

Chapter 2

Array-Based High-Throughput DNA Markers and Genotyping Platforms for Cereal Genetics and Genomics

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2.1 Introduction

During the last three decades, DNA-based molecular markers have become indispensable tools for detailed genetic analysis and molecular breeding in crop plants. Newer marker systems have been developed at regular intervals during last more than 20 years, and have been discussed by us in a series of articles (Gupta et al. 1996, 1999a, b; Gupta and Varshney 2000; Gupta et al. 2001, 2002; Gupta and Rustgi 2004; Gupta et al. 2008). For cereals, these marker systems and the corresponding molecular maps developed until eight years ago were described in an article included in our earlier edited volume '*Cereal Genomics*' (Gupta and Varshney 2004). A number of other reviews on molecular markers and their uses also appeared after the publication of our book '*Cereal Genomics*'. In particular, a book "*Molecular Marker Systems in Plant Breeding and Crop Improvement*" edited by Lörz and Wenzel (2005) contained a number of useful articles. Based on these earlier reviews, it may be recalled that the marker systems used in 1980s, 1990s and in the early years of the present century were largely either hybridization-based (without PCR; e.g., RFLPs) or PCR-based (involving slab-gel or capillary-electrophoresis based separation of PCR products). In most cases, genotyping was performed

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for individual markers, although multiple loading, multiplexing and multi mixing did improve the speed of genotyping. But this status of molecular marker technology was still far short of the kind of high-throughput that is required for genotyping of either thousands of plants with few markers or a limited number of plants with millions of individual markers. This level of high throughput is needed not only for high precision in the detection of QTLs/genes involving linkage-cum-association mapping, but also for their use in improving the efficiency of large plant breeding programs. Map-based cloning of genes/QTLs involving use of large segregating populations is another area of research, where this high-throughput is required.

During the last eight years, i.e. after the publication of the edited volume “*Cereal Genomics*” in 2004, there has actually been a revolution in the development of not only the new marker systems, but also in the development of corresponding high throughput-genotyping platforms. This led to the extensive use of molecular markers in gene discovery/cloning and molecular breeding studies in cost/time-effective fashion, thus making them routine in most laboratories around the world. Further, this has become possible particularly due to the adoption of microarrays/chips, real-time detection methods and next generation sequencing (NGS) technologies for discovery and detection of markers, which is a pre-requisite for identification of genes/QTLs associated with specific traits of interest. The marker-trait associations (MTAs) identified for a variety of simple and complex traits in a number of crops have also been effectively utilized for molecular breeding leading to the release of improved cultivars, particularly in cereals including wheat (Gupta et al. 2010a, b), rice (Singh et al. 2011) and maize (Prasanna and Hoisington 2003). The importance of molecular breeding in developing countries has also been repeatedly emphasized (Ribault et al. 2010; Anthony and Ferroni 2011).

The choice for marker systems that are now being increasingly utilized has also shifted from the first and second generation marker systems including RFLPs, RAPDs, SSRs and AFLPs to the third and the fourth generation marker systems, which include SNPs, DaRT, TDMs (including SFPs), ISBP markers (Gupta et al. 2008; Potokina et al. 2008; Paux et al. 2008, 2010, 2012), and CNVs/PAVs (Springer et al. 2009; Belo et al. 2010). Most of these latest marker systems make use of microarrays, which is the subject of this brief review. In fact, with the availability of NGS technology, and due to the possible low-cost resequencing of whole genomes in crops like rice (with a small genome), it is now possible to resolve recombination breakpoints within an average length of 40 kb. It has been estimated thus that in comparison with the PCR-based markers, map construction using sequencing based methods is now $20 \times$ faster in data collection and has $35 \times$ higher precision in determination of recombination breakpoints (Huang et al. 2009; Wang et al. 2011; also see, Seifollah et al. 2013 in this volume).

The array-based marker systems became available more than five years ago, and were discussed by us in an earlier review (Gupta et al. 2008). In this earlier review, we had briefly described four types of marker systems (SNPs, SFPs, DaRT and RAD), the corresponding genotyping platforms and their effective use in a variety of plant species. However, during the last few years, after the publication of our above review, the array-based genotyping platforms have been increasingly used in plants including cereals, thus establishing their utility in molecular breeding programs.

Array-based comparative genomic hybridization (aCGH) has also been used for detection of structural variations (SVs) including copy number variations (CNVs), presence-absence variations (PAVs) and insertions/deletions (InDels). Newer and improved array-based genotyping platforms with enhanced throughput and reduced genotyping cost are also being regularly developed (Fig. 2.1). Thus, these array-based platforms will perhaps continue to remain important for high-throughput marker discovery and genotyping, although low-cost NGS technologies are also being used now in parallel with the development of these array-based marker systems (see Seifollah et al.2013 in this volume). In this chapter, we wish to describe first the different array types and the principles/methods involved in these array-based marker systems, then discuss the results obtained in cereals during the last few years using the array-based marker genotyping platforms, and finally discuss the utility of these markers and the corresponding genotyping platforms in future molecular crop breeding programs. In order to give a comprehensive picture of the array-based marker systems, some of the information available in our earlier review will also be included in this chapter, this repetition being unavoidable. The sequencing-based marker systems and the genotyping platforms involving NGS technologies will not be included in this chapter, since these are being covered in the next chapter of this book.

2.2 Array Types for Marker Development and Genotyping

As mentioned above, microarrays were initially developed to facilitate large-scale screening of whole genomes/transcriptomes or a few genes/proteins in any living system including crop plants like cereals. These microarrays have undergone a variety of modifications in the original design and concept depending upon the aims and

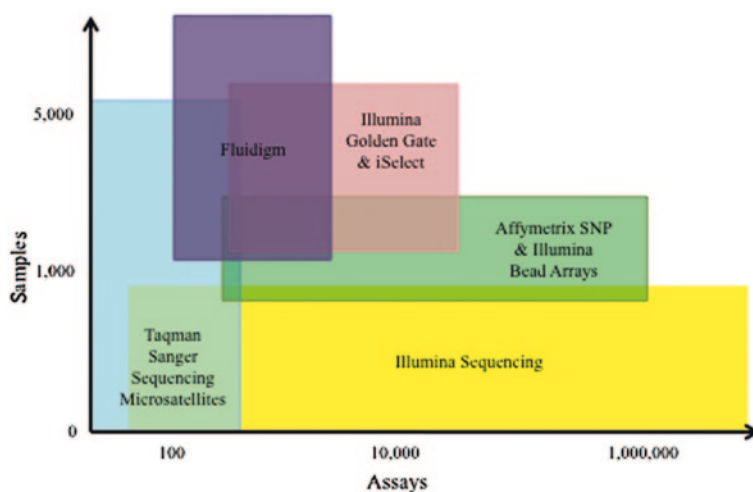


Fig. 2.1 Different platforms for genotyping, showing their relative high-throughput in terms of number of *samples* and *assays* that can be used in a single run (modified from BioScience LifeSciences)

objectives of using them, and also to achieve high throughput and cost-effectiveness. These microarrays were initially developed and continue to be used as planar arrays, where nucleic acid oligomers/probes or beads with oligonucleotides were immobilized on a flat solid surface mostly represented by a glass slide, a silicon wafer or a patterned planar substrate (for reviews, see Gupta et al. 1999a, b; Venkatasubbarao 2004). Later, microsphere-based suspension arrays were developed, where encoded microspheres (with tens of millions of particles per milliliter of suspension) with distinct optical properties were used as solid supports for biomolecules and the suspension arrays analyzed through flow cytometry; this also includes Luminex xMAP™ technology developed for rapid and high-throughput multiplexed genotyping (for reviews, see Nolan and Sklar 2002; Dunbar 2006).

Fluidigm Dynamic Arrays™ is another technology, where microfluidics with a miniaturized complex fluid-handling system is used on the chip, making it a ‘lab-on-a-chip device’. This is actually a ‘reagent in-data out’ platform, where PCR reactions also take place on the chip, followed by identification of PCR products for genotyping. As a proof of concept, using Fluidigm’s new SNPTYPE™ system a genotyping panel each of 48 SNPs was designed and validated respectively on a set of 94 rice (Ilic et al. 2011) and 46 cocoa (Ilic et al. 2012) accessions. Similarly, a panel of 96 SNPs was validated on a set of 23 *Bromus tectorum* accessions using Fluidigm and KASPar SNP genotyping platforms (Merrill et al. 2011).

Ultra-high-throughput nano-arrays/nano-chips were also developed and used for screening human genomes (Chen and Li 2007). Biomolecule conjugated quantum dots (QDs), which are semiconductor fluorescent nanocrystals, were also used for the assembly of microarrays to improve sensitivity of these microarrays (Chan and Nie 1998; Ioannou and Griffin 2010). Latest in the series are the Ion Torrent Chips, which employ a unique combination of fluidics, micromachining, and semiconductor technology to increase the high throughput further. For instance, recently an Ion Torrent Chip with a capacity to support up to 1.2 million DNA-testing wells was launched and used successfully to demonstrate sequencing of a bacterial genome in amazingly short period of time, within just two hours (Zakaib 2011). A next generation 11 million well Ion Torrent Chip was also under testing, which will allow the use of this ultra-high throughput technology in marker development and genotyping in foreseeable future.

However, not all of the above array technologies have been used in crop plants (particularly in cereals), so that we will restrict our discussion in this chapter to only those array technologies, which have been used for cereal genomes. However, a brief reference to other technologies will also be made, whenever possible and necessary.

2.2.1 DNA Chips/Microarrays/High-Density Oligonucleotide Arrays (Ordered Arrays)

The high-density oligonucleotide arrays (also described as DNA chips or microarrays) differ either in their design (featured chips vs tiling arrays) or in their production (spotting vs in-situ synthesis). Therefore, these arrays could be

classified into following two types: (1) featured chips (carrying gene sequences, or other polymorphic sequences known for an organism), and (2) tiling arrays that virtually represent the whole genome of an organism.

Among featured chips, the gene chips carry either cDNA-PCR products or long/short oligonucleotide probes (70–100 mers or 25 mers) printed on coated glass slides or silicon wafers. In featured chips with short oligonucleotide probes, for each of a number of genes, 20 pairs of oligonucleotide probes are selected from the exons located near the 3'-ends. The number of features on a single featured chip can also vary from 10,000 to > 6 million (Liu 2007). The tiling arrays, on the other hand, virtually cover the entire genome.

Although the above DNA chips/microarrays have generally been extensively used for expression analysis, but emphasis of the current review is specifically on their use in the detection/genotyping of SNPs/InDels, TDMs (GEMs and SFPs), ELPs, and CNVs/PAVs at genome-wide scale. Other featured arrays (e.g., TAM, DArT, RAD, aCGH) were also used to identify DNA polymorphisms.

