techniques are being used to detect the polymorphism in the DNA of two different individuals. Some of these are based upon the initial digestion of the DNA with restriction enzymes (as AFLPs), while others depend upon the use of different enzymatic reactions, called as the polymerase chain reaction (PCR).

Molecular marker technology in wheat: Wheat (*Triticum aestivum* L.) is a major food crop of Pakistan, grown under a wide range of environments. Plant breeders are always interested in genetic diversity among their existing germplasm with the desired objectives, like wise high grain yield, early maturity with increased grain filling period, resistance to biotic (diseases) and abiotic stresses (drought tolerance and high temperatures) through conventional breeding (hybridization), mutagenesis, tissue culture and molecular techniques. Various researchers through out the world have successfully employed the use of molecular markers in many crop species including wheat (Rafalski and Tingey, 1993; Udupa and Baum 2001; Eujayl *et al.,* 2002). Identification and registration of bread wheat varieties is currently based on morphological and physiological characteristics. Even though these descriptors are useful, they are limited in number and may be affected by environmental factors. Molecular markers allow cultivar identification in early stages of plant development, being neutral to environmental effect.

Progress in molecular genetics has resulted in the development of DNA tags, which can be used in marker-assisted selection (MAS) strategies for cultivar development (Paterson *et al.,* 1991, Dubcovsky, 2004). These markers are particularly useful for incorporating genes that are highly affected by the environment, genes for resistance to diseases, a gene to increase grain protein content that cannot be easily screened through conventional methods. Accumulation of multiple genes for resistance to specific pathogens and pests within the same cultivar can be introduced through gene pyramiding. During last decade, public researchers constructed detailed wheat genetic maps including more than 3000 molecular markers and physical maps including more than 16000 loci (http://wheat.pw.usda.gov/NSF/;) and http://wheat.pw.usda.gov/PhysicalMapping/) (Dubcovsky, 2004). Cloning of agronomical important genes has made possible to develop "perfect markers", based directly on the allelic variation responsible for the differences in the trait. Examples of the perfect markers in the wheat include the glutenin genes for glutenin strength, the waxy gene for starch properties, puroindoline genes for hardness, the vernalization genes for vernalization requirement, the Rht genes for semi-dwarf habit and the Lr10 and Lr21 genes for leaf rust resistance (Briney *et al*., 1998, Beecher *et al.,* 2002, Yan *et al.,* 2003, Yan *et al.,* 2004, Peng *et al.,* 1999, Feuillet *et al.,* 2003). Wheat researchers have also developed closely linked molecular markers to yet unidentified genes with positive effects on quality traits and resistant to insects, fungi and viruses (Anderson, 2000). The potential of utilization of marker-assisted selection has increased as more molecular markers have been identified. In wheat up to year 2000, over 400 microsatellites or simple sequence repeats (SSR) markers have been reported (Bryan *et al.,* 1997, Stephenson *et al.,* 1998, Hill-Ambroz *et al.,* 2002). The majorities of studies investigating QTLs for agronomic traits indicate inconsistent QTL detection across different experiments, environments and populations (Campbell *et al.,* 2004, Beavis and Keim, 1996). In wheat, chromosome 3A is known to contain QTLs for agronomic performance traits that are

sensitive to different environmental conditions. Campbell *et al.,* .2003, detected significant QEI for grain yield, kernels per squire meter, grain volume weight, plant height, 1000-kernel weight, spikes per squire meter and kernels per spike. A major QTL (*QGyld.unl.3A.2*) was detected for grain yield and kernels per squire meter.

Wheat Genome: Bread wheat (*Triticum aestivum*, L.), is an allohexaploid (2n=6x=42), composed of three distinct genomes, A, B, and D, which are originated from natural hybrids of three diploid wild progenitors. *T. urartu Tum.*, is recognized as the donor of the A genome, although *Aegilops speltoides* was considered the donor of the B genome, current evidence suggest that the real donor is either extinct or discovered species belonging to the *Sitopsis* section of *Aegilops* . *Triticum tauschii,* also known as *Aegilops tauschii*, is widely recognized to be the donor of D genome. Among crop plant species, wheat posses one of the largest (about 16 billion bp per haploid genome) and most complex (hexaploid) genome, with a high percentage of repetitive sequences (90%), which makes it quite challenging to study and manipulate at the molecular level. However, polyploids (as wheat) have a greater ability to tolerate loss or higher dosages of chromosomes, referred to as aneuploidy (nullisomics, monosomics, trisomics, and tetrasomics).

