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Molecular Marker Techniques in *Musa* Genomic Research

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ABSTRACT

Banana is one of the most important subtropical crops. Its genetic system is relatively unknown and complicated by inter- and intra-specific hybridization, heterozygosity, and polyploidy. Thus molecular markers have been widely used among *Musa* germplasm to detect genetic variation and relationships, duplicate accessions identification, monitoring genetic stability, analyzing irradiated genotypes and identification of trait markers for use in breeding programs. Various markers based morphology, protein, and DNA are in existence. Among them the most widely used markers suitable for marker assisted selection (MAS) are RFLP, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), and

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List of abbreviations after the text.

simple sequence repeat (SSR). This chapter compares the efficiency of available markers, its application and development for *Musa* genome analysis. High-throughput technologies based on single nucleotide polymorphisms (SNPs) or small scale indels are efficient alternatives with desired dosage sensitivity and the ability to distinguish haplotypes in polyploid crops. However, the choice of markers differ and depends on the labor required for their detection, possibility of revealing single or multiple loci, dominant or co-dominant nature, and costs. Recent developments reported in various crops like high resolution DNA melting (HRM) analysis has great potential for cultivar identification, candidate gene identification, mapping (genomic and association) studies. Thus the identified genomic regions in banana with these approaches has the potential to dramatically enhance the pace and efficiency of genetic improvement in *Musa*.

Keywords: molecular marker systems, *Musa*, genetic improvement

4.1 Introduction

Advances in molecular biology gave rise to a number of new technologies that held great promise for crop breeding. One of such innovations was the development of molecular markers for tagging genes of interest. Genetic markers are landmarks on a chromosome that assist in genome analysis (Lefebvre et al. 1995). Markers are generally described as "genetic tags" that identify particular locations within a plant's DNA sequence and are transmitted to the offspring following the laws of inheritance from one generation to the next (Drew 1997). The use of DNA-based markers has allowed researchers to mark genes or chromosome regions that are related to genetic traits such as host plant resistance to pathogens and pests (Drew 1997). Once genetic markers are defined, they can be used directly in breeding programs since they allow researchers to predict phenotypes based on the presence or absence of the marker. Alternatively, once genes for particular trait have been identified by genetic marker techniques, they can be cloned. A particular advantage of such techniques is that complex multigenic traits can be analyzed. Mapping techniques can also be used to isolate genes based entirely on their genetic behavior. Once these have been identified, sequenced and cloned, gene transfer techniques can be used to transfer them to other species (Drew 1997).

Molecular markers are based on naturally occurring polymorphisms in DNA sequences (i.e., base pair deletions, substitutions or additions, among others). Different kinds of molecular markers exist, some are morphological, or protein-based while the most common ones are DNA-dependent. Genetic markers that reveal polymorphisms at the protein level are known as biochemical markers, while DNA markers reveal polymorphisms at the DNA level. Biochemical markers are proteins, produced from gene

expression, which can be separated by electrophoresis to identify the alleles. The most commonly used protein markers are isozymes, which are variant forms of the same enzyme. Protein markers reveal differences in the gene sequence and function as codominant markers. However, their use is restricted due to their limited number in any crop species and also because they are subject to post-translational modifications (Staub et al. 1982). There are two categories of DNA markers according to how their polymorphism is expressed: hybridization-based and PCR-based polymorphisms. The hybridization-based polymorphisms include restriction fragment length polymorphisms (RFLP) (Sambrook et al. 1989) and variable number tandem repeats (VNTR) loci (Rogstad 1993; Weising et al. 1998), where probes such as random genomic clones, and probes for microsatellite (or simple sequence repeats, SSR) and mini-satellite sequences are hybridized to filters containing DNA that has been digested with restriction enzymes. The polymorphisms in the case of VNTR loci are due to a difference in the number of repeats, while RFLPs are generated due to events such as point mutations, inversions, deletions, or translocations.

