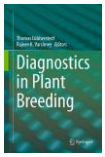

Evolving Molecular Marker Technologies in Plants: From RFLPs to GBS

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Evolving molecular marker technologies in plants: from RFLPs to GBS

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Abstract

Molecular markers have proven to be useful tools for genetics and molecular breeding of crop plants, starting with low-throughput RFLPs (restriction fragment length polymorphisms) in 1980 and culminating in ultra high-throughput SNPs at present. Molecular marker technology has continuously evolved from hybridization-based RFLPs to PCR-based RAPDs, AFLPs, and SSRs, and finally high-throughput SNPs. More recently, ultra high-throughput genotyping by sequencing (GBS) has been established. Among these molecular markers, SSRs were considered the markers of choice for several plant breeding applications because of their various desirable attributes, and are still considered inexpensive for simply inherited traits. However, more recently, SNP markers have become markers of choice due to their abundance, uniform distribution throughout genomes and high resolution as well as their amenability to high-throughput approaches. With the advent of next-generation sequencing (NGS) technologies, new sequencing tools have been found to be valuable for the discovery, validation, and application of genetic markers. These ultra high-throughput markers will not only prove useful for preparation of high-density genetic maps and identification of QTLs for their deployment in plant breeding but will also facilitate genome-wide selection (GWS) and genome-wide association studies (GWAS).

Introduction

Analysis of DNA-sequence variation (or allelic state) at a specific chromosomal location in an individual/genotype is referred to as genotyping. Variation in the DNA sequence may or may not have functional significance. For example, variation may result either in a synonymous or non-synonymous change in a codon. Such alterations may either cause a favorable change or deleterious mutations (mis-sense or non-sense) in an organism. Genetic variation may be small changes in frame (point-mutations, substitutions) or frame-shifts (insertions or deletions) (Jones et al. 2009). Nevertheless, these variations have been used as molecular markers to understand genome architecture as well as for plant breeding applications. Marker genotyping has various applications including parent genotype selection, screening mapping populations, genome mapping, trait mapping, germplasm diversity assessment, marker-assisted selection, linkage drag elimination in backcrossing and identification of genomic re-arrangements across taxa (Jain et al. 2002).

Variation in germplasm collections has been harnessed at both the morphological as well as molecular level. When morphological traits, including plant height, tillering, photoperiod, seed type, texture, leaf shape, and flower colour, have been used for assessing and utilizing genetic variation, they are referred to as ‘morphological markers’ (Tanksley 1983, Emami and Sharma 1999). As morphological markers are normally limited in number, the genetics and breeding community found a need to use enzymes and DNA polymorphisms as markers, which are referred to as biochemical and DNA-based ‘molecular markers’, respectively. Although biochemical markers are also molecular markers, the term is mostly used to refer to DNA-based polymorphisms. Molecular markers can provide genomic information for plant evaluation before entering the next cycle of selection which is critical for success in plant breeding (Bagge and Lübberstedt, 2008) and also help track polymorphisms with no obvious phenotype.

Due to advances in automation coupled with the demand of increasing throughput in a cost-effective manner, molecular marker technology has evolved during the last three decades. Based on their degree of multiplexing capacity /throughput, i.e., number of genetic loci per experiment, available molecular markers can be classified into the following categories: (i) low-throughput (100s of loci on 100s of lines), (ii) medium-throughput (from 100s up to 1000s of loci on 1000s of lines, (iii) high-throughput (1000s of loci on 1000s of lines), and (iv) ultra-high throughput marker systems (from 1000s loci up to 50,000 loci on 1000s of lines) (Figure 1). This article provides a brief overview of the different molecular markers in these categories with a major emphasis on emerging genotyping technologies including genotyping-by-sequencing (GBS). It is anticipated that new marker technologies/genotyping platforms will facilitate development of functional molecular markers (Table 1, Figure 1).

