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Research Paper

EMS Induced Mutants of Upland Rice Variety Nagina22: Generation and Characterization

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One of the approaches for understanding the functions of the genes predicted in the rice genome requires use of mutants. Mutations induced in a given genetic background provide opportunities to assign function to a given gene with a minimum of background genetic noise. This paper describes generation and initial characterization of a large set of Ethyl Methane Sulphonate (EMS) induced mutants in the upland rice variety Nagina22, through a National Initiative involving six Research Institutes namely National Research Centre on Plant Biotechnology, New Delhi; Indian Agricultural Research Institute, New Delhi; Tamil Nadu Agricultural University, Coimbatore; Directorate of Rice Research, Hyderabad; University of Agricultural Sciences, Bangalore and Punjab Agricultural University, Ludhiana, funded by the Department of Biotechnology (DBT), Government of India. The uniqueness of this collaborative effort is phenotyping for a range of traits that has led to identification of mutants for plant growth and architecture, flowering, maturity, grain number, shape and size, yield, phosphorus use efficiency, resistance to blast and bacterial leaf blight diseases, and tolerance to drought, salinity and herbicide. A set of 22, 292 mutagenised lines generated under this initiative and phenotyped for the traits enlisted above has resulted in the isolation of a few promising mutants which are being characterized. Shortly, these mutants will be registered and made available to the researchers in the country for use in studies in rice genetics, breeding and functional genomics. The mutant stock is expected to serve as a national resource for understanding rice biology as well as for use in genetic improvement of the crop.

Key Words: EMS Induced Mutation; Nagina22; Phenotyping; Rice

1. Introduction

Rice (*Oryza sativa*) is the staple food of more than half of the world's population. The importance of this crop lies in the fact that it has shaped the cultures, diets and economies of millions of people living particularly in Asia. Considering the role rice plays in providing food and nutritional security, and eradicating poverty, the United Nations designated the year 2004 as the International Year of Rice. In order to achieve stable growth in rice production, a strong push is required to boost productivity, break yield barriers and provide safety against fluctuations in climatic conditions. The International Rice Genome Sequencing Project (IRGSP, 2005) generated very high quality sequences that were used to predict the number and type of genes, and the non-genic

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regions containing repeats and mobile genetic elements. Concerted efforts are required to understand the function of individual genes, and their interactions among themselves as well as with environment, in relation to variation in traits for a directed genetic manipulation of this important crop for the benefit of the mankind.

One of the approaches to determine functions of genes employs natural mutants available in the germplasm or those induced by physical, chemical or biological agents. Mutants facilitate unveiling the causal relationships between coding/regulatory sequences and plant performance, and also cloning of the corresponding genes. Therefore a number of international efforts are underway for generation, collection and characterization of mutants for providing technological platform for functional genomics (Krishnan *et al*. 2009). In rice, mutations have been induced by using ionizing radiations and chemical mutagens (Wu *et al*. 2005), T-DNA (Jeon *et al*. 2000; Jiang and Ramachandran 2010), maize transposons *Ac/Ds* and *En/Spm* (Upadhyaya *et al*. 2002; Kumar *et al*. 2005) and retrotransposon element *Tos17* (Miyao *et al*. 2007). Induced mutations can be efficiently integrated with genomics, transcriptomics, proteomics and metabolomics studies to understand the phenome. However, limited information is available on their phenotypic evaluation and only a small subset of these mutants is freely available for unrestricted use.

The upland rice varieties such as Nagina22 possess many traits such as drought tolerance, heat tolerance, and resistance against pests and diseases, useful for climate resilient agriculture. Induction of loss-of-function mutations affecting such traits and gain of function mutations in key developmental and agronomically important genes provide opportunities for identification and isolation of the underlying genetic factors. No efforts, however, have so far been made in India to induce mutations in rice with the aim of generating national mutant resource for functional genomic studies.

The objective of the present work was to generate mutants using the chemical mutagen, ethyl methane sulphonate (EMS) in the upland rice variety Nagina22 and characterize them for a range of traits including biotic and abiotic stress tolerance, plant architecture and growth, flowering, maturity, nutrient use efficiency and yield so that they can be availed and made use of in functional genomics research including discovery of new genes and alleles in rice.

2. Materials and Methods

(a) Plant Material and EMS Mutagenesis

Pure seeds of rice variety Nagian22 were grown in the field of Indian Agricultural Research Institute (IARI) and maintained by panicle-to-progeny rows. There was no phenotypic variation observed within and between lines. Bulked seeds were used for mutagen treatment. The dry seeds were first treated with a fungicide (Bavistin-0.2%) for two hours and then washed twice with double distilled water prior to mutagen treatment. To choose the right dose, 50 seeds per each dose were treated with 0.6%, 0.8%, 1.0% and 1.5% EMS. The seeds were pre-soaked in double distilled water for 24 hrs and then treated with EMS at 28^{o}C for 12 hrs with gentle shaking at 60 rpm. The EMS solution was decanted and seeds were rinsed twice with double-distilled water. The seeds were then thoroughly washed with running tap water for two hours to terminate the residual effect of the mutagen. After completion of the treatment, the treated seeds were allowed to germinate in petri dishes and scored for percent germination. Based on this test, 0.8% was chosen appropriate and used further to treat approximately 10,000 seeds (200g) each in different batches for generating the mutant population.

