DBT propelled national effort in creating mutant resource for functional genomics in rice

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In 2007, with the help of DBT, a research project to create mutant resources for functional genomics in rice was launched through a national initiative involving ICAR-National Research Centre on Plant Biotechnology, New Delhi; ICAR-Indian Agricultural Research Institute, New Delhi; Tamil Nadu Agricultural University, Coimbatore; ICAR-Indian Institute of Rice Research, Hyderabad; University of Agricultural Sciences, Bangalore and Punjab Agricultural University, Ludhiana. Genetically well-defined material is a prerequisite for functional genomics. Thus, the project aimed to generate EMS mutants in the background of an upland and short duration aus genotype, Nagina22, characterize the mutants and use them in crop improvement. As of now, nearly 85,000 rice M2 mutant populations have been created under the project. Based on field phenotyping, gain and or loss of function mutants for tolerance to herbicide spray, drought, salinity and resistance to rice leaf and panicle blast, sheath blight and high phosphorus (P) use efficiency under low P field have been identified. Notably, the herbicide-tolerant mutant identified is under the process of registration for distribution to public and private rice breeders under appropriate material transfer agreement. Besides this, the project also aims to serve as a 'National Repository of rice EMS mutant resource' for the researchers involved in rice biology and improvement in the country.

Keywords: EMS mutagenesis, mutant resources, Nagina22, rice.

Introduction

RICE, in addition to being a major food crop worldwide, has emerged as an excellent model plant system for genomic research owing to its relatively small genome size and availability of complete DNA sequence information. Genomics is paving the way for identification and characterization of genes for traits of interest, mining of superior alleles, development of DNA markers for tagging of useful traits and their subsequent use in marker-assisted breeding, genome-wide association mapping for identification of genes and QTLs, development of transgenic crops and comparative genomics of evolutionarily closely related crop species with large and complex genomes for understanding evolution. Though there is a long way to go before we understand rice at molecular level, as a plant and as a crop in its entirety, a small but robust beginning has been made. In this article, we describe the journey India took with the help of DBT with respect to the generation and characterization of mutant resources for functional genomics in rice and where we stand today.

DBT supported venture into structural genomics

Structural genomics includes sequencing of genomes, its assembly, establishing a link between chromosomes and sequence information with the help of genetic and physical maps, and sequence-based prediction of the complete set of transcripts and proteins encoded by the genome. In 2000, the first plant genome sequence was unravelled from *Arabidopsis thaliana*, a popular model system. Soon after this, the International Rice Genome Sequencing Project (IRGSP), of which India was one of the partners, was launched and rice became the first crop genome sequenced[1]. Indian Council of Agricultural Research-National Research Centre on Plant Biotechnology (ICAR-NRCPB) and University of Delhi South Campus (UDSC) undertook the responsibility of sequencing the long arm of chromosome 11 of rice and thus entered the international arena of plant genomics research. The infrastructure created and expertise developed during rice genome sequencing have been extended by DBT to other international genome sequencing efforts namely, International Tomato Genome Sequencing Consortium and International Wheat Genome Sequencing Consortium (IWGSC). Now, India is poised to undertake crop genome sequencing projects

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independently and has already published pigeonpea and chickpea genomes[2,3] whereas mango and jute genome sequencing is near completion (N. K. Singh, pers. commun.).

Structural to functional genomics

With the structural genomics projects well established, rice is now well supported by a wealth of genomic resources such as resequencing data of 3000 rice genotypes[4], high throughput genotyping arrays[5–7] and expression arrays[8]. However, it was realized as early as 2004 by the rice research community that the greater challenge is to unravel the function of the sequence-based predicted genes in the rice genome[9]. Even after a decade, it remains a challenge which is evident from the fact that only a few rice genes have been characterized despite the availability of the complete genome sequence.

Creation of genetic material for functional genomics – international scenario

Genetically well-defined material is the key to functional genomics. The best genetic material for functional genomics could be a library of isogenic lines which can be obtained by either laborious genetic route or easier induced mutant route. Random mutagenesis for generating mutant stocks can be accomplished by use of either chemical/physical mutagens or insertional mutants (IM). Initially it was felt that insertional or knock out (KO) mutants were the best course to adopt for functional genomics[9–11]. As of 2015, at least 16 insertional mutagenesis-based rice mutant libraries were created in 7 different genetic backgrounds either using T-DNA or transposon or retrotransposon tags with one or more trap vectors[12]. The major attraction of insertional mutant libraries was the ability to generate index of flanking sequence tags (FST) of the insertion sites, thereby establishing a clear causal link between a gene and its phenotype. After a decade of experience with KO mutants, it has been understood that FST isolation is laborious and time consuming; causal links between biological function and gene could be established only when there are single copy insertions; IM mutants do have insertional bias and hence they are far from equally distributed across the genome[13]. Despite more than a dozen independent efforts to generate insertional mutant libraries, we are yet to achieve complete saturation of the rice genome[12].

