

Rice functional genomics research in China

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Rice functional genomics is a scientific approach that seeks to identify and define the function of rice genes, and uncover when and how genes work together to produce phenotypic traits. Rapid progress in rice genome sequencing has facilitated research in rice functional genomics in China. The Ministry of Science and Technology of China has funded two major rice functional genomics research programmes for building up the infrastructures of the functional genomics study such as developing rice functional genomics tools and resources. The programmes were also aimed at cloning and functional analyses of a number of genes controlling important agronomic traits from rice. National and international collaborations on rice functional genomics study are accelerating rice gene discovery and application.

Keywords: rice; *Oryza sativa*; functional genomics; agronomic traits; genome sequencing; microarray

1. INTRODUCTION

Rice is the principal food for over half of the population of the world. With a compact genome spanning approximately 430 megabase (Mb) pairs, an extensive genetic map (Harushima *et al.* 1998) and established synteny with other cereal crops (Gale & Devos 1998), the cultivated rice species *Oryza sativa* represents a model for cereals as well as other monocot plants (Shimamoto & Kyoizuka 2002). The completion of the genome sequence of rice, in December 2004, opens a new and exciting chapter in our quest to functionally characterize all of the approximately 50 000 annotated genes in rice (Feng *et al.* 2002; Sasaki *et al.* 2002; Rice Chromosome 10 Sequencing Consortium 2003). A systematic approach to characterizing these genes will allow us to dissect and understand the regulatory networks and evolutionary selection controlling such complex traits as yield, grain quality, biotic and abiotic stresses, reproductive barriers, epigenetics and flowering time. The next essential steps towards deciphering the sequenced genome are to develop complete and accurate maps of actively transcribed regions during rice development, and to generate more and more

genome-wide rice mutant resources. These will facilitate the identification of all the genes and proteins encoded in the DNA sequence. Such information will allow further analysis of their function, regulation and how they cooperate in complex biological processes in a systematic manner.

In China, the Ministry of Science and Technology has funded two rice functional genomics programmes since 1999 under the National Basic Sciences Initiatives (Xue & Xu 2002; Xue *et al.* 2003) and the '863' High-Tech Development Plan. The 863 programme was started in 2002. The Chinese Rice Functional Genomics Programme is composed of three parts: (i) development of technical platforms, (ii) functional genomics of agriculturally important traits, and (iii) molecular cloning and functional analysis of agronomic genes. The platforms are aimed at enabling high-throughput analyses and effective determination of gene functions, which consist of three major components: generation and characterization of a large mutant library; expression profiling of the predicted exons and expressed sequence tags (ESTs) of the entire genome; and isolation of full-length complementary DNAs (cDNAs). The traits targeted for functional genomics studies in this programme include grain quality, yield potential, architecture, stress tolerance, disease and insect resistance, and nutrient efficiency,

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which are also the traits targeted for gene isolation. The organized national consortium for rice functional genomics research has been established in China. This consortium includes a number of institutes and universities in Beijing, Shanghai, Wuhan, Hangzhou, Guangzhou and Yangzhou. Here, we report the progress that has been achieved in the last several years in these programmes.

2. RAPID PROGRESS IN RICE GENOME SEQUENCING

Domesticated Asian rice, *O. sativa*, which consists of two major subspecies *indica* and *japonica* partially isolated in terms of sexual reproduction, has become the most important rice crop in the world (Goff *et al.* 2002; Linares 2002). *Indica-japonica* genome comparison has been chosen as a model system for understanding the origin, speciation, domestication and genome evolution of rice. *Indica* and *japonica* cultivars can be classified based on their agronomic traits (Oka & Morishima 1997) and the use of molecular markers (Glaszmann 1987; Cheng *et al.* 2003). Rapid progress in both *indica* and *japonica* genome sequencing has been made in China (Yu *et al.* 2002, 2005). Here, we describe the completed sequence of rice chromosome 4 and whole-genome shotgun sequencing of an *indica* variety 93-11.

(a) Rice *Oryza sativa japonica* chromosome 4 sequencing as part of international efforts to sequence the entire genome

The International Rice Genome Sequencing Project (IRGSP) has adopted the clone-by-clone approach for obtaining a finished rice genome sequence, because it is modular, allows efficient gap filling, avoids problems arising from distant repetitive sequences and results in the early completion of larger contiguous segments of a genome (Sasaki & Burr 2000). As part of international efforts to completely sequence the entire rice genome, the National Center for Gene Research (NCGR) of the Chinese Academy of Sciences has finished the sequence of rice *japonica* chromosome 4 through a map-based strategy (Feng *et al.* 2002; Zhao *et al.* 2002; IRGSP 2005). This was one of the first two rice chromosomes to have been completely sequenced. The finished sequence spans 34.6 Mb and represents 97.3% of the chromosome. To characterize the blueprint of intraspecific DNA-sequence variations between *indica* and *japonica* subspecies, NCGR has conducted an *indica-japonica* chromosome 4 comparison at the nucleotide sequence level. A 22.1 Mb of the *indica* Guangluai 4 (GLA4) chromosome 4 has been sequenced, the largest map-based high-quality *indica* sequence, and compared with 23.2 Mb of the *japonica* orthologous chromosome 4 (Han *et al.* 2007 unpublished data). This comparison provided a fine-scale *indica-japonica* comparison. A sequence comparison of the collinear regions from *indica* and *japonica* chromosome 4 has revealed not only their extensive micro-collinearity in gene order and content, but also the intraspecific sequence polymorphisms in both coding and non-coding regions (Feng *et al.* 2002; Han & Xue 2003; Li *et al.* 2004). Comparative genome analysis between cultivated rice subspecies shows that there is

an overall syntenic relationship between the chromosomes and the divergence at the level of single-nucleotide polymorphisms (SNPs) and insertions and deletions. The rice genome has been well mapped both genetically and physically and has a syntenic relationship with other cereals. Identification of the sequence polymorphisms of the two subspecies has produced the greatest density of polymorphic sequence maps of the *indica* and *japonica* chromosome 4. Importantly, this comparison revealed the dynamic changes that may reflect major evolutionary events occurring throughout the domestication and natural selection of rice.

A complete sequence of a chromosome centromere is necessary to fully understand centromere function. The rice chromosome 4 centromere sequence has been completely determined and its structures have been analysed. Complete sequencing of the 124 kb rice chromosome 4 centromere revealed that it consisted of 18 tracts of 379 tandemly arrayed repeats known as CentO and a total of 19 centromeric retroelements (CRs), but no unique sequences were detected. The preferential insert of the CRs among CentO repeats indicated that the centromere-specific retroelements may contribute to centromere expansion during evolution. The presence of three intact retrotransposons in the centromere suggests that they may be responsible for functional centromere initiation through a transcription-mediated mechanism (Zhang *et al.* 2004).