Low-throughput marker systems

Restriction fragment length polymorphisms (RFLPs)

RFLPs initialized the era of DNA marker technology during the 1980s in plant genetic studies and are, therefore, referred to as 'First generation molecular markers' (Jones et al. 2009). The polymorphisms detected by RFLPs are due to changes in nucleotide sequences in recognition sites of restriction enzymes or due to insertions or deletions of several nucleotides leading to detectable shift in fragment size (Tanksley et al. 1989). RFLPs have several advantages including high reproducibility, a co-dominant nature, no need of prior sequence information, and high locus-specificity. By using RFLP markers, genetic maps have been developed in several crop species including rice (McCouch et al. 1988), maize (Helentjaris 1987), wheat (Chao et al. 1989), soybean (Keim et al. 1990), tomato and potato (Tanksley et al. 1992), barley (Graner et al. 1991), and chickpea (Simon and Muehlbauer 1997). Although these markers have also been used for trait mapping (see Varshney et al. 2005, Gupta et al.

2010), they have not been found to be very useful for plant breeding applications. This can be attributed to the tedious and time consuming procedure involving their use as well as a general inability to automate the procedure.

Medium-throughput marker systems

The revolutionary advent of PCR during the 1980s stimulated development of different molecular marker types. A brief overview over some of these markers is provided below.

Random amplified polymorphic DNA (RAPDs)

RAPDs are probably the first PCR based genetic markers that were easy to use and inexpensive (Williams et al. 1990). RAPD markers are easy-to-use and less expensive as no prior sequence information is required. They are used as universal markers for species with little or no genomic resources available. RAPD markers have been extensively used in different plant species for fingerprinting, assessment of genetic variation in populations and species, study of phylogenetic relationships among species/subspecies and cultivars, and for many other purposes including gene tagging (see Gupta et al. 1999). However, RAPD markers are dominant that cannot distinguish between homozygous and heterozygous individuals. Furthermore, due to their random nature of amplification and short primer length, they are not a preferred choice for genome mapping. In addition, these markers do not exhibit reliable amplification patterns, are not reproducible, and vary with the experimental conditions (Huen and Helentjaris 1993, Ellsworth et al. 1993).

Simple sequence repeats (SSRs)

Simple sequence repeats (SSRs) or microsatellites were developed during 1990s and provided a choice for various studies since they are amenable to low, medium and high-throughput approaches. SSRs are easily assayable by gel electrophoresis for few to hundreds of samples, which could be affordable by laboratories with limited resources. SSRs are often derived from non-coding/anonymous genomic regions, such as genomic survey sequences (GSSs) and bacterial artificial chromosomes (BACs). As a result, development of SSR markers used to be expensive and laborious. In recent years, however, due to the availability of large-scale gene/EST (expressed sequence tag) sequence information for various plant species, SSR markers can easily be developed *in silico*. Such markers have been referred to as genic SSR markers and have been developed in a very cost-effective manner (Varshney et al. 2005). The high degree of polymorphism as compared to RFLPs and RAPDs, their locus specific and co-dominant nature, make them the markers of choice for a variety of purposes including practical plant breeding (Gupta and Varshney 2000). SSR markers dominated genetics research and breeding applications, especially in plants for more than a decade. SSR markers are probably the only class of markers that have been used for almost all aspects of genetics research and breeding in a wide range of plant species (Gupta and Varshney 2000, Varshney et al. 2005).

Amplified fragment length polymorphism (AFLPs)

Amplified fragment length polymorphism is a multi-locus marker technique that combines the techniques of restriction digestion and selective PCR amplification of restriction fragments and can be applied to DNA of any origin or complexity (Vos et al. 1995). The use of AFLP markers is cost-effective, since it needs moderate amounts of DNA, and a single assay allows simultaneous detection of a large number of co-amplified restriction fragments. Moreover, AFLPs are considered to be a robust and reliable genotyping technique, as

stringent primer annealing conditions are used. The high frequency of identifiable AFLP bands coupled with a high reproducibility makes this technology an attractive tool for fingerprinting, constructing genetic maps and saturating genetic regions with low marker density (Gupta et al. 1999). In addition, the property of reliable inheritance and transferability of these markers have encouraged their application in genetic diversity analyses in several crop species like rice (Mackill et al. 1996, Zhu et al. 1998, Maheshwaran et al. 1997), wheat (Huang et al. 2000, Xu and Ban 2004, Barrett et al. 1998, Shan et al. 1999, Soleimani et al. 2002), barley (Faccioli et al. 1999, Shan et al. 1999), and also in legume species like soybean (Maughan et al. 1996, Young et al. 1999) and chickpea (Winter et al. 2000, Nguyen et al. 2003). While AFLPs have also been used for trait mapping in several instances, the conversion of associated AFLP markers into a locus-specific and user-friendly marker such as a sequence tagged site (STS) or sequence characterized amplified region (SCAR) has not always been straightforward. Therefore, use of AFLP markers has not been common for molecular breeding applications (Xu and Ban 2004).