Random mutagenesis using chemical or physical mutagens is expected to compensate for the shortcomings of the insertional mutagenesis essentially because it can give rise to allelic series and hence amenable to TILLING[12,14] and its site of action is random. The spectrum of mutants created in *Arabidopsis* using EMS suggests that the mutations are randomly distributed in the genome without any bias[15]. Moreover, generation of mutant resource is much simpler in chemical or physical mutagenesis and hence easier to saturate the genome. Chemical and or physical mutagenesis in rice for functional genomics was initiated at the International Rice Research Institute (IRRI) in the background of IR64, a superior rice variety widely cultivated across the rice-growing regions in the world, generating more than 60,000 mutants employing EMS, DEB, γ rays and fast neutrons[16]. Soon after, a chemically mutagenized population was generated in the background of a japonica cultivar, Nipponbare, to facilitate TILLING[17].

DBT's venture into rice functional genomics

India, with its freshly gained experience, having sequenced the rice genome as a partner of IRGSP, intended to enter into the era of functional genomics. The major limitation, however, was the lack of the availability of well-defined genetic stocks. To overcome this lacuna, an Indian initiative on 'Generation, characterization and use of EMS-induced mutants of upland variety Nagina22 (N22) for functional genomics in rice' was conceptualized in 2005 which was later launched in 2007 with funding support from the Department of Biotechnology (DBT), New Delhi[14]. This multi-institutional network project, which was funded for six years (2007–13), involved six institutes, viz. ICAR-NRCPB, New Delhi; ICAR-Indian Agricultural Research Institute (ICAR-IARI), New Delhi; ICAR-Indian Institute of Rice Research (ICAR-IIRI), Hyderabad; Tamil Nadu Agricultural University (TNAU), Coimbatore; Punjab Agricultural University (PAU), Ludhiana and University of Agricultural Sciences (UAS), Bangalore. Chemical mutagenesis was opted over IM in the project for the advantages it offers over IM (discussed in the previous section) and also the restrictions on growing transgenic material in field in India. The primary objective of this effort was to generate and characterize a large set of EMS-induced mutants in rice for various traits that are important from the perspective of crop breeding such as yield-related traits, nutrient use efficiency and tolerance to various biotic and abiotic stresses as well as developmental traits. Owing to the successful implementation (described later in this article) of the first phase of the project, DBT has extended its funding to support the second phase of the project in November 2015. For logistic reasons, in place of PAU, ICAR-National Rice Research Institute (ICAR-NRRI), Cuttack, has been included in the second phase of the network.

Choice of genotype for mutagenesis and creation of mutant resource

Since chemical/physical mutagenesis gives freedom as to the choice of genetic background (i.e. unlike IM, the

genetic material need not be amenable for Agrobaterium-mediated transformation), after a very careful examination, an upland and *aus* type short duration rice variety Nagina22 was chosen for EMS mutagenesis[14] (refer for treatment details). N22 has high CO_2 compensation point[18], wide compatibility[19] and tolerance to multiple abiotic stresses such as drought and heat[20–22] and to biotic stresses such as gall midge and white-backed plant hopper[23]. Approximately 85,000 M2 mutant lines have been generated and are available with the network which is being maintained as 'National Repository of Rice EMS Mutant Resource' at NRCPB, NRRI and TNAU. Of these mutants, approximately 7000 have been subjected to screening for one or more traits whereas the others are being subjected to screening for specific traits by the partner institutions. These mutant lines are also available to other scientists of the country for screening for traits of their interest. Besides, screening for forward genetics, the M2 mutant populations are also an excellent resource for TILLING. Under the aegis of DBT, the mutant resource is proposed to be rejuvenated periodically and maintained at the facility available/being created at NRRI and TNAU and NRCPB.

Mutant garden and database

Macromutants identified across partner institutes for variation in plant height, tillering, leaf type, maturity duration (Figure 1), root architecture, panicle length (Figure 2) and architecture, grain size (Figure 3), seed shattering, and stay green trait are maintained in the mutant garden at ICAR-NRCPB. The different kinds of mutant with their frequency are given in Figure 4. A database named Indian Initiative on Generation of Rice Mutants was constructed using DotNet and SQL. Current version of RMD contains the information on detailed characterization using 47 DUS traits for the macromutants. The mutant database allows search for mutants both by phenotype and mutant ID.