(b) Whole-genome shotgun sequencing of *indica* 93-11

A whole-genome shotgun sequencing approach was carried out for sequencing an *indica* variety 93-11 genome by the Beijing Genomics Institute of the Chinese Academy of Sciences (Yu *et al.* 2002, 2005). The improved whole-genome shotgun sequences for the genomes of *indica* and *japonica* rice, both with multi-megabase contiguity, gave an almost 1000-fold improvement over the drafts of 2002 (Yu *et al.* 2005). Tested against a non-redundant collection of 19 079 full-length cDNAs, 97.7% of the genes are aligned, without fragmentation, to the mapped superscaffolds of one or the other genome. Yu *et al.* (2005) introduced a gene identification procedure for plants that do not rely on similarity to known genes to remove erroneous predictions resulting from transposable elements. At least a quarter of the two sequences could not be aligned, and where they could be aligned, SNP rates varied from as little as 3.0 SNP kb⁻¹ in the coding regions to 27.6 SNP kb⁻¹ in the transposable elements. These results have revealed ancient whole-genome duplication, a recent segmental duplication on chromosomes 11 and 12, and massive ongoing individual gene duplications. More importantly, ongoing individual gene duplications provide a never-ending source of raw materials for gene genesis and are major contributors to the differences between members of the grass family.

3. INFRASTRUCTURES OF RICE FUNCTIONAL GENOMICS

(a) Rice chips

As a first attempt to decipher the rice genome, computational annotation has been successful,

although improvements are needed (Yuan *et al.* 2003). Recent efforts to verify experimentally the gene model structure by sequencing cDNA and ESTs have provided valuable information towards our understanding of gene structure and genome-coding capacity (Wu *et al.* 2002; Rice Full-Length cDNA Consortium 2003; Rensink & Buell 2004). An essential and necessary step in this effort is the determination of the coding information and expression patterns of each sequenced chromosome. However, until now only about half of the predicted genome-coding capacity had any cDNA or EST expression support. Clearly, experimental approaches complementary to computation-based genome annotation are essential for an understanding of genome structures. Owing to the presence of large amounts of unfinished sequence data, unusual compositional gradients in genes and the large size of the rice genome (Rensink & Buell 2004), there is even greater need for experimental approaches in rice genome annotation.

(i) *Rice chromosome 4 tilling chip: chromosomal level regulation*

A chromosome-scale transcriptional analysis will expand our knowledge of possible chromosomal level transcriptional regulation. One prominent feature of eukaryotic chromosomes is their organization into heterochromatic and euchromatic regions. Heterochromatin was first distinguished from euchromatin cytologically as more intensely staining nuclear material throughout the cell cycle in Bryophyta. In fact, heterochromatin has emerged as a key regulator in the epigenetic control of gene expression, chromosome behaviour and evolution. Using tilling path microarray analysis as a tool, it is now possible to perform high-throughput profiling of the transcriptional activities along an entire sequenced chromosome to examine potential connections between transcription and cytologically defined chromatin organization. Xing-Wang Deng's and Bin Han's laboratories have developed a tilling path DNA microarray consisting of overlapping PCR-amplified genomic fragments covering over 33 Mb (95.5%) of *japonica* rice chromosome 4 (figure 1). Using this array, they analysed the transcriptional activity of chromosome 4 in six representative rice chromosome 4 transcriptome organs or tissues. Chromosomal-scale transcription patterns were analysed in comparison with cytologically observed chromatin organization and the distribution of transposon-related and various other gene model groups (Jiao *et al.* 2005). Six representative rice organ types were examined using this microarray to catalogue the transcribed regions of rice chromosome 4 and to reveal organ- and developmental stage-specific transcription patterns. This analysis provided expression support for 82% of the gene models in the chromosome. Transcriptional activities in 1643 non-annotated regions were also detected. Comparison with cytologically defined chromatin features indicated that in juvenile stage rice the euchromatic region is more actively transcribed than the transposon-rich heterochromatic portion of the chromosome. Interestingly, elevated transcription of transposon-related gene models in certain heterochromatic regions was

observed in mature stage rice organs and in suspension cultured cells. These results suggest a close correlation between transcriptional activity and chromosome organization and developmental regulation of transcription activity at the chromosomal level (Jiao *et al.* 2005; Li *et al.* 2005).

(ii) *Rice cDNA chips*

A 10 K cDNA microarray was developed and used in an analysis of genetic programmes controlling pollination (fertilization and stress responses) in rice (Lan *et al.* 2004, 2005). Yongbiao Xue and his colleagues have identified 253 cDNAs that are regulated by pollination/fertilization in rice by using this cDNA microarray. To investigate this relationship, they obtained the expression profiles after dehydration and wounding treatments in this study. Venn diagram analysis indicated that 53.8% (136/253) and 21% (57/253) of the pollination/fertilization-related genes are indeed regulated by dehydration and wounding, respectively, and nearly half of the genes expressed preferentially in unpollinated pistils (UP) are responsive to dehydration. These results indicated that an extensive gene set is shared among these responses, suggesting that the genetic programmes regulating them are probably related. Among them, the genetic network of the water stress control may be a key player in pollination and fertilization. Additionally, 39.5% (100/253) cDNAs that are related to pollination/fertilization appear not to be regulated by the stress treatments (dehydration and wounding), suggesting that the existence of additional genetic networks is involved in pollination/fertilization. Furthermore, comparative analysis of the expression profiles of the 253 cDNAs under 18 different conditions (various tissues, treatments and developmental status) revealed that the genetic networks regulating photosynthesis, starch metabolisms, gibberelin and defence responses are involved in pollination and fertilization. Taken together, these results provided some clues to elucidate the molecular mechanisms of pollination and fertilization in rice.

