I.2 Genotyping Tools in Plant Breeding: From Restriction Fragment Length Polymorphisms to Single Nucleotide Polymorphisms

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1 Introduction

A series of molecular marker types are available for plant genotyping, but no single technique is generally applicable to the wide range of questions in plant genome analysis; each available technique exhibits both assets and drawbacks, thus, a decision is needed which marker system should be used for which research aim. The marker types differ in information content, number of scorable polymorphism per reaction, and degree of automation. In addition, the choice of method often depends on the genetic resolution needed as well as on technological and financial constraints. Here, we review the basic principles of the most important techniques and their suitability for molecular plant breeding.

2 Restriction Fragment Length Polymorphisms

Both the basis and techniques for restriction fragment length polymorphisms (RFLPs; Botstein et al. 1980) in plant genome mapping have been extensively reviewed (Tanksley et al. 1988) and as such will only be briefly outlined here. In complex plant genomes, RFLPs are detected upon hybridization of (mainly) single-copy DNA probes to restriction enzyme-hydrolyzed, agarose gel electrophoresis-separated and nylon membrane-bound genomic DNA. For the detection of polymorphisms, the DNAs of the genotypes to be surveyed are digested, usually with various restriction enzymes, and marker alleles are identified by size differences of the restriction fragments to which the probes hybridize. Thus, RFLP markers are specified by a clone/restriction enzyme combination. Major sources of RFLP probes are species-specific genomic DNA and cDNA sequences. The use of heterologous probes for cross-genome RFLP analysis will be discussed later in this section.

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RFLPs allowed the construction of whole-genome linkage maps in plants for the first time (Bernatzky and Tanksley 1986; Helentjaris et al. 1986) and, thus, the localization of any genetically inherited trait. Despite being 'old-fashioned' and the most time-consuming molecular marker technique, RFLP analysis still displays high efficiency for the generation of high-density transcript genetic maps. The major reason is the availability of diverse, marker-saturated mapping populations for most crop species allowing simultaneous parental survey and, thus, the mapping of as many EST (expressed sequence tag) polymorphisms as possible. In the German GABI genome project, three barley doubled haploid populations (involving populations Igri × Franka with 71 lines, Steptoe × Morex with 150 and Oregon Wolfe Barley Dom × Oregon Wolfe Barley Rec with 94) are being exploited to generate a transcript map comprising 1200 ESTs (http://pgrc.ipk-gatersleben.de/barley–proj –2001.pdf). In addition to denaturing high-performance liquid chromatography mapping of single nucleotide polymorphisms (SNPs) between EST alleles (Kota et al. 2001), the major part of EST mapping is managed through DNA gel blot hybridizations.

RFLP markers initiated a rapid evolution in the field of comparative genomics by linking plant genomes through comparative genetic maps. Especially for species from the same plant family, the use of a common set of heterologous, single-copy probes in DNA gel blot experiments allowed the detection of orthologous marker loci and, thus, the prediction of genomic localization of both qualitative and quantitative traits across related species (Gale and Devos 1998; Doganlar et al. 2002). However, for plant species belonging to different families, comparative mapping via common probes is difficult because gene similarities are reduced due to a greater evolutionary divergence time, for which reason gene probes that have remained relatively stable in both sequence and copy number will be required. Fulton et al. (2002) identified more than 1000 conserved genes which were referred to as conserved ortholog set (COS) markers by computationally comparing more than 20,000 tomato ESTs with the *Arabidopsis thaliana* genome sequence. ESTs having a single match in the *Arabidopsis* genome (criterion to avoid problems with multigene families for which orthology and paralogy cannot be differentiated) were probed against tomato DNA to ensure that these COS markers were truly single- or low-copy. The evolutionary stability of some of the COS markers was shown by the consecutive hybridization of 'garden blots' (which were composed of DNA from a wide range of plant species) with tomato ESTs and their counterpart *Arabidopsis* probes with both sets detecting (nearly) identical restriction fragments.

Due to the time-consuming multi-step protocol and the requirement of radioactivity for satisfying fragment detection, RFLPs lost their importance in marker-assisted breeding. However, the development of simple PCR markers from sequenced RFLP probes provides an opportunity to maintain useful polymorphism found in previous gene mapping studies.

