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Molecular markers: their use in tree improvement

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ABSTRACT: Earlier breeders used phenotypic selection based on morphological characteristics to improve tree varieties. These selections often take many cycles of breeding and backcrossing in order to place desired characteristics. But today the knowledge has paved the way for a much deeper understanding of the mechanics of cell biology and the hereditary process itself. Breeders are presented with numerous possibilities of altering the behaviour of existing varieties. Linkage between molecular markers can be translated to genetic linkage maps, which have become an important tool in plant genetics. They may choose to use marker-assisted approaches in order to facilitate the selection of favourable combinations of genes that occur naturally within a tree species.

Keywords: molecular markers; marker-assisted selection; genetic variation; QTLs

Classical plant breeding which was practiced for the past three thousand years with the advent of agriculture and domestication surpassed the methods used by these ancient agriculturists, and classical plant breeders came into an era where selection is to be based on genetic characteristics involving DNA markers. Earlier plant breeders used phenotypic selection based on morphological characteristics which often take many cycles of breeding and backcrossing in order to place a desired characteristic from one parent into a genotype with suitable agronomic and quality characteristics.

Today geneticists are often trained to extrapolate information about what individual genes do in a model species when observed in a controlled environment and how homologous genes are likely to behave in complex genomes in a related field environment. While this approach may hold promise for simply inherited traits, the ability to understand the genetic basis of quantitatively inherited traits relies on the use of populations evaluated directly in relevant field environments.

Plant breeders currently use marker-assisted approaches in order to facilitate the selection of favourable combinations of genes that occur naturally within a tree species (Wu et al. 2000; Li et al. 2011). They also have opportunities to adjust particular traits or phenotypes via the introduction of alien genes using transgenics (Sedjo 2006; Doty et al. 2007). In either case, it is information about genes and their functions that drives biotechnological applications in plant improvement. The introduction of molecular markers has revolutionized genetics. The use of molecular markers in the breeding programmes started in the 1980's. The range of polymorphisms (Cullis 2002) that are available is increasing and the advent of large-scale cDNA and genomic sequencing is a source of an ever-increasing set of available markers. These are gained by comparing specific regions of DNA sequence, finding differences between homologous chromosomes and then devising a high throughput test to distinguish the chromosomes based on the difference in DNA sequence. Such differences are mapped relative to other markers and traits either by reference to the complete chromosome sequence where it is known, by genetic linkage using segregating populations or by association mapping if a large number of isolates is available.

The ease with which any particular marker type can be applied to an experimental system depends, to some extent, on the amount of genomic information available for that system. However, comparative genomics is enabling a wider range of marker technology to be applied to relatively information-poor systems. The types of markers that are available in-

clude restriction fragment length polymorphisms, amplified fragment length polymorphisms, random amplified polymorphic DNA's, simple sequence repeats, single nucleotide polymorphisms and small insertions/deletions (Paterson 1996). The use of DNA polymorphisms as molecular markers has revolutionized the whole process of generating genetic maps and opened the door to genetic characterization and improvement in many species that were previously intractable. However, the presence of repetitive DNA and the size of some of the complex genomes, as well as the polyploid nature of many plant genomes can make this a difficult task. The range of methods to identify and utilize molecular markers is continuously increasing, and PCR methodology has facilitated the development of marker based gene tags, map-based cloning of agronomically important genes, synteny mapping, marker-assisted selection and quantitative trait analysis.

The ultimate utility of DNA markers is based on two characteristics of such markers. One is their insensitivity to environment and the other is the practically unlimited number that can be followed in any given population. Many of the marker systems are codominant, reducing the population sizes necessary to draw statistically significant conclusions.