High-throughput marker systems

Molecular breeding in general involves screening of large segregating populations with molecular markers. Therefore, screening of markers in a high-throughput manner can offer cost-effective marker genotyping and enhance adoption of molecular markers in plant breeding applications. In this context, genotyping of SSR markers in a high-throughput manner has been adopted by using ABI capillary sequencing electrophoresis and the Multiplex-Ready^{TM} marker technology (MRT) (Appleby et al. 2009). Despite of those high-throughput SSR platforms, , costs are still prohibitive for many breeding programs.

Single nucleotide polymorphisms (SNPs)

Single nucleotide polymorphisms (SNPs) are the most abundant sequence variation in nature (frequency varies with each organism/species) (Rafalski 2002). SNPs are mostly bi-allelic and arise either due to substitutions/point mutations (transition and transversion) or due to insertion/deletion of nucleotides and are detectable when similar genomic regions from different genotypes of same or different species are aligned. Their occurrence in coding sequence may be linked to phenotypic changes in an organism. SNPs are not only efficient in terms of reliability, reproducibility and transferability, but are also amenable to automation and high-throughput approaches.

Although initially development of SNP markers was considered expensive as it mainly involved allele-specific sequencing, the advent of NGS or second generation sequencing technologies (454/FLX, Solexa/Illumina, SOLiD/ABI) has brought sequencing cost down (Thudi et al. 2012). Very recently, the third (or future) generation sequencing technologies such as single molecule sequencing (PacBio/Pacific Biosciences (USA), HeliScope/HelicosBiosceinces (USA), and Polonator (Dover/Harvard, USA) started to emerge (Thudi et al. 2012). These third generation sequencing technologies are expected to further reduce sequencing costs drastically to levels below $1 per mega base compared to $60, $2, and $1 estimated costs for sequences generated by 454/FLX, SOLiD/ABI, and Solexa/Illumina, respectively. All these sequencing technologies are being used for whole genome *de novo* and re-sequencing studies (synteny.cnr.berkeley.edu/wiki/index.php/Sequenced_plant_genomes), reduced representation sequencing (Hyten et al. 2010a, Davey et al. 2011), targeted genomic sequencing (Delmas et al. 2011, Griffin et al. 2011), paired-end sequencing (Rounsley et al. 2009), meta-genomic sequencing (Ottesen et al. 2011), transcriptome sequencing (Cheung et al. 2006, Hiremath et al. 2011), small RNA sequencing (Gonzalez-Ibeas et al. 2011, Zhou et

al. 2009), and chromatin immune-precipitation sequencing (ChIP) (Shendure et al. 2008, Varshney et al. 2009). As a result, it has become easier and very-cost effective to quickly identify a large number of SNPs in short time in any plant species.

For genotyping SNP markers in low to medium-throughput approaches, more than 30 assays are currently available that can be classified into four reaction principles or chemistries: hybridization with allele-specific oligonucleotide probes, oligonucleotide ligation, single nucleotide primer extension, and enzymatic cleavage (Gupta et al. 2001, Syvanen 2005, Kwok 2001, Steemers et al. 2006). However, very recently additional SNP genotyping platforms from the company Illumina have been developed and discussed below in detail.