3 Microsatellites

Microsatellites also termed simple sequence repeats (SSRs), short tandem repeats (STRs) or sequence-tagged microsatellite sites (STMS) are tandem repeats of short nucleotide sequence motifs (mono-, di-, tri-, tetra- or pentanucleotide units). Microsatellites are abundant and are relatively evenly spaced throughout eukaryotic genomes (Tautz and Renz 1984). SSR loci are extremely variable in the number of repeat units among individuals of a given species (mutation rates for di- and tetranucleotide repeats: 0.001 mutations per generation, Goldstein and Pollock 1997) and can be easily typed via STS (sequence-tagged site)-PCR due to unique sequences bracketing individual microsatellites (Weber and May 1989). The opportunity to tag a fast mutating – repetitive – sequence motif as a simple PCR marker represents the ideal resource for the development of a valuable, if not the most important, marker class to be applied in practical plant breeding, particularly in species otherwise characterized by low levels of genetic diversity. As a consequence, for the most agriculturally important crops, a huge number of SSR markers are already publicly available for research (Milbourne et al. 1998; Röder et al. 1998; Cregan et al. 1999; Ramsay et al. 2000; Temnykh et al. 2000; Sharopova et al. 2002; Song et al. 2002). Furthermore, this type of repetitive sequence is also occurring within genes as has been demonstrated by searching EST databases for the presence of microsatellites (Eujayl et al. 2002; Hackauf and Wehling 2002; Thiel et al. 2003). EST-derived SSRs are expected to display slightly less polymorphisms than genomic library-derived SSRs, as there is pressure for sequence conservation in coding regions (Scott 2001). However, the availability of SSR markers from the expressed portion of the genome might facilitate their transferability across genera, compared to the low efficiency of SSR markers that have been retrieved from gene-poor areas (Peakall et al. 1998). This approach would benefit plant species with minimal resource and research expenditure.

For an efficient exploitation of SSR polymorphisms, particularly for dinucleotide repeats, in which alleles may differ by only two base pairs, analysis on a DNA sequencing instrument is recommended. This further simplifies scoring of SSR loci showing characteristic sub-banding, the so-called stutter bands.

4 Random Amplified Polymorphic DNAs

In 1990, Williams et al. and Welsh and McClelland independently described the utilization of a single, random-sequence oligonucleotide primer in a low-stringency PCR (35–45 °C) for the simultaneous amplification of several discrete DNA fragments referred to as random amplified polymorphic DNA

(RAPD) and arbitrarily primed PCR (AP-PCR), respectively. Usually, ten-base oligomers of varying GC content (ranging from 40 to 100 %) are applied. RAPDs are qualified as genetic markers, since short and inverted repeats on complementary strands to which the primer hybridize, are more or less regularly spaced throughout the genome. Polymorphism between individuals results primarily from sequence differences in one or both primer sites or from indels (insertions-deletions) exceeding the PCR-amplifiable distance, and are visible in conventional agarose gel electrophoresis as presence or absence of a particular RAPD band. RAPDs predominantly provide dominant markers, but homologous alleles can sometimes be identified with the help of detailed pedigree information.

Most advantageous for this molecular marker technique is the use of universal primers enabling the cost-effective accomplishment of various genetic analyses in a short period of time (for review: Tingey and del Tufo 1993). Nevertheless, due to frequently observed problems with reproducibility of overall RAPD profiles and specific bands, this marker class is often treated with reserve (Ellsworth et al. 1993; Muralidharan and Wakeland 1993; Schierwater and Ender 1993). In replication studies by Pérez et al. (1998), mispriming error amounted up to 60 %. To overcome this problem, Paran and Michelmore (1993) converted RAPD fragments to simple and robust PCR markers termed sequence characterized amplified regions (SCARs). Primer pairs are deduced from cloned RAPD fragments by usually extending the original decamer primer sequence with 10–15 bases.

A modified approach of the RAPD technique, DNA amplification fingerprinting (DAF; Caetano-Anollés et al. 1991) employs one or more primers as short as five nucleotides in length to produce complex banding patterns that are resolved by polyacrylamide gel electrophoresis. A further enhancement in detection of polymorphic DNA was achieved by profiling endonuclease-digested DNA (Caetano-Anollés et al. 1993). Advances in the fingerprinting of small templates up to 250 kb (e.g., BACs) were achieved with primers harboring an arbitrary sequence of only three nucleotides if a stable mini-hairpin was attached to their 5'-ends (Caetano-Anollés and Gresshoff 1994).