Whole Genome High Density Resequencing Microarrays for SNP Discovery

A variety of whole genome microarrays have been developed that are suitable for both discovery of markers (e.g., SNPs) and genotyping. These microarrays are often based on oligonucleotides, which may be (1) partially overlapping or non-overlapping and tiled end to end, or (2) spaced at regular intervals to interrogate the entire genome without annotation bias. The whole genome microarrays also include biased expression arrays, splice-junction arrays, or exon-scanning arrays, when one wishes to scan specific regions at the whole genome level. Lastly, these microarrays include tiling resequencing arrays, where each nucleotide of the reference genomic DNA sequence is represented by a set of eight oligonucleotide probes (four possible nucleotide for each strand). Some of these whole genome microarrays are shown in Fig. 2.2.

High-density, oligonucleotide microarrays are often used for detection of genome-wide DNA polymorphisms (e.g., Chee et al. 1996; Patil et al. 2001; Hinds et al. 2005). These microarrays, follow a 1-bp tiling path to query each base of the genome relative to a known reference sequence, and are therefore, described as resequencing arrays. Each base is interrogated with eight features that consist of forward and reverse strand 25-mer oligonucleotide quartets. Within a quartet, oligonucleotides are identical to the reference sequence except at the central position, where each of the four possible bases is represented in four oligonucleotides. When hybridized to labeled genomic DNA, the highest signal intensity is expected for the perfect match, thereby predicting the base in the corresponding target DNA sample. Large-scale polymorphism discovery using such resequencing arrays was first performed in humans, identifying a large fraction of common single nucleotide polymorphisms (SNPs) in the global population (Patil et al. 2001; Hinds et al. 2005). These resequencing arrays have also been used in plants including

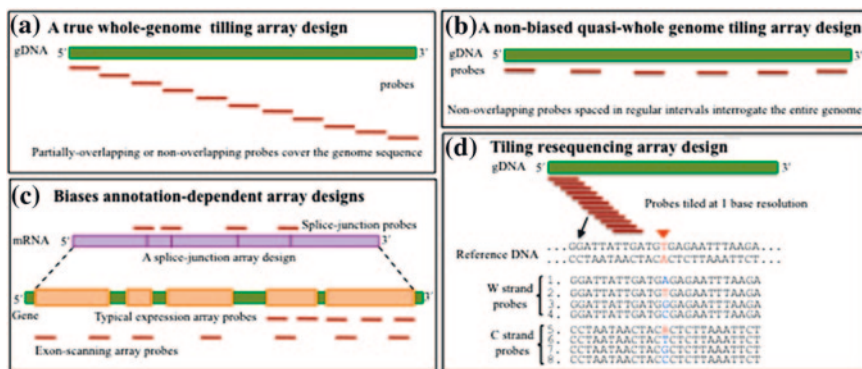


Fig. 2.2 A comparison of different whole-genome array designs. Unbiased whole-genome tiling array designs (a and b) contain oligonucleotide probes representing the entire genomic sequence. Probes may be a partially overlapping or nonoverlapping and tiled end to end or may be (b) spaced at regular intervals to interrogate the entire genome without annotation bias. c Other biased whole-genome array designs such as typical expression arrays, splice-junction arrays, and exon-scanning arrays contain only oligonucleotide probes for the known and predicted features of a genome. d Tiling resequencing arrays represent each nucleotide of the reference genomic DNA sequence with a set of eight oligonucleotide probes

Arabidopsis thaliana (Clark et al. 2007) and *Oryza sativa* (McNally et al. 2009). Re-sequencing arrays are now available for several plant systems, although high error rate (~ 50 %) makes them unreliable for identification of individual SNPs.

Gene-Based Microarrays (GeneChips) for TDMs (ELPs/GEM and SFPs)/CNVs

GeneChips have been developed by Affymetrix, Nimbelgen and Agilent in several plant species. In Affymetrix chips, a large number of genes are each represented by 11 perfect match (PF) and 11 mismatch (MM) 25-mer oligonucleotides constituting a probe set, so that thousands of such probe sets are included in a GeneChip. In contrast, Nimbelgen and Agilent chips include two to seven of 60-mer oligonucleotide probes representing each gene, although these chips have limited application in polymorphism survey in the form of markers like SFPs. The two oligonucleotides (PM and MM) in a pair differ in only one base. In wheat, as many as 55,052 transcripts (spanning all the 21 chromosomes) were used for this purpose (Bernardo et al. 2009). Genomic DNA is often hybridized to such GeneChip arrays, and a difference in hybridization intensity with two oligos (PM and MM) is recorded as a DNA polymorphism often described as an SFP. If cRNA is used for hybridization (in order to minimize the problem of large genome size and repetitive DNA), the polymorphisms recorded are described as transcript-derived markers (TDMs). Such GeneChips have been developed and used in maize, barley, wheat and rice (see Gupta et al. 2008 for details).

Microarrays for SNP Genotyping (SNP Chips)

SNP chips, sometimes also described as ‘variant detector arrays’ (VDAs) have been developed using different approaches. In one of these approaches, four oligos that differ only at the last position are used per SNP. To determine which alleles are present, genomic DNA from an individual is isolated, fragmented, tagged with a fluorescent dye, and applied to the chip. The genomic DNA fragments anneal only to those oligos to which they are perfectly complementary, which allows SNP genotyping through computer-aided identification of the position of fluorescent tags.

In another approach, the oligonucleotide on the chip may stop one base before the variable site, and typing relies on allele-specific primer extension. A DNA sample is added, which gets stuck onto the chip through base-pairing and is used as a template for DNA synthesis, with the immobilized oligonucleotide being used as a primer. The four nucleotides, containing different fluorescent labels, are added along with DNA polymerase. The incorporated base, which is inserted opposite to the polymorphic site on the template, is identified by the nature of its fluorescent signal. In a variation of this technique, the added nucleotide is identified not by a fluorescent label but by mass spectrometry, as done in MassARRAY used in Sequenom platform, launched by SEQUENOM. This platform makes use of a 384 Spectro CHIP, where PCR products are automatically transferred and utilized for mass spectrometry. However, Mass Array technology has not been used for cereal genomics on any large scale.

Illumina’s SNP chip is based on Bead Array Technology (utilized in Illumina’s iScan System), where silica beads (3-micron in size) self assemble in micro wells on either fiber optic bundles or planar silica slides. Each silica bead is covered with hundreds of thousands of copies of a specific oligonucleotide acting as the capture sequences in one of the several available Illumina’s assays.

2.2.2 Diversity Arrays for DArT Markers

A diversity array is necessarily crop-specific and consists of a large number of diverse anonymous clones. It is prepared by a proprietary method used for selection of diverse clones of genomic DNA from a sample of pooled DNA derived from a number of diverse accessions of the crop under study. These clones are characterized through sequencing and are mapped on the genome. The arrays are used for development of markers, where polymorphic DArT fragments between any two or more genotypes can be identified and used for a variety of studies.

2.2.3 Arrays of PCR Products Spotted on a Slide to be Scanned by Probes: Tagged Array Markers (TAMs)

In the approaches discussed above, the ‘target’ sequence is interrogated by each of a number of ‘probes’ arrayed on the chip. In tagged array markers (TAMs),

the situation is reversed, where the target sequences in the form of PCR products (obtained using one biotin labeled primer shared by both alleles, and two unique primers carrying allele-specific oligonucleotide tags) are spotted directly from 96 or 384-well PCR plates onto streptavidin-coated glass microarray slides. The tags attached to the primers remain single stranded due a C-18 linker between the allele specific sequence and the tag. The target sequences (allele-specific PCR products carrying unique tags) are then hybridized with fluorescent-labeled detector probes to identify alleles represented by each of the targeted sequences that are arrayed. In this way, thousands of tagged samples that are arrayed can be genotyped for a few markers in a single experiment, making it particularly useful for screening large populations for few important markers. The method was developed to score retrotransposon-based insertion polymorphism (RBIP) markers, but can also be used to score SNPs (Flavell et al. 2003).

2.2.4 Arrays for Comparative Genomic Hybridization (aCGH)

The technique of comparative genomic hybridization (CGH) was originally developed in early 1990s and involved competitive hybridization of two differentially labeled genomic DNA samples (a test and a control) on to metaphase chromosomes. The fluorescent signal intensity of the labeled test DNA relative to that of the reference DNA could then be linearly plotted across each chromosome, facilitating identification of copy number variations (Kallioniemi et al. 1992). However, the resolution of this CGH technology was limited to alterations of approximately 5–10 Mb (Lichter et al. 2000; Kirchhoff et al. 1998), and could identify only microscopic structural and numerical alterations in chromosomes (including duplications/deletions and aneuploidy).

In order to improve resolution, microarrays were designed and used for the so-called array comparative genomic hybridization (aCGH), which allowed resolution in the range of 1 Kb–3 Mb (Lucito et al. 2003). These microarrays resemble those generally designed for the detection of SFPs, except that the oligonucleotides used for aCGH are longer (generally 50–85 bp, but could be hundreds of kb as in the BACs). The spotted oligos in microarrays used for aCGH generally represent the genomic regions of interest. Digital imaging systems are used to capture and quantify the relative fluorescence intensities of the labeled DNA that is hybridized to each target. The fluorescence ratio of the test and reference hybridization signals is determined at different positions along the genome, and it provides information on the relative copy number of sequences in the test genome as compared to the reference genome.

Currently, Roche NimbleGen and Agilent Technologies are the major suppliers of whole-genome array CGH platforms. The oligonucleotides in a CGH microarray may number up to 2–4 million (2–4 M) in case of Roche/NimbleGen and up to 1 M in case of Agilent Technologies (Alkan et al. 2011).

2.3 Array-Based Molecular Markers: Classification

In our previous review on array-based markers, we grouped array-based marker systems into four classes including SNP, SFP, DaRT and RAD markers (Gupta et al. 2008). To these four classes, we may add structural variations (SVs) including copy number variations (CNVs), presence-absence variations (PAVs) and insertions/deletions (InDels). At the molecular level, each of these individual array-based marker types represents either nucleotide substitutions or duplications/InDels. In view of the nature of genotyping/detection platforms developed during the last few years, and their throughput and suitability for various applications, we classified array-based markers into two classes, (1) those based on genotyping procedure and (2) those based on the level of throughput. Later in this chapter, for a more detailed account of array-based markers, we will follow the classification based on the technique (procedure) involved.

In the first classification, which is based on the technology involved, the array-based marker platforms are placed into the following three major groups: (1) hybridization-based platforms, where SNP-specific chips or microarrays, developed for CGH, are used for hybridization; (2) real-time detection-based platforms, where an array of samples to be genotyped is used to provide templates for real-time PCR, and (3) the platforms involving both hybridization and real-time detection.

