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Advances in induced mutagenesis and mutation mapping approaches in rice

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ABSTRACT

Induced mutagenesis has been proven to be a successful strategy for the improvement of several crops including rice. In the present review, different induced mutagenesis approaches have been discussed concerning the efficient exploration for rice improvement. Significant efforts and the popular rice varieties developed through the mutagenesis approaches was also well focused. Apart from the use for direct trait improvement, mutagenesis is also important to perform forward and reverse genetics for the characterization of novel genes and biochemical pathways. In this regard, precise mapping of casual mutation has great importance. Recent development in next generation sequencing (NGS) technology has provided a great opportunity to pinpoint the causal mutation with great precision and affordable manner. Here, NGS based approaches like MutMap, MutMap+ and MutGap have been discussed. Similarly, advanced bioinformatics methods like Simultaneous Identification of Multiple Mutations (SIMM) are also highlighted. In addition, we have provided a catalogue of online database of rice mutant lines concerning efficient utilization of available resources. The information provided here will be helpful to better understand recent advances in mutagenesis research and its efficient utilization for the rice improvement program.

Key words: *Chemical mutagenesis, physical mutagenesis, mutation mapping, MutMap, rice*

INTRODUCTION

Rice is one of the most important global staple foods along with wheat and maize. It is the dominant cultivated crop plant especially in Asia (Droc et al., 2019). World population is expected to be doubled in next 50 years and this creates an urgent need to devise methods and techniques to meet the increasing global food and nutrition demands. Modern high-throughput techniques have paved the way for development of elite crop varieties with desired agronomic traits. Rice is a model crop plant as it was the first crop genome to be sequenced (Project and Sasaki, 2005). Since, rice is a major source of calories for populations in developing and under-developed nations, it is imperative to elucidate biological functions of sequenced rice genetic regions for agronomical important traits like better yield, grain quality, stress tolerance etc (Wei et al., 2013).

One of the pioneer techniques which was utilized to produce rice varieties with desirable traits was breeding. Breeding by introgression of favorable alleles providing better yield, grain and nutritional quality along with higher tolerance against different stresses from the wild germplasm is time taking. It takes time to recover the genetic background of the high yielding elite cultivar. In this regard, induced mutagenesis provides an opportunity to create allelic variations by minimum disturbances in the well optimized genetic background of the high yielding cultivar. A rather simpler approach, mutagenesis is challenging due to the randomness of

the mutations and the possibilities to get desired changes are completely based on the luck. However, such induced mutagenesis approaches provide an opportunity to identify novel genes and study their functional regulations. Since rice is a self-pollinated crop, increasing the genetic base by crossing has limitations. Mutagenes overcomes these constraints and paves the way for crop improvement. Moreover, mutagenesis methods are exempt from much regulatory scrutiny as faced by other crop improvement techniques *viz*., genetically modified crops (Parry et al., 2009). In addition, mutagenesis approaches can target oligogenic and polygenic traits in crop plants. Thus, mutagenesis is found to be an excellent tool to study plant biology which has been explored for the trait development as well as for the genetic studies in many crop plants including rice.

Approaches for the induced mutagenesis

Induced mutagenesis is termed as an essential tool for crop improvement which may create new alleles. Mutagenesis is also a powerful method to generate genetic diversity. Mutagenesis is of two types: spontaneous mutagenesis and induced mutagenesis. Spontaneous mutations occur naturally in plants but these can also be induced in plants through exposure with different types of physical or chemical agents having mutagenic properties (Mba et al., 2010). For identification of novel genes and their respective functions, there are different types of mutagenic approaches such as ionizing radiation, chemicals, RNAi mediated interference and T-DNA insertion (Tadege et al., 2009) in plant biologies. In RNA-mediated interference (RNAi), a gene complementary to the target gene, for example, artificial microRNAs (amiRNA) is transformed in plants where it gets processed through dicer pathway and produces the small silencing RNA molecules that degrade targeted gene (Gilchrist and Haughn, 2010). Insertional mutagenesis is a technique which intrudes the DNA sequences through T-DNA or transposons insertions (Topping and Lindsey, 1995). T-DNA or transposon insert between the DNA randomly, results in the formation of deformed protein or transcript (Krysan et al., 1999). On the other hand, chemical and ionizing radiation mutagenesis approach are relatively more flexible and straight forward (Wu et al., 2005). For the development of new varieties with enhanced traits, physical and chemical mutagens are successfully applied in plant breeding programs (Kodym and Afza, 2003). Most commonly used chemical mutagen is Ethyl Methane Sulfonate (EMS) which is an alkylating agent. Other chemical mutagens such as ethyl nitrosourea (ENU), EtBr (ethidium bromide) or Bromouracil (Mba et al., 2010) are also used. Gamma rays, X-rays, UV, alpha particles and fast neutrons are used to generate ionizing radiation (Kodym and Afza, 2003). EMS treatment results in point mutation whereas ionizing radiation result in both deletions as well as a point mutation (Wu et al., 2005). A detailed method for EMS-induced mutagenesis is shown in Fig. 1. Another approach which creates large deletion and chromosome rearrangement is fast neutron mutagenesis (Gilchrist and Haughn, 2010). In this, the plant is treated with neutron irradiation or through neutron bombardment which creates a mutagenized population (Li et al., 2001).