Restriction fragment length polymorphism (RFLPs): RFLP, the non-PCR based markers has been extensively used for analysis of complex plant and animal genome in the past decade. Their application includes the construction of molecular maps, synteny studies and the identification of genomes of the crops such as wheat, rice and barley (Heun *et al.*, 1991; Graner *et al.*, 1991; Hayes *et al.*, 1993). These are single locus markers also used a diagnostic tool for screening ergonomically valuable traits such as CNN resistance and boron tolerance (Kretscmer *et al.*, 1997; Jefferies *et al.*, 1999). RFLPs are co-dominant markers (generate bands which can discriminate heterozygotes) and are highly reproducible. RFLP analysis is based on the generation of DNA fragments formed by the cleavage of DNA at a particular site with restriction enzymes. Within genomes, mutations can occur which may result in the creation or destruction of restriction enzyme recognition sites and these mutations can be detected among genotypes by separating out the fragments by gel electrophoresis. Normally, 5-10µg DNA is restricted and the fragments are transferred and fixed to a nylon membrane. These membranes can be hybridized using radioactive or flourescently labeled probes and analyzed by autoradiography (Rafalski and Tingey, 1993). Up to now, the efficient use of RFLPs has been limited due to various factors, such as restriction analysis and southern hybridization methods are expensive and time consuming and requires large amount of genetic material. RFLP analysis requires large quantities of DNA, multiple steps are involved and the results are slow as compare to PCR based assays.

Polymerase Chain Reaction (PCR): The development of polymerase chain reaction (PCR) was a technological break through in genome analysis since it enabled the amplification of specific fragments from the total genomic DNA. PCR is a very simple technique and defined as the *in vitro* synthesis of DNA, based on the function of copying enzyme DNA polymerase and two oligonucleotides. PCR was developed in 1983 by Karry B. Mullis, an American biochemist who won the Nobel Prize for Chemistry in 1993 for his invention (Mansoor, 2004). Before the development of PCR, the methods used to amplify, or generate copies of recombinant DNA fragments were time consuming and labour-intensive. In contrast, a machine designed to carry out PCR reactions can complete many rounds of replication, producing billions of copies of a

DNA fragment, in only a few hours. DNA polymerase has ability to synthesize a duplicate molecule of DNA from a template DNA and the primers are hybridized to opposite strands flanking the region of interest in the target DNA. The product of duplication of the original template DNA becomes a second template for another round of duplication; repeated duplications thus lead to an exponential increase in DNA product accumulation within few hours. The real breakthrough came when a *Taq* polymerase (a thermostable DNA polymerase isolated from themus or natures) was isolated and purified, however, the basic concept of PCR was first tested with Klenow polymerase. Primers are short stretches of DNA sequence, complementary to the opposite ends of the target sequence DNA. The DNA target is defined by primer annealing sites, they anneal to the complementary sequences in the target and thus 'prime' the polymerase amplification. Since both the strands of DNA molecule run in antiparallel orientation so the primer sequences point to each other. Amplification from virtually any region of a DNA molecule is possible by selecting specific sequences as primers at both flanks of the target region (the sequence of these flanks must be known). Primers can be synthesized or brought from commercial suppliers/ companies. In typical PCR-based assay, the building blocks (nucleotides) required to synthesize a new strand of DNA are mixed with the template containing the target DNA together with primers in a tube along with thermostable DNA polymerase. They pass through cycles of differential temperatures involving template denaturation, primer annealing, and extension of the annealed primers by DNA polymerase. Several types of PCR-based markers are being used in wheat genome such as random amplified polymorphic DNA (RAPDs), sequence-tagged sites (STSs), simple sequence repeats (SSRs) or microsatellite and amplified fragment length polymorphism (AFLP).

Random amplification of polymorphic DNA (RAPD): RAPD technology, first developed by Williams *et al.,* 1990, utilizes PCR methods to amplify several loci within genome with a single random sequence oligonucleotide primer. These markers have been used as species-specific markers. These are cheaper, faster and simpler to use than RFLPs, and no prior sequence knowledge is required. The primer source is limitless which makes them useful for the study of genomes across a broad spectrum of species. RAPD is generated by using oligonucleotide GC-rich primers; usually about 10 arbitary nucleotides in length. During PCR reaction primer will infrequently bind to DNA at two regions that are separated by a short length. The sequence between these priming sites can be amplified. The use of a small oligonucleotide and low annealing temperatures ensures that the primer binds to many randomly distributed sites and gives rise to products which are usually separated on agarose gels and detected by ethidium bromide staining. The number of generated fragments is dependent on the size of genome and the length of the primer. The amplified products usually consist of 5-10 discrete bands of length between 200-2000 base pairs. The sequence differences between different individual gives rise to bands, which are present or absent. Usually these are dominant markers and will segregate from a heterozygous diploid as Mendelian alleles (Rafalsaki and Tingey, 1993). RAPDs are difficult to transfer between species due to their dominant, random nature and short primers length. Recent studies have shown that the RAPD markers are easily generated by PCR, but are not reproducible due to low annealing temperature conditions required for PCR reaction. They do not usually show co-dominance and therefore heterozygotes cannot generally be distinguished from homozygotes of one class. However, they are useful in the rapid genertion of phylogenetic relationship

data and have been used for bulk segregant analysis of cereals and many other plant species (Jones *et al.*, 1997).