(iii) *Development of a rice whole-genome oligonucleotide microarray*

With the complete genome sequence available, it is possible to design a microarray for expression profiling of all known and predicted genes in the rice genome. For this purpose, a rice 70-mer oligonucleotide microarray was designed during the period October–December 2002, in collaboration with the Beijing Genome Institute and Peking–Yale Joint Center of Plant Molecular Genetics and Agrobiotechnology. The oligonucleotide set was based on a combination of FGENESH-predicted gene models from an *indica* rice draft sequence (Yu *et al.* 2002) and the available 28 444 full-length cDNAs (Rice Full-Length cDNA Consortium 2003), 15 558 public EST-derived unigenes (Rensink & Buell 2004) and 31 776 ESTs derived from *indica* rice (Zhao *et al.* 2004) at the time. The following two criteria were used to remove redundancy. First, for gene models overlapping with cDNAs with open reading frame (ORF), cDNA sequences instead of predicted gene models were used for oligonucleotides' design. Second, FGENESH-predicted gene models were used with or without

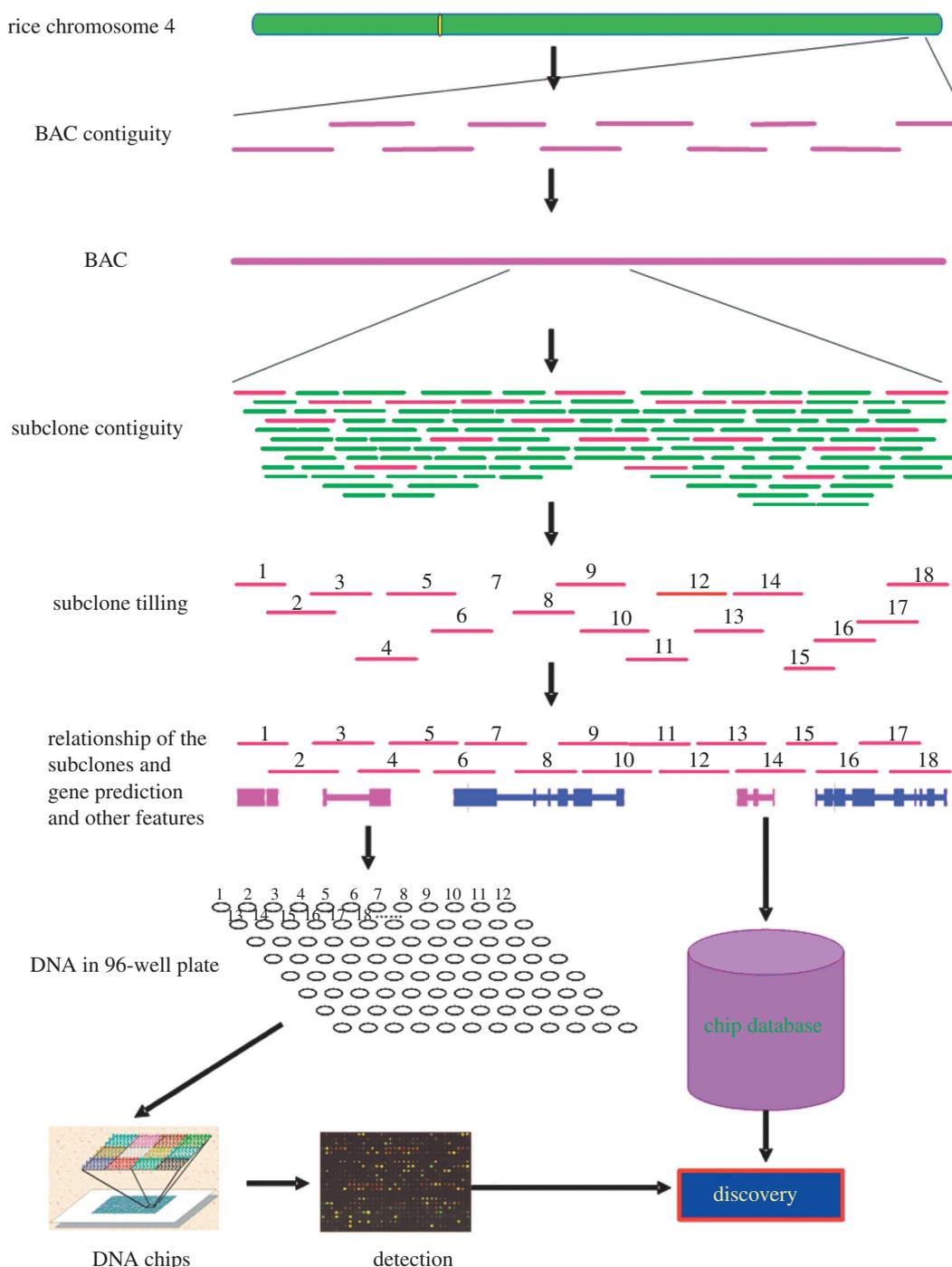


Figure 1. Diagram of rice chromosome 4 tiling chips. BAC, bacterial artificial chromosome.

partially overlapping EST supports. In total, a set of 61 123 non-redundant protein-coding genes or gene models were generated and used for oligonucleotide design. The design of oligonucleotide optimized cross-hybridization, melting temperature and GC-content minimized the potential of hairpin stem formation and biased for 3' ends of the coding region of annotated genes (Wang *et al.* 2003). A total of 58 404 oligonucleotides were designed and custom synthesized. After the recent release of the improved *indica* rice genome sequence and corresponding annotation (Yu *et al.* 2005), we aligned oligonucleotides to updated rice gene annotation. This oligonucleotide set matched 41 754 known and predicted gene models (excluding transposon-related models), including those located in the physically unassembled

rice DNA sequences (Yu *et al.* 2005). Among this group, 70-mer oligonucleotides for 4622 gene models could match another one or more gene models with a 70% identity threshold and have potential cross-hybridization. Therefore, this 70-mer oligonucleotide set covers 37 132 known and predicted genes of the most current version of the *indica* genome (Yu *et al.* 2005) with one-to-one specificity. Oligonucleotides' annotation information is hosted on BGI-RIS databases (<http://rise.genomics.org.cn>). A further alignment of these 37 132 unique oligos to the Syngenta *O. sativa* L. ssp. *japonica* (Goff *et al.* 2002) and the IRGSP *O. sativa* L. ssp. *japonica* (<http://rgp.dna.affrc.go.jp/IRGSP/Build2/build2.html>) sequences indicated that 92% (34 248) of them matched to the *O. sativa* L. ssp. *japonica* genome sequences.

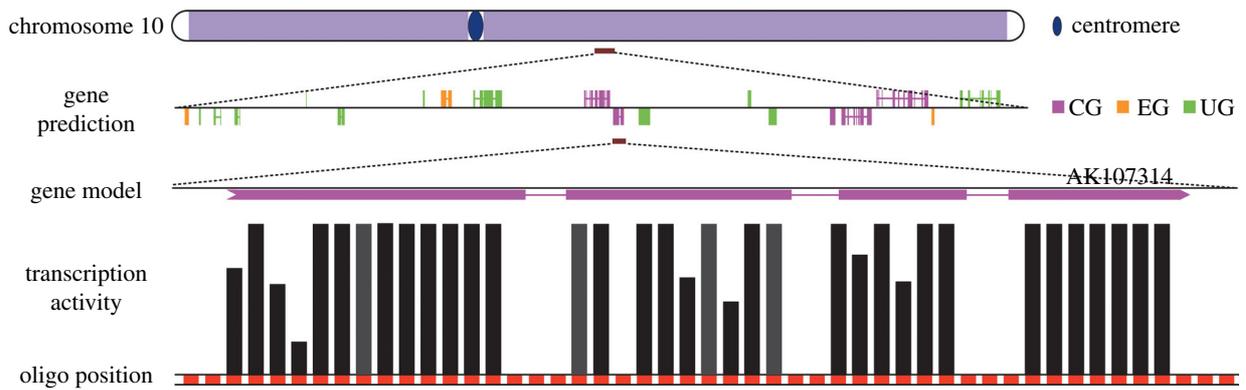


Figure 2. A transcriptional analysis of the entire rice chromosome 10 genome using high-density oligonucleotide tilling microarray.

