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The use of DNA Polymorphisms in Genetic Mapping

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Abstract

The introduction of molecular markers has revolutionized genetics. The range of polymorphisms 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. 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 include restriction fragment length polymorphisms, amplified fragment length polymorphisms, ransom amplified polymorphic DNAs, simple sequence repeats, single nucleotide polymorphisms and small insertions/deletions. The types of questions that can be addressed with these molecular markers include the generation of genetic and physical maps for the identification of interesting loci, the development of marker based gene tags, map-based cloning of agronomically important genes, synteny mapping, marker-assisted selection and quantitative trait analysis. The continued development of technology including new high throughput methods, for example those being applied to single nucleotide polymorphisms, will change to ease with which current questions can be answered as well as enable new analyses that are currently impossible to undertake.

Introduction

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 (1,2). A central effort of eukaryote genome research is the generation of integrated genetic and physical maps. 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 (3,4).

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. Molecular markers for mapping applications should be reliable and easy to detect, with a high throughput of samples being possible.

Many techniques have been used for detecting polymorphisms (5) including restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNAs (RAPD) (6), amplified fragment length polymorphisms (AFLP) (7) and simple sequence repeats (SSR) (8) or microsatellites. Although each of these techniques has particular advantages and disadvantages, all have been applied in mapping experiments. RFLPs are the most cumbersome of the markers and are generally used when they can be converted into sequenced characterized amplified regions (SCAR markers). This then allows the polymerase chain reaction (PCR) to be employed in their detection. In most cases it is the identification of the polymorphisms that are segregating in a particular cross under consideration that is the bottleneck in the analysis, rather than the ultimate characterization of the final genetic material. This has been particularly true where the genetic base of the cultivated crop is particularly narrow.

Integrated genetic and physical genome maps have proved to be valuable as sources of sequence-ready clones for genome sequencing projects as well as for map-based gene isolation and comparative genome analysis. One approach for the assembly of physical maps of complex genomes uses restriction enzymes to generate large numbers of DNA fragments from genomic sub clones, usually BAC clones (9,10). These DNA fingerprints are compared to identify related clones, and to assemble overlapping clones in contigs. These contigs are usually combined with anchor loci to construct high-quality integrated physical and genetic maps of complex genomes (10,11).

Marker systems

RFLPs

These markers are generally detected by the hybridization of a probe to restriction-digested genomic DNA. Many of the probes are derived from single copy sequences with EST sequences being one rich source of potential probes. The detection can be time-consuming, and the material involved in the mapping experiments has to be screened with possible probes to determine which are polymorphic. The generation of large numbers of polymorphisms is, to some degree, specific to the genetic material under consideration, and the wider applicability of any of the probes is not assured.

AFLPs

These markers are generated by the PCR amplification of RFLPs. Essentially adaptors are added to the ends of restriction fragments, and these adaptors are then used as primers in a PCR reaction. The amplified bands are separated on gels or through sequence readers and differences in the patterns generated identified. The particular band differences can be cloned and sequenced to identify the source of the polymorphism. This method constitutes a rapid screening for polymorphisms. AFLPs can also be used to compare the hyper- and hypo-methylated regions of the genome using methylation sensitive and insensitive restriction enzyme isoschizomers facilitating the detection of possible epigenetic effects.

RAPDs

PCR amplification using random ten base primers has been very successful in generating large numbers of polymorphisms (6). The methodology is rapid and can be used on material about which little else is known and can sample areas of the genome

inaccessible to RFLPs. The results using this technique make it relatively easy to distinguish between a large number of individuals and genetic loci. A major drawback of the technique is that the information is not easily extended to related material, and, in general, for every different individual a new map has to be generated. An additional drawback is that the results appear to be somewhat difficult to generalize because the reproducibility between laboratories is low.

