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Improvement of rice variety for biotic and abiotic stress resistance using the technique of allele mining

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Abstract
During the crop evolution, humans were interested to select desirable plants for cultivation that leads to the bottleneck of genetic domestication. In this way, domestication and modern breeding strategies reduce the genetic variation of the crop. As a result, the modern-day breeding program concentrates only on the limited genetic resources for crop improvement. Research concentrates to increase genetic variability either by unused genetic resources or induced variation by mutation. Allele mining is one of the techniques to unlock the variation in genetic resources which remain to be the unexploited resources for various agronomic traits. Allele mining identifies the role of an allele with a phenotype that paves the way to develop allele-specific markers and these will be useful in the Molecular Breeding program. This review describes progress made in allele mining and its impact on a rice improvement program.

Keywords: Rice, mutation, allele mining, haplotype, genome sequencing

Introduction
Rice (*Oryza sativa* L.) is one of the cereal crops which supplies essential nutrient, vitamins and minerals to the human diet for their proper growth and development. India is the second-largest producer of rice next to china, about 65 per cent of the country's population depends on rice as a major source. Thus, rice production is an important pillar for the food security of India. During 2017-2018, the productivity of rice in India was 112.91 million tonnes [1]. Breeders are consistently developing new crop varieties of high yielding, good agronomic traits and ideal grain quality character to fulfil the requirement of farmers and consumers by utilizing the available genetic resources *viz*., Germplasm, Landraces, Mutant, Obsolete varieties and Wild species [2-4]. Breeders need genetic variation in concerned crop species and the absence of variation there is no possibility of selection or to develop the new varieties. In earlier days, humans have selected the plants based on their requirements without any sound knowledge of Science and Technology. Unfortunately, this procedure leads to ignoring the reservoir of genetic resource (wild species) as it paves the way to the genetic bottleneck of domestication, narrow down a genetic base and crop attain yield plateau [5-8]. The ultimate challenge is to break the yield plateau in modern varieties and unlock the variation in wild species with the help of the latest technologies *viz*. Marker-Assisted Selection, Genomic Selection, Genome Sequencing technologies and Allele Mining. In this review, we will discuss various aspects of the allele mining technique and its impact on the improvement of the characters of the rice crop.

Allele Mining
It can be defined as the strategy to identify and validate the specific allele related to phenotype for concerned trait using the Polymerase Chain Reaction (PCR) and Next Genome Sequencing (NGS) technologies. It facilitates to discover the Single Nucleotide Polymorphism (SNP), InDel marker in unexploited genetic resources for the various biotic resistance, abiotic resistance, and ideal grain quality parameters. It helps to transfer the desirable allele from wild species into high yielding genotype without linkage drag or to assess the genetic variation of agronomic desirable traits across various genotypes; study the evolution of gene and introduce into the gene pool to increase genetic diversity; identify new haplotype and develop an allele-specific marker for crop improvement programme as well [9-12].
Rice was the first sequenced crop in the collaboration with different countries [13] by taking the advantage of its low genome size (430 Mb), diploid in nature, availability of genetic resources and synteny relationship to the crops under Poaceae family. Recently, IRRI has completed the project of 3000 K in collaboration with various Institutes and to find out allelic diversity in

various genomic resources. Researchers can use the data to find out a genetic variation within species and between species and these variations determine haplotype or nucleotide base occurs at each position. This knowledge provides an opportunity to further enhance and exploit allelic variation for trait enhancement [14-17].

