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Review

Single nucleotide polymorphism genotyping and its application on mapping and marker-assisted plant breeding

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The nucleotide diversity across a genome is the source of most phenotypic variation. Such DNA polymorphism is the basis for the development of molecular markers, an indispensable tool in genetic mapping studies. In general, the high resolution fine mapping of genes is often limited by lack of sufficient number of polymorphic molecular markers. This problem is compounded with traits controlled by multi-genes because in several such studies, QTL cannot be resolved to a workable resolution that could be feasible for predicting the candidate gene(s) associated with traits of interests. The availability of abundant, high-throughput sequence-based markers is the key for detailed genome-wide trait analysis. Single-nucleotide polymorphisms (SNP) are the most common sequence variation and a significant amount of effort has been invested in re-sequencing alleles to discover SNPs. In fully sequenced small-genome model organisms, SNP discovery is relatively straight forward, although high-throughput SNP discovery in natural populations remains both expensive and time-consuming. Here five central biochemical reaction principles that underlie SNP-genotyping methods specifically for large panel sizes and an intermediate number of SNPs are reviewed.

Key words: SNP, QTL mapping, marker assisted breeding.

INTRODUCTION

Molecular genetics is the field of biology which studies the structure and function of genes at a molecular level (Alberts et al., 2007). Molecular genetics employs the methods of genetics and molecular biology. In this realm of knowledge, methods and techniques may be divided into two groups; reverse genetics and forward genetics.

Reverse genetics is a particular approach in discovering the function of a gene that usually goes in the opposite direction of what is called forward genetic screens associated with classical genetics. To put it simply, while forward genetics has the goal of trying to find the genetic basis of a phenotype or trait, reverse genetics is aimed at finding the possible phenotypes that may be derived from a specific genetic sequence that is detailed in a DNA sequencing (Pekosz et al., 1999). Forward genetics has been responsible for our understanding of many biological processes and is an excellent method for identifying genes that function in a particular process. Forward genetics refers to a process where studies are initiated to determine the genetic underpinnings of observable phenotypic variation. It begins with a well-characterized phenotype and then works toward identifying the gene(s) responsible for the phenotype. In many cases the observable variation has been induced using a DNA damaging agent (mutagen) such as T-DNA tagging, transposon tagging and gene or enhancer traps which require inserting foreign DNA into a host genome. Genetic mapping approaches such as quantitative trait loci (QTL) mapping and association mapping are also forward genetic approaches and are often used because gene transfer is not required (Tierney and Lamour, 2005; White et al., 2007).

QUANTITATIVE TRAIT LOCI

The objectives of QTL mapping analysis comprise: 1.) To provide knowledge towards a fundamental understanding of individual gene and interactions; 2.) To enable positional cloning; 3.) To improve breeding value estimate and selection response through marker assisted selection in plants and animals (Collard et al., 2005; Holland, 2004).

QTL mapping analysis is the statistical study of the alleles that occur in a locus and the phenotypes (physical forms or traits) that they produce. Because most traits of interest are governed by more than one gene, defining and studying the entire locus of genes related to a trait gives hope of understanding what effect the genotype of an individual might have in the real world (Zeng, 1994).

ELEMENTS NECESSARY TO DETECT QTLS

Availability of a population segregating for the trait(s) of interest

The construction of a linkage map requires a segregating plant population (that is, a population derived from sexual reproduction). The parents selected for the mapping population will differ in one or more traits of interest. Population sizes used in preliminary genetic mapping studies generally range from 50 to 500 individuals. However larger populations are required for high-resolution mapping. If the map will be used for QTL studies (which is usually the case), then an important point to note is that the mapping population must be phenotypically evaluated (that is, trait data must be collected) before subsequent QTL mapping. Generally in self-pollinating species, mapping populations originate from parents that are both highly homozygous (inbred). In cross pollinating species, the situation is more complicated since most of these species do not tolerate inbreeding. Many cross pollinating plant species are also polyploid (contain several sets of chromosome pairs). Mapping populations used for mapping cross pollinating species may be derived from a cross between a heterozygous parent and a haploid or homozygous parent (Tanksley, 1993; Young, 1994).