Microsatellites and simple sequence repeats (SSRs)

Microsatellites or simple sequence repeats (SSRs) are genetic markers that are derived from short (usually <6 base pairs) tandemly repeated sequences such as $(GA)_n$, $(AAT)_n$, $(GT)_n$. This type of sequence is widely dispersed through the genome and polymorphisms are due to the variability in the number of repeats at a given site. SSRs can be isolated from genomic libraries, or enriched genomic libraries (12) or generated from analysis of cDNA sequences. They can be converted into sequenced tagged sites using primers designed in unique regions surrounding the repeat, and have become an important source of genetic markers for many eukaryotic genomes (12), especially when the primers are designed in a conserved region of a transcribed sequence, making them applicable over a wide range of taxa.

Single nucleotide polymorphisms (SNPs)

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 (13,14). 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 every polymorphism was to be inherited independently then this would generate a exceptionally large number of haplotypes. However, this is not the observed result. Relatively few haplotytpes 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 form 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 the use of specific SNPs as individual molecular markers.

Targeted Mutations

Many resources are becoming available to isolated individuals in which a mutation has been produced in a specific gene, either by insertional mutagenesis or by mutagens generating point mutations. Insertional mutants can be identified using a pair of specific primers, one from the gene of interest and the other from the DNA that is used as the insertional mutagen (15). Point mutations in a specific gene, however, have been more difficult to identify. Generally, a mutant indivudual (showing a phenotype) has been identified and map-based cloning used to isolate the specific gene. A new procedure, TILLING can now be used in conjunction with sequenced genomes in order to isolate

individuals with point mutations in a specific gene, irrespective of any phenotype, known or otherwise, associated with that mutation (16).

Uses of molecular markers

The primary use of molecular markers has been of the generation of genetic maps. These have been made using RFLPs, AFLPs, RAPDs and SSRs. 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). However, while there has been spectacular success at identifying the genes responsible for traits inherited in a Mendelian fashion (a change in the observable features (phenotype) that has arisen as a consequence of a mutation in one (dominant) or both (recessive) copies of the gene) the identification of the genes underlying quantitative trait loci (QTLs) or multifactorial disorders has been much less successful (17). Since Mendelian inheritance probably only accounts for less than 10% of all traits of interest, the ability to use molecular markers to understand and identify the underlying control of QTLs is especially important (18).

Genetic Maps

The use of molecular markers made it possible to map, for the first time, an almost unlimited number of randomly distributed polymorphic loci in a single population and provided the foundation for efficient, whole-genome studies at the molecular level. The initial application of RFLP technology for genetic mapping was pioneered in humans (19).

RFLPs, AFLPs, RAPDs and SSRs have been used in the generation of genetic maps. AFLPS and RAPDs have been especially useful in generating maps in outcrossing species where there is little previous information, such as in cassava (20), Kiwifruit (21) and pineapple (22).

The initial application of the molecular marker maps was to facilitate map based cloning, almost exclusively of Mendelian genes. A second use of these maps has been in marker assisted selection (17). 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 (23, 24,25, 26). As marker typing costs reduce, 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 individuals in order to measure their phenotype. Studies in dairy cattle populations indicate that marker assisted selection is economically efficient under realistic assumptions regarding costs and benefits (23).

The integration of physical and genetic maps is advanced for very few organisms so that map based cloning is still a difficult proposition for most organisms. The density of markers surrounding most map positions is still very limited so large areas of the genome still have to be isolated and characterized in order to identify the gene. As will be returned to later, the possible conservation of clustering of genes may aid in the transferability of information between well studied species to those with much less information available.

Quantitative Trait Loci.

QTLs are generally considered to be the chromosomal locations of individual genes or groups of genes that influence complex traits. Since QTLs are defined by a chromosomal position, they can be either a single genetic determinant or represent multiple genetic factors closely linked, that can be resolved into multiple genetic factors by recombination (27, 28). For the manipulation of QTLs in a breeding program, it may not be important to determine the nature of the QTL. However, if cloning of specific QTLs is the goal, then the location must be reduced to a manageable piece of DNA in order to identify the causative gene (29).