Approaches of allele mining

Technologist follows different strategies to mining the allele for various important traits *viz*., TILLING (identify single base-pair allelic by artificial means of chemical treatment); Eco-TILLING (discover the allelic variation in natural genomic resource); Next Generation Sequence (Genome sequence technology helps to identify the association between allele and phenotype variation) and Sequence-Based Allele Mining (with the help of PCR and Sequencing technology it is possible to construct haplotype)

I. Tilling (Target Induced Local Lesion in Genome)

It is one of the techniques to find out gene function which follows reverse genetic strategy either by a screening of mutation in a gene with known sequence or allow rapid mutational screening to obtain lesion in the gene of interest. Mutation creates a new type of variability which does not occur in nature. Spontaneous mutation can alter the characters of the individual either by substitution, addition or deletion of allele but the problem of spontaneous mutations is that occurrence of mutation in the organism is very low (10^{-5} to 10^{-8} per locus) in frequency, so it is not possible to use in the crop improvement program. For this reason, the development of variation through physical/chemical mutagen is considered to be a viable option. Both mutagens have its advantage and disadvantage as comparatively chemical mutagen is preferable because it induces nucleotide substitution and mutation frequency [17, 18, 19]. The procedure is as follows:

a. Development of the mutagenized population through chemical mutagenesis method.
b. Extraction of DNA samples of desirable plants from M_2 generation.
c. The pooling of eight DNA samples and conducts the Polymerase Chain Reaction using fluorescent dyes.
d. Identify gene sequence and variation with the help of the Li-COR analyser

a. Development of mutagenized population through chemical mutagenesis method

The protocol begins with the selection of disease-free seed (virus, fungi, and bacteria), free from insect infection and discards if any shrivelled seed from the seed lot. The most commonly used technique starts with immerse of the seed in at least 2-3 times the volume of the water for at least 10 hours. The pre-soaked seeds are incubated in different concentration of chemical mutagen (Ethyl Methanosulphonate (EMS) is most commonly used mutagen because it induces base substitution of G/C to A/T thereby it influences genetic and morphological traits of the crop) in the combination of 0.1 M sodium phosphate buffer (pH 7.0) for 16 hours at room temperature with gentle agitation (50 rpm) to determine Lethal Dose 50 (LD 50); It is a common parameter to decide the effective dose of chemical mutagens and based on the survival rate of different concentration of EMS with control, the optimum concentration (Letha Dose 50%) will be used for the conduct the experiment. After the incubation period with mutagen, the seeds are continuously washed with water to remove traces of mutagen from the surface of seed [20-23].

The EMS treated seed (M_1 population) will be sown in the field immediately after treatment or if it is not possible for sowing due to external factors then delays sowing by the store the seed in godown with 13% moisture content to avoid any biotic damage to seeds. M_1 plants are in heterozygous condition (only dominant allele will be expressed) for the target gene so the breeder has to harvest M_1 plant either by plant to row or spike to row to develop segregating population of the dominant or recessive allele in M_2 generation. In M_2 generation, Breeder has to select and study the mutant plant carefully since this generation contains more variable mutants either by visual, mechanical, and physical screening. The plant characters *viz*., vigorousness, plant stature, resistance to disease and seed size, shape, and weight can be evaluated through the method of visual screening and mechanical/ physical methods respectively. The ultimate aim is to identify the desirable mutant plant in segregating the population whether the mutant plant possesses a unique character than the original strain variety or combination of characters [24-26].

b. Extraction of DNA samples of desirable plants from M_2 generation

The molecular biology studies require high-quality DNA from desirable mutants of M_2 generation to study the function of genes. The most commonly followed method is to isolate genomic DNA from various plant sources is that CTAB (Cetyl Trimethyl Ammonium Bromide) method as modified [27].

c. The pooling of eight DNA samples and conducts the Polymerase Chain Reaction using fluorescent dyes

