I.3 A Model Crop Species: Molecular Markers in Rice

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1 Introduction

Molecular markers were being used in the study of rice genetics even before the emergence of techniques for easy manipulation of DNA. Morphological markers had relatively limited applications, but isozyme markers were used extensively to study rice systematics (Second 1982; Glaszmann 1987). The development of restriction fragment length polymorphism (RFLP) markers and, subsequently, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and microsatellite or simple sequence repeat (SSR) markers allowed the genetic mapping of many important traits. Use of these markers for rice has been recently reviewed (Mackill and Ni 2001; Temnykh et al. 2001; Xu 2002).

Model species have been used extensively in biology. The first model plant species chosen was *Arabidopsis*, and its complete genome sequence has been published recently (Kaul et al. 2000). Rice is the second model plant species, and it is a representative of monocot plants, in addition to its immense agricultural importance. Rice has many practical advantages for use in molecular genetics research, which include its small genome size and relatively low amount of repetitive DNA, its diploid nature, and its ease of manipulation in tissue culture. Table 1 lists the major milestones in the development of rice as a model crop species. The completion of a high-quality draft of the rice genome sequence by the International Rice Genome Project was announced on 18 December 2002 (http://rgp.dna.affrc.go.jp/rgp/Dec18–NEWS.html).

The term "molecular markers" usually signifies the use of DNA fragments from locations in the genome to map and follow the segregation of these fragments or observe underlying genetic variation. A useful feature of molecular markers is that the fragments themselves need have no function; their utility lies in the ease with which they can be assayed and the amount of information relative to genetic variation they impart. In this sense, a major value of markers is that they can be applied easily in any crop. Nevertheless, the enormity of the accumulated genetic information on rice offers unique opportunities for the development and deployment of molecular markers for breeding applications and advanced biological studies.

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Table 1. Milestones in the molecular genetic analysis of rice

Milestone	References
First RFLP map	McCouch et al. (1988)
Transgenic japonica rice	Toriyama et al. (1988); Zhang et al. (1988); Zhang and Wu (1988)
Transgenic indica rice	Datta et al. (1988)
Major gene mapping	Yu et al. (1991)
RAPD markers	Zheng et al. (1991)
Microsatellite markers (SSR)	Zhao and Kochert (1992, 1993); Wu and Tanksley (1993)
QTL mapping	Ahn et al. (1993); Wang et al. (1994)
Agrobacterium transformation	Hiei et al. (1994)
Positional cloning	Song et al. (1995)
AFLP markers	Cho et al. (1996); Mackill et al. (1996)
Rice YAC library	Umehara et al. (1996)
Rice BAC library	Jiang et al. (1995); Wang et al. (1995)
Rice genome draft	Goff et al. (2002); Yu et al. (2002)

Several useful applications of molecular markers are facilitated or enhanced by the availability of the rice genome sequence, and these will be greatly augmented with the discovery of functionally important genes. These advantages are already being realized in *Arabidopsis*. The sequence information itself can be used to identify new microsatellite markers in particular regions for saturation mapping at high resolution (Casacuberta et al. 2000). This approach has been used to identify 2537 of the 2740 experimentally verified SSR primer pairs now available (McCouch et al. 2002). Orthologous genomic sequences from two or more sources can also be used to develop single nucleotide polymorphisms (SNPs) that can be used to map or identify candidate genes through association mapping (Buckler and Thornsberry 2002; Rafalski 2002a, b). High-throughput genetic mapping using multiplexed SSRs and small mapping populations can be used to rapidly map important genes (Ponce et al. 1999) and determine their sequence in relatively small positional cloning experiments (Lukowitz et al. 2000).

The identification of the function of known genes will follow from the annotation of the sequence of the entire rice genome. Those genes having only "hypothetical" as the rationale for their annotation will need to be identified through the efforts of functional genomics. Following assignment of function, the most difficult and important part of this process will be the discovery of useful allelic variation for genes that affect economically important traits. Techniques that allow the mining of these useful alleles will produce the most useful molecular markers. These represent DNA sequence changes that confer improved phenotype in plants, and they can be used directly to follow segregation of these genes in segregating populations.