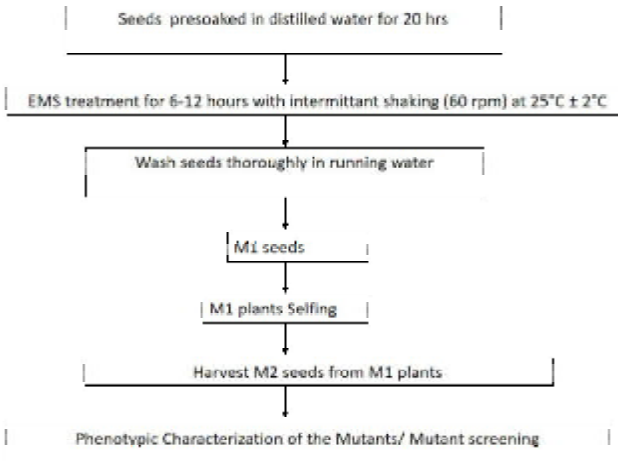

Fig. 1. Generalized flowchart showing steps involved in EMS mutagenesis in rice.

Mutagen dosages to raise mutant population in rice

The frequency to induce mutation differs from plant to plant and it also depends on the amount of mutagen to which it is exposed. The correct dose of mutagen prior to treatment must be known to get desired results. It can be taken from previous experiments but if no prior work is done then pilot experiments need to be performed to know about the optimal dosage for induction of mutation. For example to know about the optimal dosage for EMS-induced mutagenesis, rice

seeds are treated with different concentrations of EMS such as 0.5%, 0.75%, 1%, 1.5%, and 2%. Thereafter, the desired optimal dose is calculated which kills 50% of the population; this is known as the lethal dose. After calculation of lethal dosage, seeds are treated in bulk with the optimal EMS concentration. These treated seeds are sown to get M1 population. Subsequently, selfing of M1 plants produce M2 lines which segregate. Then, homozygous plants are selected from M3 lines. The system from induced mutation to stable improved breeding lines generation is shown in Figure 2.

Significant induced mutagenesis efforts in rice

Different methods like chemical mutagenesis, irradiation methods, T-DNA insertion, Tos retrotransposons and transposable elements Ac/Ds have been extensively used in rice improvement programs (Wei et al., 2013). A great progress made in mutagenesis is clearly visible from the efforts of various researchers which help in understanding the molecular biology. Moreover, a lot many rice pre-breeding lines with improved traits have been developed through this mutation approaches which are being used in rice breeding. For instance, utilization of T-DNA insertion mutagenesis produced a large population of 18,358 fertile lines from 22,090 transgenic plants which will be useful for discovery of novel genes in rice (Jeon et al., 2000). Similarly, another seminal study developed a new T-DNA vector, Pga2715 through which 13,450 mutant lines were generated; these lines can be used to analyze promoter activities as the promoter-less beta-glucuronidase (GUS) found to be reporter gene is present in it (Jeong et al., 2002). T-DNA mutants are advantageous as the method creates low copy number of insertions (Radhamony et al., 2005). In addition, techniques like transposon, chemical and irradiation mutagenesis have their respective advantages.

Transposon mutagenesis in rice utilizes *Tos, Ac/Ds* and *En/Spm-sSpm* system among other methods. A number of studies have been conducted to analyze the specificity of various retrotransposons. In one such study, analysis of 4316 *Tos17* mutant rice lines proved its significance in insertion mutagenesis at genic regions as compared to intergenic regions (Miyao et al., 2003). Similar studies have been undertaken to assess mutant sites and discussed the utility of different selectable markers, site-specific recombination mechanisms in rice for *Ac/Ds* and *En/Spm-sSpm* system as well. A study conducted in *Oryza sativa* ssp.