The advances in molecular linkage mapping have changed the landscape of possibilities for studying genotype-phenotype relationships. Molecular maps have been constructed for numerous plant species over the last 15 years. These maps have been used to facilitate genetic analysis and have been used to develop QTL analysis using molecular markers (30). The first use of a complete RFLP map for this purpose was reported in tomato (*Lycopersicon* sp.) (31). Marker-based technology already is a powerful approach for identifying and mapping QTLs there is still a great scope for the development of novel approaches to rapidly identify individual genes responsible for specific QTLs.

Important traits such as those related to crop yield are frequently difficult to select for due to genotype-by-environment interaction, (i.e. the differential reaction of genotypes to environmental changes) and the fact that such traits are often controlled by a large number of genes. Therefore it can be difficult to analyze their genetic basis. However, the ability to monitor the genotype directly can greatly increase the efficiency of selection for such traits. This direct monitoring can result from a knowledge of the genes responsible for the trait, or by following markers that are tightly linked to the relevant loci. The assignment of the map positions of the QTLs is achieved by scanning the markers on an already ordered linkage map for individuals from a population segregating for the trait of interest (32, 33). The association of specific chromosomal regions with the trait values locates the position of the QTLs. These types of associations indicate the number of chromosomal locations that have a measurable effect on the trait, but do not necessarily confirm the presence of only a single gene in any given region. The assignment of these QTLs also results in information of the contribution each region makes to the observed phenotype as well as their interactions. The genotype by-environment interaction of each of thes regions containing a QTL can be estimated by performing the mapping studies across a variety of environments. The possibility of using 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. An example of this is the cloning of a fruit-size-determining gene in tomato (34)

Molecular markers, whether they represent single genes or complex loci, can be used in either marker-assisted selection or marker assisted introgression. Marker assisted selection uses follows a series of markers at the genotypic level to identify the individuals that are likely to have a superior performance, often in the absence of detailed phenotypic evaluation. Marker-assisted introgression is the incorporation of a desirable fragment, frequently carrying a disease resistance locus, from an exotic germplasm source into breeding material by repeated backcrossing. Monitoring the progeny using markers specific to the donor and the recipient genomes in the successive generations can greatly reduce number of generations required to recover a

useful introgression line in comparison with the classical procedure. Thus the availability of molecular markers has opened up the possibility of utilizing new germplasm much more efficiently and therefore broadening the genetic base of many of the major crops. Again molecular markers have revealed that the germplasm of wild relatives of the major crops, either still available in the wild or conserved in gene banks, is vastly broader than the narrow gene pools of cultivated plant species and constitutes a much needed resource, perhaps “unlocking the genetic potential from the wild” (35)

Evolutionary comparisons

Polyploid ancestry in all biological kingdoms is probably more universal than once was believed (36). This appears to be particularly true in higher plants. The development of RFLP maps that identified the position of genes and QTLs along the chromosomes also highlighted the presence of duplicated chromosome segments within many plant genomes. Such regions of duplication have been described in detail in maize (*Zea mays*) (37) and the Brassicaceae family, of which Arabidopsis is a member, and have emphasized the themes of genome duplication and rearrangement (38, 39). The timing of duplication events and the types of selection pressures affecting particular genes or larger regions of the chromosomes of plant, animal, and microbial genomes over the course of evolution are being clarified by the detection of genome-wide and fine-scaled duplication as a result of the development of finer scale genetic maps and the inclusion of physical, and sequence-based mapping studies (40).