2 Gene Mapping with Molecular Markers in Rice

2.1 Molecular Maps

Rice benefited early from the development of RFLP maps, largely because of the coordinated rice biotechnology program of the Rockefeller Foundation and later the Rice Genome Program of Japan. The first genetic map was published in 1988 (McCouch et al. 1988), and this was followed by much denser maps (Causse et al. 1994; Harushima et al. 1998). RFLP markers were used to map several important traits in rice. These markers were very useful because of their reliability and well-defined map location. However, PCR-based markers such as RAPDs, AFLPs, and SSRs became more popular because of their ease of use. AFLP markers were particularly useful because of the large number of markers that could be determined with few reactions (Cho et al. 1998). They have also been useful in fine-scale mapping because of the potentially large number of markers available (Xu et al. 2000). However, SSR markers have proven to be the most popular because of their high polymorphism and codominant inheritance (Chen et al. 1997; Temnykh et al. 2000). Primers and map positions for thousands of SSR markers are now available with an experimentally verified SSR placed on average every 157 kb of sequence (McCouch et al. 2002), and researchers can develop their own markers from the sequence data.

Early mapping studies relied on F_2 or F_2-derived F_3 ($F_{2:3}$) populations, which are still useful for major gene traits because they are easy to develop. However, these populations have limited use beyond the initial study and they cannot be easily regenerated or maintained. Fixed populations developed by repeated self-pollination (recombinant inbred lines, RILs), anther culture (doubled haploids, DHs), or backcrossing (near-isogenic lines, NILs; substitution lines, near-isogenic introgression lines, NIILs) are preferred because they can be replicated indefinitely and used in many studies. Xu (2002) listed 14 permanent mapping populations being used in rice, but undoubtedly more have been developed. Molecular mapping data are available for these populations, and it is therefore easy to add new traits, assuming that genetic variation exists for these traits.

2.2 Mapping Useful Genes

The mapping of important traits, including those controlled by major genes or quantitative trait loci (QTLs), has been reviewed previously (Yano and Sasaki 1997; Mackill 1999; Mackill and Ni 2001; Xu 2002). Genetic mapping has resulted in much useful information on important major genes such as those for disease resistance and morphological traits. This information is particularly helpful in clarifying the allelism of genes conferring similar phe-

notypes. Linked markers are already being used in marker-assisted selection (MAS) programs for developing improved rice cultivars (Chen et al. 2000; Hittalmani et al. 2000; Sanchez et al. 2000; Gregorio et al. 2002).

The accumulated information is particularly interesting for identifying QTLs. One problem of mapping QTLs is that those with a relatively small effect (i.e., most of them) are difficult to accurately identify and map with good resolution. However, when several traits are being mapped in different populations and/or different environments, the nature of QTL variation in different germplasm can be assessed. Xu (2002) has provided examples of QTLs mapped across different populations. These comparisons indicate that some QTLs are important in diverse varieties and environments, while others are specific to a particular cultivar or location.

One of the implications of this finding is that the results from a single QTL study do not reveal a complete picture of the genetic control of a trait. Most QTL studies have used a parent with a high expression of the trait of interest. QTLs with the largest effects are the most interesting to breeders. However, different QTLs may show a strong effect in different donors, or under different conditions. Molecular approaches for identifying this type of variation (i.e., allele mining) will be described below. A sequential backcrossing approach, in which up to 200 "donors" representing maximum genetic diversity are crossed to one or a few elite cultivars, was described by Li (2001). Selection is practiced during the backcrossing stage to maximize the occurrence of desirable genotypes and allow the identification of chromosomal segments associated with improved phenotype. This approach will sample a larger number of alleles than standard QTL analysis, and will also allow the identification of alleles that express well in a desirable genetic background.

3 Positional Cloning

3.1 Examples of Positional Cloning in Rice

The positional cloning approach is appropriate for situations in which a gene is identified based on phenotype, but its function is unknown (forward genetics approach). This is the situation for most genes affecting agronomic traits. This approach has been used extensively in *Arabidopsis* and increasingly in rice.