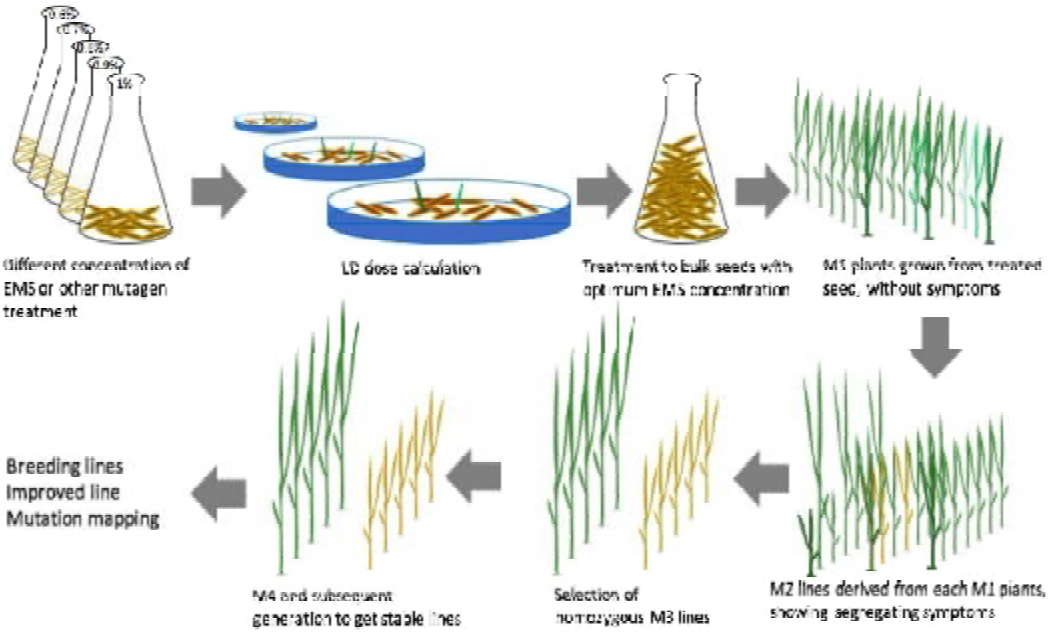

Fig. 2. Generalized steps involved in mutagene dose optimization and subsequent development of mutant population in rice.

Table 1. List of significant efforts made towards the development of rice mutant population

Technique	Description	Examples
Chemical mutagenesis	Ethyl Methyl Sulfonate (EMS), ethyl methane sulfonate (MMS), N-nitroso-N-methyl urethane (NMU), hydroxyl amine (HA), sodium azide (NaN(3))	Evaluation of effect of EMS in 340 mutant rice families from single cultivar (Luz et al., 2016) Investigation of azide mutagenesis in rice (Rao and Reddi, 1986) Characterization of EMS induced mutants in Nagina22 rice variety (Mohapatra et al., 2014)
Physical mutagenesis	Gamma rays, X-rays, Fast neutrons	Effects of physical and chemical mutants evaluated in Tellahamsa and IR-24(Rao and Rao, 1983) Gamma rays used to breed reimei variety with mutations for culm length and lodging resistance (Futsuhara, 1968) Comparative study of ion beams and gamma rays efficiency in rice mutagenesis (Yamaguchi et al., 2009)
Insertional mutagenesis	T-DNA, Ac/Ds and En/Spm-sSpm	Epigenetic regulation of Tos 17 by gene silencing discussed (Cheng et al., 2006) Gene tagging efficiency of Ac/Ds system evaluated (Chin et al., 1999) Development of En/Spm system for mutagenesis in rice (Kumar et al., 2005)
Nuclease based mutagenesis	TALEN, ZFN, Crispr/Cas	Heritable multiple base mutations and deletions induced by TALEN in rice (Zhang et al., 2016) Zinc finger nucleases in rice target gene identification (Cantos et al., 2014) OsMYB1 gene targeted in rice using CRISPR /Cas(Mao et al., 2013)