The markers involving hybridization based platforms for genotyping can be further classified as follows: (1) transcript derived markers [TDMs, including expression level polymorphisms (ELPs) or gene expression markers (GEMs) and single feature polymorphisms (SFPs)] (Potokina et al. 2008), (2) diversity array technology (DaRT) markers (Jaccoud et al. 2001; Wenzl et al. 2004), (3) tagged microarray markers (TAMs) (Jing et al. 2007), (4) restriction site-associated DNA (RAD) markers (Miller et al. 2007a, b), (5) GoldenGate SNP genotyping assays (Steermers and Gunderson 2007); and (6) copy number variations (CNVs) and presence-absence variations (PAVs). Similarly, the real-time detection-based platforms include (a) *KASPar* SNP genotyping system (<http://www.kbioscience.co.uk/reagents/KASP.html>), and (b) high-resolution melting (HRM) curve analysis (Hoffmann et al. 2007). Illumina's GoldenGate and Infinium assays, on the other hand, rely on a 'hybrid technology' involving both hybridization and real-time detection (Steermers and Gunderson 2007).

In the second classification that is based on the level of throughput and suitability for different applications, the assays involving array-based markers are placed into the following three groups: (a) assays involving genotyping an individual with an array of marker-specific probes, (b) assays involving genotyping an array of individuals with a single marker, and (c) the flexible platforms involving both of the above situations. Based on this classification, hybridization-based platforms (including SFPs, DaRT and RAD) represent the first group, real-time detection-based platforms (viz. *KASPar* and HRM) represent the second group, and Illumina's GoldenGate and Infinium assays represent the third group (for details, see later).

2.4 Array-Based Molecular Markers: Principles and Methods

As mentioned above, a majority of array-based markers are hybridization-based (sometimes including real-time detection method), which will be described in relatively greater detail in this section.

2.4.1 *Microarray-Based Markers Involving Hybridization*

The microarray-based markers rely on hybridization of genomic DNA, cDNA, cRNA and/or mRNA to GeneChips, oligonucleotide tiling arrays, diversity arrays, and/or glass microarray slides. Even though, these methods were quite successful in detecting/determining polymorphism at the genome-wide scale, they suffer from several associated limitations including the following: (1) High background noise owing to cross-hybridization, and a limited dynamic range of detection because of both background and saturation of signals. (2) High experimental cost, since many replications are required per experiment to increase statistical confidence of the observations. (3) Dependence upon the existing sequence (genome/transcriptome) information. (4) Requirement of comparing expression levels across different experiments, which is often difficult (Wang et al. 2009). These hybridization-based markers (except CNVs and PAVs) were described in greater detail in our previous review (Gupta et al. 2008) and therefore, will be only briefly summarized here.

Transcript Derived Markers (TDMs)

The TDMs constitute a major class of microarray-based markers, detected upon hybridization of transcripts/cDNAs on microarrays or GeneChips. These markers were successfully used for cereals including maize, barley and wheat (for details, see Sect. 2.6.3). The TDMs are gene-specific markers (due to SNPs/InDels) and include both ELPs/GEMs and SFPs. ELPs/GEMs represent expression level differences (total absence to differences in transcript abundance) recorded on chips in the form of difference in signal intensity observed for different samples under study. But the observed signal intensity for a particular sample is consistently shown by all the features (oligos) representing a particular gene on the chip. In contrast, SFPs represent differences in hybridization intensity observed between two samples, denoted by only one of the many features representing a gene on the microarray (c.f. Gupta et al. 2008). Both kinds of molecular markers (ELPs/GEMs and SFPs), if applied on a mapping population, will allow grouping a population into two discrete classes (presence or absence of expression), in contrast to the cases where the population shows a continuous distribution for the transcript abundance of a particular gene, which can be recorded as an e-trait to be mapped as expression QTL(s).

Diversity Array Technology (DArT)

DArT is a high-throughput microarray hybridization-based technique that allows simultaneous typing of several hundred polymorphic loci spread over the entire genome without any prior sequence information about these loci (Jaccoud et al. 2001; Wenzl et al. 2004). DArT is an extension of ‘garden blots’ prepared using the genomic DNA of different plant species. DArT involves development of a ‘discovery array’, which is developed from the metagenome (pool of genomes representing the diverse germplasm of interest), that was subjected to complexity reduction to reduce the level of repetitive DNA, since repetitive sequences interfere with DArT assays (Kilian et al. 2003, 2005). For a ‘discovery array’, individual clones from a genomic representation library are amplified and spotted onto glass slides (www.diversityarrays.com). Labelled genomic representations of individual genomes that were earlier included in the metagenome pool, were then hybridized to this ‘discovery array’, and polymorphic clones (called DArT markers) thus detected are assembled into a ‘genotyping array’ for routine genotyping work. These markers are biallelic and dominant (presence vs absence) or co-dominant (two doses vs one dose vs absent) in nature, and were successfully used in rice, barley, wheat and maize (see [Sect. 2.6.2](#) for details).

Tagged Array Markers (TAMs)

TAMs allow high-throughput distinction between predicted alternative PCR products. Typically, the method is used as a molecular marker approach for determining the allelic states of individual single nucleotide polymorphisms (SNPs) or insertions/deletions (InDels) in multiple individuals. Biotin-labeled PCR (unpurified), products are spotted, onto a streptavidin-coated glass slide and the alternative products are distinguished by hybridization to fluorescent detector oligonucleotides that recognize corresponding allele-specific tags on the PCR primers. There are several advantages of this method, which include high throughput (thousands of PCRs are analyzed per slide), flexibility of scoring (any combination, from a single marker in thousands of samples to thousands of markers in a single sample, can be analyzed) and flexibility of scale (any experimental scale, from a small lab setting to a large project). The TAM technology was initially adopted in pea to genotype retrotransposon-based insertional polymorphism (RBIP) markers on a dot assay, which was later made fully automated for handling thousands of samples. The basic RBIP method has been developed for high-throughput applications by replacing gel electrophoresis with array hybridization to a filter (Flavell et al. 1998; Jing et al. 2007).

CNVs and PAVs

Copy number variations (CNVs) and presence-absence variations (PAVs) are the latest markers developed recently. They have been extensively used in humans and are now being used in plants also. These markers are detected through the use of

microarrays, which are specially developed for each individual plant species, and are then used for comparative genomic hybridization (CGH). The genomic DNAs of test sample and the reference sample are differentially labeled (with C3 and C5) and are then hybridized on to the CGH microarray. The ratio of the fluorescent signal intensity of the labeled test DNA to that of the reference DNA is used to detect CNVs, PAVs and InDels (Schrider and Hahn 2010).

2.4.2 Real-Time Detection Based Markers: KASPar Genotyping System

The KBioscience competitive allele-specific PCR (KASPar) genotyping system is a modification of TAM technology that does not require a hybridization step; instead it involves real-time detection of the product, giving it an advantage in terms of steps and time involved in the detection process. This makes KASPar a simple, cost-effective and flexible way for determining both SNP and InDel genotypes, since the assays can be adjusted according to the needs to 48, 96, 384 and 1536-well plate formats. The technology utilizes a unique form of allele specific PCR that is different from the conventional amplification refractory mutation system (ARMS), which makes use of four primers, two allele specific and two locus specific primers. The KASPar chemistry involves two competitive allele specific tailed forward primers and one common reverse primer. The KASPar[®] assay system relies on the discrimination power of a novel form of competitive allele specific PCR to determine the alleles at a specific locus. To improve the performance of the detection platform, KBioscience perfected this technique by incorporating (1) a 5'-3' exonuclease cleaved *Taq* DNA polymerase (the engineered *Taq* increases its discrimination power) and (2) a homogeneous Fluorescence Resonance Energy Transfer (FRET) detection system. The two allele-specific primers of a SNP are designed such that they incorporate a unique 18 bp tail to the respective allele specific products, which in later cycles allow incorporation of allele specific fluorescent labels to the PCR products (with the help of corresponding labelled primers). The dual emission modules of the detection system offer great advantage to read the internal standard (ROX) and the allele specific dyes (FAM or VIC) together, making it a qualitative SNP genotyping assay technique.

2.4.3 High-Resolution Melting (HRM) Curve Analysis

The HRM analysis is a recently developed promising technology used for the detection of variations in DNA. The thermal stability of a DNA fragment is determined by its base sequence. When the DNA fragment contains an altered sequence, the duplex stability is changed, leading to different melting behavior, which can be identified with HRM analysis. During HRM analysis, melting curves are produced

using intercalating DNA dyes (*SYBR Green*) that fluoresce in the presence of double-stranded DNA and a specialized instrument designed to monitor fluorescence during heating. When the temperature increases, the DNA-intercalating dye is released from the DNA and the fluorescence decreases. This process produces a characteristic melting profile that can be monitored with precision. Changes in the sequence within the DNA fragment, as in SNPs or InDels, alter the melting profile. The HRM assay has successfully been used to detect point mutations in crop plants. The technique has also served as an alternative to agarose gel electrophoresis to score for the presence/absence of amplicons to detect insertion/deletion polymorphisms (IDPs) with or without prior knowledge of the presence of a polymorphism. For instance in bread wheat insertion site-based polymorphism (ISBP) were amplified by PCR in the presence of *SYBR Green I* and subsequently used for melting curve analysis on an *ABI_PRISM 7900HT*. The HRM analysis of 711 ISBP markers allowed assignment of these markers to deletion bins by scoring for the presence/absence of the amplicon in chromosome 3B aneuploid lines and also allowed evaluation of polymorphism between the parents of five mapping populations (Paux et al. 2010).

2.5 High Density Arrays-Based Resequencing for SNP Discovery

High-density whole genome oligonucleotide tiling arrays have been used for resequencing whole genomes of model systems like *Arabidopsis* and rice, leading to discovery of millions of SNPs. *Arabidopsis* was the first plant to enjoy the advent of these whole genome approaches, where high-density microarrays were used to describe sequence diversity in the entire *Arabidopsis* genome (Clark et al. 2007). In this study, hybridizing genomic DNA from 20 divergent *Arabidopsis* strains to tiling arrays with almost one billion different oligonucleotides increased the number of known SNPs to 1,074,055, and provided the foundation for the first haplotype map among organisms outside mammals. The extensive number of polymorphisms identified in this study eliminated the need for polymorphism discovery, and made mapping and tracking of genes controlling complex traits feasible by the development of very high-density genetic linkage maps. A similar study was also conducted in rice (*O. sativa*), where genomes of 50 accessions of cultivated rice allowed detection of 6.5 million SNPs (Xu et al. 2012); however both of these studies were quite successful but marked the end of an era of microarray-based resequencing, when NGS technologies became available.