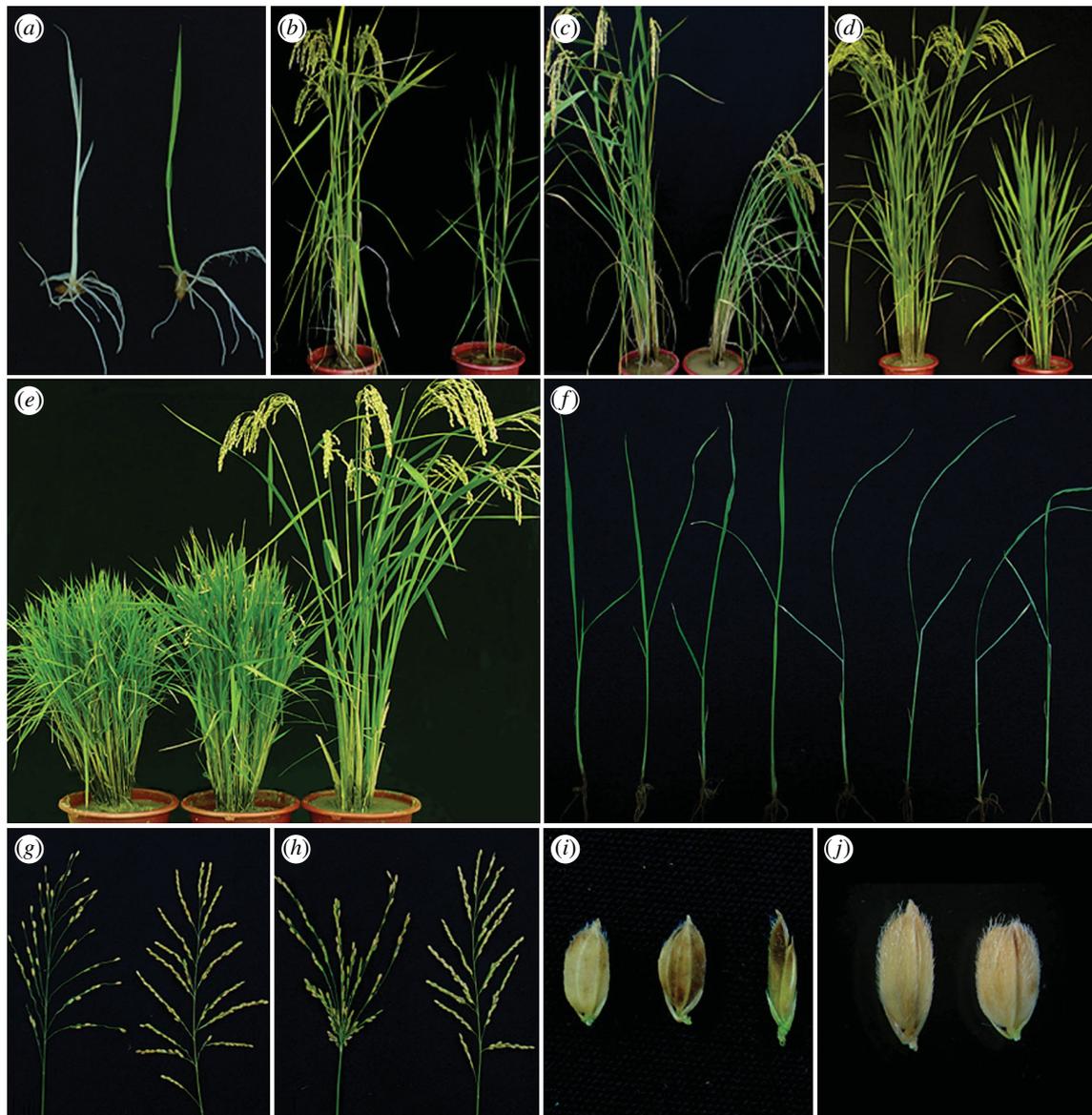


Figure 3. Phenotypes of the various mutants observed in the T-DNA insertion library during field planting (photos provided by Changyin Wu's group): (a) albino seedling, (b) semi-dwarf, fewer tillers with branches on the culm, (c) softer stems, (d) semi-dwarf and later flowering, (e) bushy tillers, (f) more sensitive to cold stress, (g) smaller number of grains per panicle, (h) change in panicle morphology, (i) thinner grains and (j) thicker and shorter grains.

(iv) *Unbiased transcriptional analysis of rice genome using high-density oligonucleotide tilling microarray*
Recent completion of both *indica* and *japonica* genome sequence analysis now allowed an unbiased

interrogation of transcriptional activity of the whole genome and experimental verification of computational genome annotation. The Maskless Array Synthesizer (MAS) platform was chosen for a high-density

oligonucleotide microarray analysis of the complete genomic sequences. Xing-Wang Deng and his colleagues interrogated the non-repetitive sequence of the *indica* and *japonica* genomes using sets of 34 or 32 MAS microarrays probed by labelled cDNA from representative tissues to detect transcriptional activity without any presumption of gene structures. In a pilot test, one of the arrays containing approximately 390 000 individual probes representing a portion of the last 11.2 Mb sequence of chromosome 10 was analysed in detail based on a mathematical model developed (Stolc *et al.* 2005). More recently, a comparative tilling analysis of the entire chromosome 10 of both *indica* and *japonica* genomes has been completed (figure 2; Li *et al.* 2005). This analysis detected expression of about three quarters of the unsupported gene models without previous experimental evidence in both subspecies. This tilling analysis also identified 549 new models for the *japonica* chromosome, representing an 18% increase in the annotated protein-coding capacity. This analysis also revealed that a chromosomal level transcriptional regulation largely coincides with the heterochromatin and euchromatin domains.