MARKER SYSTEMS

RFLPs

A Restriction Fragment Length Polymorphism is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA sample into pieces with restriction enzymes and then analyzing the size of the restricted fragments by gel electrophoresis. It is the sequence that makes DNA from different sources different, and RFLP analysis is a technique that can identify some differences in sequence (when they occur in a restriction site). RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. RFLP markers that are used for high density genomic mapping (Botstein et al. 1980) provided a new technique which overcame some of the problems associated with isozymes and proteins. RFLPs, being codominant markers, can detect the coupling phase of DNA molecules, as DNA fragments from all homologous chromosomes are detected. They are very reliable markers in linkage analysis and breeding and can easily determine if a linked trait is present in a homozygous or heterozygous state in an individual, which is information highly desirable for recessive traits. However, their utility has been hampered due to the large amount of DNA required for restriction digestion and Southern blotting. The requirement for radioactive isotope makes the analysis relatively expensive and hazardous. The assay is time-consuming and labour-intensive and only one out of several markers may be polymorphic, which is highly inconvenient especially for crosses between closely-related species. Their inability to detect single base changes restricts their use in detecting point mutations occurring within the regions at which they are detecting polymorphism.

AFLPs

AFLP (Amplified Fragment Length Polymorphism) is a highly sensitive method for fingerprinting genomic DNA within any organism (Vos et al. 1995). Restriction endonucleases such as Mse1 and EcoR1 are used to digest the DNA before amplification. A subset of resultant fragments representing many loci is then ligated to synthetic adaptors and amplified with specified primers which are complementary to a selective sequence on the adaptors. The amplified fragments are then to be analyzed using denaturing polyacrylamide gel electrophoresis which generates fingerprints to be compared as polymorphisms. The particular band differences can be cloned and sequenced to identify the source of the polymorphism. This method constitutes a rapid screening for polymorphisms. These polymorphisms are typically inherited in a Mendelian genetics fashion, enabling their use for typing, identification and mapping of genetic characteristics. AFLP analysis depicts unique fingerprints regardless of the origin and complexity of the genome. Most AFLP fragments correspond to unique positions on the genome and hence can be exploited as landmarks in genetic and physical mapping. Applications of this technique reach far beyond agricultural applications, ranging from agronomic trait analysis, diagnostics, pedigree analysis, forensics, parental heritage and may be used as a universal fingerprinting system (Pereira et al. 2010).

RAPDs

A Random Amplified Polymorphic DNA (RAPD) technique is based on the polymerase chain reac-

tion and has been one of the most commonly used molecular techniques to develop DNA markers. RAPD markers are amplification products of anonymous DNA sequences using single, short and arbitrary (10 mers) oligonucleotide primers (Welsh, McClelland 1990; Williams et al. 1990). RAPDs are much simpler and less expensive to work with than RFLPs because no prior knowledge of sequences is required and there is no need for radioactive probes. RAPDs produce DNA profiles of varying complexity, depending on the primer and template used. The primer amplifies pieces of DNA between 200 and 2,000 kb long, which lies between two inverted copies of itself, one copy binding to each strand of the DNA. The methodology is rapid and can be used on material without prior information on genome. A major drawback of the technique is that because of the random nature of their generation, and short primer length, they cannot be easily transferred between species. An additional drawback is that of poor reliability and reproducibility, and their sensitivity to experimental conditions (Devey et al. 1995).

SSRs (Microsatellites or Simple Sequence Repeats)

These are short segments of DNA that are derived from short (usually < 6 base pairs) tandemly repeated sequences such as (GA) n, (AAT) n, (GT) n and are known as microsatellites. Many of them are located in centromeric regions, telomeric regions and in the roots of chromatin loops, whilst others are thought to play a role in pairing and synapsis of chromosomes. Though these markers are neutral and codominant, yet the high development cost is a major impediment to the routine application of SSRs in the genetic study of non-commercial species and for identifying markers located in chromosomal regions of interest. Point mutation in the primer annealing sites in some species may lead to the occurrence of null alleles, where microsatellites fail to amplify. To develop the locus-specific SSR markers, the isolation and characterization of individual loci and the construction and screening of a DNA library with microsatellite-specific probes, followed by DNA sequencing of positive clones are required (Roder et al. 1998). Because these regions are hypervariable, RFLP analysis with probes for micro- and minisatellites gives multilocus patterns which have resolved variation at the levels of populations and individuals (Marquardt, Echt 1995; Butcher et al. 1999). SSR markers are considered highly polymorphic as the number of repeats varies due to slippage of DNA polymerase during replication and unequal crossing over and thereby allowing us to detect many different alleles for that marker. The frequency of SSRs is higher in transcribed regions than in non-transcribed regions. SSRs are the markers of choice due to their codominant expression, multiallelism and high PIC value.