GoldenGate assays

Illumina's GoldenGate assay provides SNP genotyping for genome-wide marker profiling. Thus, one can select any number of SNPs (for each of the samples to be genotyped) and the throughput level best suited for a study. GoldenGate assays may be developed for any crop species using either BeadArray, or Veracode technology (Thomson et al. 2011). On the basis of level of multiplexing and through-put, GoldenGate assays can be classified into: (i) GoldenGate BeadArray allowing simultaneous genotyping of 96-, 192-, 384, 768-, 1536- and 3,072 SNP loci in a fairly large collection of samples, (ii) GoldenGate Veracode (*BeadXpress*) allowing genotyping of 48-, 96-, 192-, and 384-plexes, and (iii) GoldenGate Indexing allowing genotyping of 96 to 384 SNPs simultaneously. Among these GoldenGate Indexing screen up to 16 times more samples per reaction than one can do with the standard GoldenGate assay thereby decreasing costs of the genotyping assay. These assays are used for a variety of applications such as association mapping, linkage mapping, and diversity analyses in crops like rice (see McCouch et al. 2010, Thomson et al. 2011), wheat (Akhunov et al. 2009, Chao et al. 2010), barley (Rostoks et al. 2006, Close et al. 2009, Druka et al.

2011), maize (Yan et al. 2010, Mammadov et al. 2011), soybean (Hyten et al. 2008, Hyten et al. 2009), common bean (Hyten et al. 2010b), pea (Deulvot et al. 2010), and cowpea (Muchero et al. 2009).

Competitive Allele-Specific PCR (KASPar) Assays

Above mentioned GoldenGate (GG) assays by Illumina seem are superior for genotyping a a large number of SNPs/sample for several samples. This makes KASPar a simple, cost-effective and flexible genotyping system, since the assays can be adjusted with a range of DNA samples. However some molecular breeding applications such as marker-assisted selection (MAS) or marker-assisted backcrossing (MABC) employ genotyping of large number of lines with only few SNPs. In such cases, new genotyping assays that involve competitive allele-specific PCR for a given SNP, followed by SNP detection via Fluorescence Resonance Energy Transfer (FRET) have been developed (Chen et al. 2010). These assays for the target SNPs are being developed and used for genotyping commercially by Kbioscience UK (http://www.kbioscience.co.uk/) and are referred as **K**BioScience **A**llele-**S**pecific **P**olymorphism (KASP) or KASPar assays. One of the advantages of using KASPar assays is that there is no need of sequencing to identify SNPs for assay development, instead SNP flanking sequences already known while developing different types of genotyping assays (e.g., Illumina) can readily be used for primer design (one common and two allele-specific primers) for KASPar assays (for review see McCouch et al. 2010). Although KASPar genotyping assays have come to the market very recently, they have started to be used for genetic diversity studies (Maughan et al. 2011, Cortes et al. 2011) and genetic mapping (Allen et al. 2011).

Diversity array technology (DArT)

Diversity array technology (DArT) is a high-throughput microarray hybridization based assay involving genotyping of several hundred polymorphic loci simultaneously spread over the genome without prior sequence information (Jaccoud et al. 2001). DArT markers are bi-allelic and behave mostly in a dominant (presence *vs* absence) or sometimes in a co-dominant (2 doses *vs* 1 dose *vs* absent) manner. These markers usually detect polymorphisms due to single base-pair changes (SNPs) within restriction sites recognized by endonucleases, or due to insertion/deletion (InDels) or rearrangements (Jaccoud et al. 2001). The technique is reproducible and cost-effective, and has become available for >70 species of both plants and animals (http://www.diversityarrays.com/genotypingserv.html). In plants, DArTs have been already developed in all major crop species including rice (Jaccoud et al. 2001), wheat (Akbari et al. 2006, Semagn et al. 2006, White et al. 2008, Peleg et al. 2008, Jing et al. 2009), sorghum (Mace et al. 2008, Mace et al. 2009), rye (Bolibok-Brągoszewska et al. 2009), oat (Newell et al. 2011), triticale (Badea et al. 2011) and more than 30 other plant species (Jing et al. 2009). It is important to note that for wheat alone more than 50,000 samples (>95% as service at ~1 cent per marker assay), >350 mapping populations have been processed, which resulted in preparation of >100 genetic maps with ~7,000 markers assigned to chromosomes (A. Kilian, personal communication). DArT markers have been extensively used for diversity studies, genetic mapping, bulked segregant analysis (BSA), QTL interval mapping, and association mapping.