2.6 Array-Based Genotyping Platforms

2.6.1 Array-Based High-throughput SNP Genotyping Platforms

Microarray based genotyping platforms are increasingly becoming popular for genome-wide genotyping since they offer highly multiplexed assays at a relatively

low cost per data point. These high-throughput platforms offer large-scale genotyping for dozens to thousands of SNPs in one or more genomic DNA samples (see Syvanen 2005; Fan et al. 2006a, b; Gupta et al. 2008; McCouch et al. 2010). Both low to high resolution platforms are available to meet the different needs of research communities in different crop plants. Some of the important SNP genotyping platforms reported for low to high through-put SNP genotyping include the following: (1) Illumina's GoldenGate platform (Fan et al. 2003), (2) Illumina's BeadChip™ based Infinium platform (Steemers and Gunderson 2007), (3) GenomeLab™ SNPstream Genotyping System, (4) MegAllele genotyping system based on Affymetrix ParAllele's Molecular Inversion Probe (MIP) Technology, (5) GeneChip™ technology and ASO tiling arrays based on Affymetrix GeneChip platform, (6) TaqMan by Life Technologies (Livak et al. 1995), (7) OpenArray platform (TaqMan OpenArray Genotyping System, Product Bulletin), and (8) Competitive Allele Specific PCR (KASPar) by KBiosciences (<http://www.kbioscience.co.uk/index.html>).

Based on the methodology involved, majority of the above SNP genotyping assays have been classified into the following groups: (1) allele-specific hybridization, (2) primer extension, (3) oligonucleotide ligation (4) invasive cleavage, (5) allele-specific PCR amplification, (6) DNA conformation methods, and (7) enzymatic cleavage method to include the invader assay (for details, see Xu 2010). The details of these platforms have been described elsewhere in several earlier reviews (see Fan et al. 2003; Syvanen 2005; Gunderson et al. 2006; Steemers and Gunderson 2007; Gupta et al. 2008; Appleby et al. 2009; Ragoussis 2009). However, among all these genotyping platforms, the most popular high-throughput genotyping assays among researchers working on cereal crops included Illumina's GoldenGate and Infinium assays, and KBiosciences KASPar assay. Therefore, in this section, we will briefly discuss the development and use of Illumina's GoldenGate and Infinium assays and KBioSciences' KASPar assay for world's major cereal crops.

Illumina's GoldenGate Assay

Illumina's GoldenGate assay, which makes use of customized oligonucleotide pool assays (OPAs), is one of the most widely used genotyping platforms for cereals at present. It provides low to mid-plex genotyping for genome profiling and validation studies. This genotyping platform is extremely flexible and allows researchers to select for number of SNPs (for each of the samples to be genotyped) and the throughput level that best suit their experimental requirements. The system can be utilized for any crop species using either Bead Array, or VeraCode technology (http://www.illumina.com/applications/detail/snp_genotyping_and_cnv_analysis/custom_low_to_mid_plex_genotyping.ilmn). Based on the level of multiplexing and throughput, GoldenGate assays can be classified into: (1) GoldenGate Bead Array (2) GoldenGate Veracode and (3) GoldenGate Indexing. GoldenGate assay, which is common to all the three technologies involves use of two allele specific

oligonucleotides (ASOs) and a locus specific oligonucleotide (LSO) for each SNP. All the three oligonucleotides are supplemented with non-template specific universal primer sites; the LSO also carries an anti-tag sequence corresponding with a particular bead type on the BeadArray. The specific primers (ASOs and LSO) bordering each SNP allow allele specific primer extension and universal primers allow labeling and detection of the product (for details, see Fan et al. 2006a, b). A comparison of three assays has been presented in Table 2.1.

1. **GoldenGate BeadArray Assays (Bead Array, iScan).** This assay allows simultaneous genotyping of 96–3,072 (96-, 192-, 384, 768-, 1536- and 3,072) SNP loci in a fairly large collection of samples (up to 384 samples) in parallel. This is one of the most popular SNP genotyping platforms providing cost effective assays (per genotype cost \$0.03). These assays are now becoming available in all major cereals including wheat (Akhunov et al. 2009; Chao et al. 2010), rice (McCouch et al. 2010), barley (Rostocks et al. 2006; Close et al. 2009; Druka et al. 2011) and maize (Mammadov et al. 2012). Among cereals, barley is the first crop where GoldenGate assay for 1,536 SNPs (selected on the basis of EST mining) was developed and used for the study of population structure and the level of LD exhibited in elite Northwest European barley (Rostoks et al. 2006). Later, it was also used for molecular characterization, genetic diversity analysis, preparation of integrated maps, consensus maps, bulk segregant analysis (BSA), identification of QTL, linkage disequilibrium (LD) studies, association mapping, joint linkage–linkage disequilibrium (LD) mapping approaches, etc. A summary of SNP studies conducted in different cereals using Illumina's GoldenGate (GG) assays is presented in Table 2.2.
2. **GoldenGate VeraCode (VeraCode Bead Plate BeadXpress):** BeadXpress involving Veracode technology is considered as most flexible and low- to mid-plex GoldenGate SNP genotyping assay. In this genotyping platform, custom SNP assays are ordered from Illumina in 48-, 96-, 192-, and 384-plex (GoldenGate Kits), 1-144-plex (Universal Capture Bead Sets) and 1–48-plex (Carboxyl Bead Sets) formats, and the DNA samples are processed in a 96-well format (Table 2.1). BeadXpress involving 384-SNP OPAs is very useful for cereal breeding/genetics

Table 2.1 Comparison of various GoldenGate SNP genotyping assays

Features	GoldenGate BeadArray	GoldenGate VeraCode	GoldenGate Indexing
Multiplexing	96–1536-plex	48–384-plex	96–384-plex
DNA needed	~250 ng	~250 ng	~250 ng
System used	iScan system	BeadXpress system	iScan system
Array type	Bead Array	VeraCode	Bead Array
Through-put	~288 samples/day	~288 samples/day	>2,000 samples/day
Reaction	ASPE	ASPE	ASPE
Suitability for MAS	Less	More	Less
Suitability for mapping	More	Less	More

ASPE = allele specific primer extension

community since this is reliable and requires little technical adjustments after their designing and optimization. As against, BeadArray technology, VeraCode makes use of a VeraCode Bead Plate, which carries addresses for SNP alleles to be detected by the anti-tags carried by LSO. The use of VeraCode Bead Plate in place of BeadArray reduces the cost per sample, when lower-plex genotyping is needed. This genotyping platform is suitable to assay hundreds or thousands of genotypes in a short span of time. Several 384-SNP BeadXpress assays have already been developed and used in cereals (see Table 2.2).

3. **GoldenGate Indexing (iScan):** Illumina's GoldenGate Indexing is a recent high-throughput, low cost technology involving low to mid-plex genotyping of 96–384 SNPs simultaneously. This will allow researchers to pool multiple samples, thus increasing the number of samples in a single run (http://www.illumina.com/documents/products/datasheets/datasheet_goldengate_indexing.pdf). One can screen up to 16 times more samples per reaction than one can do with the standard GoldenGate (GG-BeadArray) assay (see above) and therefore, increases throughput from 288 samples/day (in GG-BeadArray assay) to >2,000 samples/day, thereby decreasing cost (Table 2.1). This system has not been used in plants so far, but it is anticipated that this emerging technology will soon find its application in plants also.

Illumina's Infinium Assays (SNP-CGH)

Illumina's BeadChipTM based Infinium assay, involving array-comparative genomic hybridization (aCGH), is a high-density SNP genotyping technology for whole-genome genotyping that allows genotyping of fixed sets of hundreds of thousands of SNPs simultaneously. It allows simultaneous measurement of both signal intensity variations and changes in allelic composition (Varshney 2010). In this assay, BeadChips with 12-, 24-, 48- or 96 sections can be used simultaneously with each section of a BeadChip containing 1.1 million beads carrying decoded oligonucleotides (for further details consult, Syvanen 2005; Gundersson et al. 2006; Steemers and Gundersson 2007; Gupta et al. 2008).

With the advent of next-generation sequencing technologies, high density SNPs have been discovered in all important crop plants including cereals; this facilitated the development of Infinium assays in these crops. For instance, in soybean, 44,299 informative SNPs were used to develop '*Illumina Infinium iSelect SoySNP50 chip*' that was later used to dissect and resolve the issue of origin of genomic heterogeneity in soybean cultivar, Williams 82. The CGH analysis for >2,03,000 loci revealed the consequences of this heterogeneity in terms of structural and gene content variants among individuals of the cultivar, Williams 82 (Haun et al. 2011). Similarly, efforts are being made to design a 50 K SNP Illumina Infinium assay and use it to analyze each of the 18,603 cultivated and 1,116 wild soybean accessions from the USDA soybean germplasm collection and 1,000 RILs from each of the two mapping populations of soybean (Williams

Table 2.2 A summary of SNP genotyping studies conducted in some important cereal crops

Crop and Platform	Silent features of the study	Reference
<i>1. Wheat</i>		
GG (1536 SNPs)	878 loci assigned to 7 linkage groups at a maximum resolution of 0.087 cM. Map comparisons with rice and sorghum revealed 50 inversions and translocations	Luo et al. (2009)
GG (96 SNPs)	53 tetraploid and 38 hexaploid wheat lines were genotyped at 96 SNP loci to demonstrate utility of GoldenGate assay for polyploids	Akhunov et al. (2009)
GG (1536 SNPs)	Studied LD and population structure in a panel of 478 spring and winter wheat cultivars from USA and Mexico	Chao et al. (2010)
GG	53 ISBP-derived SNPs markers were used to genotype 96 hexaploid wheat varieties, 96 individuals from a Chinese Spring x Renan F ₂ population, and aneuploid lines	Paux et al. (2010)
Illumina beadexpress (768 SNPs)	275 new SNPs reported; 157 SNPs mapped in one of two mapping populations (Meridiano × Claudio and Colosseo × Lloyd) and integrated into a common genetic linkage map	Trebbi et al. (2011)
<i>2. Rice</i>		
(1,536 SNPs) GG	Captured variation within and between <i>O. sativa</i> subpopulations	Zhao et al. (2010)
	Captured variation within temperate <i>japonica</i> cultivars	Yamamoto et al. (2010); Nagasaki et al. (2010)
		Thomson et al. (2012)
Illumina BeadXpress (384-plex)	Evaluated variation between various rice species/subspecies: <i>indica</i> and <i>japonica</i> , <i>indica</i> and <i>aus</i> , US tropical <i>japonica</i> , <i>indica</i> and <i>O. rufipogon</i> , <i>japonica</i> and <i>O. rufipogon</i>	
Affy (44,100 SNPs)	Evaluate diversity within and between sub-populations of <i>O. sativa</i>	Tung et al. (2010)
Illumina BeadXpress (384-plex)	372 SNPs unraveled the <i>indica-japonica</i> subspecific differentiation and geographic differentiation within <i>Indica</i> and <i>Japonica</i> in 300 rice inbred lines	Chen et al. (2011)
Affy (1 M SNPs) Resequencing 6.5 M SNPs	Evaluate diversity within and between <i>O. sativa</i> , <i>O. rufipogon/O. nivara</i> , <i>O. glaberrima</i> and <i>O. barthii</i>	Xu et al. (2012)
<i>3. Barley</i>		
GG BeadArray (SNPs in 1,524 barley unigenes)	Studied genome-wide molecular diversity, population substructure, and LD in elite Northwest European barley cultivars	Rostocks et al. (2006)
GG (two 1,536-SNPs assays)	Use of high-throughput SNP genotyping platform for the development of a consensus map containing 2,943 SNP loci covering a genetic distance of 1,099 cM	Close et al. (2009)