The first gene cloned by this approach in rice (and in any monocot) was the *Xa21* gene for resistance to bacterial blight disease (Song et al. 1995). Several additional genes have been cloned by a map-based approach (Table 2), and many ongoing projects will result in new genes in the near future. While most of these projects involve major genes, two QTLs that control heading date, *Hd1* and *Hd6*, have been cloned. *Hd1* is an allele of the photoperiod-sensitive gene *Se1*, which is inherited as a major gene in many cultivars, but

Table 2. Positional cloning of genes in rice reported in the literature

Gene	Trait	Source of allele	Description of gene	Population size for map	Description of how gene was isolated	Reference
Xa21	Bacterial blight resistance	*O. longistaminata*	Receptor kinase with LRR	386	RFLP marker RG103 cosegregating with *Xa21* was used to probe BAC subclones which were transformed into TP309	Song et al. (1995)
Xa1	Bacterial blight resistance	IRBB1, Kogyoku	NBS-LRR	4225	Selected by homology from 7 cDNA sequences that cosegregated with *Xa1*	Yoshimura et al. (1998)
d1	Dwarf	FL2 (mutant)	α subunit of GTP binding protein	13,000	Identified from a cDNA fragment cosegregating with *d1*	Ashikari et al. (1999)
Pib	Blast resistance	Tohoku IL9 (from source Engkatek)	NBS-LRR resistance gene	3305	Transcribed gene with NBS was found in 80-kb region determined by recombination	Wang et al. (1999)
Hd1	*Se1*, photoperiod sensitivity	Niponbare-Kasalath	Transcription factor homologous to *CONSTANS*	>9000	Region limited to 12 kb by recombination found to contain the *CONSTANS* homolog	Yano et al. (2000)
Pi-ta	Blast resistance	Tadukan	Cytoplasmic receptor, NBS	Not reported	Candidate with NBS was identified in sequences of BAC clones spanning about 850 kb	Bryan et al. (2000)
Hd6	Heading date QTL	Niponbare-Kasalath	α subunit of protein kinase CK2	2807	Only one EST found in the 26.4-kb region delimited by recombination	Takahashi et al. (2001)
Spl7	Spotted leaf lesion mimic	KL210 mutant line	Heat stress transcription factor (HSF)	2944	Gene prediction from 3-kb region delimited by recombination	Yamanouchi et al. (2002)
Sd1	Semidwarf gene	DGWG	Gibberellin 20 oxidase	3477	1 ORF identified in 6-kb interval	Monna et al. (2002)
Hd3	Heading date QTL	Niponbare-Kasalath	Protein similar to flowering locus (FT) of *Arabidopsis*	2207	Region limited to 20 kb by recombination found to contain the gene similar to FT	Kojima et al. (2002)

not in the genetic background (Nipponbare/Kasalath) from which it was cloned (Yano et al. 2000).

The usual approach for positional cloning is to develop a low-resolution map, followed by a high-resolution map with around 3000 or more F_2 progeny. Bryan et al. (2000), however, did low-coverage sequencing through a bacterial artificial chromosome (BAC) contig of about 1.5 cM representing about 850 kb of DNA to identify the location of the *Pi-ta* resistance gene for rice blast. For most traits, the large population is screened for the trait of interest. DNA pools can be used to reduce the number of marker assays (Ahn et al. 2002). Selected progeny can be screened for flanking markers obtained from the low-resolution map. However, phenotyping of the entire large population is not necessary. When measuring the trait is difficult, flanking markers from the low-resolution map can be used to screen the large population before plants are phenotyped (Xu et al. 2000). A subset of plants showing recombination between markers that flank the gene of interest is then screened for more markers. In some cases, AFLP markers are screened on these progeny. Other types of markers can be used, such as CAPS (cleaved amplified polymorphic sequence) or EST (expressed sequence tag) markers. A physical map is created using large-insert clones such as YACs (yeast artificial chromosomes) or BACs. One or two of the clones can be sequenced, and there is an attempt to narrow the location of the gene to as small a fragment as possible by determining where the closest recombination events occur. Candidate genes from this segment are ultimately evaluated through transformation. However, if a strong candidate can be identified in a small interval flanked by recombination events around the gene, sequence differences characteristic of the particular phenotype would be a good indication that the candidate is the gene of interest, and supporting evidence could be provided by expression data.