5 Amplified Fragment Length Polymorphisms

Like RAPD, amplified fragment length polymorphism (AFLP; Vos et al. 1995) is a universal, multi-locus marker technique that can be applied to genomes of any source or complexity. The method is based on the selective PCR amplification of restriction fragments from a total double-digest of genomic DNA under high stringency conditions. Oligonucleotide adaptors are ligated to the restricted DNA, which serve with the restriction site sequences as target sites for primer annealing. By using primers having extensions into the restriction fragments, the specific amplification of only those fragments matching the

primers is achieved. The option to permute the order of the selective bases and to recombine the primers with each other theoretically discloses the chance of the gradual collection of all restriction fragments from a particular enzyme combination showing a suitable size for DNA fragment analysis from a genotype. AFLP products need to be separated in high-resolution electrophoresis systems. Thus, a well-balanced number of amplified restriction fragments ranges from 50–150. A major improvement has been made by switching from radioactive to fluorescent dye-labeled primers for the detection of fragments in gel-based or capillary DNA sequencers in which fluorescently labeled fragments pass the detector near the bottom of the gel/the end of the capillary, resulting in a linear spacing of DNA fragments and, therefore, increasing the resolution over the whole size range (Schwarz et al. 2000).

A two-step amplification strategy was developed for complex genomes (10^9–10^{10} bp) using six- (*Eco*RI, *Pst*I, *Hin*dIII) and four-base (*Mse*I, *Taq*I) cutters for AFLP template preparation and three selective nucleotides on both primers. The first PCR amplification step utilizes primers each having a single selective nucleotide and reduces the overall complexity of the mixture up to 16-fold, allowing the target sequence to become the predominant species. PCR products from preamplification are diluted and used as templates for a second amplification round using primers with full base extensions. Although the complexity is reduced with each additional selective nucleotide, the selectivity is maintained with nucleotide extensions to a maximum of three selective bases. For small plant genomes (10^7–10^8 bp), two to three selective nucleotides on the 3'-end of each primer are sufficient to reveal polymorphism. Another widespread enzyme system substitutes six-base for eight-base cutting enzymes, such as *Sse*8387I or its isoschizomer *Sda*I, and usually employs two to three selective bases on both primers (Law et al. 1998; Hartl et al. 1999).

Due to its capacity to reveal many polymorphic bands in a single reaction, the AFLP technique has been extensively used with plant DNA for the construction of both whole-genome (Becker et al. 1995; Keim et al. 1997; Haanstra et al. 1999; Vuylsteke et al. 1999; Chalmers et al. 2001) and high-resolution maps around loci that control agronomically important traits (Thomas et al. 1995; Büschges et al. 1997; Simons et al. 1998; Schwarz et al. 1999; Ballvora et al. 2001). Furthermore, AFLP markers have been proven to be reliable and reproducible not only within, but also among mapping populations, for which reason allele-specificity of comigrating AFLP markers has been used for aligning genetic maps from different genotypes (Rouppe van der Voort et al. 1997; Waugh et al. 1997a; Groh et al. 2001; Singrün et al. 2003). Consequently, AFLP provided an ideal tool for estimating genetic variation of both cultivated and natural populations (Travis et al. 1996; Barrett and Kidwell 1998).

The AFLP technique can be modified so that one primer is obtained from a known multi-copy sequence to detect sequence-specific amplification polymorphisms (S-SAP). This approach has been used successfully to generate

genome-wide *Bare-1* retrotransposon-like markers in barley (Waugh et al. 1997b) and diploid *Avena* (Yu and Wise 2000) as well as in alfalfa by making use of consensus sequences from long terminal repeats (LTRs) of *Tms1* retrotransposon (Porceddu et al. 2002). Based on the LTR regions of retrotransposable elements *Tps12* and *Tps19* of pea (Pearce et al. 2000) and *Ty1-copia* of sweetpotato (Berenyi et al. 2002) S-SAP systems were established for their use in genetic diversity studies.

The cDNA-AFLP technique (Bachem et al. 1996), which applies the standard AFLP protocol on a cDNA template, was used to display transcripts whose expression was rapidly altered during race-specific resistance reaction (Durrant et al. 2000), for the isolation of differentially expressed genes from a specific chromosome region using aneuploids (Money et al. 1996; Kojima et al. 2000) and for the construction of genome-wide transcriptome maps (Brugmans et al. 2002).