Japonica cv. Nipponbare showed higher transpositional events in earlier generations as opposed to later mutant generations with *Ac/Ds* mutagenesis (Greco et al., 2003). Further, chemical and physical mutagenesis is a quicker approach which uses lesser number of mutant populations (Henikoff and Comai, 2003). Wu et al. (2005) developed 60,000 IR64 mutants through chemical as well as physical mutagenesis for forward and reverse genetic studies. Additional methods employed for mutagenesis include TALEN (Transcription activator-like effector nucleases, CRISPR/Cas (clustered regularly interspersed short palindromic repeats), ZFN (zinc-finger nuclease) among others. These mutagenesis techniques are being used to deduce gene functions and regulation in plants. In this regard, a study was undertaken to explore role of SS1Va enzyme in starch biosynthesis by introduction of double-stranded breaks using ZFN in rice (Jung et al., 2018). Similarly, TALEN based studies have also helped in developing rice mutant varieties with better qualities; the developed rice variety through TALEN approach showed better resistance to bacterial blight disease (Li et al., 2012). Recently, utility of CRISPR/Cas has been analyzed in model crop plants like rice (Miao et al., 2013). Hence, mutagenesis techniques are being continuously developed which are enlisted in Table 1. The mutation technique has provided a wide array of applications in improving crop plant varieties for various traits.

Rice varieties developed through mutagenesis

Numerous novel varieties have been developed using mutagenesis in rice as well as other crop plants for higher yield, better grain qualities and stress tolerance. For example, rice variety MR 219 from Malaysia was mutated using gamma rays for tolerance to blast disease, higher yield and less water requirement in 2015. Similarly, a high yielding mutant variety, *Si Denok* from Indonesia was approved in 2011. Rice mutant lines have also been developed in India *viz*., ADT-41 developed in 1994 using gamma rays had features like semi-dwarfness, large grains, mild aroma, high yield, but was susceptible to major pests and diseases. Likewise, another variety, Anashwara, was developed in India using gamma rays in 2006 which is a photoperiod sensitive semi-tall rice variety and suitable for *rabi* (post-rainy season). To get descriptive information about the varieties, an extensive mutant variety database (https://mvd.iaea.org/) has been developed. Here, we have provided the information of a number of rice varieties developed through mutagenesis by retrieving the data from mutant variety database (Table 2). The

Table 2. Rice varieties developed by mutagenesis.

Variety	Registration year	Trait	Country
NMR 152	2015	Minimal water requirement, Tolerant to blast disease, High yield, and Longer panicle length	Malaysia
PandanPutri	2010	High yield, early maturity, tolerance to BLB and good quality	Indonesia
Zhejing 41	2009	Medium maturity japonica rice variety, medium tillering ability, large panicle, high yield, good grain quality, the resistance to blast and bacterial leaf blight and resistance to brown plant hopper	China
Jianuo 1 You No.6	2009	High yield, good grain quality, good agronomic traits	China
Jianuo1 You No.3	2009	High yield, good panicle, resistance to blast, good quality	China
Yixiang 907	2009	Early maturity, high yield, good panicle and good quality	China
Zhefuliangyou 12	2009	Seed production traits, high yield, good quality and resistance to disease	China
VN24-4	2009	Bigger panicles, stiff culms, strongly seedling vigor, high tolerance to pest & diseases (BPH & GSV) and adverse condition	Vietnam
Peiza 130	2008	High yield, resistance to fungal diseases and early maturity	China
Peizahangxiang	2008	High yield, resistance to bacterial leaf blight, good eating quality and early maturity	China
Liangyouhang 2	2008	High yield, resistance to fungal diseases, resistance to blast and bacterial blight and good grain quality	China
Neiyouhang 148	2008	High yield, blast resistance and late maturity	China
Zhejing 28	2008	High yield and good grain quality	China
Hangxiang 18	2008	Late maturity and resistance to fungal diseases.	China
Guangyinruanzhan	2008	High yield, high quality, resistance to blast and bacterial leaf blight	China
Jahesh	2008	Short stature, early maturity, high yield and tolerant to stem borer and blast disease	Iran
Partou	2008	Short stature, early mature, high yield, tolerant to stem borer and blast disease	Iran
Bestari	2008	High yield, early maturity, tolerant to BLB and good quality	Indonesia
VN121	2008	Better plant type, high yield, good quality (aroma, long grain, no chalkiness) and tolerant to BPH, BL, GSV	Vietnam

previous experience of rice community with development of mutant variety was particular whereas existing variety targeted to modify a monogenic trait looks satisfying. However, with the advent of new technique, the causal mutation can map easily to add up value to the program which has dual targets of achieving trait improvement as well as genetic understanding.