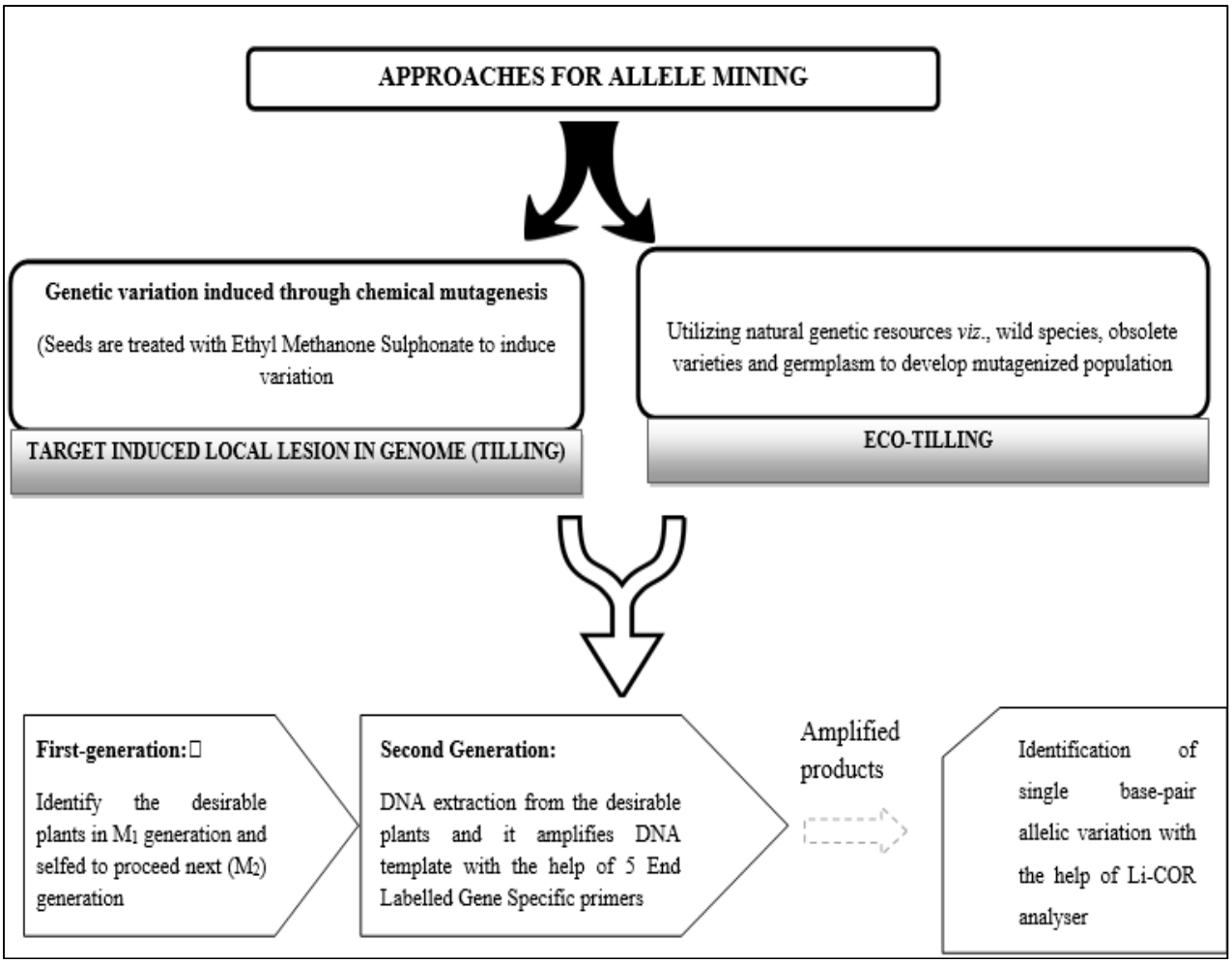
The protocol begins with the pooling of eight extract DNA samples of desirable mutant plants into 96 well micro-titer plates for Polymerase Chain Reaction Analysis (PCR). PCR amplifies a DNA template to produce specific DNA fragments *in vitro* with gene-specific primers fluorescence dyes [28, 29]. The amplified products are denatured (separation of double-stranded DNA) by heating and annealing (joining of double-stranded DNA) by cooling so that it leads to the formation of heteroduplex.

d. Identify gene sequence and variation with the help of the Li-COR analyser

The heteroduplex molecules became the substrate for enzymatic mismatch cleavage by endonuclease (Cel I, S1 nuclease and Mung Bean Nuclease) [30, 31, 32] and the allelic variation in the target gene is examined through the Li-COR. PLACE, Plant CARE, JASPAR, MEME, DB, BioEdit, *etc*. are commonly used bioinformatics tools in allele mining to study, compare the allelic variation to reference genome thereby identify markers of Single Nucleotide Polymorphism and InDEL [33, 34].