The use of molecular markers has greatly improved breeding approaches since these markers directly reveal genetic variability through DNA analysis (Staub et al. 1996) and therefore their detection is not influenced by environmental effects. The development of numerous molecular markers for plant genome analysis makes it possible to select the genotype instead of the phenotype, leading to the concept of marker-assisted selection or MAS (Paterson et al. 1991). The most widely used markers suitable for MAS are RFLP, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) (Barone 2004). The choice of the most suitable markers for MAS however differs and depends on the labor required for their detection, possibility of revealing single or multiple loci, dominant or codominant nature, and costs.

Molecular markers have been widely used in *Musa* for detecting genetic variation and genetic relationships in banana germplasm, identification of duplicate accessions in field and tissue culture germplasm banks, monitoring genetic stability of tissue culture material, analyzing irradiated banana genotypes and identification of trait markers for use in breeding programs. This chapter examines some of the more common markers used in *Musa* and mentions some new marker systems that may be potentially useful in *Musa*.

4.2 Isozyme Markers

Many studies in banana and plantain were undertaken to characterize isozymes as genetic markers for estimating genetic diversity (Bhat et al. 1992a) within the genus, to measure among others somaclonal variation,

to identify protoplast fusion products, and for clonal identification (Bhat et al. 1992b).

However, the sensitivity of this technique is a function of the number of polymorphic loci that can be resolved. The use of isozymes as genetic markers for variety identification in banana dates back to 1980s and received considerable attention (Tanksley and Orton 1983). Few studies conducted to examine the peroxidase isozymes in various banana clones and species noted a lack of polymorphism (Bonner et al. 1974). Rivera (1983) examined the peroxidase and polyphenoloxidase systems for polymorphism and was able to distinguish between the "Saba" (ABB/BBB) and "Bluggoe" (ABB) banana genome groups. Jarret and Litz (1986a, b) studied the efficiency and application of various isozymes such as *shikimate dehydrogenase* (*SKDH*), *malate dehydrogenase* (*MDH*), *peroxidase* (*PRX*), *phosphoglucomutase* (*PGM*) and *glutamate oxaloacetate transminase* (*GOT*). Some isozymes were useful in discriminating clones in some genomic groups. Simmonds (1966) used *esterase* (*EST*), *tetrazolium oxidase* (*TO*), *6-phosphogluconate dehydrogenase* (*6PGDH*), *malic enzyme* (*ME*), *phosphoglucose isomerase* (*PGI*), *alcohol dehydrogenase* (*ADH*), *triosephosphate isomerase* (*TPI*), *galactose dehydrogenase* (*GDH*), *succinate dehydrogenase* (*SUDH*), *sorbitol dehydrogenase* (*SDH*) and *glutamate dehydrogenase* (*GUDH*) to classify bananas. Espino and Pimentel (1990) indicated that *MDH* was useful in distinguishing the AAB and ABB cultivars from those with BB/BBB genomes. The isozymes of *MDH*, *PRX* and *GOT* were used to analyze the variation of 100 Indonesian *Musa* cultivars belonging to the AA, AAA, AAB, ABB, and BB genomic groups (Megia et al. 2001). A high degree of polymorphism was recognized for *MDH* and *PRX*. *GOT* was the least polymorphic isoenzyme. The numbers of cultivars that exhibited specific isozyme profiles were 16 for *MDH*, 20 for *PRX*, and 8 for *GOT*. These isozyme profiles were able to distinguish 31 cultivars. Isozymes were also useful for assessing the induction of genetic variability by γ-radiation in banana (Megia et al. 2001). Isozymes were used to identify resistance to *Banana Bract Mosiac Virus* (BBrMV) (Dhanya et al. 2006). The *BBrMV*-infected samples showed the presence of a definite single peroxidase band that was absent in non-infected samples.

Most isozyme markers used in banana research helped elucidating clonal identification in phylogenetic studies. Nevertheless, the use of isozymes as genetic markers will provide estimates of genetic diversity within the cultivated and wild relatives of the genus *Musa*, facilitate clonal identification and should provide valuable evidence on the origins of triploid cultivars. Differentiation of somatic mutations from their parental clones may occasionally be detected by isozyme analysis (Brettell et al. 1986).