Genotype of all individuals constituting the segregating population using genetic markers

Genetic markers represent genetic differences between individual organisms or species. Generally, they do not represent the target genes themselves but act as ‘signs’ or ‘flags’. Genetic markers that are located in close proximity to genes (that is, tightly linked) may be referred to as gene ‘tags’. Such markers themselves do not affect the phenotype of the trait of interest because they are located only near or ‘linked’ to genes controlling the trait. All genetic markers occupy specific genomic positions within chromosomes (like genes) called ‘loci’ (singular ‘locus’) (Collard et al., 2005).

There are three major types of genetic markers: 1.) morphological (also ‘classical’ or ‘visible’) markers which themselves are phenotypic traits or characters; 2.) biochemical markers, which include allelic variants of enzymes called isozymes; and 3.) DNA (or molecular) markers, which reveal sites of variation in DNA morphological markers and are usually visually characterized phenotypic characters such as flower color, seed shape, growth habits or pigmentation. Isozyme markers are differences in enzymes that are detected by electrophoresis and specific staining. The major disadvantages of morphological and biochemical markers are that they may be limited in number and are influenced by environmental factors or the developmental stage of the plant. However, despite these limitations, morphological and biochemical markers have been extremely useful to breeders (Tanksley, 1993; Winter and Kahl, 1995).

DNA markers are the most widely used type of marker predominantly due to their abundance. They arise from differrent classes of DNA mutations such as substitution mutations (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA. These markers are selectively neutral because they are usually located in non-coding regions of DNA. Unlike morphological and biochemical markers, DNA markers are practically unlimited in number and are not affected by environmental factors and/or the developmental stage of the plant. Apart from the use of DNA markers in the construction of linkage maps, they have numerous applications in plant breeding such as assessing the level of genetic diversity within germplasm and cultivar identity. These markers can be broadly classified in the following four groups: 1.) hybridization based markers (e.g. RFLP); 2.) PCR based markers (e.g. RAPD, SSR); 3.) molecular markers based on PCR followed by hybridization (RAPD/MP-PCR) and; 4.) sequencing and DNA chip based markers (e.g. SNP) (Paterson et al., 1991; Gupta, et al., 1999; Jones et al., 1997).

Phenotype of all individuals constituting the segregating population for all traits measured

The accuracy of the estimated M-QTL genetic parameters, such as their effects, genomic locations and gene actions, vary considerably, depending largely on errors in phenotyping. The nature and size of mapping populations and use of replications in phenotyping play a key role in reducing phenotyping errors (Young, 1994).

Linkage analysis

The final step of the construction of a linkage map involves coding data for each DNA marker on each individual of a population and conducting linkage analysis using computer programs. Missing marker data can also be accepted by mapping programs. Although linkage analysis can be performed manually for a few markers, it is not feasible to manually analyze and determine linkages between large numbers of markers that are used to construct maps; computer programs are required for this purpose. Linkage between markers is usually calculated using odds ratios (that is, the ratio of linkage versus

no linkage). This ratio is more conveniently expressed as the logarithm of the ratio, and is called a logarithm of odds (LOD) value or LOD score (Risch, 1992). LOD values of >3 are typically used to construct linkage maps. An LOD value of 3 between two markers indicates that linkage is 1000 times more likely (that is, 1000:1) than no linkage (null hypothesis). LOD values may be lowered in order to detect a greater level of linkage or to place additional markers within maps constructed at higher LOD values. Commonly used software programs include Mapmaker/EXP (Lincoln et al., 1993) and MapManager QTX (Manly et al., 2001), which are freely available from the internet. JoinMap is another commonly-used program for constructing linkage maps (Collard et al., 2005; Stam, 1993).

MARKER ASSISTED SELECTION

In contrast to past decades, when almost no markers were available and breeding was mostly based on the selection of phenotype, advances in molecular genetics have enabled the partial dissection of the "black box" of quantitative traits. The use of molecular genetics rests on the ability to determine the genotype of individuals using DNA analysis (Newbury, 2003).

Markers are effective aids to selection in three ways. First, markers can aid selection on target alleles whose effects are difficult to observe phenotypically. Examples include recessive genes, multiple disease resistance gene pyramids combined in one genotype (where they can epistatically mask each other's effect), alleles that are not expressed in the selection environments (e.g. genes conferring resistance to a disease that is not regularly present in environments) or genes whose phenoltypic assays are more expensive that marker assays (e.g. some end-use or grain quality traits) (Holland, 2004).