Approaches for the mapping of casual mutation in the rice genome

The availability of efficient, rapid and cost-effective mapping technology is of utmost importance to exploit the application of mutagenesis for the crop breeding and improvement. The advent of high throughput Next Generation Sequencing (NGS) technologies has pushed the bar high for all sorts of genetics mapping techniques. Some of the techniques like Mutmap, MutMap+, Mutmap-Gap and Genome-wide sequencing (GWS) are based on sequencing platforms and are very popular approaches to map the mutations. However, such approaches are not yet routinely used by the rice community and rather restricted to few labs. Extensive training, online resources and interdisciplinary collaborations are some of the aspects that can help to explore these advances at its full capacity. In this research we have discussed the recent advances in mutation mapping approaches which can be useful for rice researchers.

Mutmap and related mapping methodologies

MutMap is a very effective forward genetic approach to study mutation in small or medium size crop genome. It is based on inducing a mutation in the parental line. As shown in Fig. 2, plants with homozygous recessive desired phenotypes are backcrossed with its wild type parents that are followed by selfing of the F_1 population.

Table 3. Details of online database for rice mutants.

Institution	mutagen	Mutated Loci	*FSTs/Screen	*FST Lines Availability	Genotype	Database Web link
CSIRO Plant Industry, AU	Ac-Ds GT/ET	16,000	611	Approximately 50% lines no seed	Nipponbare	http://urgi.versailles.inra.fr/OryzaTagLine
EU-OSTID, EU	Ac-Ds ET	25,000	1,380	1300	Nipponbare	http://orygenesdb.cirad.fr/
NIAS, JP	Tos17	500,000	34,844	34,844	Nipponbare	http://tos.nias.affrc.go.jp
Zhejiang University, CN	?-ray EMS	40,000	-----	Selected lines	Kasalath SSBM	http://www.genomics.zju.edu.cn
Gyeongsang National University, KR	Ac-Ds GT	30,000	4,820	4,820	DongjinByeo	KRDD http://www.niab.go.kr/RDS

*FST- Flanking sequence tag.

It can be theorized if the causal mutation is recessive in nature then it is expected to segregate in the ratio of 3:1 in the wild type and mutant population, respectively. The DNA of the plants with desired or the mutant phenotype are sequenced and aligned with the reference genome of parental cultivar. The genome sequence of parental line is prepared by replacing the known SNP in the reference genome of that cultivar. MutMap technique targets the SNP induced in the mutant population as the SNP of the desired phenotype will segregate in the ratio of 3:1 whereas, the SNP which are not related to our desired phenotype would show the segregation ratio of 1:1. Many such studies have been successfully conducted in rice to target the mutant by MutMap technique (Abe et al., 2012; Takagi et al., 2015). Mutmap technique is not suitable to study lethal or sterility based studies as the mutants with these types of desired phenotypes are not suitable for crossing. Such kind of studies can be carried out with the specialized type of MutMap technique which is known as MutMap+. In this method the heterozygous plants, are selfed and the DNA from mutant and wild type parents are bulk sequenced and aligned to identify the sterility or lethality based genes. Mutmap+ has been effectively used to identify the Hit9188 gene which is responsible for early stage lethality in rice. Many such studies can be carried out based on the application of this technique (Fekih et al., 2013). Another improvised technique of Mutmap is Mutmap-Gap. Sometimes gaps are present in the reference genome of parental cultivar in the region where our desired mutation is located. In such conditions, sequencing techniques are used to reconstruct the reference sequences. Takagi et al. (2013) located a blast resistance gene Pii in rice cultivar, Hitombare based on Mutmap-Gap.

Simultaneous Identification of Multiple Mutations (SIMM)

SIMM (simultaneous identification of multiple mutations) or popularly known as an advanced bioinformatic methodology to identify causal mutation in the crop plant. A study was conducted using this methodology very recently in rice from which seven new preferred mutant alleles were mapped. This approach effectively narrows down the causal mutations to one or few SNPs which are responsible for the desired phenotype in the mutating population. The preparation of plant material in this process is exactly similar to the mutmap. The F_2 mapping population having desired mutations in homozygous state are harvested and DNA libraries with the insert size of 200-500bp are prepared and sequenced. The sequence reads are aligned to reference Nipponbare genome and SNP indexing is performed. Various filters like removal of adapters, allele index, euclidean distance etc. are involved in the analysis to remove noise from the background (Yan et al., 2017).