II. Eco-Tilling

It refers to develop the TILLING population from the available genetic resources *viz*., wild species, landraces, mutant, obsolete varieties, and germplasm without the use of any chemicals. The logic behind to use of Eco-TILLING is that in most of the crops are not a response to chemical mutagenesis, in that case with this help of technology to identify allelic variants and its association with this phenotype [35, 36]. Eco-TILLING utilizes modern genotyping technologies *viz*., Li-COR, WAVE-HS liquid chromatography, ABI 377 sequencer to identify and tap the unexploited natural novel candidate gene either SNP or In DEL from the natural population [37-39].



III. Next-Generation Sequencing (NGS) for Allele mining:

Next Generation sequence technology *viz.* Illumina/Solexa sequencing, Roche/454 sequencing and Applied Biosystem/SOLiD sequencing can be used to assess the allelic variation in the targeted sequence region based on Massive Parallel Sequencing (automated protocol for preparation and construction of the barcoded libraries). DNA Re-sequencing technologies and analysis of automated NGS data have paved the way to discover the variation in candidate genes for important traits which is essential for the breeding program [40-43].

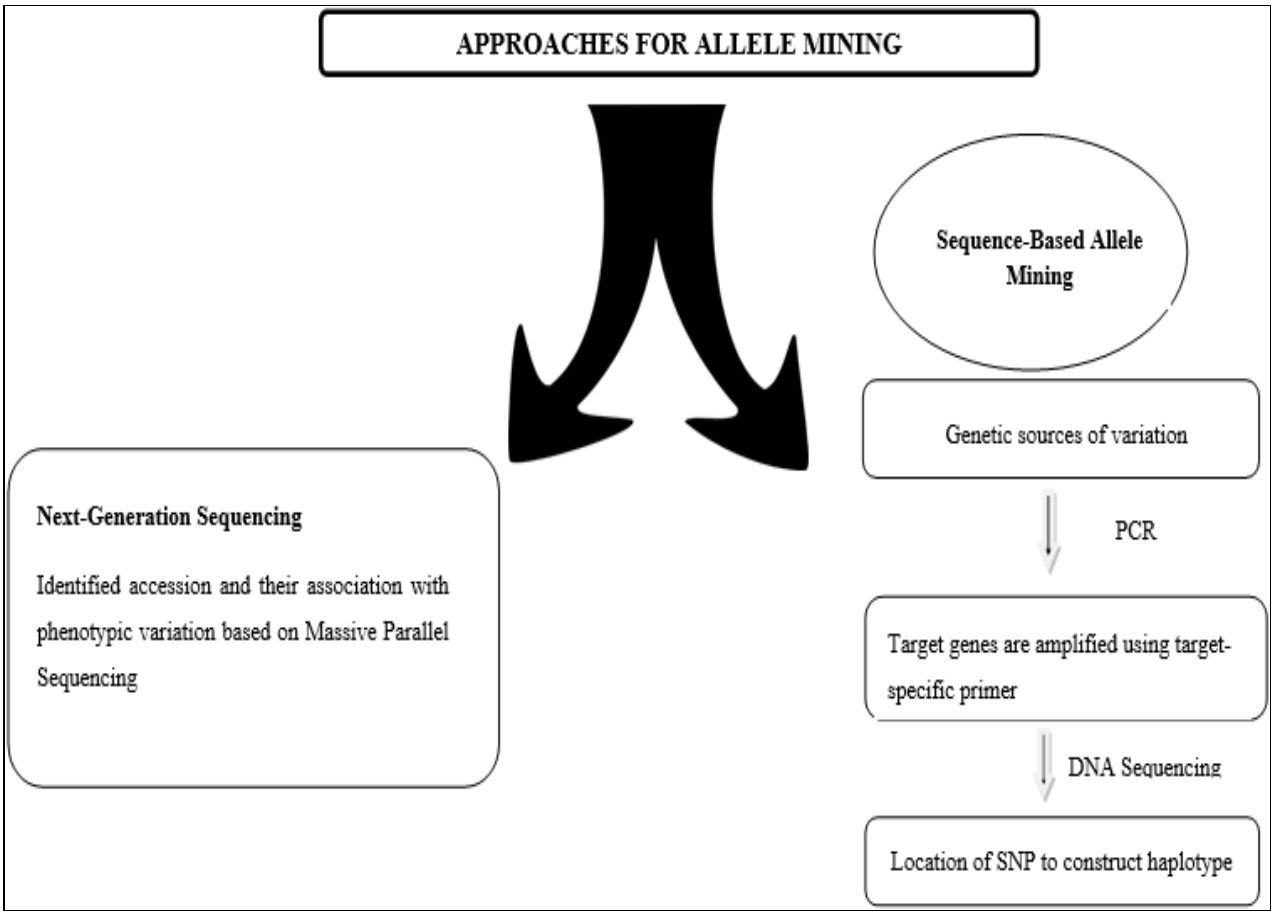
IV. Sequencing Based on Allele mining

This approach using the technique of Polymerase Chain Reaction (PCR) and Next Genome Sequencing technologies is to reveal the allelic variation in a natural population. The technique can be used to sequence and identify the mutation or the presence of Single Nucleotide Polymorphism (SNP) or observed the insertion/deletion as InDEL in target sequence to develop a Haplotype. This method defines the haplotype structure of the genome and discovers the relationship between the genome region and its phenotypic variation of the trait and it also serves as a viable solution to develop allele-specific markers [44-46].

Application of allele mining for the improvement of various characters in the rice