3.2 Future Use of Positional Cloning

With a small genome size and good transformation protocols, rice has been a good system for positional cloning. The relatively few cases reported so far (Table 2) should be augmented greatly in the near future. These projects have not been trivial undertakings, and they usually involve several years of intensive work. It would be expected that the genome sequence information would greatly accelerate the process of positional cloning as has been the case with *Arabidopsis* (Jander et al. 2002).

A formula was devised for determining the number of progeny required to identify crossovers on either side of a candidate gene in *Arabidopsis* (Durrett et al. 2002):

$$P = 1 - [1 + NT/(100R)]e^{-NT/(100R)}$$

where R is the kb/cM ratio for the region, N is the number of gametes to sample (number of testcross progeny or twice the number of F_2 plants, and P is

the probability of finding a minimum of two crossovers, one on each side of the gene, at a physical distance less than Durrett et al. (2002) point out that, assuming that a probability of 0.95 is needed for crossovers within a BAC clone, the population size would be about 600 F_2 plants. This was computed for a region where 250 kb corresponded to 1 cM genetic distance, which is approximately the average for the rice genome. However, the formula assumes constant recombination within the target region, and this would not be the case over the entire rice genome.

Positional cloning efforts in rice typically rely on large populations of more than 3000 F_2 individuals for fine-scale mapping (Table 2). By the above formula, using a population of 3000 F_2 plants, an interval of 10 kb could be delimited by recombination with a probability of 0.69 assuming that R=250 kb/cM, or a probability of about 0.34 if a more conservative R of 500 is used. In the future, however, such large population sizes should not be needed. Annotation of the sequence will be improved markedly with data from large-scale expression studies using microarrays and large mutant collections. The strategy of map-based cloning will merge with the other methods of functional genomics. This will be particularly important for identifying genes underlying QTLs, for which map position is more difficult to determine (Wayne and McIntyre 2002). Molecular markers are used to define the position of the genes controlling a particular trait and to detect selection for a trait in artificial or natural populations, and potential candidate genes can be identified by reference to genes of known function and expression data. Even in such a strategy, it will be helpful to have good resolution of the genes or QTLs. On average, a 1-cM genetic distance would correspond to nearly 30 potential candidates.

4 Array-Based Markers

To date, most efforts using microarrays in rice have focused on expression profiling. Studies have been published for expression analyses using rice ESTs for responses during salt stress (Kawasaki et al. 2001) and resistance to blast disease (Rao et al. 2002), oligonucleotides for 21,000 rice genes for nutrient partitioning during grain filling (Zhu et al. 2003), and full-length cDNA clones (Kikuchi et al. 2002) for monitoring chemical induction of disease resistance (Shimono et al. 2003). The full-length cDNA microarray developed by Kikuchi and collaborators has also been used to analyze gene expression during Fe-deficiency stress in barley (Negishi et al. 2002). Kikuchi and collaborators have recently published their rice expression database (Yazaki et al. 2002; http://red.dna.affrc.go.jp/RED/), which will serve as the entry point for microarray analyses from a consortium of more than 50 laboratories.

Nevertheless, microarrays promise to be a convenient route for genotyping and mapping. Current applications of array-based markers involve the use of

either genomic clones as in the diversity array technology or DArT (Jaccoud et al. 2001) or oligonucleotides as probes. The latter technique has centered around the use of GeneChips, such as those produced by Affymetrix (Fodor et al. 1991, 1993; Pease et al. 1994), wherein oligonucleotides are synthesized directly on the array substrate. Such arrays typically involve tens if not hundreds of thousands of oligonucleotide "features".

DArT was designed as a means to produce diversity fingerprints via DNA/DNA hybridization between reduced-complexity genomic clones and target germplasm. This is a sequence-independent method that does not rely on sequence information to identify the clones on the array. Hence, this approach is amenable to any organism for which little or no sequence data exist. Jaccoud et al. (2001) developed this technique using a panel of nine *Oryza sativa* lines as proof of concept for the procedure. If the clones on the diversity array are anchored to a genetic map, the arrays can then be used not only for fingerprinting diversity, but also to provide molecular markers for mapping. We are currently implementing DArT at IRRI to characterize rice genetic diversity, while, at CIAT, diversity arrays are under development for *Phaseolus* spp. (J. Tohme, pers. comm.).