(b) Development of a T-DNA tagging system for functional analysis of the rice genome

Reverse genetics aims to determine the functions of genes. One of the best strategies for reverse genetics is based on insertional mutagenesis using T-DNA or transposon as a mutagen (Ito *et al.* 2002). As a powerful strategy for functional genomics study, T-DNA tagging was widely used in many species. The main advantages of using T-DNA tags are that T-DNA insertions are stable and their copy number is low (Delseny *et al.* 2001). Several strategies such as inverse PCR and thermal asymmetric interlaced-PCR (TAIL-PCR) have been used to obtain the flanking sequences of T-DNA or transposons inserted in a plant genome. With their flanking sequences, T-DNA tags can be mapped *in silico* to the chromosomes to reveal the distribution pattern in the tagged genome (Chen *et al.* 2003b; Yang *et al.* 2004).

The Chinese Rice Functional Genomics Programme aimed at a T-DNA insertion mutant library of 200 000 independent transformants. The T-DNA vector adopted three strategies for analysing the rice genome: insertional mutation, enhancer trapping and ectopic expression (Wu *et al.* 2003). Three major groups in Beijing, Shanghai and Wuhan have been involved in this work, and collectively these groups have generated approximately 270 000 independent transformants using a *japonica* cultivar Zhonghua 11. With an average of 2.1 copies of T-DNA insertions per genome, this amounts to a total of over 500 000 independent insertions, which is 10 times the predicted 50 000 genes. A total of 20 000 flanking sequences have been isolated currently. Large-scale screening of mutations is in progress for agronomical important traits including stress tolerance/resistance (rice blast, bacterial blight, temperature, drought and salinity), grain quality, plant architecture, fertility, tillering, nutrition efficiency, photosynthesis, etc. (figure 3). High-throughput platform for quality assays via infrared, in which amount of starch, amylase, protein and fat could be quantitatively measured, has been established. Expression profiling of

the enhancer trap lines has also been conducted by detecting the reporter gene expression in various tissues of the lines in the mutant library. Such screening has produced a large number of mutants that are now tagged for gene isolation and lines with specific expression patterns for identification of regulatory elements such as promoters and enhancers. A centralized database (<http://rmd.ncpgr.cn>) has been developed for this mutant library (Zhang *et al.* 2006).

(c) Full-length cDNA cloning

Full-length cDNAs are essential for the correct annotation of genomic sequences and the functional analysis of genes at both transcriptional and translational levels. Although over 28 000 *japonica* full-length cDNAs have been identified (Rice Full-Length cDNA Consortium 2003), more cDNAs are needed to be cloned since there are approximately 50 000 genes predicted in rice. Additional *indica* cDNA clones may be particularly useful for comparative analysis between *indica* and *japonica* subspecies, improvement of genome sequence annotation and identification of lineage-specific genes because *indica*-specific genes have been found (Zhang *et al.* 2005). The NCGR has built-up full-length cDNA libraries from *O. sativa indica* Guangluai 4 for large-scale collection of *indica* rice full-length expression sequences. Now, five full-length cDNA libraries have been constructed from the germinated seed, seedling, root, filling, etiolated seedling and plants grown under various stresses, respectively (X. Liu *et al.* 2006, unpublished data). The 5'-end ESTs of 100 000 cDNAs have been clustered into 16 000 non-redundant cDNA groups. The 3' end of the 16 000 non-redundant cDNA is under sequencing. First, the highest quality RNAs were prepared in their full-length form, not in truncated form. Second, Superscript II reverse transcriptase, which has a good rate of processing activity, was used in cDNA synthesis in order to obtain cDNAs in their full-length forms, but not in truncated forms. Full-length mRNA and cDNA were selected by the 5' caps. Ligation, transformation and propagation are more efficient for shorter cDNAs than longer ones, thus introducing size bias in cDNA library generation. In our approach, duplex cDNA was size fractionated. Different cDNAs with size ranges greater than 2 kb, 1–2 kb, 0.5–1 kb and 250–500 bp were ligated to the cloning vector, respectively, in order to reduce size bias and improve clone representation. Each full-length cDNA library from plants at various developmental stages and from various plant tissues contains over 106 primary non-amplified clones. Over 90% of the clones are full-length cDNAs containing the complete protein-coding region as well as the 5'- and 3'-untranslated regions (UTRs). The promoter sequences can be obtained by comparing the 5'-end sequences of cDNAs with the rice genome sequences.

In addition, 10 828 full-length cDNAs from another *indica* cultivar Minghui 63 have been characterized (Xie *et al.* 2005). Comparison with the databases showed that 780 of them are new rice cDNAs with no match in *japonica* cDNA database. Totally, 9078 of the full-length cDNAs contained predicted ORFs matching with *japonica* full-length cDNAs, with 6543 finding

homologous proteins. Moreover, 53% of the matched full-length cDNAs had longer 5' UTR than the *japonica* homologues. *In silico* mapping showed that 9776 (90.28%) of them had matched genomic sequences in the *japonica* genome and 10 046 (92.78%) had matched genomic sequences in the *indica* genome. The average nucleotide sequence identity between the two subspecies is 99.2%. More than 60% of the new cDNAs isolated in this study had no homology to known proteins.

Depending on the expression of the cellular mRNA, mRNA can be defined as abundant, intermediate and rare. In a typical cell, 5–10 species of abundant mRNA comprise at least 20% of the mass mRNA, 500–2000 species of intermediately expressed mRNA comprise 40–60% of the mass mRNA and 10 000–20 000 rare mRNA may account for less than 20–40% of the mRNA mass. Sequencing cDNAs from standard cDNA libraries is ineffective for discovering rarely expressed genes, when highly and intermediately expressed cDNAs would be sequenced redundantly. Normalization and subtraction procedures were introduced into the construction of the full-length cDNA libraries to reduce the frequency of highly expressed mRNA in the library and to enhance the discovery of new cDNAs. Subtraction procedures were based on hybridization of the first-strand cDNA with RNA drivers from highly expressed cDNAs and previously sequenced cDNAs or DNA primers designed from already known cDNA of *japonica*. Large-scale new gene discovery was accelerated by using these procedures.

4. CLONING AND FUNCTIONAL IDENTIFICATION OF GENES OF AGRONOMIC SIGNIFICANCE

Cloning and functional analysis of the agronomic important traits including plant architecture, stress tolerance/resistance (bacteria blight and salinity), grain quality, fertility and nutrition efficiency are important targets for China Rice Functional Genomics Programmes. Here, we highlight recent progresses in these studies.