4.3 Hybridization Based Systems

4.3.1 Restriction Fragment Length Polymorphisms (RFLP)

RFLPs are variations in DNA fragment length banding patterns of electrophoresed restriction digests of DNA samples. These variations are frequent due to the presence of a restriction enzyme cleavage site at one site in the genome of one individual and the absence of the site in another individual. RFLP is a codominant marker meaning that it is able to distinguish homozygotes and heterozygotes. RFLP is robust, easily transferred between laboratories, and requires no prior sequence information about an organism for its use. It is, therefore, useful for constructing genetic linkage maps, especially in *Musa*.

As it is locus-specific, estimations of conservation of synteny are possible. Using single locus probes it allows discrimination at the population level. The disadvantages are that it is expensive to develop, requires large amounts of DNA, is not possible to automate as compared to AFLP and diversity array technology (DArT) or multiplexing of SSRs, needs a suitable probe library, may require radioactive labeling and is laborious and time consuming. RFLPs have been used in all *Musa* mapping projects to date and most recently have been linked to polymorphisms in resistance gene analogs (Hippolyte et al. 2010).

RFLPs are considered highly repeatable and specific since a probe of known sequence is used. RFLPs of diverse germplasm have been used to study the taxonomy and phylogeny of *Musa* species (Gawel and Jarret 1991a, b; Gawel et al. 1992; Jarret et al. 1992) and variation in the chloroplast genome (Gawel and Jarret 1991a, b; Nwakanma et al. 2003). However, there is only one report of their usefulness in distinguishing closely related material (Bhat et al. 1995). The relatively high cost and technically demanding nature of this technique makes it inappropriate for routine breeding applications.

4.3.2 Variable Number of Tándem Repeats (VNTR)

VNTRs are tandem repeats of short DNA sequences and include minisatellites (10–45 bp) and microsatellites (2–6 bp). While initially VNTRs were detected involving radioactive probes presently they can be detected by employing PCR technology. VNTRs are abundant and ubiquitous in the eukaryotic genomes (Tourmente et al. 1994). These sequences hybridize to multiple loci and are hypervariable in nature, thus are highly informative for genetic analysis. Tandem repeats are usually classified into microsatellites (the length of the repeat unit being 5 bp or less) and minisatellites (more than 5 bp and up to 100 bp in length) (Buard and Vergnaud 1994). Crouch et al. (1998; 1999a, b) used VNTR analysis of microsatellite loci to compare the genetic similarity of full-sib 2*x* and 4*x* plantain-banana hybrids and their parental genotypes.

4.4 PCR-based Marker Systems

All PCR-based molecular markers appear to detect a high level of polymorphism within a range of *Musa* breeding populations. PCR-based assays are amenable to the large-scale throughput demands of screening breeding populations. Thus, researchers have concentrated on the applications of PCR for *Musa* genome analysis. Various molecular markers, especially different PCR-based molecular markers including AFLP, RAPD, microsatellites, have been frequently used for assessing genetic diversity and phylogenetic relationship in wild banana and cultivated accessions (Grapin et al. 1998; Loh et al. 2000; Wong et al. 2001; Carreel et al. 2002; Ude et al. 2002a, b, 2003; Creste et al. 2003a,b; Nwakanma et al. 2003).

A large variety of PCR amplification methods has emerged since its discovery. These include single primer amplification reaction (SPAR) (Gupta et al. 1994; Weising et al. 1995). The SPAR approach to minisatellite analysis has been described as direct amplification of minisatellite-region DNA (DAMD), which directs the amplification to regions rich in minisatellite repeats by using the core sequence of minisatellites as single primer (Heath et al. 1993; Zhou et al. 1997). Single-strand conformation polymorphism analysis (SSCP) is a rapid method for detection of minor sequence changes in polymerase chain reaction-amplified DNA (Orita et al. 1989). RAMPO combines arbitrarily or semispecifically primed PCR with microsatellite hybridization to produce several independent and polymorphic genetic fingerprints per electrophoretic gel (Richardson et al. 1995). Recently the sequence-related amplified polymorphism (SRAP) technique, aimed for the amplification of open reading frames (ORFs), was used to analyze the genetic variation and relationships among 40 *Musa* accessions (Muhammad et al. 2010).