6 Single Nucleotide Polymorphisms

The next generation of genetic markers is based on SNPs, which are defined as single nucleotide positions in a given DNA stretch at which there are variations between different individuals within a species. Thus, single base insertion/deletion (indels) variants would not formally be considered to be SNPs (Brookes 1999). In general, SNPs are the most common form of DNA sequence polymorphisms and, therefore, sufficiently abundant for comprehensive haplotype analyses. In the human genome SNPs occur once every 100–300 bases, but up to now there is not much information about the frequency of SNPs in plant genomes. Only for *Arabidopsis thaliana*, a collection of 37,344 predicted SNPs between the whole genome sequences of two ecotypes is documented, and estimated to occur at one out of every 3.3 kb (http://www.arabidopsis.org/cereon/). In addition, the binary (di-allelic) character and stability from generation to generation make SNPs amenable to automated, high-throughput genotyping and, therefore, an attractive tool for quantitative trait loci (QTL) mapping studies and marker-assisted selection in plant breeding programs.

The basic method for determination of SNP genotypes has been Sanger dideoxysequencing. Since sequencing generates more information than necessary, misses SNPs when the DNA template is heterozygous and, thus, is time-consuming and very expensive, other gel-based assays were applied for SNP genotype detection. A widely used agarose gel-based SNP detection technique is PCR-RFLP (Fig. 1A), also referred to as cleaved amplified polymorphic sequence (CAPS; Konieczny and Ausubel 1993). A target DNA segment containing a SNP is amplified by PCR and the product is incubated with an appropriate restriction enzyme, which cuts the molecule if it contains the SNP variant creating the recognition site, but not if it contains the other.

Though CAPS analysis is performed routinely in many laboratories, the time-consuming post-PCR digestion step limits its application for small-scale experiments. Allele-specific PCR (AS-PCR, Fig. 1B) is a further agarose gel-based method to assay SNPs by using oligonucleotide primers with 3'-nucleotides complementary to the SNP site (Ugozzoli and Wallace 1991). Allele-specific primers perfectly match only with the corresponding alleles, whereas they have 3'-mismatches with the alternative alleles. Since in many cases a single base pair change at the 3'-end of alleles is not sufficient for robust allele discrimination, AS-PCR procedure was improved by utilizing competing polymorphism-detecting primers (Mohler and Jahoor 1996; See et al. 2000; Ellis et al. 2002) or by introducing artificial mismatches at 3'-subterminal positions of the allele-specific primers (Drenkard et al. 2000). Another important technology for SNP scoring is single-stranded conformation polymorphism analysis (SSCP; Orita et al. 1989; Fig. 1C). Allele discrimination relies on the secondary structure being different for single-stranded DNA derived from PCR products that differ by one or more internal nucleotides. PCR products are denatured and electrophoretically separated in native polyacrylamide gels. Differences in electrophoretic mobility between amplicons from the wild type and mutant genotype, respectively, will suggest the presence of SNP(s). Even though SSCP analysis is simple, accurate and relatively inexpensive, the polyacrylamide gel-based assay format prevents its use for high-throughput allele typing.

The limitations of the above-mentioned techniques for high-throughput SNP genotyping forced the development of new technology platforms utilizing the reaction principles of minisequencing, heteroduplex analysis and allele-specific hybridization.

The robust detection of known mutations using minisequencing (Fig. 1D) bases on oligonucleotides which anneal immediately upstream of the query SNP to be extended by single dideoxynucleotides (ddNTPs) in cycle sequencing reactions. The fidelity of thermostable proof-reading DNA polymerases guarantees that only the complementary ddNTP is incorporated. Several detection methods have been described for the discrimination of primer extension (PEX) products. Most popular is the use of dideoxynucleotide terminators which are labeled with different fluorescent dyes. The differentially dye-labeled PEX products can readily be detected on CCD camera-based DNA sequencing instruments. For minisequencing in parallel, solid-phase assays with detection by fluorescence scanning, also denoted arrayed primer extension (APEX) were developed, in which one detection primer per SNP is covalently immobilized on glass slides. Prior to analysis, single-stranded templates are prepared through degrading one strand of PCR products by digestion with an exonuclease enzyme (Shumaker et al. 1996). Both gel- and array-based PEX approaches were applied for SNP analysis in barley (Kanazin et al. 2002). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is another well-suited method for detection and discrimination of small DNA molecules. Due to the inherent molec-