(continued)

Table 2.2 (continued)

Crop and Platform	Silent features of the study	Reference
GG (1,536 SNPs)	Reported GWA mapping of 15 morphological traits across ~500 cultivars genotyped with 1,536 SNPs, and fine-mapped anthocyanin pigmentation to a 140-kb interval containing 3 genes	Cockram et al. (2010)
GG (1,536 SNPs)	Defined the genetic location of 426 morphological mutants using 3,072 SNPs	Druka et al. (2011)
GG	Diversity analysis using 1,301 SNPs on a set of 37 barley accessions revealed high polymorphism rate between 'Haruna Nijo' and 'Akashinriki'. A DH population was derived from them, and genotyped using 1,448 SNPs, of which 734 showed polymorphism and integrated into the linkage map. 98 RCLSs developed from the same cross were also genotyped using SNPs	Sato et al. (2011)
4. Maize		
GG (1536 SNPs)	Genotyping global maize collection of 632 inbred lines and estimation of genetic diversity, population structure, and LD	Yan et al. (2009)
GG (1,536 SNPs)	Molecular characterization of global maize breeding germplasm involving study of genetic diversity	Lu et al. (2009)
GG (1536 SNP)	Genotyping of Nested Association Mapping (NAM) population (4,699 RILs) with 1,106 SNPs to develop integrated linkage map	McMullen et al. (2009)
Illumina_BeadArray™ (768 SNPs)	Mapping of 591 markers on IBM2 genetic map covering ~88 % genome	Jones et al. (2009)
Sequenom-based typing of 1,359 SNPs	Mapping of phenotypic mutants using a combination of quantitative SNP-typing and bulked segregant analysis	Liu et al. (2010)
GG (1,536 SNPs)	Construction of a high-density linkage map containing 662 markers (1,673.7 cM)	Yan et al. (2010)
GG (two 1536 SNP assays)	Conducted linkage and LD based mapping for detection of drought tolerance QTLs	Lu et al. (2010)
GG assay (1,000 SNPs; ~700 loci)	Mapped 604 SNPs distributed on ten maize chromosomes	Mammadov et al. (2010)
GG (1,536 SNPs)	Genotyping of 2–3 comparable generations of twenty maize accessions conserved in five genebanks	Wen et al. (2011)
GG	Used 695 highly polymorphic SNPs for genotyping with Illumina's GoldenGate/Infinium, TaqMan and KASPar	Mammadov et al. (2012)

(continued)

Table 2.2 (continued)

Crop and Platform	Silent features of the study	Reference
GG (2 OPAs of 1,536 SNPs each)	An integrated map spanning 1,346 cM was constructed using 1,443 molecular markers, including 1,155 SNPs. A 100-fold difference in recombination frequency was observed between different chromosomal regions	Farkhari et al. (2011)
GG (1,536 SNPs)	1,006 polymorphic SNPs grouped 80 lines in 6 subgroups. Pairwise LD and association map- ping with phenotypic traits investigated under water-stressed and well-watered conditions showed rapid LD decline within 100–500 kb	Hao et al. (2011)
Illumina MaizeSNP50 BeadChip (56,000 SNP)	Joint-linkage mapping and GWAS revealed that kernel composition traits are controlled by 21–26 QTLs. Numerous GWAS associations were detected, including several oil and starch associations in <i>acyl-CoA:diacylglycerol acyltransferase 1-2</i> , a gene that regulates oil composition and quantity	Cook et al. (2012)
5. <i>Sorghum</i> GG (384 SNPs)	Genotyped 125 sorghum genotypes to perform whole genome association mapping for height and brix (stem sugar)	http://maizeandgenetics.tamu.edu/presentations

GG = GoldenGate

82 × PI468916 and Essex × Williams 82) with the Illumina BeadStation 500 to obtain ultra-high resolution genetic maps of soybean (http://www.soybeancheckoffresearch.org/DetailsbyPaperid.php?id_Paper = 991). These Illumina Infinium genotyping assays have now been used even in non-model plant species. For instance, using Infinium assays, 622 loblolly pine trees sampled from 167 locations were genotyped using SNPs across 3,059 functional genes. This allowed a study of population structure and environmental associations to aridity in loblolly pine (Eckert et al. 2010).

In cereals, some of the applications of Infinium assay included the following: (1) A 50 K SNP Infinium chip in maize covers approximately two-thirds of all maize genes and also includes additional SNPs spread over most of the remaining maize genome resulting in an average marker density of approximately one marker every 40 kb (Ganal et al. 2011). (2) A set of 618 gene-based SNPs were successfully converted into different genotyping assays including Infinium assay in maize (Mammadov et al. 2012). The study also demonstrated the conversion of SNPs from GoldenGate assays into Infinium assays with a success rate of ~89 %. The commercial availability of these high-density SNP platforms will undoubtedly facilitate the application of SNP markers in molecular breeding (Mammadov et al. 2012). (iii) A pilot 9 K SNP Infinium assay (http://129.130.90.21/IWSWG/sites/default/files/9K_assay_available_updated.docx) was developed recently in a USA/Australia collaborative project and used to genotype tetraploid and hexaploid wheat lines and cultivars. The assay includes SNPs discovered from the transcriptomes generated from a set of 27 US/Australian lines. Preliminary results showed that more than 90 % of SNPs produce high-quality genotype calls.

Cost Effectiveness of GoldenGate (GG) Assays and Their Suitability for Molecular Breeding

In maize, genotyping using GoldenGate (GG) assay was found 100-fold faster than gel-based methods. When the cost of genotyping for preparation of two linkage maps was compared, it was found that there was a cost saving of ~75 % in GG-based SNP genotyping relative to gel based methods used for SSRs. In addition, SNP genotyping with GG assays allows development of molecular maps with 2–3 times higher density in a fraction of time required for the development of SSR-based maps (Yan et al. 2010). However, while comparing with DArT markers, GG assays were found to be 3 times more expensive (Mantovani et al. 2008).

However, GG assays have not been favored for molecular breeding, although both GG and Infinium assays have been applied for rapid construction of genetic linkage maps, gene/QTL mapping and GWAS. This may be due to the requirement for multiplexing to bring down the assay cost per data point, which will be a bottleneck for their use in molecular breeding involving use of only a few SNPs closely linked to the gene/QTL of interest. If an associated SNP belongs to the set of SNPs (OPA) included in a GG or Infinium assay, the breeder prefers to convert the desired SNP(s) into another user-friendly high-throughput assay (e.g. KASPar or TaqMan) that does

not require multiplexing and are still cost effective. However, several issues may crop up while converting one SNP assay into another and may jeopardize the application of a particular marker in MAS (Mammadov et al. 2012).

Fluidigm SNP Genotyping

In addition to several genotyping platforms by Illumina discussed above, BioScience LifeSciences™ “Fluidigm SNP genotyping system” is also one of the important SNP genotyping platforms gaining popularity in plant molecular breeding community. It uses the innovative integrated fluidic circuit (IFC) and is used for studies requiring ultra-low cost and high-sample throughput for low- to mid-multiplex SNP genotyping™ (<http://www.lifesciences.sourcebioscience.com/genomic-services/genotyping/snp-genotyping-using-the-fluidigm-ep1-system.aspx>). The system involves the use of 48.48, 96.96 and more recently 192.24 type of arrays. The 192.24 array is called “Dynamic Array™ IFC” because it is designed to genotype 192 samples against 24 SNP assays in a single run, thus greatly increasing the sample throughput with only few selected important SNPs for molecular breeding. The platform has already been used in rice (Ilic et al. 2011), cocoa (Ilic et al. 2012), grain amaranths (Maughan et al. 2011) and *Bromus tectorum* (Merrill et al. 2011).

Competitive Allele Specific PCR (KASPar) Assays

GoldenGate (GG) and/or Infinium assays have been widely used for rapid genotyping of a large number of SNP markers in all major crop species including cereals (see Table 2.2). However, for genotyping a population for few SNPs, where GG assays are not cost-effective (Chen et al. 2010), KASPar (KBioScience Allele-Specific Polymorphism, KBioscience, UK) system provides a promising alternative. The method involves competitive allele-specific PCR, followed by SNP detection via Fluorescence Resonance Energy Transfer (FRET; for review see McCouch et al. 2010).

KASPar genotyping may be of particular interest to breeders and researchers who are interested in analyzing a small number of targeted SNPs in a large number of samples. Therefore, KASPar genotyping assays may be used for a variety of purposes including the following: (1) genetic diversity studies; (2) genetic mapping and saturation of already prepared maps; (3) fine-mapping of QTLs; (4) detection of functional SNPs within a subset of germplasm; (5) marker-assisted breeding, and (6) retaining target regions in NIL development (see McCouch et al. 2010). This genotyping system has already been used for a large number of species including cereal crops like rice, maize and wheat. In wheat, the technique has been used for rapid generation of a linkage map containing several hundred SNPs (Allen et al. 2011). Similarly, in maize, a set of 695 highly polymorphic gene-based SNPs from a total of 13,882 GG-validated SNPs were selected and converted into KASPar genotyping assay with a success rate of 98 % (Mammadov et al. 2012).