4.4.1 Random Amplified Polymorphic DNA (RAPD)

RAPD has the advantage that it is easy to carry out with relatively little costs, but reproducibility is sometimes limited and reliability depends on the skill of the operator. Different results have been reported when RAPD analysis is carried out in different laboratories using identical PCR parameters and conditions. The RAPD technique requires no prior knowledge of the genome of an organism since it depends on the use of short arbitrary primers. The advantages of the RAPD technique include its speed, low DNA template requirement and technical simplicity. It is a convenient tool for detecting genetic variation within the germplasm. RAPDs have been widely used to examine genetic relationships in banana germplasm (Uma et al. 2006; Jain et al. 2007; Nsabimana and van Staden 2007) for identification of duplications among accessions in field and tissue culture germplasm banks

and monitoring of genetic stability of tissue culture material (i.e., somaclonal variation) (Bairu et al. 2006, 2008; Ray et al. 2006; Lakshmanan et al. 2007), analyzing and differentiating irradiated banana genotypes (Toruan-Mathius and Haris 1999; Finalet et al. 2000; Imelda et al. 2001; Hautea et al. 2004) and identification of trait markers for use in cross- and mutation-breeding programs (Damasco et al. 1996). DNA profiling would also be important for policing plant patents and for legal protection of newly bred cultivars in asexual crops like *Musa* (Kaemmer et al. 1997). Highly informative RAPD markers can be converted to sequence characterized amplified regions (SCAR) primers for specific applications. RAPD assays have proven to be powerful and efficient means of marker-assisted introgression and backcross breeding. Specific RAPD markers for the A and B genomes of *Musa* have been identified and were used routinely (Pillay et al. 2000; Oselebe et al. 2006; Pillay et al. 2006).

However, RAPD analysis has several disadvantages including the dominant nature of the marker system and reproducibility problems, which may limit their application in marker-assisted selection. The RAPD technique has been successfully used to distinguish diverse *Musa* germplasm (Howell et al. 1994; Bhat and Jarret 1995; Pillay et al. 2001; Uma et al. 2006; Jain et al. 2007; Nsabimana and van Staden 2007). In addition, a molecular linkage map has also been developed using a variety of marker systems including RAPD (Faure et al. 1993). RAPD analysis has also been used to differentiate *Musa* genome groups (Howell et al. 1994; Pillay et al. 2000), closely related *Musa* germplasm (Bhat and Jarret 1995) and full-sib hybrids in plantain breeding populations (Crouch et al. 1998, 2000).

True-to-the-type clonal fidelity is one of the most important prerequisites in the micropropagation of any banana species. A major problem often encountered with *in vitro* cultures is the presence of somaclonal variation amongst subclones of one parental line. RAPD and inter-simple sequence repeat (ISSR) were adopted for evaluation of clonal fidelity in banana plantlets (Bennici et al. 2004; Carvalho et al. 2004; Martins et al. 2004; Ray et al. 2006). The somaclones confirmed through PCR-based tests were relatively stable without generally reverting to normal phenotypes (Ramage et al. 2004). A stringent RAPD technique called high annealing temperature (HAT)-RAPD was used with Thai banana cultivars (Sitthipron et al. 2005). Despite the criticism of the technique, RAPD is still being used in genomic analysis of banana as evidenced from recent publications (Das et al. 2009; Miri et al. 2009; Khatri et al. 2009).

4.4.2 Simple Sequence Repeats (SSR)

Limitations in RAPD markers led to the development and utilization of microsatellite primers for *Musa* (Jarret et al. 1994; Kaemmer et al. 1997;

Grapin et al. 1998; Creste et al. 2003a, b). Microsatellite or SSR loci are abundant, randomly dispersed, locus specific, codominant and multi-allelic markers. They are tandemly repeated DNA motifs (generally less than or equal to 4–6 bp) with an overall length in the order of tens of base pairs. Microsatellites or SSRs have been reported to be highly abundant and randomly dispersed throughout the genomes of many plant species (Crouch et al.1998). Variation in the motif is thought to arise through slippage errors during DNA replication. SSRs were shown to be reproducible markers in other systems due to their abundance, polymorphism and reliability (Campbell et al. 2003).