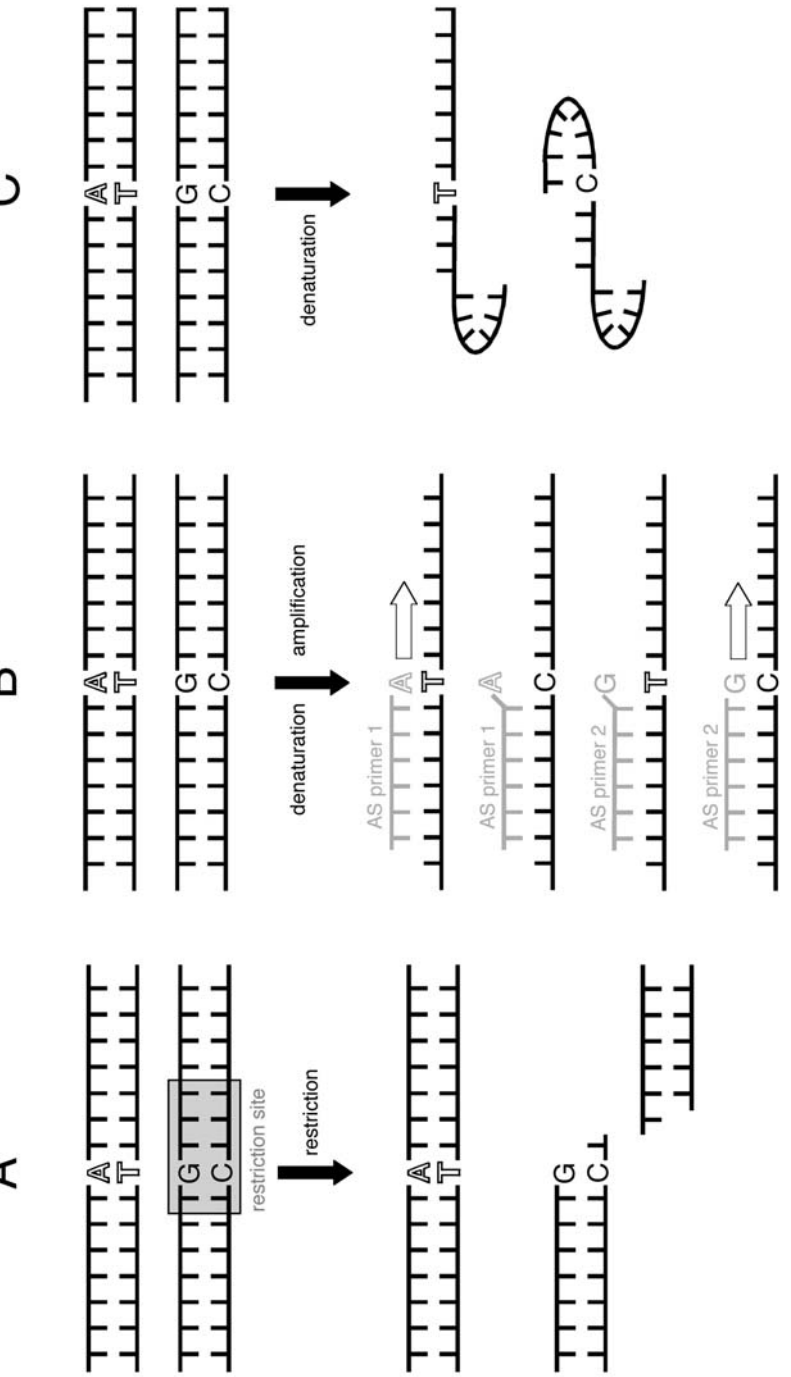