Second, markers can be used to select for rare progeny in which recombinations near the target gene have produced chromosomes that contain the target allele and as little possible, surrounding DNA from donor parent. Young and Tanksley (1989) demonstrated that large amounts of donor parent chromosomal material can remain around a target gene even after many generations of conventional backcrossing. Since this surrounding material may contribute to "linkage drag" especially if the donor parent is a wild relative or exotic germplasm source, minimizing the size of introgressed segments from the donor parent is often critical to the successful backcross breeding of a new cultivar.

Third, markers that are unlinked to a target allele can also be useful in marker-assisted backcrossing, by permitting selection for those progeny with higher proportions of the recurrent parent genetic background. After two backcrosses, for example, progeny are expected to have recurrent parent alleles at on average 87.5% loci unlinked to the target gene. However, variation around this average proportion exists among the progeny and "background markers" can identify those progeny that are most similar to the recurrent parent. This can reduce the number of generations needed to obtain a genotype with 98 or 99% genetic similarity to recurrent parent for a fixed sample size (Frisch et al., 1998).

SNP GENOTYPING

In general, popular techniques employ markers based on length differences, such as SSR (Provan et al., 1996; Milbourne et al., 1998), on alterations within restriction sites of DNA cutting enzymes, such as RFLP (Paterson et al., 1988; Tanksley et al., 1989), AFLP (Vos et al., 1995) and CAPS (Konieczny and Ausubel, 1993) and on short polymorphic sequences, such as gene- and allele-specific markers (SCAR) (Paran and Michelmore 1993) and DALP (Desmarais et al., 1998). Combinations of these principles often are applied to increase the number of useful polymorphisms detected in a limited number of steps. While having the advantage of being applicable at the species level and also in less-studied genomes, the common drawback of all these marker technologies is their dependence on the distribution and frequency of redundant, global features across a genome. A global marker technique that relies on the recognition site of a specific restriction enzyme can maximally detect all the corresponding restriction sites within a genome. In contrast, every SNP in context with its surrounding genomic sequence is unique. SNPs can mark functionally important allelic differences and SNPs that flag individual alleles of known genes have been used widely as molecular markers (Nakitandwe et al., 2007).

SNPs are naturally occurring variants that affect a single nucleotide. These markers are the most common form of genetic variation between individuals and occur once every 1,000 bases or so (Perkel, 2008). They are most commonly changes from one base to another - transitions and transversions-, but single base insertions and deletions (indel) are also SNPs. Some authors regard two-nucleotide changes and small indels up to a few nucleotides as SNPs, in which case the term simple nucleotide polymorphism may be preferred (Batley et al., 2003).

The study of the distribution of genetic variants, including SNPs, lie within the domain of population genetics and the study of the relationship between SNPs and phenotypic variation lies in the domain of quantitative genetics. Genomics methods have led to a renaissance of interest in both fields of enquiry. While the earliest applications of genomics were in the mapping of Mendelian loci, the emerging importance of SNPs lies in the mapping and identification of quantitative trait loci, which are loci that contribute to polygenic phenotypic variation. On one hand, SNPs provide the wherewithal to scan genomes for linkage to QTL; on the other hand, the vast majority of QTL effects are almost certainly due to as yet unidentified SNPs. For both reasons, characterization

of the distribution of SNP variation is a major goal of most genome projects (Greg and Muse, 2004).

Due to the abundance of SNPs and development of sophisticated high-throughput SNP detection systems, it has recently been proposed that SNP markers will have a great influence on future mapping research studies and MAS (Rafalski, 2002; Koebner and Summers, 2003). The following is a summary of central biochemical reaction principles that underlie SNP-genotyping methods:

Restriction site cleavage

Single base differences between alleles generate restriction sites and can be used as CAPS markers (Michaels and Amasino, 1998). PCR-RF-SSCP (PRS), which combines cleaved amplified polymorphic sequence (CAPS) and single-strand conformation polymorphism (SSCP), is expected to be a useful technique for DNA polymerphism analysis (Sato and Nishio, 2003).

SSCP analysis is a widely accepted and useful application for detecting mutations or DNA polymorphisms based on the ability of a single (or multiple) nucleotide change to alter the electrophoretic mobility of a single-stranded DNA molecule under non-denaturing conditions (Bernat et al., 2002; Nikolausz et al., 2008).