For oligonucleotide arrays, several genotyping examples as well as their use in mapping have now been published on nonhuman model species. These studies include work on yeast (Winzeler et al. 1998, 2003; Steinmetz et al. 2002), *Arabidopsis* (Cho et al. 1999; Spiegelman et al. 2000; Borevitz et al. 2003), mouse (Lindblad-Toh et al. 2000), and zebrafish (Stickney et al. 2002). Two approaches have been used for the design of the oligonucleotide arrays in these studies. The first approach is the variation detector array, in which 16 features are routinely synthesized for each locus (the coding and noncoding strands for two alleles with all four combinations of bases for the polymorphic site of each allele). The advantage of this approach is the accurate identification of the SNP at the locus, whereas the main disadvantages are the need to know the base constitution for the alleles to be queried and the high production costs for the design, optimization, and synthesis of the arrays. Other approaches for using SNPs as markers are described in the next section.

The second approach uses existing oligonucleotide arrays developed for expression analyses to identify genetic variation as measured by differential hybridization intensities to features on the array (Borevitz et al. 2003; Winzeler et al. 2003). The polymorphisms discovered in this manner have been termed "single-feature polymorphisms" or SFPs since the specific identity of the nucleotides leading to the differential hybridization may not be known. A recent development in oligonucleotide array technology is the maskless array system pioneered by NimbleGen Systems, Inc. (Singh-Gasson et al. 1999; Nuwaysir et al. 2002). This method of array synthesis uses a digital-micromirror system to direct light at specific elements during each round of synthesis, thus allowing for quick turnaround in array design and optimization. A pilot project to develop rice oligonucleotide arrays for profiling abiotic and biotic stress-related genetic diversity and expression using the

NimbleGen approach has begun between David Frisch at the Genome Center of the University of Wisconsin and IRRI.

5 Candidate Genes as Markers

A dense microsatellite map has been developed in rice, and most mapping studies now rely on these markers. They have also proven to be popular for applications in marker-assisted breeding. The many advantages of SSR markers have been described, and foremost is their high level of polymorphism, which allows them to be used in a wide range of germplasm. Yet, they can still be problematic to use in closely related germplasm. For example, in temperate japonica cultivars, genetic diversity can be relatively low despite marked differences in phenotypes (Mackill et al. 1996; Ni et al. 2002). However, with such an abundance of microsatellite markers, estimated to exist at one per 8000 bp on average from sequence data (Goff et al. 2002), even these limitations could be overcome to develop markers at specific locations in the genome.

The outcome of functional genomics research should allow the identification of gene function for all the rice genes, and this will provide a means of manipulating these genes directly for cultivar improvement. However, this is seen as a long-term objective, and the development of useful products will depend on many factors, such as how a useful phenotype can be produced by manipulating the genes. In the shorter term, these candidate genes can be used directly in identifying favorable alleles and following their inheritance in segregating populations. The use of gene sequences as selectable markers has several advantages over the use of SSR or other markers that are obtained from linkage maps. Linked markers will always have the problem of recombination, often necessitating the use of flanking markers for selection. Furthermore, identification of the sequence change that imparts a desirable phenotype will allow the development of a marker specific for the favorable allele. In addition to avoiding any problem with recombination, this would also allow the use of the marker in nearly any population, and also as a general screen of germplasm or elite breeding lines for genes of interest.

Markers specific for alleles of a gene are most likely those that detect single nucleotide polymorphisms. Alignment of genomic sequence from the *japonica* and *indica* subspecies as well as cDNA or EST sequences from other varieties will allow SNPs to be located and primers designed for the target alleles or intervals. Numerous experimental techniques are available for SNP detection (for recent reviews see Gut 2001, Kwok 2001, Syvänen 2001 and Kirk et al. 2002). Basically, existing SNP detection methods rely on four reaction principles: hybridization with allele-specific probes, oligonucleotide ligation, single nucleotide primer extension, or enzymatic cleavage. The separation step(s) of the assays can occur: (1) on a solid support (microarray, microtiter plate, or