Ultra high-throughput marker systems

Some modern genetics and breeding approaches like genome-wide association studies (GWAS) and genome-wide selection (GWS) or genomic selection (GS) require genotyping

of large populations with a large number of markers. Such studies require ultra-high throughput marker systems (Figure 1, Figure 2).

Infinium assay for whole-genome genotyping

Illumina's Infinium assay based on BeadChip^{TM} technology is a high-density SNP genotyping technology for whole-genome genotyping allowing for genotyping of hundreds of thousands of SNPs simultaneously. One of the advantages of this system is that it allows simultaneous measurement of both signal intensity as well as changes in allelic composition (Gupta et al. 2008, Varshney 2010). This assay involves the use of 12-, 24-, 48-, or 96-sectioned BeadChips simultaneously, where each section of a BeadChip contains 1.1 million beads carrying oligo-nucleotides with known functions (Syvanen 2005, Gunderson et al. 2005, Steemers and Gunderson 2007). The challenge for the development of infinium assays in plants was the availability of a sufficient number of SNPs. This problem has been solved with the advent of NGS technologies, which allowed discovery of sufficient high density SNPs for infinium assays. Infinium assays have already been developed and used in crop plants. For instance in soybean, the *Illumina Infinium iSelect SoySNP50 chip* containing 44,299 informative SNPs was used to resolve the issue of origin of genomic heterogeneity in William 82 cultivars (Haun et al. 2011). In maize, a 50K SNP Infinium chip containing SNPs in approximately two-thirds of all maize genes providing an average marker density of ~1 marker every 40 kb was developed (Ganal et al. 2012). Infinium genotyping assays have been developed in tree species like loblolly pine to study population structure and environmental associations to aridity (Eckert et al. 2010). The commercially availability of these high density SNP platforms will undoubtedly facilitate the application of SNP markers in molecular plant breeding (Mammadov et al. 2011).

Genotyping-by-sequencing (GBS)

Recent advances in NGS technologies have helped us in providing unmatched discovery and characterization of molecular polymorphisms e.g. SNPs. However, before assaying the identified polymorphisms, there is a need to develop the genotyping platform. Genotyping-by-sequencing (GBS) is an approach that identifies and genotypes the SNPs simultaneously. GBS is a robust, cost-effective, highly multiplexed sequencing approach considered a powerful approach for association studies and also to facilitate the refinement (anchoring and ordering) of the reference genome sequence while providing tools for GAB. With the continuous increase in NGS machine output, thereby continuous reduction in cost/sample, GBS will clearly become the marker genotyping platform of choice in coming years. Unlike other SNP discovery and genotyping platforms, GBS overcomes the issue of ascertainment bias of SNPs in a new germplasm. Keeping the cost/sample in view, it is also believed that GBS will provide an attractive option for genomic selection applications in breeding programs where cost per sample is considered a critical factor (Huang et al. 2010, Elshire et al. 2011, Poland et al. 2012).

GBS approach involves the use of restriction enzymes (REs) for reducing the complexity of genomes followed by targeted sequencing of reduced proportions, so that each marker can be sequenced at high coverage across many individuals at low cost and high accuracy. Overall, the process of GBS involves the following sequential steps: (i) isolation of high quality DNA, (ii) selection of a suitable RE and adaptor, (iii) preparation of libraries for NGS, (iv) single-end sequencing of either 48-plex or 96-plex library on NGS platforms like Genome Analyzer II or HiSeq 2000 of Illumina Inc. (www.illumina.com/systems.ilmn), (v) sequence quality assessment/filtering, (vi) sequence reads alignment, (vii) calling of SNPs. The complete

procedure of GBS has been described elsewhere (Elshire et al 2011) and a modified approach has been also developed and tested in wheat and barley recently (Poland et al. 2012). A workflow of GBS has been presented in Figure 3. Comparison of GBS approach with other marker systems has also been presented in Table 1.