Microsatellites have been widely used to detect the genetic diversity of plant species. These markers have been widely used in plants for fingerprinting, mapping, and genetic analysis. Simple sequence repeat length polymorphism (SSRLP) analysis has been shown to detect a high level of polymorphism between individuals of *Musa* breeding populations (Crouch et al. 1998, 2000). However, isolation of microsatellites is time consuming and expensive. Nonetheless, with the availability of automated DNA sequencing facilities, improved techniques for the construction of genomic libraries enriched for SSR and improved techniques for the screening of appropriate clones, the isolation of SSR is becoming routine. This has recently allowed the rapid isolation of several hundred microsatellites from the *Musa* B genome (Buhariwalla et al. 2005). To date, several hundred SSR markers have been identified across *M. acuminata* and *M. balbisiana* material (Jarret et al. 1994; Kaemmer et al. 1997; Crouch et al. 1998; Creste et al. 2003a, b; Wang et al. 2009), with numbers likely to increase with advances in genomic technologies.

Numerous uses exist for such markers, although perhaps their most important applications reside in genetic map saturation and MAS, following co-localization with specific traits. For example, 352 SSR loci were recently discovered in *M. acuminata* Calcutta 4 bacterial artificial chromosome (BAC) end-sequences (Cheung and Town 2007). In comparison with other crop species, however, novel SSR loci available today for genetic analyses remain relatively limited in *Musa*, given that alleles can be absent or monomorphic when applied across cultivars. Over 40 microsatellite loci have recently been identified from five fully sequenced *M. acuminata* Calcutta 4 BAC consensi datasets (Genbank accession numbers AC186748, AC186749, AC186954, AC186747 and AC186750), with validation for polymorphism conducted on genotypes contrasting in host plant resistance to Sigatoka diseases (Miller et al. 2010).

4.4.3 Inter-Retrotransposon Amplified Polymorphism (IRAP)

IRAP is a complementary dominant marker system used to detect polymorphism in retro-transposon insertions. Retrotransposons are abundant and dispersed components of most plant genomes comprising over 50% of the nuclear DNA content in many species (Kumar 1999).

They act as mutagenic agents thereby providing putative sources of biodiversity (Heslop-Harrison 2000). The major classes of retroelements include long interspersed elements (LINES), short interspersed elements (SINES), copia and gypsy-like elements and retroviruses. Teo et al. (2005) used IRAP markers for identification and characterization of banana cultivars and classification of *Musa* genome constitutions. Ty1-copia-like retrotransposons have been extensively used as molecular marker in other plant species (Kalender et al. 1999; Pearce et al. 2000; Yu and Wise 2000). Balint-Kurti et al. (2000) suggested that Ty3-gypsy-like retrotransposons were introduced into *Musa* genus prior to the divergence of *Musa acuminata*, *M. balbisiana* and *M. vellutina* and they were able to distinguish the A and B genome using Ty3-gypsy-like retrotransposons. Aert et al. (2004) found highly homologous sequences to the "monkey" sequence from diploid *M. acuminata* Pisang Mas and *M. acuminata* Calcutta 4 and pointed out the relationship between A genomes in these three cultivars. Nair et al. (2005) used IRAP markers to identify the genome composition of bananas in India and identified a PCR-based molecular marker specific for the B genome. The B-specific band was absent in the AA and AAA cultivars (except in one AAA and AAB cultivar) but present in all other AB, AAB and ABB cultivars. Four cultivars with ABB genomes were identified as AAB, and one AAA cultivar showed a pattern similar to cultivars with the ABB genome. The authors suggested that the plants were misidentified.