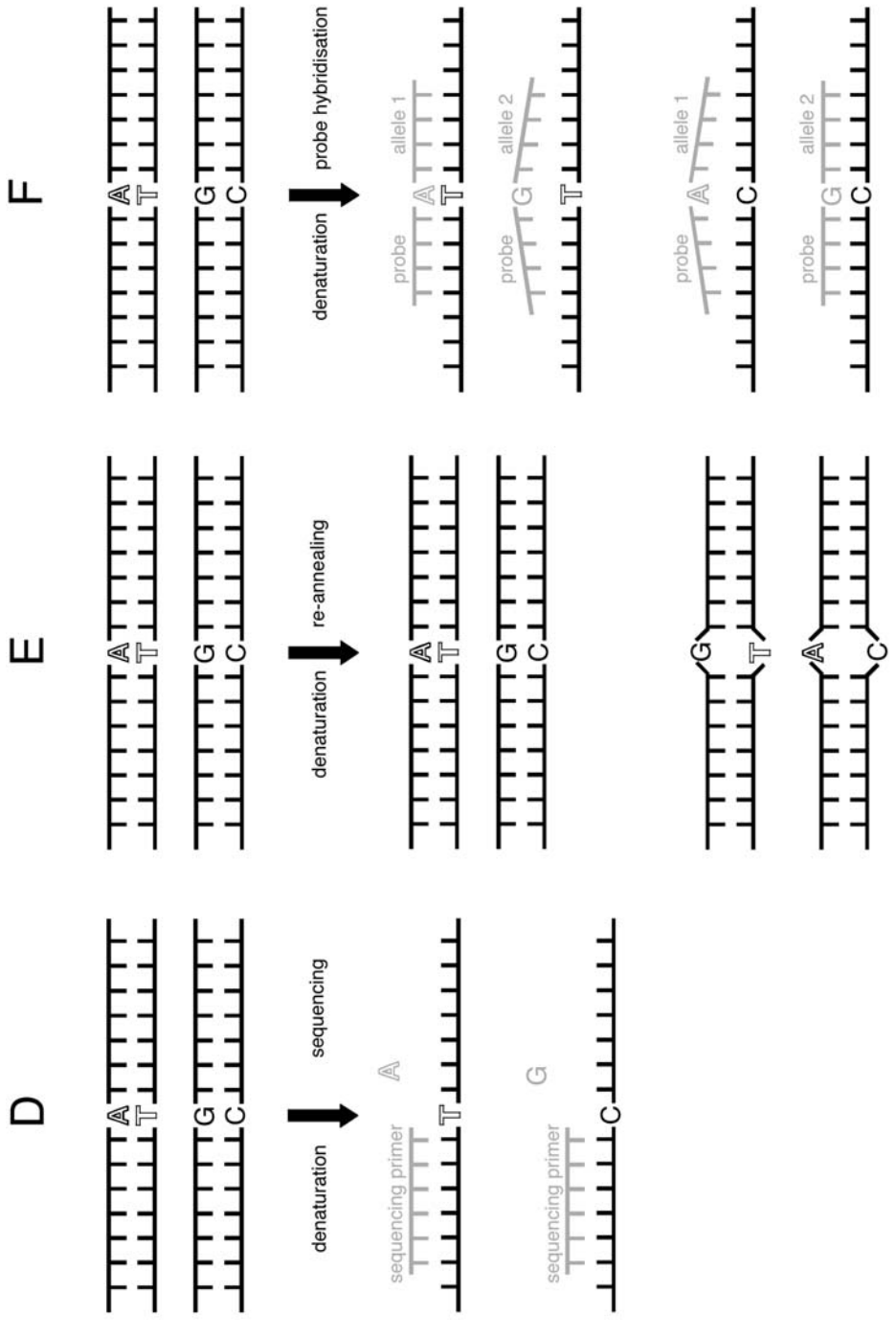