microspheres); (2) in liquid phase by electrophoresis, flow cytometry, or denaturing high-performance liquid chromatography; or (3) in liquid phase with no requirement on separation. Products are then detected by indirect colorimetry, mass spectrometry, fluorescence, fluorescence resonance energy transfer, fluorescence polarization, or chemiluminescence. The choice of any particular method will be determined by the ease of automation, sensitivity of the assay, feasibility of multiplexing, or whether de novo sequencing is a prerequisite. Recently, Nasu et al. (2002) identified 2800 SNPs located in 417 regions from three *Oryza sativa* subsp. *japonica* cultivars, two *indica* cultivars, and one wild *O. rufipogon* accession by sequencing and aligning about 250 kb. From these SNPs, they established a set of 213 codominant SNP markers suitable for use in molecular breeding.

The targeted local lesions in genomes (TILLING) approach developed for reverse genetics (Colbert et al. 2001; Till et al. 2003) is also applicable for SNP genotyping and discovery. Genomic loci are chosen for querying, and differentially labeled primers are produced for this locus. These primers are used to amplify PCR products from pools of chemically mutagenized plant lines. Following denaturation/renaturation, the PCR products are treated with the enzyme CEL-I that only cuts mismatched base pairs as small as a single base. If, for the target locus, a chemically induced mutation occurred in one or more lines, cleaved products can be visualized on automated genotypers as new bands. If, instead of pools of chemically mutagenized plants, heteroduplexes are formed between a reference cultivar and a query cultivar, genetic variation in the form of SNPs or indels can be detected, and this application has been termed "EcoTILLING" (Comai et al. 2004).

SSR markers could still be used after the identification of candidate genes. Their convenience, codominant inheritance, and high polymorphism may make SSRs preferable over SNPs identified from the favorable allele. SSR markers adjacent to or within genes can serve for this purpose. An example is the *waxy* gene, which contains a microsatellite within it (Bligh et al. 1995). For this SSR locus, differences in amylose content are associated with repeat length (Ayres et al. 1997).

6 Conclusions

The impact of the DNA sequence of rice is just being felt and undoubtedly many functional genes will be identified in the next few years. In addition to using this information to improve rice and other crops through a transgenic approach, there will be a need for mining alleles of important genes from the largely underused germplasm collections. As an entry point for this effort, a core collection of 11,200 accessions from the International Rice Genebank Collection (IRGC) has recently been developed at IRRI. The set of accessions chosen for the core collection was based on species, variety group, ecocul-

tural type, source location, estimates of possible deployment based on tracing pedigrees in the International Rice Information System crop information database, and characterization data.

The level of coverage of accessions in the IRGC is about 11 % for the cultivated species *O. sativa* and *glaberrima* and from 100 to 20 % for the wild species (depending on the number of accessions per species in the IRGC). These levels of coverage seem adequate such that the core collection will encompass most of the diversity contained in the entire collection. This set of germplasm is currently being processed to produce an archive of lyophilized tissue and the genomic DNA. SSR and/or DArT fingerprinting of the core collection materials will be carried out in the near future to define population structure as a prerequisite for association mapping. In addition, candidate genes for a wide variety of biotic and abiotic stresses and nutritional factors are being identified, and markers for these target loci established. PCR-based methods such as EcoTILLING, locus-specific SNP detection, or length polymorphisms will be used to identify alleles in the core collection. Pooling strategies enabling the detection of infrequent alleles will be used to increase throughput as much as possible.

This process seems likely to identify numerous novel alleles; accessions carrying these alleles will be phenotyped for traits appropriate to the physiological function of the candidate genes. The analysis of these data by association mapping and/or linkage disequilibrium will identify those alleles that make a positive contribution to the phenotype of interest. Marker-assisted breeding programs can then benefit by an infusion of new alleles with markers that are the underlying genes or are located within the haplotype block determined by the extent of linkage disequilibrium at that locus. Furthermore, the identification of novel alleles from a wide range of germplasm will lead to the pyramiding of alleles in favorable genetic backgrounds by breeding schemes using multiple donors that previously would have been difficult to devise. Through allele mining, the products of functional genomics will be delivered to the ultimate end-users, farmers in the developing world, in the form of new rice varieties with enhanced nutrition and improved tolerance for biotic and abiotic stresses.

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