Minisatellites

A minisatellite (also referred as VNTR) is a section of DNA that consists of a short series of bases about 10–60 bp in length. Minisatellites consist of repetitive, generally GC-rich, variant repeats. These variant repeats are tandemly intermingled, which makes minisatellites ideal for studying DNA turnover mechanisms and have been used extensively in many areas of genetics. Minisatellites have been associated with chromosome fragile sites and are proximal to a number of recurrent translocation breakpoints. The hypervariable minisatellites are 9–24 bp long and are found mainly at the centromeric regions whereas the telomeric minisatellites are 6 bp long with repeated sequences in telomeres.

Cleaved Amplified Polymorphic Sequences (CAPS)

CAPS are DNA fragments amplified using specific primers, which are afterwards digested by restriction enzymes. Sequence polymorphisms result in the cutting of products in different places, and these variants are revealed as length differences on agarose gels. The CAPS approach is sometimes known as restriction fragment length polymorphism (RFLP) PCR, and the technique bears similarities to the non-PCR-based older RFLP method. CAPS can be applied to organism-specific nuclear sequences, or to organellar DNA using universal primers. As with SSRs, sequencing is generally required in the former case in order to develop primer pairs. Similarly to SSRs, CAPS assess variation at one locus only in a particular PCR (Kadu et al. 2006).

SNPs (Single Nucleotide Polymorphisms)

SNPs markers are known as the third generation markers, which are nowadays extensively used in various genomic studies for individual genotyping. Single nucleotide polymorphisms or SNPs (pro-

nounced "snips") are DNA sequence variations that occur due to point mutations when a single nucleotide (A, T, C, or G) in the genome sequence is altered (Gupta et al. 2001). Single nucleotide polymorphisms are single base changes in the genome that occur at a frequency of about 1% and can be particularly useful in linkage mapping. They are derived from sequence information, particularly where DNA sequence (either from genomic DNA or cDNA) is available from more than one individual. Informatics tools can be used to compare the sequences and identify variations. These variable nucleotides are subsequently checked to determine whether or not the SNP is real or an artifact. The use of such informatics tools often depends on the availability of appropriate sequence data such as sequence trace files. If each polymorphism were to be inherited independently, then this would generate an exceptionally large number of haplotypes. However, this is not the observed result. Relatively few haplotypes are observed, indicating that perhaps the rate at which SNPs arise is somewhat akin to the rate at which recombination occurs across these small regions leading to a much lower number of haplotypes than would be expected from the number of polymorphic sites. Therefore, SNPs are most likely to be useful as defining haplotypes, rather than for their information individually, and so the use of SNPs is likely to involve linkage disequilibrium studies using the haplotype rather than specific SNPs as individual molecular markers.

ESTs (Expressed Sequence Tags)

An Expressed Sequence Tag or EST is a short (300–500 bp) sub-sequence of a cDNA sequence. The cDNAs used for EST generation are individual clones from a cDNA library which are complementary to mRNA, so the ESTs represent portions of expressed genes. ESTs are used to identify gene transcripts, and play an important role in gene discovery and gene sequence. Since the ESTs are often partial sequences that correspond to the same mRNA of an organism, they are assembled into contigs so as to reduce the number of expressed sequence tags for downstream gene discovery analysis.

Uses of molecular markers

Modern biotechnology provides new tools such as molecular markers that can facilitate the development of improved plant breeding methods and augment our knowledge of plant genetics. Molecular markers can be used to study natural, managed and cultivated tree stands, and can measure the extent to what individuals and populations are connected to each other. They are able to establish the breeding systems of populations, to determine relationships among different taxa, to assess hybridization and other interactions between species. The primary use of molecular markers has been the generation of genetic maps. The initial purpose of these maps has been to localize the position of genes of interest (those underlying a specific identifiable trait) for either positional cloning or marker-assisted selection (MAS). The various uses of molecular markers are:

Characterization of breeding populations

Breeding populations can be characterized by quantifying the levels and organization of genetic variation within and between different breeding groups. Marker-based systems have been used to study and compare the levels of random genetic variation throughout the different cycles of a breeding programme, thus allowing much greater flexibility and control over the rate of reduction of genetic variability (Lia, Wua 2007). RAPD markers were successfully used to characterize a wide range of genetic variation in the germplasm bank of *E. globulus* and thereby assist in the designing of further seed collections (Nesbitt et al. 1995). The application of molecular markers for directional selection is still an unfulfilled promise due to the recent domestication of tree crops resulting in the wide genetic heterogeneity of breeding populations and the inability to develop inbred lines at least on a short-term basis and also to allow a more precise understanding of genetic architecture of quantitative traits. Different markers like ISSRs and SSRs can be used to study the genetic relationship between different accessions (Mittal, Dubey 2010).

Genetic linkage maps

Segregation analysis is important for the population that is derived from a common set of ancestors. Markers that co-segregate (either are always present or absent together) must be linked, i.e. they must be located in each other's vicinity on the genome. In some cases, however, due to recombination events, the linkage between the markers may be lost. The frequency with which the linkage between co-seg-

regating markers is broken is an indication of the genetic distance between the markers. An extensive analysis of the linkage between a large number of molecular markers yields information on their arrangement on the genome (Binelli, Bucci 1994; Byrne et al. 1995). Such an analysis can finally result in the construction of a genetic map, on which all markers are arranged in separate linkage groups or chromosomes. The main applications of genetic maps to plants are to provide the basic knowledge of genomic structure and mapping and detection of complex quantitative trait loci (Sewell, Neale 2000). Nowadays, software for the calculation of genetic maps has brought marker analyses that aim at the construction of genetic maps. Genetic linkage maps can provide a more direct method for selecting desirable genes via their linkage to easily detectable molecular markers (Tanksley et al. 1989; Butcher et al. 2000).

The initial application of the molecular marker maps was to facilitate map-based cloning, almost exclusively of Mendelian genes. Another use of these maps has been in marker-assisted selection. Having closely linked markers to the trait of interest greatly reduces the effort and number of progeny needed to introgress a gene of interest, especially if that gene has a large, measurable effect in the progeny. As the costs of marker typing decrease, the value of their use in breeding programs will be dominated by their returns, rather than by their costs. Under the appropriate conditions, markers can replace phenotypic selection, thereby removing the need for growing or rearing of individuals (Chen et al. 2010).

Quantitative trait loci

Genetic factors that are responsible for a part of the observed phenotypic variation for a quantitative trait can be called quantitative trait loci (QTLs). Although similar to a gene, a QTL merely indicates a region on the genome, and could be comprised of one or more functional genes (Falconer, Mackay 1996). QTL are identified via statistical procedures that integrate genotypic and phenotypic data. QTL are assigned to chromosome locations based on the positions of markers on a linkage map. QTL are located to regions of the genome at specified levels of statistical probability. In a process called QTL-mapping the association between observed trait values and the presence/absence of alleles of markers that have been mapped onto a linkage map is analyzed. Mapping QTL in tree species is not as simple as mapping the gene that affects a qualitative trait. The major obstacle is the time necessary to complete a breeding generation. Tree breeding is made even more difficult by the changes that occur during the transition from juvenility to maturity. When it is significantly clear that the correlation that is observed did not result from some random process, it is proclaimed that a QTL is detected (Collins et al. 2008). Also the size of the allelic effect of the detected QTL can be estimated. A breeder can analyze QTL occurrences and use this knowledge to his advantage, for instance by using indirect selection (Tschaplinski et al. 2006). When selection is based on genetic information retrieved through the application of molecular markers, this is called marker-assisted selection. Once these traits are identified and mapped, map-based cloning becomes more practical to identify the gene(s) responsible for a QTL as the density of markers increases in the regions containing QTLs increases. QTL mapping in trees has been carried out using both three-generation outbred pedigrees and two-generation pedigrees involving crosses between highly heterozygous parents (pseudo-testcross strategy). Based on a detailed understanding of the molecular architecture of quantitative traits, current applications include yield-oriented advanced backcross QTL (ABQTL) systems as well as accelerated line conversion following trait introgression by marker-assisted backcrossing (MABC).