4.4.4 Amplified Fragment Length Polymorphisms (AFLP)

AFLP is a DNA fingerprinting technique based on selective PCR amplification of DNA restriction fragments under stringent conditions (Vos et al. 1995). It can be used for DNA of any origin and complexity and is reported to be both reproducible and reliable (Vos et al. 1995). AFLP combines the reliability of RFLP with the power of PCR. AFLP is based on the selective amplification of restriction fragments obtained from the digestion of total genomic DNA. Given their dominant and biallelic nature, AFLP markers have been increasingly applied to various plants, mainly due to its capability of detecting a very high number of polymorphisms in a single assay, considerable repeatability and reasonable coverage of the

genome (Vos et al. 1995; Cervera et al. 1998; Vuylsteke et al. 1999; Shim and Jørgensen 2000). AFLP has been used to detect the genetic diversity of both cultivated accessions and wild progenitors of *Musa* (Wong et al. 2001; Ude et al. 2002a, b, 2003; Opara et al. 2010). Unfortunately, the information content of these banding patterns is restricted, as they must initially be treated as dominant markers. However, when AFLP analysis is applied to large populations, they can be detected as codominant markers in a segregating population (Masiga and Turner 2004). Software has been developed to distinguish the nature of individuals (homozygotes or heterozygotes) on the basis of band intensity. AFLP assays are also technically demanding and expensive in that they require a number of DNA manipulations and a complex visualization procedure. In addition, they require relatively large amounts of reasonably high quality DNA as the use of poor quality DNA may lead to incomplete digestion, which can result in spurious polymorphisms. Using AFLP markers, Lheureux et al. (2003) found that 10 markers were co-segregating with the presence or absence of banana streak badnavirus infection in *Musa* hybrids. AFLPs and SSRs are now being used to identify markers for fruit parthenocarpy, dwarfism and apical dominance in banana and plantain. Microsatellite markers and AFLP analysis appear to be the most appropriate technologies for marker-assisted breeding in *Musa* (Crouch et al. 1999; Hautea et al. 2004).

An AFLP-based technique for surveying cytosine methylation at CCGG sites has been developed (Reyna-Lopez et al. 1997; Fraga and Esteller 2002). DNA methylation plays an essential role in regulating plant development through its influence on gene transcription, and is involved in a number of specific biological processes such as gene silencing or mobile element control (Finnegan et al. 1996). These methylation-based molecular markers are different from others regarding their genetics, transmission of characters, and the evolution/mutation process (Xiong et al. 1999; Cervera et al. 2002). These markers can target different genomic regions and help in understanding epigenetic processes, which could be involved in phenotypic diversity of plantain landraces. However, as no strong correlation exists between major phenotypic and genotypic traits (Crouch et al. 2000) and methylation diversity in plantain, the question of predictability of characters through crosses involving plantains as parents is still unresolved. Even under the hypothesis of methylation–phenotype correlation, evolution, transmission and control process of methylation is unclear and different from random mutation process (Cervera et al. 2002) has to be revealed. AFLP bands can also be sequenced and converted to codominant sequence characterized amplified region (SCAR) markers for PCR analysis for use with agarose gel electrophoresis (Bradeen and Simon 1998; Xu et al. 2001). These markers can also be used to screen pooled BAC

libraries. AFLP has been shown to be a useful tool to generate linkage maps in *Musa* and has been used in the generation of the M53 (selfed) and the AFCAM (AFCAM refers to a F_2 population from a "Calcutta 4" x "Madang" cross) mapping populations.

4.4.5 Diversity Arrays Technology (DArT)

DArT is a DNA hybridization-based genotyping technology, which enables low-cost whole-genome profiling of crops without prior sequence information. DArT reduces the complexity of a representative sample (such as pooled DNA representing the diversity of *Musa*) using the principle that the genomic "representation" contains two types of fragments: constant fragments, found in any "representation" prepared from a DNA sample from an individual belonging to a given cultivar or species, and variable (polymorphic) fragments called molecular markers, only found in some but not all of the "representations" (www.diversityarray.com). DArT markers are biallelic and may be dominant (present or absent) or codominant (2 doses vs. 1 dose or absent). This approach enables quick profiling of segregating populations for quantitative trait loci (QTL) analysis with hundreds of polymorphic markers. Sequenced DArT markers can also be cheaply and rapidly anchored into BAC contigs, and therefore can facilitate the construction of high quality physical maps of the banana genome, which is a critical step in a sequencing project. Within the framework of the Generation Challenge Program, ca. 1,500 DArT markers have been developed using a wide array ("metagenome") of *Musa* accessions (Kilian 2007) and are now being used for the *Musa* framework map being developed at CIRAD additionally, 380 of these markers have recently been used in the construction of the BORLI map at CIRAD (Hippolyte et al. 2008, 2010). The application of DArT for bulked segregant analysis has been demonstrated in a *Hordeum* array (Wenzl et al. 2007). The disadvantage of this technology is that it depends on the availability of the array, a microarray printer and scanner, and computer infrastructure to analyze, store and manage the data produced. However, the markers are sequence ready and therefore if sequenced, can be developed for a PCR analysis using standard electrophoresis equipment.