(a) *A gene controlling rice tillering*

Tillering in rice is an important agronomic trait for grain production, and also a model system for the study of branching in monocotyledonous plants. Rice tiller is a specialized grain-bearing branch that is formed on the unelongated basal internodes and grows independently of the mother stem (culm) by means of its own adventitious roots. Rice tillering occurs in a two-stage process: the formation of an axillary bud at each leaf axil and its subsequent outgrowth. Although the morphology and histology and some mutants of rice tillering have been well described, the molecular mechanism of rice tillering remains to be elucidated. Jiayang Li and his colleagues reported the isolation and characterization of *MONOCULM 1 (MOC1)*, a gene that is important in the control of rice tillering (Li et al. 2003a,b). The *moc1* mutant plants have only a main culm without any tillers owing to a defect in the formation of tiller buds. *MOC1* encodes a putative GRAS family nuclear protein that is expressed mainly in

the axillary buds and functions to initiate the axillary buds and promote their outgrowth. To identify genes involved in the control of rice tillering, they have screened for mutants with altered tiller numbers from collections derived from spontaneous mutations or γ -ray radiation and ethyl methanesulphonate (EMS) mutagenesis. A spontaneous *moc1* mutant is of particular interest, because *moc1* plants nearly completely lose their tillering ability, producing only one main culm, in contrast to the multiple tillers in wild-type plants. Genetic analysis with reciprocal crosses between *moc1* and wild-type plants revealed that *moc1* possesses a recessive mutation in a single nuclear locus. Allelic tests between the *moc1* mutant and five recessive tillering mutants with reduced culm number (*rcn1-rcn5*) indicated that *MOC1* is a previously unknown locus that is involved in the control of rice tillering.

(b) *Cloning of GS3, a major quantitative trait locus (QTL) for grain length and weight and a minor QTL for grain width and thickness*

Grain size is a major determinant of grain weight, one of the three components (number of panicles per plant, number of grains per panicle and grain weight) of grain yield. Grain size is also a highly important quality trait in rice (figure 4). Although the preference for rice grain characteristics varies with consumer groups, long and slender grain is generally preferred for *indica* rice by the majority of consumers in China, the United States and most Asian countries. For example, a length/width ratio of 2.8 is adopted as an enforced threshold for a national quality standard of *indica* rice in China (http://www.knowledgebank.irri.org/regionalSites/china/10_CodesStandards/default.htm). Thus, understanding the genetic and molecular basis of grain size is extremely important for rice improvement programmes.

Many QTLs associated with rice grain size were identified in the last decade. Among them, a QTL with major effect on grain size was consistently detected around the centromeric region of chromosome 3 in numerous studies across different genetic backgrounds and environments (Yu et al. 1997; Tan et al. 2000; Xing et al. 2002). Thus, this gene, referred to as *GS3* hereafter, can be very useful for improvement of both yield and quality in rice breeding programmes.

Fan et al. (2006) isolated *GS3* by map-based cloning. Near isogenic lines of *GS3* were developed by successive crossing and backcrossing of Minghui 63 (large grain) with Chuan 7 (small grain), using Minghui 63 as the recurrent parent. Analysis of a random population of 201 individuals from the BC3F2 progeny confirmed that the *GS3* locus explained 80–90% of the variation for grain weight and length in this population. In addition, this locus was resolved as a minor QTL for grain width and thickness. Using 1384 individuals with recessive phenotype (large grain) from a total of 5740 BC3F2 plants and 11 molecular markers based on sequence information, *GS3* was mapped to a DNA fragment approximately 7.9 kb in length. A full-length cDNA corresponding to the target region was identified, which provided complete sequence information for the *GS3* candidate. This gene consists of five exons and encodes 232 amino



Figure 4. Grains of six cultivars, showing great diversity in grain length, width, thickness and overall size.

acids with a putative PEBP-like domain, a transmembrane region, a putative TNFR/NGFR family cysteine-rich domain and a VWFC module. Comparative sequencing analysis identified a nonsense mutation, shared among all the large-grain varieties tested in comparison with the small grain varieties, in the second exon of the putative *GS3* gene. This mutation causes a 178-amino acid truncation in the C-terminus of the predicted protein, suggesting that *GS3* may function as a negative regulator for grain size. Cloning of such a gene provided the opportunity for fully characterizing the regulatory mechanism and related processes during grain development.

(c) Control of rice root formation

Adventitious root formation in rice is involved in the whole development of rice root. Ping Wu and his colleagues identified a novel rice mutant, *arl1* (*adventitious rootless1*), which completely lacks adventitious root during the whole developmental period (Liu *et al.* 2005). They have done cross-sections at the base of the stem of 5-day-old seedlings and found that formation of adventitious primordium is impaired in the *arl1* mutant. Both α -naphthylacetic acid and ethephon at the optimal concentrations to promote adventitious root growth cannot rescue the defect of *arl1*. They cloned and demonstrated that the *ARL1* gene was responsible for the *arl1* mutant phenotype. Subcellular localization of *ARL1* suggests that *ARL1* is a nuclear protein. Yeast two-hybrid experiments suggest that as a nucleic protein, *ARL1* may homodimerize and activate reporter gene. In addition to the characterization of the expression pattern of *ARL1* gene, the response of *ARL1* gene expression to different hormones was also investigated in this study (Liu *et al.* 2005).

(d) Genes related to rice mechanical strength

Plant mechanical strength is an important agronomic trait. To understand the molecular mechanism that controls the plant mechanical strength of crops, Jiayang Li and his colleagues characterized the classic rice mutant *brittle culm1* (*bc1*) and isolated *BC1* using a map-based cloning approach (figure 5). *BC1*, which encodes

a COBRA-like protein, is expressed mainly in developing sclerenchyma cells and in vascular bundles of rice. In these types of cells, mutations in *BC1* cause not only a reduction in cell wall thickness and cellulose content, but also an increase in lignin level, suggesting that *BC1*, a gene that controls the mechanical strength of monocots, plays an important role in the biosynthesis of the cell walls of mechanical tissues. The plant cell wall, a strong fibrillar network that provides mechanical support to cells, tissues and the entire plant body, is a highly organized composite that may contain many different polysaccharides, aromatic substances and proteins. The structure and composition of plant cell walls are ideally suited to the functions they perform. For example, parenchyma cells, which consist of primary walls, provide the main structural support in growing regions of the plant body. Sclerenchyma cells, which have both primary walls and thick secondary walls, provide the major mechanical support in non-elongating regions of the plant body (Carpita & McCann 2000). Cellulose usually constitutes 20–30% of the dry weight of the primary walls and 40–90% of the secondary walls, depending on the cell type (Taylor *et al.* 1999). In some cells, lignin may be incorporated into the cell wall, enhancing its mechanical strength. Despite extensive descriptions of the chemical and physical structures of cell walls, the mechanisms that regulate the deposition of cell wall materials and that determine cell wall strength remain to be elucidated. To understand the mechanisms that regulate the mechanical strength of the plant body and the biosynthesis of plant cell walls, mutants defective in stem strength have been isolated and characterized.