Marker-Assisted Selection

Marker-Assisted Selection (MAS), sometimes also called Marker-Aided Selection, is a relatively new tool for plant breeding which is primarily based on the phenotypic selection of superior individuals among segregating progenies resulting from hybridization. In its simplest form it can be applied to replace the evaluation of a trait that is difficult or expensive to evaluate. When a marker is found that co-segregates with a major gene for an important trait, it may be easier and cheaper to screen for the presence of the marker allele linked to the gene, than to evaluate the trait. In trees the long generation times along with poor juvenile-mature trait correlations have promoted interest in marker-assisted selection (Grattapaglia et al. 2004).

From time to time the linkage between the marker and the gene should then be verified. When more complex, polygenic controlled traits are concerned, the breeder is faced with the problem how

to combine as many as possible beneficiary alleles for the QTLs that were detected. In this case the breeding material can be screened for markers that are linked to QTLs. Based on such an analysis, specific crosses can be devised for the creation of an optimal genotype, combining beneficiary QTL alleles from different sources. With the development and availability of an array of molecular markers and dense molecular genetic maps in crop plants, MAS has become possible for traits governed by both major genes and quantitative trait loci.

Marker-Assisted Selection may be used to facilitate the controlled inflow of new genetic material. 'Wild' or unadapted material often carries desired components that may be missing in cultivated material. Such components can be transferred to elite cultivated material by repeated backcrossing in the unadapted material. In a backcross program, the presence of the desired QTL-alleles can be verified continuously by observing linked markers. At the same time, markers provide information on the origin of the remaining genome, allowing selection within the backcross material for genotypes that have lost the majority of unwanted donor DNA. Usually the application of this marker-assisted backcross procedure will also result in a reduction of the number of backcross generations that are required, thereby speeding up the breeding program.

Microarray-based mapping

DNA microarray technology has given rise to the study of functional genomics. The entire set of genes of an organism can be microarrayed on an area as small as a fingernail and the expression levels of thousands of genes are simultaneously studied in a single experiment (Gupta et al. 1999). Microarrays have demonstrated significant power for genome-wide analyses of gene expression, and recently have also revolutionized the genetic analysis of segregating populations by genotyping thousands of loci in a single assay (Drost et al. 2009). DNA microarray technology allows comparisons of gene expression levels on a genomic scale in all kinds of combinations of samples derived from normal and diseased tissues, treated and non-treated time courses, and different stages of differentiation or development. Further computational analysis of microarray data allows the classification of known or unknown genes by their mRNA expression patterns. Global gene expression profiles in cells or tissues will provide us with a better understanding of the molecular basis of phenotype, pathology, or treatment of diseases.

The markers of choice, at present, for this type of analysis are SNPs. MALDI-TOF can be used to screen large numbers of samples at many loci. This can also be used to determine allelic frequencies in phenotypically defined pools of individuals. As the number of SNPs increases and their placement on the genetic map is achieved, the number of genotypes that are needed to confirm an association will be reduced.

CONCLUSION

The potential benefits of using markers linked to genes of interest in breeding programmes, thus moving from phenotype-based towards genotype-based selection, have been obvious for many decades. However, realization of this potential was limited by the lack of markers. Molecular markers make it possible to accelerate the plant breeding process because it is possible to generate high density linkage maps of traits and markers and use them in many genetic backgrounds as required in a breeding program. Having access to thousands of markers linked to traits makes it possible to select the genotype of interest based on markers and so to limit field and breeding activities to plants with the genotype of interest. Some traits and markers are conserved across related species and so comparative genomics of traits and markers between crops also helps in a breeding process.

Molecular markers and marker mapping are a part of the intrusive new genetics that is pushing its way into all areas of modern biology, from genomics to breeding, from transgenics to developmental biology, from systematics to ecology, and even, perhaps especially, into plant and crop physiology. However, because genes do not function as single entities, it is necessary to know how numerous genes function together. This, in turn, requires the knowledge of the potential and constraints of biological functions of plants. The understanding of the interaction between genes, organs and environmental factors, which include other organisms, is a major challenge for plant biologists. To obtain this information, it is important to exploit the tools of classical and molecular genetics.

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