4.4.6 Single Nucleotide Polymorphisms (SNPs)

On average, SNPs will occur in an organism's DNA more than 1% of the time (www.biotech.iastate.edu). Most SNPs are found outside the regions of genes of interest because about 3–5% of an organism's DNA codes for proteins (Sherry et al. 2001). SNPs found in a gene of interest are of particular interest to researchers because they are directly associated with

a desired trait. Because of the recent advances in technology, SNPs are playing a greater role in selection and analysis of genetic traits. SNPs have now been added to the repertoire of useful markers, especially when they appear in coding regions and hence in the messages (so called expressed SNPs or eSNPs, or cSNPs for SNPs as part of a cDNA), since only then can they be expected to have an impact on protein function, and consequently on the phenotype of the organism (Brookes 1999). To date, not one report has appeared on detection of SNPs in the banana genome.

4.4.7 Ecotilling

Till et al. (2010) has applied ecotilling for discovering polymorphisms in the *Musa* genome. They provide the following account of ecotilling, which is a high-throughput method for the discovery and characterization of SNPs and small insertions/deletions (indels) (Till et al. 2010). It is an adaptation of the enzymatic mismatch cleavage and fluorescence detection methods originally developed for the targeting induced local lesions in genomes (TILLING) reverse-genetic strategy (Colbert et al. 2001; Comai et al. 2004). The technique was first described for *Arabidopsis* ecotypes (it was therefore named ecotilling). It is an accurate, low-cost and high-throughput method for the discovery and evaluation of nucleotide diversity and has been used in many organisms. About 700–1,600 bp gene target regions are amplified using gene-specific primers that are fluorescently labeled for ecotilling using enzymatic mismatch cleavage. After PCR, samples are denatured and annealed, and heteroduplexed molecules are created through the hybridization of polymorphic amplicons. Mismatched regions in otherwise double-stranded duplex are then cleaved using a crude extract of celery juice containing the single-strand specific nuclease CEL I. Cleaved products are resolved by denaturing polyacrylamide gel electrophoresis (PAGE) and observed by fluorescence detection (Till et al. 2006).

Denaturing PAGE provides base pair resolution allowing grouping of accessions based on shared banding patterns indicative of haplotype grouping (Comai et al. 2004). Sequence validation can be performed on only one or a small number of samples to provide base polymorphism data for the whole group, providing a savings in cost and informatics load over sequencing approaches. Alternatively, banding patterns alone can be used to evaluate genetic diversity and similarity between accessions on a gene-specific scale. When samples are screened alone, ecotilling provides a catalog of heterozygous nucleotide diversity between samples. Reference DNA can be added to each sample prior to screening to uncover homozygous polymorphisms. Additionally, the high sensitivity of the assay allows for pooling of multiple samples for the specific discovery of rare polymorphisms.

4.5 Efficiency of Molecular Marker Systems

In a molecular breeding study on *Musa*, Crouch et al. (1999) reported poor correlation between estimates of genetic similarities derived from different types of markers. The main reason is that data inconsistency stemmed from differences among molecular techniques that selectively screened complementary, but not overlapping, regions of the genome. Therefore, integration of genetic estimates from different molecular techniques was proposed to provide a clearer picture of *Musa* genetic relationship and generate highly accurate estimates of genetic similarity in germplasm analysis (Crouch et al. 1999; Wong et al. 2001). A better understanding of the population structure in *Musa* can, therefore, be obtained with simultaneous use of a different marker system, which will be very informative. Thus, it is necessary to utilize a range of marker systems in order to generate highly accurate estimates of genetic similarity in genetic diversity studies.