Amplified fragment length polymorphism (AFLP): AFLP has provided an alternative technique for the generation of highly reliable polymorphisms in eukaryotes genomes. DNA fragment patterns may be generated from any DNA source without prior sequence knowledge using a small amount of source material. AFLPs are often more informative markers than RFLP markers, as has been demonstrated in barley, where the diversity index using AFLPs is three times more than that of RFLPs (Russell *et al.*, 1997). AFLP technology has been extensively used for the generation of polymorphism in cereals (Qi et. al., 1996; Powell *et al.*, 1997) and generally used as bridging markers for the analysis of mapping populations (Becker *et al.*, 1995). AFLPs are generally dominant markers, however co-dominant to map the same genetic locus across varieties in some species. AFLPs have been successfully used to map agronomic traits such as barley leaf rust (Qi et. al., 1996).

The procedure to generate AFLPs is called selected restriction fragment amplification and is based on the selective PCR amplification of restriction fragments from a total digest genomic DNA (Liu *et al.*, 2002). The template DNA is usually restricted with a combination of a restriction enzymes i.e., four-base cutter and six-base cutter, which result in to enough fragments of sizes suitable for analysis. Generally *Msel* and PstI are the restriction enzymes of choice for achieving highly reproducible results. The combination of *Msel* and Pst I has also been used successfully, and generate thousands of AFLP bands. As the analysis of these numerous bands is not possible, therefore the specific primers are designed which typically generate 50-100 bands. The genetic variation of the restricted template is detected by selective PCR amplification with primers that extend in to the restricted fragments. The fragments only, which, the primer extension matches the sequence flanking the restriction sites will be amplified (Vos *et al.*, 1995). The fragments can be labeled with radioisotopes or fluorescent dye and are separated by electrophoresis on polyacrylamide "sequencing" gels. The bands can be scored using autoradiography or sophisticated genetic analysis systems, such as the system developed by PE Applied Biosystems (ABI). A single AFLP reaction can allow the detection of many loci simultaneously on multiple banding patterns. This provides breeders with more information and offers a powerful diagnostic tool to accelerate the backcrossing selection strategies. The breeding lines ultimately, with desired agronomic traits could be selected more efficiently, and about 50% time could be saved required releasing the new crop varieties (Jafferies *et al.*, 1997). The specific AFLP fragments associated with the traits can be excised by polyacrylamide gels and cloned, sequenced and converted to sequence tagged sites (STSs) or allele specific amplification (ASAs) and marker assays.

Microsatellites or Simple Sequence Repeats (SSRs): Genomes of eukaryotic organisms are rich with repetitive sequences known as the microsatellites or simple sequence repeats (SSRs). These sequences consist of tandem repeats of very short motifs and are randomly dispersed throughout the chromosomes. Microsatellites are highly mutable loci, composed of tandem repeats of 2-6 such as di-nucleotides (AT)n, (GT)n, (AC)n; tri-nucleotides (ATT)n, (TCT)n, (TTG)n; tetra-nucleotides (GACA)n,(TATG)n and so on DNA core sequences (base pairs) which are present through out the many sites in eukaryotic genome. These are highly polymorphic (can make up to 40% of genomes), even among closely related genotypes, due to mutations causing variation in the number of repeating units (Levinson

and Gutman, 1997). The flanking sequences at each SSR may be unique, which allowing the design of PCR primers that amplify the intervening SSRs (like sequence-tagged microsatellites).

These are usually single locus (because of the high mutation rate are often multi-allelic). The main advantage is that, they are co-dominant markers of the observed polymorphisms (which means the homozygous A and B, as well as heterozygous AB can be identified) and can be detected by PCR (non-hybridization based) assay. These are also transferable between genotypes within a species and most map to single locus, however, unlike RFLPs, microsatellites are not generally transferable between genomes (eg. between wheat and barley). Microsatellite markers have become a popular DNA marker system in wheat (Cook *et al*., 2004, Bryan *et al*., 1997). A microsatellite map developed by Roder *et al* 1995 demonstrates that microsatellite loci are well distributed across the wheat genome providing suitable coverage for marker analysis. Recently, several microsatellite have been identified which are ranked to both insect and disease resistant genes in wheat (Chantret *et al*., 2000, Anderson *et al*., 2000, Liu *et al*., 2002). The PCR products are usually run on polyacryl-amide gels and visualized using ethidium bromide staining, silver staining, autoradiography, or by using fluorescently labeled primers and automated analysis technology (Jones *et al*., 1997). Variability for each microsatellite locus can be measured using the 'Polymorphism Index Content' (PIC).