2.6.2 Diversity Array Technology (DArT) Markers in Cereal Crops

Diversity array technology (DArT) is a high throughput microarray hybridization-based technique that allows genotyping for several hundred polymorphic loci spread over the whole genome without any prior sequence information (Jaccoud et al. 2001). The technique is reproducible and cost-effective (Wenzl et al. 2004), and therefore, has been used in a number of crop species (including cereals) as evident from the trends in the number of papers published during the last decade (Fig. 2.3). This trend of papers published using DArT platforms is continuously increasing each year along with papers being published using GoldenGate assays. It is estimated that for the discovery of polymorphic markers ~5,000–8,000 genomic loci are typed in parallel in a single-reaction assay using a small quantity (50–100 ng) of highly purified genomic DNA. Polymorphic markers once discovered on a discovery array (prepared using metagenome of a crop species) are combined into a single array called “genotyping array” to be used for routine genotyping work (Huttner et al. 2005). The detailed method used for the development of DArT markers was described in our earlier review (Gupta et al. 2008). However, with the success of array based DArT markers over the past ~12 years, it was realized that the number of polymorphic markers can be increased by involving the use of next-generation sequencing (NGS), so that the cost of producing sufficient number of tag counts dropped to the commercially viable levels. In this platform, genome complexity reduction for genotyping has been combined to next

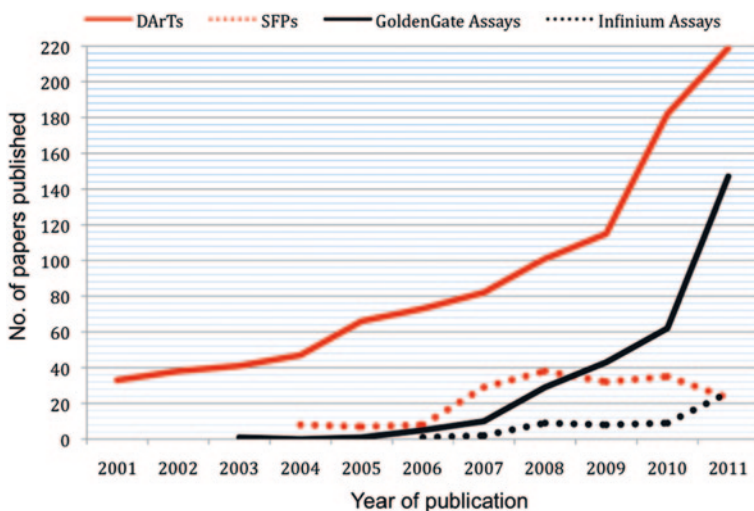


Fig. 2.3 Trends in publications related to GoldenGate assays, Infinium assays, DArT and SFPs in crop plants. The publications on DArT, GoldenGate and Infinium assays are increasing every year at fast rate, while as only a limited number of publications have become available on SFPs. (Source Google Scholar, March 2, 2011)

generation sequencing (NGS) technologies. Such a strategy has been used for rapid SNP discovery in different organisms. This was also proposed as a method for genotyping with RAD (Restriction-site Associated DNA) sequencing and by another similar method generally termed GbS (Genotyping-by-Sequencing). Therefore, a new platform was developed for >30 organisms now and it was shown that there is 3-fold (or more) increase in marker number on the new platform compared to arrays (Andrzej Kilian, personal communications; Sansaloni et al. 2011).

Nature of DArT Markers

DArT markers are biallelic and dominant in nature (presence *vs* absence); only rarely, these can be co-dominant (2 doses *vs* 1 dose *vs* absent). The software DArTsoft is used for the analysis of hybridization intensities. The efficiency of identification of polymorphic DArT markers depends on the level of genetic diversity within a crop species and the nature of diversity in metagenome constituting the discovery array. DArT markers usually detect polymorphisms due to single base-pair changes (SNPs) within the restriction sites recognized by endonucleases, or due to insertion/deletion (InDels)/rearrangements (Jaccoud et al. 2001). The type of polymorphism detected by DArT markers depends on the complexity reduction method applied to DNA samples of various genotypes/populations. For instance, a methylation sensitive restriction enzyme like *Pst*I will identify markers having both sequence variation (SNPs and InDels) and DNA methylation polymorphism (Kilian et al. 2005).

DArT Markers in Breeding for Cereal Crops

DArT assays are available for a fairly large number of plant species, including some orphan crops, for which no molecular information is available (Huttner et al. 2006; for details see Table 2.3). It is interesting to note that within a short span of time, DArT markers have become popular among researchers and have now become available for >70 species involving plants and animals (<http://www.diversityarrays.com/genotypingserv.html>). This technology provides a good alternative to currently available marker techniques including RFLP, AFLP, SSR and SNP in terms of cost, speed/amount of data generation. DArT markers are particularly useful for organisms, where no SNP arrays are publicly available (Mace et al. 2009). In addition, DArT markers are sequence-independent and non-gel based in nature. Also DArT assays involve automation and allow discovery of hundreds of high quality markers in a single assay making DArT markers the marker of choice for resource poor and underutilized orphan crops. The cost per data point (a few cents per marker assay) is reduced by at least an order of magnitude compared to gel-based technologies (Mace et al. 2009). The initial proof of concept of DArT technology was provided by using relatively simple genome of rice (Jaccoud et al. 2001). Later barley and other crops with more complex genomes were also used to demonstrate the

Table 2.3 A summary of DArT genotyping studies conducted in some important cereal crops

Crop involved & salient features of the study	Reference
<i>1. Wheat</i>	
First successful report of DArT in bread wheat (<i>T. aestivum</i>). Mapped DArT, AFLP, SSR and STM markers using 90 DH lines	Akbari et al. (2006)
Developed complexity reduction methods to generate large number of diverse clones for genotyping arrays. Constructed a framework linkage map using 93 DH lines and DArT, AFLP and SSR markers	Semagn et al. (2006)
Used >1,000 DArT markers and developed a linkage map with 90 SSR and 543 DArT markers using 176 RILs	Wenzl et al. (2007b)
242 DArT markers used to study association with resistance against stem rust, leaf rust, yellow rust, powdery mildew, yield and yield contributing traits	Crossa et al. (2007)
Studied genetic diversity of UK, US and Australian wheat varieties	White et al. (2008)
Studied genetic diversity to elucidate the genetic relationships among the selected spring and winter wheat lines/cultivars using DArT and SSR markers	Badea et al. (2008)
A linkage map was prepared using SSRs and 209 DArT loci; QTL were identified for powdery mildew resistance and their correspondence with adult plant rust resistance loci <i>Lr34/Yr18</i> and <i>Lr46/Yr29</i> was established	Lillemo et al. (2008)
An integrated DArT-SSR map including 162 SSRs and 392 DArT loci (2,022 cM) developed; DArT markers were also used to profile a panel of durum accessions; the genetic relationships based on DArT and SSR markers were compared	Mantovani et al. (2008)
High density genetic map including 197 SSR and 493 DArT loci developed	Peleg et al. (2008)
Genetic map with SSR and DArT markers was used for identification of QTLs for grain fructan concentration	Huynh et al. (2008)
DArT markers were used to evaluate gene bank accessions of spelt for genetic diversity and for resistance to aluminium toxicity and for low <i>PPO</i> activity	Raman et al. (2009)
DArT markers were developed for <i>T. monococcum</i> to assess genetic diversity, compare relationships with hexaploid genomes, and construct a genetic linkage map integrating 274 DArT and 82 SSR markers	Jing et al. (2009)
Comparison of genetic and cytogenetic maps of <i>T. aestivum</i> using SSR and DArT markers. Extended number of DArT markers on the wheat array that can be used for mapping by determining their chromosomal location in reference to SSRs	Francki et al. (2009)
Developed high-density genetic map of chromosome 3B containing 939 markers (779 DArT and 160 other markers)	Wenzl et al. (2010)
Developed a genetic map with 429 DArT and few SSR markers. DArT markers <i>wPt-3049</i> (2.9 cM) and <i>wPt-0289</i> (4.6 cM) respectively showed associations with tan spot resistance genes <i>Tsr1</i> and <i>Tsr6</i>	Singh et al. (2010)
Used DArT and SSR markers for an updated map with higher marker density; QTL analysis for stripe, leaf and stem rust resistance	Prins et al. (2011)
Use of DArT in the study of population structure, LD and association with 20 agronomic traits (including grain yield, grain quality and disease resistance)	Neumann et al. (2011)

(continued)

Table 2.3 (continued)

Crop involved & salient features of the study	Reference
Crop involved & salient features of the study	
Studied genetic diversity and population structure using 1,637 DArT markers among 111 genotypes from northern China	Zhang et al. (2011)
567 spring wheat landraces were genotyped with 832 DArT markers to identify QTLs for resistance against <i>P. tritici-repentis</i> races 1 and 5	Gurung et al. (2011)
843 polymorphic DArT markers were used to genotype wheat lines, and genotypic data was used to assess genetic relationships among the accessions	Yu et al. (2010)
A whole genome map was developed using 676 polymorphic DArT markers. The map was used to find genomic regions associated with citrate efflux using single marker regression and interval mapping. A major QTL, <i>Qce-4BL</i> was identified on the long arm of chromosome 4B by both of the methods	Ryan et al. (2009)
195 Western European elite wheat varieties genotyped using 159 SSRs and 634 DArT markers to evaluate the effect of population structure in association tests for three major genes involved in plant height, heading date and awniness	Le Couvreur et al. (2011)
2. <i>Rice</i>	
Initial proof of concept and validation of DArT using AFLP like complexity reduction methods involving nine rice cultivars	Jaccoud et al. (2001)
Evaluated genetic diversity in a general purpose rice gene pool and validated DArT for rice genotyping	Xie et al. (2006)
3. <i>Barley</i>	
Constructed a genetic map with ~385 unique DArT markers (1,137 cM), using barley cultivars Steptoe and Morex	Wenzl et al. (2004)
Constructed high-density consensus linkage map with ~ 3,000 loci including 2,085 DArT loci	Wenzl et al. (2006)
Used DArT/SSR markers for QTL mapping of <i>Fusarium</i> head blight (FHB) resistance	Rheault et al. (2007)
Mapped >600 DArT markers covering ~2,000 cM; identified 15 clustered loci for multiple resistance	Aslop et al. (2007)
Mapped <i>Ryp1</i> on 3H and <i>Ryp2</i> , and <i>Ryp3</i> on 1H	Lee and Neate (2007)
Constructed high-density genetic map with 558 SSR and 442 DArT markers	Hearnden et al. (2007)
Tested suitability of DArT for bulk segregant analysis in barley; validated an aluminum tolerance locus on chromosome 4H	Wenzl et al. (2007a)
Detected associations of DArT markers (on 5H) with stem rust resistance in <i>Aegilops sharonensis</i> and wild barley	Steffenson et al. (2007)
Mapping of quantitative trait loci (QTL) associated with net blotch resistance in a DH population using DArT markers	Grewal et al. (2008)
Constructed a composite map using SSR, RFLP and DArT markers and used it for identification of QTLs for water logging tolerance	Li et al. (2008a)
QTL analysis for seedling and adult-plant resistance to spot and net blotch using an SSR, AFLP and DArT based map	Grewal et al. (2012)
1,130 DArT markers were used on a diverse barley collection to scan their genomes for associations with yield components	Comadran et al. (2009)

(continued)

Table 2.3 (continued)