Rice Blast (Causal organism: *Magnaporthae grisea*) is considered to be a destructive disease which impedes the productivity of the crop. Breeding varieties with genetic resistance is an economical, effective, and sustainable approach to combat this problem. However, the widespread cultivation of resistant variety leads to accelerate the evolution of new virulent strains against resistant gene most commonly termed as Boom-Bust cycle. So, the ultimate objectives of the disease-resistant breeding program are to develop the variety by utilizing allele mining. Scientific communities study the sequence variation in the blast resistance gene *viz*., *Pib*, *Pita* and *Pi54* among various cultivated and wild relatives and develop allele-based markers and it could be used in gene pyramiding *i.e*., by utilizing the various resistance sources of allele into single variety to increase durability [47-49].

Rice Yellow Mottle Virus (RYMV) induces the characteristic symptom of yellow or orange leaf discolouration, stunting, sterility empty spikelet's and at the extreme condition yield loss up to 100% [50]. Recently various allelic variations in *RYMV1*, *RYMV2*, and *NLRRYMV3* found in the wild accession of *Oryza glaberrima* [51]. In future rice breeding program, there will be the possibility to develop resistant variety by utilizing the haplotype-based markers for the RYMV resistant gene.



Drought, salinity, and cold stress are going to major constraints for rice cultivation which not only affect the yield but also rice crop productivity. Recently various rice communities were identified and characterized the genes of *DREB2*; *DREB1*, *DREB2, HKT1* and *HKT 2* for drought-tolerant, cold tolerant and salinity respectively. To develop tolerant varieties to this abiotic stress, Breeders have to screen more variants in germplasm and discover the association of SNP with abiotic stress tolerant genes [52-54].

Conclusion

With the advancement of technologies and importance to enhance the diversity of different agronomic traits, allele mining has become most commonly practised in the almost international and national institute. Allele mining not only explores the genetic diversity but also the identification of novel alleles, haplotypes-based markers which maybe become an effective tool in the rice crop improvement program. In future days, breeders have the choice of using genetic resources in combination with allele mining to utilize a diversity of germplasm to develop ideal variety which feeds to the ever-increasing human population.

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