SSRs are co-dominant markers, well dispersed, reasonably polymorphic and also transferable between cultivars. The closely related varieties, which present high similarity coefficients, could be discriminated using selected microsatellites. The studies have shown that SSRs in plants can be up to 10 fold more variable than other markers system such as RFLPs (Rodder *et al*., 1995; Udupa et. al., 1999, 2001; Eujayl *et al*., 2002). Their origin and evolution is not fully understood however, it has been proposed by Levinson and Gutman, 1997, that unequal crossing over, and slip strand miss pairing events are involved in their formation. They have been used to construct maps in numerous plant species such as wheat, barley, maize, soybean, sorghum and tomato; analyze genetic diversity and genetic relationship within a crop and also recently used as diagnostic markers for the traits of economic values.

Hexaploid bread wheat is one of the worlds most important crop plants and displays a very low level of intraspecific polymorphism. Microsatellites, or simple sequence repeats (SSRs,) have become the markers of choice for genetic studies with many crop species including wheat. Currently an international effort is underway; to enrich the repertoire of available sequence tagged microsatellite site (STMS) markers in wheat. SSR markers can provide a cost effective and rapid screening procedure for agronomic traits. They can be multiplexed to allow the analysis of more than one trait in a single screen.

Some other marker systems: Some of the RFLP and AFLP marker systems can be converted to alternative markers, which are generally dominant markers (can not discriminate heterozygotes) such as STS (Sequence Tagged Site), CAPS (Cleaved Amplified Polymorphic Sequence) and ASA (Allele-Specific Amplification) (Rafalsaki and Tingey, 1993). The conversion of RFLP markers to PCR based assay can also reduces the cost and the time involved in the analysis of genomes. These markers are also transferable within wheat and barley genomes and the polymorphism can be easily detected by PCR methods (Erpelding *et al*, 1996). The STS markers are relatively more reliable and generated by designing the primers to the known sequence of a RFLP probe or to short

known sequence of a RFLP probe on specific locations in chromosomes. The polymorphism may be detected when PCR products of STS markers are cleaved with endonucleases restriction enzymes (usually four base pair cutters), and the digested fragments may be analyzed on agarose or polyacrylamide gels.

ASA marker system has been successfully applied in the development of probes linked to CNN resistance in wheat (Williams *et al.*, 1996). The polymorphism can be detected by using allele specific primers designed from alleles containing one or more mutations. These are also dominant markers, single band can be amplified from individuals possessing the appropriate allele. They can be analyzed by using ethidium bromide or fluorescent spectrophotometry techniques eliminating the need or gel electrophoresis.

Applications of molecular markers in plant breeding: Although conventional plant breeding has made remarkable progress in many crop species including wheat through out the world, however, it is often vulnerable by difficulties in selection of agronomically important traits, especially when they are influenced by the environmental stressed conditions. There are several applications of molecular marker technology; some of these are as follows:

DNA fingerprinting: DNA fingerprinting is proved to be a valuable tool of molecular markers for establishing varietal purity, genomic characterization and estimating the genetic diversity and genetic relationship among existing pure and segregating germplasm.

Genetic mapping of target trait (s): Genetic mapping of the target trait involved in phenotypic expression can be easily done through this technique. Genes or quantitative trait loci (QTLs) are identified and characterized via two ways 1) construction of suitable segregating population by crossing two parental lines contrasting for the target trait 2) identification of markers closely linked to the gene (s) of interest for further allelic manipulation. The number of genes or QTLs significantly involved in the expression of target trait, the effect (additivity, dominance) of the identified genomic regions and their impact on phenotypic expression of the trait can also be identified.

Linkage map: When target traits are governed or influenced by several genetic factors, a genetic linkage map of the complete genome must be developed and a QTL analysis conducted to associated traits with markers over the complete genome. Once a suitable number of markers have been identified, they are used to determine the allelic composition for all genotypes in the segregating population. Segregation ratios are based on Mendel's laws of segregation and independent assortment. If the two loci are linked, significant deviation from the expected segregation ratios can be observed and confirmed statistically by performing Chi-square tests. In an F2 population, a dominant marker should segregate 3:1, whereas a co-dominant marker that would allow the identification of hetero- zygotes should segregate 1:2:1. If a recombinant inbred line (RIL) or a doubled haploid (DH) population is used, segregation ratios should be 1:1 irrespective of whether the marker is dominant or co-dominant.

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