To distinguish between alleles without restriction sites that differ by a single nucleotide, Michaels and Amasino (1998) designed the following method thus: The region of DNA containing the base change is amplified using primers which contain one or two mismatches. The introduction of these base changes into the PCR product and together with the base change between alleles creates a unique restriction site in one of the alleles. However, the majority of single base changes do not generate restriction site differences.

PCR-based methods

Allele specific primers: The method operates on the basis of the specific amplification of a target allele by the polymerase chain reaction with extension primers designed such that their 3' end is placed at the mutation site. When this base is complementary to that of the specific allele, the DNA segment is amplified; when it is not complementary, the polymerase chain reaction cannot proceed (Okayama et al., 1989).

Sequence Polymorphism-Derived (SPD) markers: These markers proved highly efficient for fingerprinting of individuals possessing a homogeneous genetic background. SPD markers are obtained from within non-informative, conventional molecular marker fragments that are screened for SNPs to design allele-specific PCR primers. The method makes use of primers containing a single, 3'-terminal locked nucleic acid (LNA) base which is a bicyclic nucleic acid where a ribonucleoside is linked between the 2'-oxygen and the 4'-carbon atoms with a methylene unit. Locked and by "locking" the molecule with the methylene bridge, the LNA™ is constrained in the ideal conformation for Watson-Crick binding. LNA™ therefore makes the pairing with a complementary nucleotide strand more rapid and increases the stability of the resulting duplex. LNA™ nucleosides give rise to increased thermal stability and discriminative power of duplexes resulting in various unique features (Nakitandwe et al., 2007).

Targeting induced local lesions in genomes (TILLinG): Targeting induced local lesions in genomes is a recently developed method for detecting mutations in a high-throughput manner available to plant geneticists. TILLinG is a non-transgenic reverse genetics approach that is applicable to all animal and plant species which can be mutagenized, regardless of its mating/pollinating system, ploidy level or genome size. This approach requires prior DNA sequence information and takes advantage of a mismatch endonuclease to locate and detect induced mutations. Ultimately, it can provide an allelic series of silent, missense, nonsense and splice site mutations to examine the effect of various mutations in a gene. TILLinG has proven to be a practical, efficient and effective approach for functional genomic studies in numerous plant and animal species. EcoTILLinG, which is a variant of TILLinG, examines natural genetic variation in populations and has been successfully utilized in animals and plants to discover SNPs including rare ones (Barkley and Wang, 2008).

To discover nucleotide changes within a particular gene, PCR is performed with gene-specific primers that are end-labeled with fluorescent molecules. After PCR, samples are denatured and annealed to form heteroduplexes between polymorphic DNA strands. Mismatched base pairs in these heteroduplexes are cleaved by digestion with a single-strand specific nuclease. The resulting products are size-fractionated using denaturing polyacrylamide gel electrophoresis and visualized by fluorescence detection. The migration of cleaved products indicates the approximate location of nucleotide polymorphisms. Throughput is increased and costs are reduced by sample pooling, multi-well liquid handling and automated gel band mapping. Once genomic DNA samples have been obtained, pooled and arrayed, thousands of samples can be screened daily (Till et al., 2006).

One possible limitation of this procedure is that it would be ideally done on homozygous lines. If there is doubt, the assay should be conducted with just the DNA from each recombinant inbred line; no SNPs should be detected (Collard et al., 2008).

Minisequencing primers: In the minisequencing primer extension reaction, a DNA polymerase is used specifically to extend a primer that anneals immediately adjacent to the nucleotide position to be analyzed with a

single labeled nucleoside triphospate complementary to the nucleotide at the variant site. The reaction allows highly specific detection of point mutations and SNPs. Because all SNPs can be analyzed with high specifically at the same reaction conditions, mini-sequencing is a promising reaction principle for multiplex high-throughput genotyping assays. It is also a useful tool for accurate quantitative PCR-based analysis (Syvänen, 1999). The technique is based on the annealing of a single primer adjacent to the polymorphic target site. The 3' primer is extended by a DNA polymerase in a cycle sequencing reaction using fluorescently labeled dideoxynucleotide (ddNTP) and the other deoxynucleotides (dNTPs). The DNA polymerase will extend the minisequencing primer until a ddNTP is incorporated when it stops. The product size varies according to the primer size and the nucleotide sequence that is adjacent to it. Tails of different sizes are added to each primer to allow the type resolution of several SNPs in the same reaction. The minisequencing products are then visualized using an automatic fluorescent DNA sequencer (Carvalho et al., 2005).