The choice of an appropriate RE is a critical factor in GBS approach for masking the repetitive regions of the genomes and, thereby, increases the chance of sampling markers from hypo-methylated gene rich regions of the genome. In the original GBS approach used in case of maize and barley, only one RE "*Ape*KI" (methylation-sensitive enzyme) was used to reduce the complexity and to select hypo-methylated regions of genome for sequencing (Elshire et al. 2011). However, recently, two REs (one''rare-cutter'' and one ''common-cutter'')-based GBS protocol has been developed and used for a species without a reference genome sequence. The two REs approach has advantages of generating suitable and uniform complexity reduction of complex genomes and has been earlier successfully tested in sequencing pools of BAC libraries for construction of physical maps (van Oeveren et al. 2011). Such GBS protocol has recently been used for genotyping bi-parental populations of wheat and barley for developing a genetically anchored reference map of identified SNPs and tags. This approach resulted in identification and mapping of >34,000 SNPs and 240,000 tags onto the Oregon Wolfe Barley reference map, and 20,000 SNPs and 367,000 tags on the Synthetic W97846 X Opata85 (SynOpDH) wheat reference map (Poland et al. 2012). In addition to above, Ion Torrent NGS platform has been also used for GBS in maize (http://www.invitrogen.com/etc/medialib/images/agricultural-biotechnology/pdf.Par.20344.File.dat/Maize-Genotyping-by-Sequencing-on-Ion-Torrent.pdf). This involves a two-step GBS protocol for genotyping of maize inbreds/RILs at up to a few hundred pre-defined SNPs in only two working days. The method in brief involves: i)

amplification (via multiplex PCR) of Genotyping by Multiple Amplicon Sequencing (GBMAS) targets, and ii) Addition of unique barcodes to the PCR products from each individual RIL and pooling of all the PCR products for Ion library construction and sequencing.

In summary, GBS is a highly multiplexed approach that can typically lead to the discovery of thousands of SNPs in one experiment and may be suitable for population studies, germplasm characterization, high-density genetic mapping, genomic selection and other breeding applications in diverse organisms (Huang et al. 2010, Elshire et al. 2011, Poland et al. 2012). The GBS approach can be used even in those plant species that do not have the reference genome available. In such cases, the sequence tags can be treated as dominant markers for kinship analysis. Moreover, availability of the genome sequence in a given species helps in increasing the number of marker loci analyzed through imputation.

Cost effectiveness of different high-throughput markers

One of the critical requirements of deployment of markers in molecular breeding programs is their cost effectiveness. While comparing different high-throughput markers systems, the DArT marker system offers the lowest costs per marker data point. The cost per marker assay in commercial service offered by Triticarte P/L is ~US$ 0.02 (or approximately US $50 per genotype; Mantovani et al. 2008), which may be >6 times lower than the costs of SSR genotyping. A similar comparison with Illumina GoldenGate assays indicate, that DArT assays are only ~3 times cheaper (Yan et al. 2010). However, GoldenGate assay based-SNP genotyping is 100-fold faster than gel-based SSR methods leading to cost savings of ~75% (Yan et al. 2010). GBS available in -48, -96, and -384 array-plexes may further reduce the cost of genotyping and may become the method of choice for future plant genotyping. The

continuous reduction in costs of GBS is due to increases in multiplexing, and, thus, lower labor, reagent, and sequencing costs. For instance, the labor cost was decreased from ~$2.00 for 48 to ~$0.50 for 384-plexes, while sequencing costs decreased from ~$33.00 for a 48-plex to ~$9.00 for a 384-plex assay. It is, therefore, obvious that the increase in throughput of markers is coupled to a reduction in their costs. Therefore, advances in NGS technologies will continuously help in reducing the costs of sequencing and, thus, the reduction in the cost of marker development and application (Davey et al. 2011).

Summary and outlook

As is evident from the discussion above, that varying levels of throughput (low to ultra-high) are available. Thus, an appropriate marker system can be selected based on the need. For instance, Illumina's GoldenGate assays and Infinium assays as well as DArT markers are suitable for the construction of genetic linkage maps and GWAS studies, but these marker systems may not be suitable for molecular breeding applications such as marker-assisted selection (MAS), or marker-assisted backcrossing (MABC). One of the reasons for this is that genotyping costs for all the SNPs present in GoldenGate or Infinium assays is in lieu of only few informative SNP markers that are linked to the traits of interest. Alternatively, the associated markers present in GoldenGate or Infinium assays need to be converted into a user-friendly assay like KASPar or TaqMan assays. KASpar assays have become very cost effective in case of large populations (Figure 2).