Figure 1. Early flowering mutant identified in the EMS mutagenized population of Nagina22. ***a***, Nagina22; ***b***, Early flowering mutant which matures in 65 days bearing a single productive tiller.

Screening mutants for various traits of interest

Two different kinds of selection strategies were followed namely, selective screening and general screening, for identification of gain- or loss-of-function mutants for the trait of interest. Under selective screening, trait-specific selection pressure is applied to identify a mutant. For identification of mutants tolerant to herbicides (Glyphosate and Imazethapyr), this strategy was followed wherein a M2 population consisting of about 100,000 M2 plants was grown infield and sprayed with herbicide repeatedly and over generations. Following this, a mutant tolerant to Imazethapyr spray has been identified, the causal genes are being characterized and the trait simultaneously introgressed in several popular varieties of rice. This strategy can work only for presence/absence or survival related traits.

In the second strategy, the mutants were raised in field and screened for traits such as tolerance to low phosphorus (P), drought, salinity and resistance to bacterial leaf blight and blast[14]. This exercise has given rise to more than 100 drought-tolerant mutants, the details of one have been published[24], 10 mutants tolerant to low P in soil, 1 heat tolerant mutant[25], 4 stay green mutants[26] and 2 blast-resistant mutants. These mutants are being subjected to detailed molecular analysis.

Use of mutants in functional genomics – achievements at a glance

Drought tolerant mutant

In brief, after screening of about 1100 mutant population in field, raised brick bed for root studies and polyethylene glycol (PEG) in hydroponics, a mutant named enhanced water deficit stress tolerant (*ewst1*) at all levels to drought was identified. Morphological, physiological and anatomical studies revealed *ewst1* had higher membrane stability, chlorophyll content, relative water content (RWC), number of closed stomata, and root length under drought as compared to the wild type but it was shorter, had fewer panicles and lesser panicle length and seed test weight. Overall, *ewst1* was similar to WT in 35 of the 38 morphological parameters studied under ambient growth conditions. Comparative genome-wide expression

Figure 2. Representative variation for grain size in the EMS mutagenized population of Nagina22. Upper panel, Long grain mutants; Lower panel, Short grain mutants.

Figure 3. Representative variation for panicle length in the EMS mutagenized population of Nagina22. Left panel, Nagina22 with short panicle mutant; Right panel: Nagina22 with long panicle mutant. Note that the grain size is the same as that of Nagina22.

analysis of *ewst1* and WT helped identify a candidate gene. Efforts are underway to map and identify the causal gene[24].

Another mutant which is tolerant to drought with variation in root morphology (reduced root volume) under drought stress has also been identified and characterized (Mohapatra, pers. commun.).

Heat tolerant mutant

One of the drought tolerant mutants (NH-219) identified from mutagenized population, after a prolonged exposure to drought in field conditions, when tested in ambient field conditions and poly-house structures for heat tolerance was found to be more heat tolerant than WT. Characterization of NH-219 and WT revealed that the mutant had increased plant height under heat stress and lesser percentage reduction in pollen viability, spikelet fertility, chlorophyll content as compared to WT. Mapping using a F4 biparental population derived from NH-219 and IR64, a heat-susceptible variety led to identification of SSR markers linked to leaf senescence and yield attributes[25].

Mapping efforts under the project

During the first phase of the project, two mutants, one heavy tillering type and another short grain mutant have been mapped by crossing them with contrasting rice genotype(s). The tiller mutant was a single gene mutant in *OsCCD7* which is a homologue of *Arabidopsis MAX3* gene involved in outgrowth of auxiliary buds[27]. The short grain mutant was a two-gene mutant (Mohapatra, pers. commun.). The mutants identified for yield-related traits and tolerance to biotic stresses, and low P are also being validated in the project and mapping efforts have been initiated.

The major disadvantage of random mutagenesis is that gene isolation is tedious and one has to follow laborious map-based cloning strategy for the same. MutMap, a method based on whole-genome resequencing of pooled DNA from mutant plants identified from a segregating population of WT and mutant cross was recently proposed to hasten fine mapping with mutants obtained from EMS mutagenesis[28]. This approach is particularly amenable when single or at the most two gene changes have given rise to the mutant type. A major pre-requisite for this approach is availability of high quality sequencing data and assembled genome of WT. Efforts are underway to generate such a high quality sequence for Nagina22