Allele-specific ligation probes

DNA ligase catalyzes the ligation of the 3' end of a DNA fragment to the 5' end of a directly adjacent DNA fragment. This mechanism can be used to interrogate an SNP by hybridizing two probes directly over the SNP polymorphic site, whereby ligation can occur if the probes are identical to the target DNA. In the oligonucleotide ligase assay, two probes are designed; an allele-specific probe which hybridizes to the target DNA so that it's 3' base is situated directly over the SNP nucleotide and a second probe that hybridizes the template upstream (downstream according to the complementary strand) of the SNP polymorphic site providing a 5' end for the ligation reaction. If the allele-specific probe matches the target DNA, it will fully hybridize to the target DNA and ligation can occur. Ligation does not generally occur in the presence of a mismatched 3' base. Ligated or unligated products can be detected by gel electrophoresis, MALDI-TOF mass spectrometry or by capillary electrophoresis for large-scale applications (Rapley and Harbron, 2004).

Hybridization-based methods

Allele-specific oligonucleotide probes: An alternative and simpler technique was described by Ji et al (2004). The genotypes of a given SNP were differentiated by hybridization with a pair of allele-specific probes labeled with dual-color fluorescent (Cy3, Cy5) (Liu et al., 2007). The probes are allowed to base pair with the target DNA that contains the SNP at conditions in which only perfectly matched probe-target hybrids are stable, and hybrids that contain a mismatch are unstable (Syvänen, 2001).

Single-feature polymorphism (SFP): A polymorphism detected by a single probe in an oligonucleotide array is called a single-feature polymorphsim (SFP), where a feature refers to a probe in the array (Cui et al., 2005). SFPs were first identified in yeast as significant differences in hybridization intensity between strains when genomic DNA was hybridized to high-density oligonucleotide expression arrays (Winzeler et al., 1998). Subsequently, the method was used in the considerably more complex context of *Arabidopsis thaliana* genome (Borevitz et al., 2003). The two main advantages of SFPs are that standard expression arrays are used in lieu of specialized genotyping technology and that no prior knowledge of SNPs is required. SFP typing is currently being applied to a wide range of organisms such as mosquito (Turner et al., 2005) and barley (Rostoks et al., 2005) with some decrease in the signal-to-noise ratio as genome size increases. Replicating the arrays improves the accuracy with which polymorphisms are detected, but with a consequent trade-off in terms of cost (Kim et al., 2006). Each SFP is scored by the presence or absence of a hybridization signal with its corresponding oligonucleotide probe on the array (Barrett et al., 2004).

Invader probe

The invader assay uses a structure-specific flap endonuclease (FEN) to cleave a three-dimensional complex formed by the hybridization of allele-specific overlapping oligonucleotides to target DNA containing an SNP site (Olivier, 2005). Flap endonucleases isolated from archaea (Lyamichev et al., 1999) is an endonuclease that catalyzes structure-specific cleavage. This cleavage is highly sensitive to mismatches and can be used to interrogate SNPs with a high degree of specificity (Olivier, 2005).

In the basic invader assay (Third Wave Technologies), a FEN called cleavase is combined with two specific oligonucleotide probes that together with the target DNA can form a tripartite structure recognized by cleavase. The first probe, called the invader oligonucleotide is complementary to the 3' end of the target DNA. The last base of the invader oligonucleotide is a non-matching base that overlaps the SNP nucleotide in the target DNA. The second probe is an allele-specific probe which is complementary to the 5' end of the target DNA, but also extends past the 3' side of the SNP nucleotide. The allele-specific probe will contain a base complementary to the SNP nucleotide. If the target DNA contains the desired allele, the Invader and allele-specific probes will bind to the target DNA forming the tripartite structure. This structure is recognized by cleavase, which will cleave and release the 3' end of the allele-specific probe. If the SNP nucleotide in the target DNA is not a complementary allele-specific probe, the correct tripartite structure is not formed and no cleavage occurs. The invader assay is usually coupled with a fluorescence

resonance energy transfer (FRET) system to detect the cleavage event. In this setup, a quencher molecule is attached to the 3' end and a fluorophore is attached to the 5' end of the allele-specific probe. If cleavage occurs, the fluorophore will be separated from the quencher molecule generating a detectable signal (Syvänen, 2001; Olivier, 2005).

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