SNP markers that are transferable across different genotyping chemistries will serve as flexible selection tools for plant breeders in marker-assisted selection (MAS). However, technical issues may jeopardize the conversion and application of a particular marker for MAS (Mammadov et al. 2011). Recently, a set of 695 putative functional GoldenGate assay

based-SNP assays were identified in maize and converted into Infinium, TaqMan, and KASPar chemistries with a high efficiency ranging from 89% for GG-to-Infinium to 98% from GG-to-KASPar (Mammadov et al. 2011). As a result of this conversion, a set of 162 highly polymorphic, putative functional and versatile SNP assays were identified and will be universally utilized in molecular genetics and breeding projects.

In contrast, low to moderate through-put marker systems like SSRs can be deployed for selection of targeted genomic loci in breeding populations without any difficulty. While comparing the value of SSR with DArT markers, it was found that SSR markers can be preselected and may, therefore, represent whole genome coverage, which is not the case of DArT markers (Yan et al. 2010). The other obstacle is that one cannot use a selected DArT marker, identified through QTL interval mapping or association mapping, directly for marker-assisted selection (MAS) procedures. For using an associated DArT marker in MAS, the marker needs to be converted to a user-friendly assay. For instance, five robust SCARs were developed from three non-redundant DArTs, that co-segregated with crown rust resistance gene "*Pc91*" in oat (McCartney et al. 2011). However, the conversion of the associated DArT marker to a PCR-based marker is not always possible especially in cases where sequence data for DArT clones are not available.

For marker genotyping of a large number of marker loci for applications such as genome-wide association studies (GWAS) and genomic selection (GS), the GBS approach seems to be the best approach in terms of costs as well as throughput. With the increasing availability of reference genome sequences in a range of crop species, GBS is going to be the approach of choice in majority of the plant species in the coming years. It is anticipated that availability and routine use of GBS technology may re-orient molecular breeding programmes from MAS

to GS, which will allow the realization the full potential of genomics-assistsed breeding in crop improvement.

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Table 1. Comparison between different marker systems

Sl. No.	Attribute	Low-throughput markers	Medium-throughput markers			High-throughput markers				Ultra high-throughput markers	
		RFLP	**RAPD**	**AFLP**	**SSR**	**KASPar**	**GoldenGate Beadchip**	**GoldenGate Beadarray**	**DArT assays**	**Infinium assays**	**GBS**
1	DNA amount/reaction	2-10µg	5-10 ng	~1 µg	10-20ng	≥5ng	+	+	50-100ng	+	100ng
2	DNA quality	high	moderate	moderate	moderate	moderate	moderate	moderate	high	moderate	high
3	Cost	high	less	high	high	low	high	high	Cheapest	high	Low/moderate
4	Reproducibility	very high	low	high	high	high	high	high	high	high	high
5	Radioactivity	yes	-	yes	-	-	-	-	-	-	-
6	Markers assayable	<100	<100	>100	>500	as per requirement	48-384	3,072	96-5,000	1,000-5,000	>1,000 -
7	Technical procedure	tedious	simpler	tedious	simpler	automated	automated	automated	simpler	automated	automated
8	Sample size	<50-100	<100	<100	48-384	<100-1,000	>1,000-3,072	48-1,000	100-500	>1,000	>1,000
9	Sequence information	-	-	-	yes	yes	yes	yes	-	yes	yes
10	Multiplexing	difficult	difficult	possible	possible	possible	possible	possible	No	possible	possible

Figure legends

1. **Low to ultra high-throughput cost-effective marker assay platforms for genotyping**

   Horizontal axis indicates number of loci that can be assayed in a single experiment, while the vertical axis indicates the number of lines/samples that can be genotyped in high-throughput manner at low cost

2. **Marker assay platforms for plant genetic analysis**

   A diagrammatic representation of utilization of different sequencing platforms for marker discovery and their subsequent use in plant genetic analyses.

3. **A workflow for genotyping-by-sequencing (GBS) approach**

   A schematic representation of various steps involved in GBS approach, adapted from Elshire et al. (2011) and Poland et al. (2012), has been shown.