Whole genome sequencing of mutant rice lines

Whole genome sequencing is a fast and powerful technique to map the mutations in all sorts of organisms worldwide. Commonly, recessive and monogenic traits are mutated and studied by mutation breeding due to the convenience in its traceability. Hence, many studies were conducted in rice which is based on monogenic traits of agricultural importance. In this methodology the DNA is extracted from all the F_2 mapping population

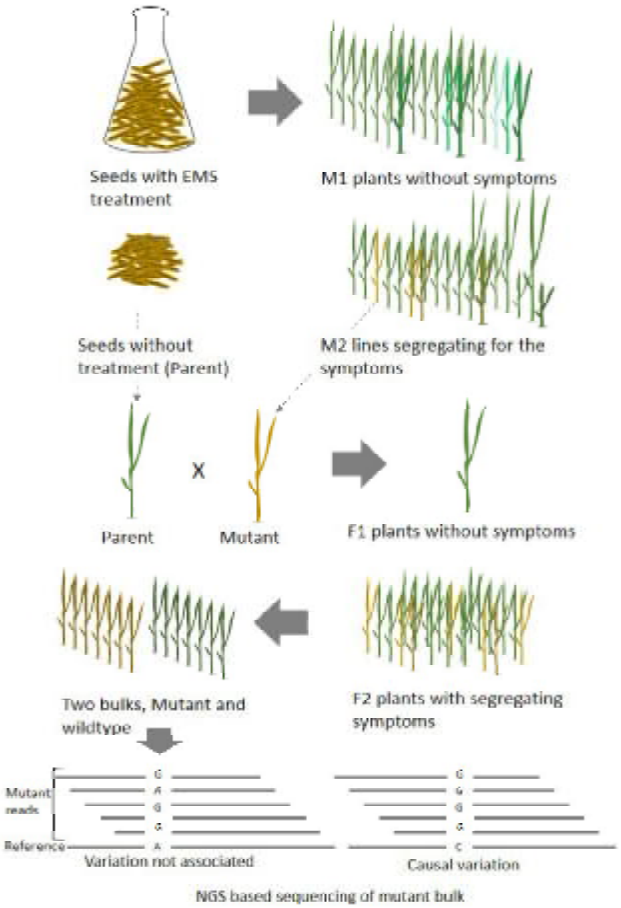

Fig. 3. Steps involved in mapping of causal mutation using next generation sequencing based Mut-map approach.

expressing the desired mutation/phenotype in homozygous state. The diversity in the SNPs is found minimum at the mutant regions while it increases as the distance from the causal mutant site due to linkage disequilibrium. The mapping procedure exploits this theory. The DNA sample extracted from the mutant plants are sequenced and aligned to the reference genome to figure out the complete pattern of sequence variants in the alignment pattern. Several efforts have been employed to sequence the entire genome of mutant genotypes to develop sequence resources. The entire genome sequencing of the mutants are excellent approach to get a clear picture about distribution and type of mutations impacted with particular mutagens. To better understand the mutagenesis processes, whole genome sequencing is a promising way.

Online resources for the rice mutants

Several online databases providing seeds of mutant genotypes as well as functional annotations of the genes are available for rice (Table 3). Some of the database also provides phenotypic data which has great importance in the identification of novel genes and biochemical pathways. Similarly, the flanking nucleotide sequences (FNS) provided in the most of the popular databases for rice offer information to priorities the candidate genes from QTL (Quantitative Trait Loci) mapping and GWAS (Genome-Wide Association mapping) studies (Sonah et al., 2015; 2016).

CONCLUSION

Though induced mutagenesis has been explored for the development of novel cultivars in rice and many other crop species in past century, over the times the mutation breeding becomes outdated. However, recent advancement in mutation mapping techniques have made mutagenesis is a choice of technique particularly for the reverse genetic approaches. The success of newly invented mutation mapping techniques like MutMap, MutMap+, MutGap and whole genome sequencing has been evident from recent studies. Direct trait improvement through mutagenesis is more interesting to rice breeder community with the objectives like identification and characterization of novel genes. This multipurpose mutagenesis programs will be more efficient, complementary, and affordable. The information provided in this review, will be very much helpful to plan the future mutagenesis programs for rice improvement appropriately.

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