Fig. 1. Biochemical reaction principles for SNP genotype determination: **A** restriction endonuclease digestion, **B** allele-specific primer PCR, **C** single-stranded conformation polymorphism, **D** minisequencing, **E** heteroduplex analysis, **F** allele-specific oligonucleotide probe hybridization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ular weight difference of DNA bases, incorporated nucleotides are identified by the increase in mass of extended primers. The MALDI-TOF method is particularly advantageous for detection of PEX products in multiplex (Ross et al. 1998; Paris et al. 2002). In the pyrosequencing format, real-time monitoring of PEX relies on the bioluminometric detection of inorganic pyrophosphate released upon incorporation of deoxynucleotide triphosphate (Ahmadian et al. 2000). The genotype of a SNP is determined by the sequential addition (and degradation) of nucleotides. The yielded light is proportional to the amount of incorporated nucleotides, for which reason pyrosequencing is qualified for the quantitative estimation of allele frequencies in pooled DNA samples. Furthermore, pyrosequencing proved to be an appropriate method for genotyping SNPs in polyploid plant genomes, such as potato, because all possible allelic states of binary SNPs could be accurately distinguished (Rickert et al. 2002).

Heteroduplex analysis (Fig. 1E) uses the different electrophoretic mobility behaviors of homoduplex and heteroduplex DNA molecules. The most straightforward method is denaturing high-performance liquid chromatography (DHPLC), an ion-pair reversed-phase chromatography method, which allows the detection of single mismatches in PCR products by injecting them into an adequately preheated mobile phase that results in partial denaturation of the DNA molecules (Oefner and Underhill 1998). Owing to reduced melting temperatures, heteroduplexes will be eluted earlier from the column than the homoduplexes. Besides scoring of known SNPs, DHPLC can be applied for the rapid and cost-effective scanning of unknown mutations in DNA amplicons (Cho et al. 1999). DHPLC was reported to have greater than 99 % sensitivity in detecting unknown SNPs and mutations between two *Arabidopsis thaliana* ecotypes (Spiegelman et al. 2000).

Oligonucleotide hybridization probes (Fig. 1F) are also an attractive tool for SNP genotyping. Under optimized assay conditions, the SNP discrimination is solely based on differences in melting temperature (T_m) of the two probe–template hybrid classes. For high reliability of SNP genotype calling, T_m differences have to be maximized by using probes as short as possible. Originally, allele-specific hybridization (ASH) used the dot blot format where probes hybridized to membrane-bound genomic DNA or PCR fragments. Meanwhile the advanced, PCR-based dynamic allele-specific hybridization (DASH) method in a microtiter plate format is available (Howell et al. 1999). Since one of the PCR primers is biotinylated at the 5'-end, the PCR products can be bound to streptavidin-coated wells and denatured under alkaline conditions. An oligonucleotide probe, complementary to one allele, is added to the single-stranded target DNA molecules. The differences in melting curves are measured by slowly heating and observing the change in fluorescence of a double-strand-specific, intercalating dye. Large-scale scanning of SNPs in a huge number of loci by allele-specific hybridization can be performed on high-density oligonucleotide chips (Wang et al. 1998). Another interesting option for SNP scoring by hybridization is provided by the use of fluoresc-

ently labeled allele-specific oligonucleotides such as Taqman (Livak et al. 1995) and Molecular Beacon (Tyagi and Kramer 1996) DNA probes. Both systems use two fluorogenic probes for allele discrimination, one for each allele. Typically, the probes are labeled with different reporter dyes at the 5'- and a quencher dye at the 3'-end. In the intact Taqman probe, fluorescence of the reporter dye is quenched by the close proximity of the 3'-dye label due to fluorescence resonance energy transfer (FRET). After hybridization of the probes to the target alleles, degradation of probes by the 5'3' exonuclease activity of the processing *Taq* DNA polymerase during PCR extension steps interrupts the interaction of the dye molecules resulting in a fluorescent signal. Contrary to linear Taqman probes, Molecular Beacons form a hairpin structure due to complementary 5'- and 3'-ends which brings the reporter and quencher into immediate vicinity of one another. Hybridization of the probe to the target sequence during PCR annealing steps results in linearization and subsequent emission of fluorescence signals.

Besides Taqman and Molecular Beacon probes, other allele-specific hybridization assays were developed for SNP genotype determination: Scorpion assays combine forward primer and probe in a single molecule (Thelwell et al. 2000), Padlock assays use oligonucleotide probes to be ligated into circles upon target recognition and isothermal rolling circle amplification (Nilsson et al. 1997) and invasive cleavage assays involve a FLAP 5'-endonuclease that is specific for a three-dimensional structure formed by two (the Invader and the primary SNP detection probe) overlapping oligonucleotides (Lyamichev et al. 1999).

7 Conclusions

A wide range of marker techniques is now available for genotyping plant genomes. Meanwhile, markers are employed not only in plant breeding research, but also in practical plant breeding. In order to exploit the full power of each marker class, one has to make its special character consistent with the desired application. Unfortunately, highly informative marker types like SSRs and SNPs have been elaborated for only a few well-studied crop plants. Due to the lack of sequencing and mapping data, genotyping in 'undiscovered' plant genomes still has to be performed using universal marker techniques like RAPD and AFLP. However, the strong synteny between closely related species will allow, to a certain extent, the transfer of marker information thereby increasing the molecular marker pool in genomes of plant families. Finally, reducing genotyping costs for high-throughput techniques, e.g., microarrays, is a major challenge for the comprehensive integration of markers into plant breeding programs.

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