Crop involved & salient features of the study	Reference
1,000 polymorphic DArT markers were used to study genetic diversity, population structure, and extent of LD in 170 Canadian barley genotypes	Zhang et al. (2009)
Preparation of integrated map with SSR, AFLP and DArT markers and identification of QTLs for β -glucan content	Li et al. (2008)
Association mapping for malting quality traits using 91 elite two-rowed malting barleys; 27 DArT markers were found to be associated with malting quality	Beattie et al. (2010)
Association mapping for spot blotch resistance using Wild Barley Diversity Collection (WBDC) using 558 DArT and 2,878 SNP markers; 13 QTLs identified	Roy et al. (2010)
DArT markers were used to find out if the accessions with multiple resistance (MR) from the Vavilov nursery were genetically related to accessions with MR from Ethiopia	Bonman et al. (2011)
Genetic maps were developed for four populations and 607 new DArT markers were integrated in a consensus map with 3,542 markers	Alsop et al. (2011)
253 DArT markers were used on a set of 183 varieties, which clearly distinguished between spring and winter types and classified them into five subgroups	Matthies et al. (2012)
4. <i>Sorghum</i>	
Genotyped of two mapping populations of sorghum	Mace et al. (2007)
Mapped 330 non-redundant DArT markers covering whole genome	Bouchet et al. (2007)
DArT markers were used to identify genomic regions associated with yield and adaptation	Jordan et al. (2007)
DArT markers were used for genetic diversity study and for construction of a genetic linkage map	Mace et al. (2008)
A genetic linkage map was constructed using 36 SSR, 117 AFLP and 148 DArT markers. QTLs for ergot resistance and two pollen traits were identified	Parh et al. (2008)
Developed consensus map including 1190 DArT loci (58.6 % of total mapped loci)	Mace et al. (2009)
5. <i>Rye</i>	
Determined genetic relationships between rye varieties and inbred lines using 1,022 DArT markers. Chromosomal location of 1,872 DArT markers was also determined, providing an average density of one unique marker every 2.68 cM	Bolibok-Bragoszewska et al. (2009)
564 RILs from 5 mapping populations were genotyped using DArT markers and subjected to linkage analysis. A consensus map was constructed using a total of 9,703 segregating markers. The average chromosome map length ranged from 199.9 cM (2R) to 251.4 cM (4R) and the average map density was 1.1 cM	Milczarski et al. (2011)

(continued)

Table 2.3 (continued)

Crop involved & salient features of the study		Reference
<i>6. Triticale</i>		
21 linkage groups assigned to the A, B, and R genomes using 155 SSR, 1,385 DArT, and 28 AFLP markers		Tyrka et al. (2011)
Evaluated DArT markers for transferability from rye and wheat to triticale; DArT technology used for diversity analyses on a set of 144 triticale accessions		Badea et al. (2011)
Consensus map, constructed out of six segregating populations, incorporated 2,555 DArT markers (2,602 loci) spanning 2,309.9 cM with an average number of 123.9 loci per chromosome and an average marker density of 1 locus/1.2 cM		Alheit et al. (2011)
<i>7. Oat</i>		
1,010 new DArT markers were used to saturate earlier genetic map. A set of 1,295 markers was used to analyze genetic diversity		Tinker et al. (2009)
Seedling crown rust resistance gene “ <i>Pc9J</i> ” was mapped to a linkage group with DArT markers. Five robust SCARs were developed from three non-redundant DArTs that co-segregated with <i>Pc9J</i>		McCartney et al. (2011)
1,205 lines and 402 DArT markers were used for population structure and LD studies in oat germplasm and determine their implications for GWAS		Newell et al. (2011)
A high density map was prepared using 974 DArT, 26 SSR, 13 SNP, and 4 phenotypic markers		Oliver et al. (2011)

usefulness of DArT technology (Wenzl et al. 2004, 2006, 2007; Hearnden et al. 2007; also see Table 2.3). For instance, DArT markers are now available for diploid wheat (Jing et al. 2009), tetraploid wheat (Peleg et al. 2008), hexaploid wheat (Akbari et al. 2006; Semagn et al. 2006; White et al. 2008), sorghum (Mace et al. 2008, 2009), rye (Bolibok-Brągoszewska et al. 2009) and more than 30 other plant species (Jing et al. 2009). In case of wheat alone, more than 50,000 samples (>95 % as service at ~1 cent per marker assay) involving >350 mapping populations were processed, which resulted in preparation of >100 maps having ~7,000 markers assigned to specific chromosomes (A. Kilian, personal communication). Chromosome specific (3B) and individual chromosome arm (1BS)-specific DArT markers have also been developed using flow sorted chromosome/chromosome arm. A total of 553 of the 711 polymorphic 3B-derived markers (78 %) were mapped on chromosome 3B, and 59 of the 68 polymorphic 1BS-derived markers (87 %) were mapped to 1BS, thus confirming the efficiency of the chromosome-sorting approach in DArT technology. A consensus map of chromosome 3B using 19 mapping populations, including some that were genotyped with the 3B-enriched array was also prepared and the map is probably the densest genetic map of 3B available to date; the map contains 939 markers including 779 DArT markers and 160 other markers (Wenzl et al. 2010). Also, DArT markers are now available on large scale for all major cereal crops and were extensively utilized for the study of genetic diversity, preparation of integrated framework linkage maps, QTL interval mapping, association mapping, etc. (Rheault et al. 2007; Wenzl et al. 2007a). The physical map of wheat genome is also being constructed using a chromosome-by-chromosome approach, where individual laboratories developed maps for individual chromosomes (Feuillet and Eversole 2008). These laboratories can now develop saturated DArT maps for their specific chromosomes in an affordable and targeted manner.

Cost-Effectiveness of DArT Assays and Their Suitability for Molecular Breeding

DArT marker assays have shown to be cheaper than any other marker system available at present. The cost per marker assay in commercial service offered by Triticarte P/L is ~US\$ 0.02 (or approximately US\$ 50 per genotype for ~2,500 DArT markers; Mantovani et al. 2008), which is >6 times cheaper than the cost of SSR genotyping, and ~3 times cheaper than Illumina GG assay (Yan et al. 2010).

The DArT markers on the wheat array are now being assigned to chromosomal bins by deletion mapping. This deletion mapping of DArT markers will provide a reference to align genetic and cytogenetic maps and estimate the coverage of DArT markers across genome (Francki et al. 2009). However, the associated DArT markers identified through QTL interval mapping or association mapping cannot be directly used in marker-assisted selection for the improvement of a desired trait in a crop species. In order to overcome this limitation, the sequences of DArT markers (usually those linked to traits of interest) can be obtained from Triticarte service and can be converted into user-friendly PCR-based markers. For instance,

five robust SCARs were developed from three non-redundant DArT markers that co-segregated with crown rust resistance gene “*Pc91*” in oats. These SCAR markers were developed for different assay platforms: agarose gel electrophoresis, capillary electrophoresis, and TaqMan single nucleotide polymorphism detection (McCartney et al. 2011).

2.6.3 Single Feature Polymorphisms (SFPs) Genotyping in Cereal Crops

Single feature polymorphisms (SFPs) represent another high-throughput array-based genotyping technology. It involves use of oligonucleotides (features), which represent segments of individual genes. Affymetrix (<http://www.affymetrix.com>) GeneChips or Nimblegen (<http://www.nimblegen.com>) arrays with small probes (25 bp) capable of detecting sequence polymorphism are the most widely used arrays for SFP genotyping (see Table 2.4). Majority of studies involving discovery and genotyping for SFPs have been conducted in model organisms like yeast, mouse and *Arabidopsis*, whose genomes have been sequenced and characterized (Brem et al. 2002; Borevitz et al. 2003; Kumar et al. 2007). In plants, SFP technology was first applied for *A. thaliana* using Affymetrix expression array (Borevitz et al. 2003). The technique was later used for all important crops including cereals. However, in crops with complex large genomes, a suitable complexity reduction method is used for sample preparation and replicating arrays are used for hybridization. Therefore, SFPs became available for all major cereal crops including barley (Cui et al. 2005; Rostoks et al. 2005), rice (Kumar et al. 2007), maize (Kirst et al. 2006; Gore et al. 2007) and wheat (Coram et al. 2008; Banks et al. 2009; Bernardo et al. 2009). SFPs have actually been used for a variety of studies including the following: (1) genetic mapping (Zhu et al. 2006; Somers et al. 2008; Banks et al. 2009; Bernardo et al. 2009), and (2) QTL interval mapping leading to detection of main effect QTLs and eQTLs (Potokina et al. 2008; Kim et al. 2009). A summary of SFP studies conducted in some of the major cereal crop species is presented in Table 2.4.

2.6.4 Use of Sequenom MassARRAY System for SNP Genotyping in Cereals

The MassARRAY platform has successfully been used to genotype SNPs in mammals (Vogel et al. 2009). More recently, it has also been used in cereals including rice, maize, barley and wheat. For instance, in wheat, SNPs identified in homoeologues of gene for acetohydroxyacid synthase conferring resistance against imidazolinone herbicides were successfully converted, and used on Sequenom MassARRAY system (Dr. Divya Neelam, BASF personal communication). Sequenom-based SNP-typing assays were also developed for 1,359

Table 2.4 A summary of SFP genotyping studies conducted in some important cereal crops

Plant species and resources used	Salient features of study	References
<i>1. Wheat</i>		
Affymetrix GeneChip Wheat Genome Array and cRNA	More than 1,500 SFPs were placed on genetic maps using 64 DH individual lines	Banks et al. (2009)
Affymetrix GeneChip (Probe sets from 55,052 Microarray analysis of 71 RILs identified 955 SFPs; 877 were mapped with 269 SSR transcripts) and cRNA	Identified 100's of SFP markers and integrated them into an existing SSR map	Bernardo et al. (2009)
Affymetrix GeneChip® Genome Array	Identified 100's of SFP markers and integrated them into an existing SSR map	Somers et al. (2008)
55 K Affymetrix Wheat GeneChip (61,127 probe sets) and cRNA	297 SFPs were identified between NILs for stripe rust resistance	Coram et al. (2008)
Affymetrix Genome Array (61,127 probe sets) and cRNA	208 high variance probe sets (HVPs) assigned to wheat chromosome arm 1BS	Bhat et al. (2007)
Wheat oligonucleotide array and cRNA	44 SFPs for 7E (<i>Thinopyrum</i> and <i>Lophopyrum</i>) identified using alien substitution/addition lines	Buescher et al. (2007)
Affymetrix GeneChip (38,577 probe sets) and cRNA	SFPs identified in 948 genes using two wheat varieties ('Eltan' and 'Oregon feed wheat')	Ling et al. (2006)
<i>2. Rice</i>		
Affymetrix Genome Array (55,515 probe sets) and cRNA	5,376 SFPs in 'LaGrue' (<i>japonica</i>), and 25,325 SFPs in 'RT0034' (<i>indica</i>), when compared with Cypess (<i>japonica</i>)	Kumar et al. (2007)
Affymetrix rice Genome Array and cRNA	1208 SFP probes were detected between two presumed parental genotypes of a RIL population segregating for salt tolerance	Kim et al. (2009)
GeneChip Genome Array (57,381 probe sets) and cRNA	6,655 SFPs between two rice varieties representing 3,131 rice unique genes	Xie et al. (2009)
GeneChip Genome Array (57,381 probe sets) and cRNA	1,632 SFPs and 23 markers were placed into 601 recombinant bins, spanning 1,459 cM. Map was used to identify 26,051 eQTLs assigned to 171 eQTL hotspots for 16,372 e-traits	Wang et al. (2010)
<i>3. Barley</i>		
Barley1 GeneChip (22,840 probe sets) and cRNA	64/46 SFPs detected from shoot/root datasets, when comparisons were made among 'Golden Promise' and 'Maythorpe'	Walia et al. (2007)
Barley1 GeneChip and cRNA	924 of 1,257 genes assigned to chromosomes with the help of SFPs	Bilgic et al. (2007)