(e) Rice bacterial blight resistance gene

Rice bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most serious rice diseases worldwide. It is not only widespread throughout Asia, but is also reported to occur in Australia, the United States and several rice-growing countries of Africa and Latin America. Yield loss due to the disease ranges from 2 to 74%. A rice gene, *Xa26*, conferring resistance against *Xoo* at both seedling and adult stages was isolated from

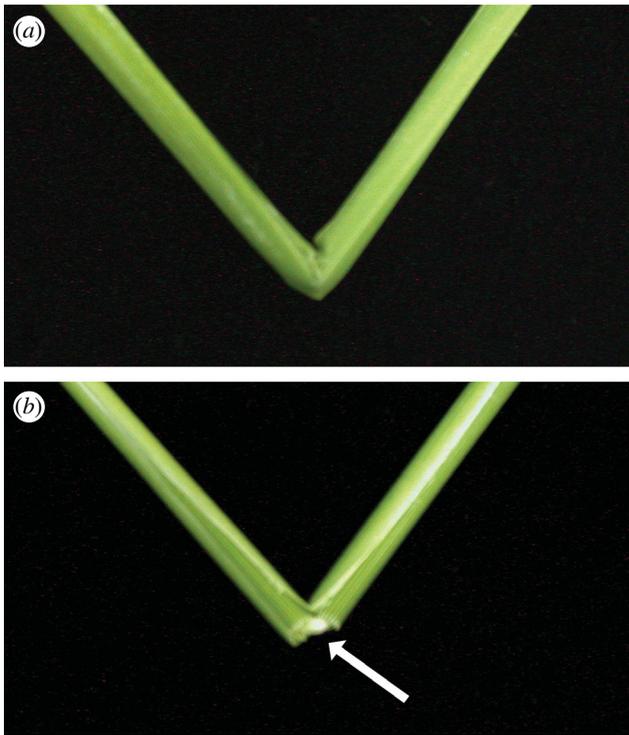


Figure 5. Phenotypes and physical properties of wild-type and *bc1-2* mutant rice plants. (a) A wild-type culm. (b) An easily broken *bc1-2* culm, as indicated by the arrow.

the rice cultivar Minghui 63 using a map-based cloning approach by Shiping Wang's group (Yang *et al.* 2003; Sun *et al.* 2004). *Xa26* encodes a leucine-rich repeat receptor kinase-like protein consisting of 1103 amino acids. The resistance conferred by *Xa26* is influenced by the genetic background such that transgenic plants with a Mudanjiang 8 background carrying the *Xa26* gene had broader spectrum and higher level of resistance than the donor parent. The function of *Xa26* is also dosage dependent. Overexpression of *Xa26* could enlarge the resistance spectrum of rice to different *Xoo* strains. Sequence analysis showed that *Xa26* belongs to a multigene family. Another rice gene, *Xa4* for bacterial blight resistance, is likely to be a member of the *Xa26* family. Comparative sequence analysis of four rice varieties from the two subspecies of cultivated rice, *indica* and *japonica*, showed that the *Xa26* family consists of 4–12 members in different rice haplotypes. This family is formed by tandem duplication followed by diversification through recombination, deletion and point mutation during evolution. The haplotype polymorphism and positive selection for diversification of the *Xa26* family provide sequence reservoir and evolutionary force to generate rapid variation for novel resistance specificities.

(f) Functional analysis of a rice cytoplasmic male sterility/restorer system

Cytoplasmic male sterility (CMS) is a widespread phenomenon observed in over 150 flowering plant species (Laser & Lersten 1972). CMS is a maternally inherited trait and often associated with unusual ORFs in the mitochondrial genomes; in many instances, the male fertility of CMS plants can be restored by specific nuclear genes, called fertility restorer (*Rf*)



Figure 6. NIL (*SKC1*) seedlings (right) are more tolerant to salt than Koshihikari (left) under salt stress (125 mM NaCl for 32 days). It indicated that the QTL *SKC1* is involved in salt tolerance.

genes (Schnable & Wise 1998). Therefore, the CMS–restoration interaction represents a striking example of the genetic interaction and cooperative function of the mitochondrial and nuclear genomes in plants. CMS and nucleus-controlled fertility restoration are widespread plant reproductive features which provide useful tools to exploit hybrid vigour in agriculture. In rice, several CMS/restorer systems defined by the different origins of CMS cytoplasm with distinct genetic features have been identified, including CMS-BT (Boro II), CMS-WA (wild abortive) and CMS-HL (Honglian; Zhu 2000). These systems have been widely used for hybrid rice in China and other Asian countries since the latter half of the 1970s. Hybrid rice usually has yields 20–30% higher than inbred varieties, greatly contributing to the increase in rice production (Li & Yuan 2000). Recently, four *Rf* genes have been cloned from rice (Komori *et al.* 2004). However, very few CMS gene candidates have been functionally tested. The rice male sterile BT-cytoplasm has been primarily identified in a variety, Chinsurash Boro II, of the *indica* subspecies and transferred into a number of *japonica* and *indica* varieties by backcrossing. The mitochondrial genome of the BT-cytoplasm contains two duplicated copies of the *atp6* gene encoding a subunit of the ATPase complex, N-*atp6* and B-*atp6*, and they are transcribed constitutively to produce different lengths of mRNAs, of which B-*atp6* mRNA has an additional sequence downstream of the *atp6*-coding sequence (Iwabuchi *et al.* 1993). In the downstream sequence of B-*atp6*, an ORF, *orf79*, was predicted (Akagi *et al.* 1994). The roles of B-*atp6* and *orf79* in the CMS and the mechanistic connection between the *Rf* gene's function and the fertility restoration are unclear, although it was proposed that the editing extents of B-*atp6* mRNA or lower levels of the antisense transcripts might be related to the CMS and restoration.

Yaoguang Liu and his colleagues identified that, in the BT-type of CMS rice, an abnormal mitochondrial ORF, *orf79*, which is cotranscribed with a duplicated copy of *atp6* (*B-atp6*), encodes a cytotoxic peptide and confers gametophytic CMS (Wang *et al.* in press). Two fertility restorer genes, *Rf1a* and *Rf1b*, are identified at the classical locus *Rf-1* as members of a multigene cluster encoding pentatricopeptide repeat (PPR) proteins. The restorer genes function to restore the male fertility by silencing *orf79* via either endonucleolytic cleavage or degradation of the dicistronic *B-atp6/orf79* mRNA. They further demonstrated that *Rf1a* has a role in promoting the RNA editing of *atp6* independent of the cleavage activity. The results revealed a novel binary post-transcriptional controlling mechanism in plant CMS/restorer systems and have general implications on the function of PPR proteins. Mitochondrial genomes encode only a fraction of the genetic information required for their biogenesis and function. Consequently, a large number of genetic and biochemical features displayed in plant mitochondria arose in the context of coevolution and coordinated gene functions between the mitochondrial and nuclear genomes (Wang *et al.* 2006).