Not only have molecular markers highlighted internal duplications within the genome of a particular species, but have also been used to identify chromosomal segments that have been conserved through evolution. Comparative mapping is particularly advanced in the Solanaceae family, for example with the 12 chromosomes of potato and tomato being largely collinear (41) . The Gramineae family has also been comprehensively studied with comparative maps developed using low-copy cDNA anchor probes (42, 43). The overall synteny maps may obscure the level of rearrangement that underlies the divergence of the species. Thus there are many exceptions where linked markers do not map to the predicted locations within collinear regions (44, 45, 46). One of the aims of syntenic mapping is to be able to use the information developed in a reference species to clone a gene in a less well described species by its positional “coordinates”. The level of conservation at the individual gene level will obviously be very important in determining the proportion of such efforts that will ultimately be successful. An unsuccessful attempt to use comparative information between barley and rice for the stem rust resistance gene (47) may have as much to do with the rapid rate of evolution of plant resistance genes as to divergence between the chromosomal segments genes (48). The advent of fully sequenced genomes, for example Arabidopsis, and the sequencing of suspected syntenic regions in a species of interest will be a useful test of the degree of conservation and ultimately the likelihood of being able to clone specific genes from detailed genomic information of a close or distant relative.

Microarray-based mapping

Markers useful in mapping generally have to be polymorphic so that different forms can be distinguished. However, partitioning of the genome can allow the mapping of invariant sequences. One example of this was the mapping of human chromosomes using somatic cell genetics and at a finer scale, radiation hybrids (49). A wrinkle on this type of

technology is the development of oat corn hybrid addition lines in which each of the corn chromosomes is added to oat complement. Therefore any corn sequence that has diverged sufficiently for its oat homolog can be placed on a chromosome (50). The continued development of lines with smaller segments of corn chromosomes will facilitate the use of microarrays as mapping tools.

Whole genome scans

Emphasis, especially in the human health arena with the "completion" of the human genome sequence has shifted to the analysis of variation on both individual and a population levels. The markers of choice, at present, for this type of analysis are SNPs. Mass spectrophotometry especially matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) can be used to screen large numbers of samples at many loci. This technology not only determines the genotypes, but can also be used to determine allelic frequencies in phenotypically defined pools of individuals (51). As the number of SNPs increase, and their placement on the genetic map is achieved, then the number of genotypes that aare needed to confirm an association will be reduced. If a whole human genome scan is needed for an unknown condition, then it would be necessary to complete 1,000 cases and 1,000 controls with between 50,000 to 300,000 SNPs. This results in the need to complete 100 to 600 million genotypes. The current technology can generate about one million genotypes per day but this is likely to increase as technology improves. However, the whole genome will only need to be scanned when nothing is known about any associations. As soon as the region(s) of the genome of interest are narrowed, the targeted scans will be much more realistic. However, if increases in throughput for sequencing is any guide, the pace of technological innovation may be such that such genome scans on many individuals may not be far over the horizon.

THE FUTURE

The developments described above imply that in plant breeding the paradigm has changed from selection of phenotypes toward selection of genes, either directly or indirectly. Plant breeders try to optimize the use of the genetic variation in nature by bringing together in one genotype alleles that maxi-mize yield, resistance to stress, etc. However, because genes do not function as single entities it is necessary to know how numerous genes function together. This, in turn, requires 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 or-ganisms, is a major challenge for plant biologists. To obtain this information, it is important to exploit the tools of classical and molecular genetics. To these disciplines a set of technologies summarized as "genomics" has recently been added. Knowledge of the factors that limit the functioning of plants is essential and may be used to design the ideal plant type. When the factors that are limiting the optimal functioning of plants are known, relevant genes can be identified to enable repair of the "defect." Thereafter, such genes could be searched for in the available germplasm. To this end, it is important to have access to all existing genetic variation both within and outside the species. The possibility to transfer genes across almost all taxonomic borders by molecular techniques has expanded the potential re-sources available to plant breeders enormously. It is becoming generally accepted that a multidisciplinary approach to plant biology will lead to the disappearance of borders between disciplines and the irrelevant difference between classical and modern (molecular) plant breeding. In the same vein, the differences between transgenic

and non-transgenic crops should become irrelevant when the focus of plant breeding is on achieving maximal production in a sustainable way to feed the growing human population.

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