The relative advantages and disadvantages of the various molecular markers are summarized in Table 4-1.

4.6 Conclusion

New DNA technologies are constantly being developed especially for human genomic research and some of them are being used in plant research (Pillay et al. 2011). High-throughput technologies based on SNPs or small scale indel are efficient alternatives for traditional markers (RFLP, RAPD or AFLP) because of their greater abundance, high polymorphism, ease of measurement and ability to reveal hidden polymorphisms where other methods fail (Dillon et al. 2007). SNPs also allow easy and unambiguous identification of alleles or haplotypes.

A good marker system for polyploid crops should be dosage sensitive and have the ability to distinguish heterozygous genotypes with multiple haplotypes (de Koeyer et al. 2010). High resolution DNA melting (HRM) analysis has been shown to have several advantages over other genotyping methods (Montgomery et al. 2007; Reed et al. 2007; Erali et al. 2008). The advantages include a short analysis time and the absence of post-PCR sample processing or separation (de Koeyer et al. 2010). The three ways in which HRM can be used for genotyping and or variant screening are discussed in de Koeyer et al. (2010). HRM has been used in many crops and holds great potential for cultivar identification especially in polyploids, mapping, polymorphism discovery, mapping candidate genes, and in combination with QTL or association studies for identifying genomic regions involved in important traits. Such techniques have not been utilized in *Musa* as yet and eagerly awaited by scientists.

Table 4-1 Molecular markers, level of polymorphism and factors influencing their use (from Pillay and Tripathi 2007).

Markers	Quantity of DNA required	Level of polymorphism	Locus specificity	Reproducibility	Labor intensity	Operational costs	Development costs	Amenability to automation
Isozymes	-	Low	Yes	High	Low	Low	Low	No
RFLP	High	Medium	Yes	High	High	High	Medium-high	No
Minisatellite	High	High	No/yes	High	High	High	Medium-high	No
RAPD	Low	Medium	No	Low	Low	Low	Low	Yes
SSR	Low	High	Yes	High	Low	Low-medium	High	Yes
SNPs	Low	Low	Yes	Medium	Low-medium	Low-medium	Low	No
AFLP	Medium	Medium	No	High	Medium	Medium	Medium	Yes

Source: Pillay and Tripathi (2007)

Abbreviations

6-PGDH	:	6-phosphogluconate dehydrogenase
ADH	:	alcohol dehydrogenase
AFLP	:	amplified fragment length polymorphisms
BAC	:	bacterial artificial chromosome
BBrMV	:	Banana Bract Mosiac Virus
CIRAD	:	Centre de Coopération Internationale en Recherche Agronomique pour le Dévelopement
EST	:	esterase
GDH	:	galactose dehydrogenase
GOT	:	glutamate oxaloacetate transminase
GUDH	:	glutamate dehydrogenase
LINES	:	long interspersed elements
MAS	:	marker-assisted selection
MDH	:	malate dehydrogenase
ME	:	malic enzyme
PAGE	:	polyacrylamide gel electrophoresis
PCR	:	polymerase chain reaction
PGI	:	phosphoglucose isomerase
PGM	:	phosphoglucomutase
PRX	:	peroxidase
QTL	:	quantitative trait loci
RAPD	:	random amplified polymorphic DNA
RFLP	:	restriction fragment length polymorphisms
SCAR	:	sequence characterized amplified repeat
SDH	:	sorbitol dehydrogenase
SINES	:	short interspersed elements
SKDH	:	shikimate dehydrogenase
SSR	:	simple sequence repeats
SUDH	:	succinate dehydrogenase
TILLING	:	Targeting Induced Local Lesions IN Genomes
TO	:	tetrazolium oxidase
TPI	:	triosephosphate isomerase
VNTR	:	variable number tandem repeats

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