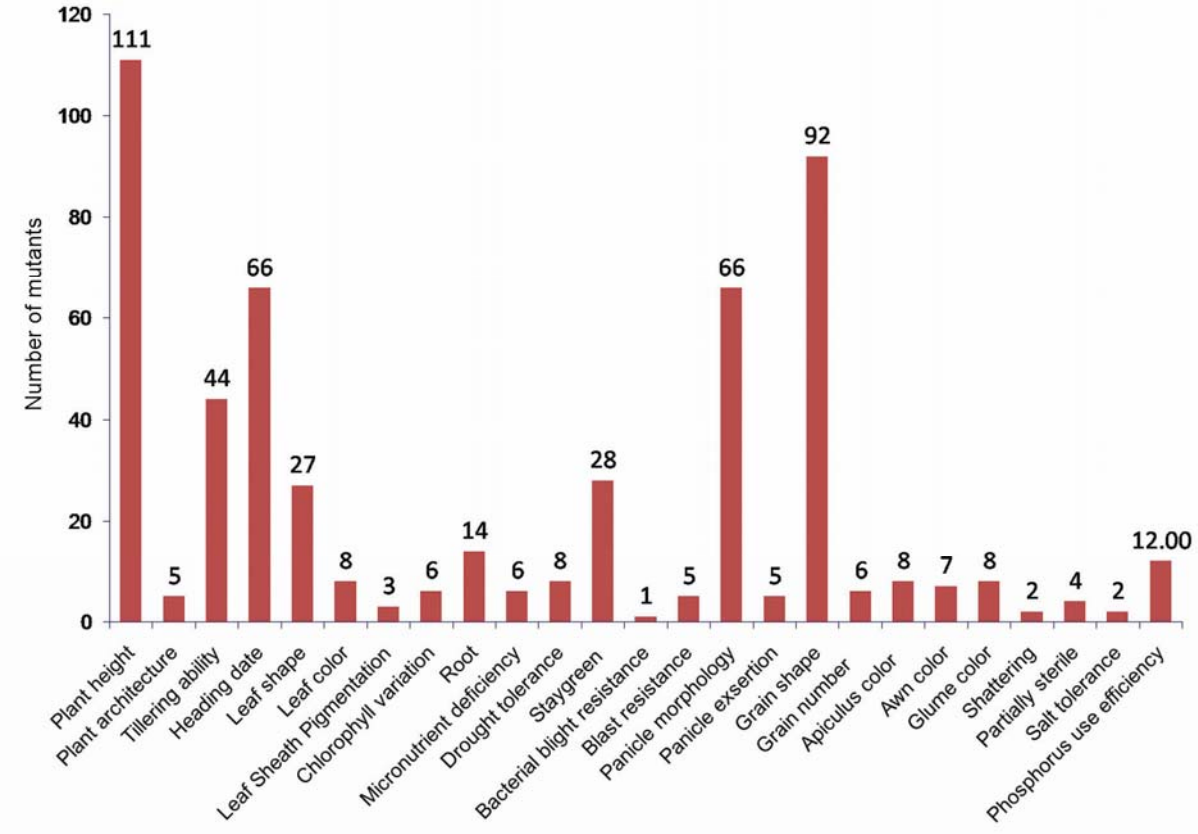

Figure 4. Frequency of different macromutants maintained in the mutant garden.

under the project. MutMap has been successfully applied to seven mutants of a Japanese elite rice cultivar which identified the unique genomic positions most probable to harbour mutations causing pale green leaves and semi dwarfism[28]. Later the same procedure or its modification[29] helped identify the causal genes for male sterility[30], blast resistance[31] and salinity tolerance[32]. In the present network project, Mutmap and Mutmap$^+$ populations are under development for mutants showing tolerance to drought, heat and low P, early flowering, staygreen, root architecture and blast resistance.

Mutants as a genetic resource in rice breeding

Some of the promising mutants identified in the project have been included in station or advanced varietal trials (AVT). Notably, the best low P-tolerant mutant was also tolerant to multiple abiotic stresses and had a high zinc concentration (16–20 ppm) in polished seeds and is in the third year of multilocation testing (AVT-2) of the All India Coordinated Rice Improvement Project (AICRIP)–Biofortification field trials. Most importantly, the herbicide-tolerant mutant identified in this project is now being deployed across partner institutions in breeding commercial varieties for Imazethapyr tolerance. We are in the process of registering the herbicide-tolerant mutant after which it will be made available to all public and private rice breeders under appropriate Material Transfer Agreement (MTA).

Major outcomes and way forward

The major outcome of this endeavour is the establishment of a 'National Repository of Rice EMS Mutants' for the rice research community in the country containing about 85,000 mutant population. The next important step is to screen the mutants for various traits. However, screening is a time consuming and laborious process. As this is a huge facility harbouring nearly 85,000 mutants, it will be rewarding, if rice researchers across the country can join hands and develop innovative, quick and sound screening procedures to screen mutants for traits of their interest and collaborate to identify novel genes and their function.

Author conflict of interest statement

There is no conflict of interest among the authors.

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