(continued)

Table 2.4 (continued)

Plant species and resources used	Salient features of study	References
Barley 1 GeneChip and cRNA	Mapped >2,000 transcript derived markers (TDMs); including both SFPs and GEMs and 23,738 eQTLs	Potokina et al. (2008)
Barley 1 GeneChip and cRNA	>4,000 SFPs identified between ‘Step toe’ and ‘Morex’, and segregation studied using DH population (Step toe × Morex)	Luo et al. (2007)
Barley 1 GeneChip and cRNA	10,504 SFPs identified between ‘Golden Promise’ and ‘Morex’; also compared with known SNPs	Rostoks et al. (2005)
Barley 1 GeneChip and cRNA	2007 SFPs identified between ‘Step toe’ vs ‘Morex’, ‘Morex’ vs ‘Barke’, and Oregon Wolfe Barley Dominant vs Recessive; 80 % were confirmed by direct sequencing	Cui et al. (2005)
<i>4. Maize</i>		
GeneChip Genome Array (17,555 probe sets; 17,477 probe sets with 15 probe pairs, and 78 with 14 or less probe pairs)	Assessment of various target preparation and hybridization methodologies (e.g., cRNA, methylation filtration, high C ₀ t and AFLP) using three diverse maize inbred lines	Gore et al. (2007)
GeneChip Genome Array	34,034 SFPs identified and mapped using an intervarietal mapping population; mapped loci validated through sequencing	Zhu et al. (2006)
Affymetrix CornChip0 (8403 probe sets) and cRNA	36,196 SFPs identified among ‘B73’ (reference genotype) and three US maize lines: Mo17, Wf9-BG and W23	Kirst et al. (2006)

maize SNPs identified via comparative next-generation transcriptome sequencing. Approximately 75 % of these SNPs were successfully converted into genetic markers that can be scored reliably and used to generate a SNP- based genetic map by genotyping recombinant inbred lines derived from the popular cross B73 \times Mo17 (Liu et al. 2010). In barley, in order to determine identity of 60 Australian varieties, a high-throughput multiplexed SNP genotyping assay was developed using Sequenom MassARRAY and iPLEXTM Gold genotyping systems. As a result, a unique identifier (barcode) of up to 20 SNPs was established for each of the 60 studied varieties (Pattemore and Henry 2008). Similarly, in rice identity of the functional polymorphism in genes influencing different aspects of salt tolerance was determined by combining genetic mapping and transcriptome profiling of bulked RILs (having extreme phenotypes) using Sequenom MALDI-TOF MassARRAY system (Pandit et al. 2010). In wheat, iPlex has been used to genotype 47 wheat SNPs on 1,314 lines (Berard et al. 2009). The agreement of the genotypes obtained by iPlex with the results obtained by different validation methods (sequencing or SNPlexTM) was 96 % showing that it can be used successfully in polyploid plants. Mass spectrometry was also used to evaluate the SNP diversity within genes related to bread making quality (*Glu* and *SPA*) on a set 113 lines (Ravel et al. 2007). Similarly, iPlex was tested in a tetraploid wheat, *Triticum durum* \times *T. dicoccoides* F₂ population, and was shown to be efficient even for discrimination of heterozygotes (Paux et al. 2012).

2.6.5 Restriction-Site Associated DNA (RAD) Markers in Cereals

RAD markers have witnessed a switch from the low cost microarray-based genotyping platforms to next-generation sequencing based detection procedures. This shift is mainly attributed to the drop in sequencing cost, ease and time for genotyping. RAD sequencing is a form of genotyping by sequencing method, which has recently been put to a variety of applications including genetic mapping and QTL analysis in wide range of organisms (Rowe et al. 2011). The RAD sequencing has provided a method for the discovery of thousands of SNPs. For instance, in barley, a total of 530 SNPs were identified from initial scans of the Oregon Wolf Barley parental inbred lines, and scored in a 93 member doubled haploid (DH) mapping population. RAD sequence data from the DH population was used for genetic map construction. The assembled RAD-only map consists of 445 markers with an average interval length of 5 cM. Sequenced RAD markers are distributed across all seven chromosomes, with polymorphic loci originating from both coding and noncoding regions in the genome (Chutimanitsakun et al. 2011). Similarly, in *Lolium perenne* SSR and STS markers were combined with the RAD markers to produce maps for the female (738 cM) and male (721 cM) parents, and QTLs were identified for resistance to stem rust caused by *Puccinia graminis* subsp. *graminicola* (Pfender et al. 2011). RAD tags were also generated from the

genomic DNA of a pair of eggplant mapping parents. The resulting non-redundant genomic sequence dataset consisted of ~45,000 sequences, of which ~29 % were putative coding sequences and ~70 % were common between the mapping parents. The shared sequences allowed the discovery of ~10,000 SNPs and nearly 1,000 indels, equivalent to a SNP frequency of 0.8 per Kb and an indel frequency of 0.07 per Kb (Barchi et al. 2011).

2.6.6 Use of CNVs and PAVs as Markers in Cereals

CNVs, PAVs and InDels are a new class of markers that are based on microarrays (making use of array-based CGH) and have been extensively used in humans. These relatively new marker types are now being increasingly used in cereals also, and are likely to be preferred over other marker systems in future. Some of the studies already conducted involving these new marker types are briefly reviewed in this section.

CNVs and PAVs in Rice

A high-density oligonucleotide aCGH microarray (containing 7,18,256 oligonucleotide probes) was used in rice to estimate the number of CNVs between the genomes of two cultivars, Nipponbare and Guang-lu-ai4. These CNVs involved known genes, and may be linked to variation among rice varieties, thus contributing to species-specific characteristics (Yu et al. 2011).

CNVs and PAVs in Maize

Whole-genome aCGH was also used for the analysis of CNVs and PAVs in maize. In one study, Mo17 was compared with B73 (Springer et al. 2009) using a microarray with 2.1 million probes developed by Roche NimbleGen, and in another study, 13 inbred lines were compared with the same standard genotype B73 (Belo et al. 2010). For this purpose, high-density microarrays developed by Roche NimbleGen (2.1 million probes) and Agilent (~60,500 probes) were utilized. The two studies revealed a fairly high level of structural diversity between the inbred lines. Several hundred CNVs and thousands of PAVs, distributed over all the chromosome arms, were identified. In yet another study, aCGH was used to compare gene content and CNVs among 19 diverse maize inbreds and 14 genotypes of the wild ancestor of maize, teosinte. CNVs in hundreds of genes were identified, and it was shown that no strong selection for or against CNVs/PAVs accompanied domestication (Swanson-Wagner et al. 2010), although these were shown to contribute to significant quantitative variations.

2.7 Summary and Outlook

The use of array-based genotyping platforms and next-generation sequencing methods for the development and use of third and fourth generation markers has already overwhelmed the plant breeding programs. This became possible due to the generation of data by these platforms in a cost and time-effective manner. These methods have revolutionized plant biology, by bringing precision to whole genome association and linkage studies. These advances in marker-technology equipped the plant breeders with tools to engineer cultivar genotypes with the desired attributes following the concept of 'Breeding by Design' (Peleman and van der Voort 2003). The array-based markers have been put to a variety of applications including genetic linkage and association mapping, diversity and LD studies, gene/QTLs cloning studies, and more recently to anchor BAC-based physical maps with the genetic linkage maps.

The use of array-based platforms for physical and comparative mapping has also improved our understanding of gene and genome organization in major cereals. For instance, Rustenholz et al. (2010) explored the possibility of using barley transcript genetic maps as a surrogate to anchor and order the wheat physical contigs by hybridizing 60 three-dimensional (plate, row, column) BAC pools representing the minimal tiling path (MTP) of wheat chromosome 3B onto barley Agilent 15 K unigene microarray. This has allowed localization of genes along chromosome 3B. The results showed that such barley-wheat cross-hybridizations represent a high throughput cost-efficient approach for anchoring genes on wheat physical maps and for performing comparative genomics studies between wheat and other grass genomes. This study has also led to fine mapping of 738 barley orthologous genes on wheat chromosome 3B. In addition, comparative analyses revealed that 68 % of the genes identified were syntenic between the wheat chromosome 3B and barley chromosome 3H and 59 % were syntenic between wheat chromosome 3B and rice chromosome 1. Later, a subset of 9,216 BACs representing the MTP of the new version of the 3B physical map was pooled into 64 three-dimensional (plate, row, and column) pools and hybridized onto a newly developed wheat NimbleGen 40 K unigene microarray. This not only improved the physical map of chromosome 3B but also allowed mapping of almost 3,000 genes on this chromosome. The expression pattern of these genes was also studied in 15 different conditions. This transcription map of chromosome 3B confirmed that 70 % of the genes are organized in islands that are responsible for an increasing gradient of gene density observed from the centromere to the telomeres. By studying their expression, and putative function, it has been concluded that the gene islands are enriched significantly in genes sharing the same function or expression profile, thereby suggesting that genes in islands acquired shared regulation during evolution (Rustenholz et al. 2011). Similarly, a Morex BAC library in barley consisting of 147,840 clones was pooled into 55 Super Pools (SPs) having seven 384-well plates per SP. The plate, row, and column pools from each SP were further pooled, respectively, into five Matrix Plate Pools (MPPs), eight Matrix Row Pools (MRPs), and 10 Matrix Column Pools (MCPs), giving a

total of 23 Matrix Pools (MPs), which were hybridized on to an Agilent 44 K barley microarray, representing 42,302 expressed genes (Liu et al. 2011). These BACs from multidimensional pools of BAC clones were also incorporated into the HICF physical map of barley. By using array hybridization in combination with next-generation sequencing, and systematic exploitation of conserved synteny with model grasses (rice, sorghum and *Brachypodium distachyon*) 21,766 of the estimated 32,000 barley genes were assigned to individual chromosome arms and their linear order was determined (Mayer et al. 2011). More recently, the array-based markers have also facilitated fine mapping and cloning of several genes contributing to a number of morphological traits in barley. For instance, cloning of *Mat-A* (Zakhrabekova et al. 2012) and *Intermedium-C* (Ramsay et al. 2011) genes respectively responsible for short-season adaptation and spikelet fertility, respectively and fine mapping of *ANT2* (Cockram et al. 2010), *TRD1*, *VRS1*, *UZU*, *NUD1* and *WAXY* genes (Druka et al. 2011) facilitated by the use of GG technology.

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