(g) Waxy gene and its regulation

Starch is the major constituent and energy reserve in many important foods, including the seeds of cereal grains and the potato tuber. Starch consists of two types of polysaccharides: amylose and amylopectin. Amylose makes up 0–30% of the starch in the cereal grains. The amylose content varies widely in different plant species and even in different cultivars of same species. Studies on starch mutants of maize, rice and barley have shown that the enzyme granule-bound starch synthase, encoded by the *Waxy* (*Wx*) gene, catalyses the synthesis of amylose. Mengmin Hong and Zongyang Wang have identified that *Wx* transcripts could be detected only in the endosperm, pollen and embryo sac, indicating that the expression of the *Wx* gene is probably regulated at the level of transcription. They previously cloned and sequenced the *Wx* gene of rice and demonstrated that the sequences within the first intron of the gene enhance its expression. Additional studies revealed that post-transcriptional events play important roles in regulating the concentrations of *Wx* protein and the amylose in rice seeds. It was also demonstrated that a sequence (860–640) upstream from the rice *Wx* gene is a positive regulator of its expression. The results of electrophoretic mobility shift assays showed that a 31 bp nucleotide sequence (840–810) located within the 860–640 region is the binding site for nuclear proteins isolated from developing rice seeds. Furthermore, experiments performed on transgenic rice plants containing a 3-glucuronidase (GUS) reporter gene controlled by the *Wx* promoter with the 31 bp sequence present synthesized 2–3 times the amount of GUS as plants carrying the reporter gene and a *Wx* promoter with the 31 bp sequence deleted. These results indicated that the 31 bp sequence plays an important role in the transcriptional regulation of the rice *Wx* gene (Chen *et al.* 2003a; Yang *et al.* 2002; Zhu *et al.* 2003).

(h) Genes involved in tolerance to phosphate starvation in rice

Ping Wu reported that a novel transcription factor was involved in tolerance to phosphate (Pi) starvation in rice (Yi *et al.* 2005). This transcription factor contains a bHLH domain and was designated as *OsPTF1*. The expression of *OsPTF1* is phosphate (Pi) starvation induced in roots while it is constitutively expressed in shoots. Transformation of promoter : GUS fusion construct shows that *OsPTF1* is expressed in phloem cells of primary root and in all cells of lateral roots. Overexpression of *OsPTF1* enhanced tolerance to Pi starvation in transgenic rice. Tillering ability, root and shoot biomass, and P content of transgenic rice plants were approximately 30% higher than those of the wild-type plants under Pi-deficient conditions in the hydroponic experiment. In soil pot and field experiments, approximately 20% increase in tiller number, panicle weight and P content was observed in transgenic plants compared with the wild-type plants under Pi-deficient conditions. Under Pi-deficient conditions, transgenic rice plants showed significantly higher instantaneous Pi uptake rate over their wild-type counterparts. Microarray result demonstrates that *OsPTF1* is involved in the efficient Pi recycling under Pi-deficient conditions, which is important in alleviating phosphorus deficiency in rice under Pi-starvation conditions.

(i) Mapping and map-based cloning of QTL for rice salt tolerance

Soil salinity is a major abiotic stress in crop productivity worldwide (Zhu 2001). It is estimated that saline soils cover from 400 to 950 million hectares of Earth's surface. Improving the salt tolerance in rice is one of the most important objectives of rice breeding programmes in coastal areas. To facilitate the development of new varieties with high level of salinity tolerance, understanding of the genetic control mechanisms for salt tolerance is required. Salt tolerance is a complex trait controlled by QTLs and is the final manifestation of several components, such as Na⁺ uptake, K⁺ uptake, ions balance, ions compartmentation, etc. To map QTLs for rice salt tolerance, we constructed an F2 and an equivalent F3 population derived from a cross between a high salt tolerance *indica* variety, Nona Bokra, and a susceptible elite *japonica* variety, Koshihikari (Lin *et al.* 2004). Eight QTLs responsible for the variations in their K⁺ or Na⁺ content under salt stress were mapped. Out of these QTLs, a major QTL, *SKC1*, that explained 40% of the phenotypic variation in shoot K⁺ content under salt stress was mapped on chromosome 1. To understand the molecular basis for QTL related to ion transport or salt tolerance, we cloned the *SKC1* by using advanced backcross progeny and map-based cloning. It showed that *SKC1* encodes an ion transporter (Ren *et al.* 2005). The complementation test of the candidate *SKC1* gene was successful (figure 6). Physiological analysis suggested that *SKC1* is involved in the long-distance K⁺/Na⁺ transport (from the root to the shoot) thereby regulating K⁺/Na⁺ homeostasis in the shoots under salt stress, suggesting a potential gene for improving salt tolerance in rice.

Trehalose is a non-reducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress in bacteria, fungi and invertebrates. In plants, it also plays important roles in the development and tolerance against various abiotic stresses. Trehalose-6-phosphate phosphatase catalyses the final step in the trehalose synthesis pathway, thereby contributing much to abiotic stress tolerance. We cloned a novel gene for coding trehalose-6-phosphate phosphatase (*OsTPPA*), which is transiently and remarkably induced immediately after 140 mM NaCl treatment (Chao *et al.* 2005). Analysing cDNA and genomic sequences of *OsTPPA* shows that it contains 12 exons, and an initial codon is located in the third exon. Microarray, RT-PCR and northern blotting results show that *OsTPPA* was rapidly upregulated under salt, drought and cold stress in roots and shoots of the salt tolerance variety. To further study its functions and exploit its application potential, we constructed *35S-OsTPPA-sense* and *35S-OsTPPA-RNAi* vectors and transferred them into rice. Salt tolerance of T1 progeny containing *35S-OsTPPA-sense* transgene significantly increased compared with the vector control under salt stress. Drought and cold tolerance of T1 progeny containing *35S-OsTPPA-RNAi* transgene significantly reduced compared with the vector control. These results indicated that *OsTPPA* plays important roles in rice abiotic stress tolerance.

5. CONCLUSION

Rice has become an important model flowering plant for studying many aspects of plant biology. The completion of the rice genome has afforded an unprecedented opportunity for systematic studies of plant gene function. The rice genome sequence provides a complete catalogue of genes that are important for improving not only rice but also other cereals, as functionally important sequences are conserved and may be identified by their similarity. The high-throughput tools and approaches have been developed and used in rice functional genomics research. National and international collaborations (Fischer *et al.* 2000) on rice functional genomics study have accelerated the gene discovery and sharing of the rice genomic resources. There is no doubt that rice functional genomics research will impact on development of a sustainable agriculture in China and worldwide.

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