(b) Phenotypic Characterization of the Mutants

The M1 seeds were grown in the field and M2 seeds were harvested from the main panicle of each M1 plant. The M2 seeds were distributed among the participating centres for advancing the generation, phenotyping and isolation of mutants. Morphologically distinct mutants identified in the M2 or later generations were maintained separately. In case of lines giving no identifiable morphological mutants, seeds from the main panicle of five to ten

plants per line were bulked and used to advance the generation. The phenotype data was recorded through visual assessment at three different stages, viz. seedling, vegetative and reproductive stages following Distinctness, Uniformity and Stability (DUS) test guidelines. The characteristics that required measurements were done according to the usual procedure. The plant height was measured from the base of the plant to the tip of main panicle. The grain data such as grain size, shape, color, etc were recorded after the harvesting and drying of the material. The characters like presence of apicular pigment, absence of awns, grain and panicle morphology, leaf and stem characters and sheath color were visually scored. Data for quantitative characters like plant height, panicle length, tiller number, spikelet fertility and 100 grain weight were also recorded.

For identifying short root mutants, a row of eight seeds per progeny were germinated on a thermo-cool board of 1 cm thickness on linear holes which were plucked with sterile sponge to hold the seed. The tray was floated on a trough of water with essential nutrients as described by Yoshida *et al*. (1976). The troughs were kept under glass house condition with temperature ranging from 25-36^{0}C and humidity of 70%. On 15th day the thermo boards were lifted and the early root length was measured in cm.

For identifying mutants with resistance to Bacterial leaf blight, plants were inoculated with the bacterial suspension at a density of 10^9 cells/ml at maximum tillering stage using the clip inoculation method (Kauffmann *et al*. 1973). Disease reaction was scored 14 days after inoculation. Plants with an average lesion length up to 6 cm were considered resistant and those with above 6 cm were scored as susceptible. A natural hot spot region at Gulalur (11º31'N; 76º28'E; 1500m MSL) in Tamil Nadu was selected for undertaking the screening for blast incidence under natural endemic condition during wet season. The progenies were sown on raised bed measuring 1m width. Each progeny was sown in a single line and per every 10 rows, a susceptible check, CO39 was repeated to create an even disease load across the bed. Two observations, on 40th day after sowing and 65th day after sowing, were recorded. Blast incidence was recorded based on IRRI-Standard Evaluation System (SES) scores ranging from 0 to 9.

In order to identify mutants exhibiting enhanced level of tolerance against salinity stress, the Yoshida nutrient solution was salinized on the 22nd day, after observing the root characters, by adding NaCl upto required concentration (100 mM). The pH of the solution was adjusted to 5.0 and monitored daily. Putative gain-of-function mutants exhibiting enhanced tolerance against 100 mM NaCl stress was identified based on the extent of leaf rolling, wilting and drying symptoms. The mutant lines were also tested for their ability to germinate under high salinity conditions by germinating twenty five seeds (3 replications) of each mutant line in the presence of 100, 150, 200 and 250 mM NaCl in petridishes. Emergence of radicle and plumule was considered as a criterion for germination.

To identify mutants that have higher phosphorous use efficiency (PUE), they were evaluated under low phosphorus (Olsen value of P<2 ppm) field conditions and also under hydroponic conditions. A line with higher percentage of survival under low P as compared to original parent was considered tolerant to low phosphorous. Besides, P was also measured in roots and shoots of the tolerant lines to study PUE.

Drought tolerance screening was carried out using 25% PEG as well as by withholding irrigation in case of lines gown in pots, one meter long tubes, root structures, and also in the field with or without rain-out shelter. Extent of survival under severe dehydration due to PEG or after withholding irrigation, staying green for long, leaf rolling, root length and biomass, carbon isotope discrimination, spikelet sterility and yield were used to assess drought tolerance.

An attempt was made to identify plants with herbicide tolerance. The experiment on screening for herbicide tolerance was conducted both in nursery and main field during *kharif* 2011. Approximately a total of 100,000 M2 plants were maintained in the main field through space planting (25 x 25 cm) and

another 100,000 plants were raised in nursery bed. Herbicides viz., Glyphosate and N,N'-dimethyl-4, 4'-bipyridinium dichloride (for nursery screening) and Glyphosate and Imazethapyr (for main field screening) were used separately. Treatment was given on 45th day after sowing (DAS) in the main field and 25th DAS in the nursery using knapsack hand sprayer. Thrice the level of concentration (15 ml/lit for glyphosate and N,N'-dimethyl-4, 4'-bipyridinium dichloride; 3ml/lit for imazethapyr) of the herbicides was sprayed on M2 plants for identification of the most tolerant plants.

3. Results and Discussion

The present effort is an Indian Initiative on "Generation, characterization and use of EMS induced mutants of upland variety Nagina22 (N22) for functional genomics in rice" initiated with funding support from the Department of Biotechnology (DBT), Government of India, New Delhi. The programme involved six partner institutions namely National Research Centre on Plant Biotechnology (NRCPB), New Delhi; Indian Agricultural Research Institute (IARI), New Delhi; Directorate of Rice Research (DRR), Hyderabad; Tamil Nadu Agricultural University (TNAU), Coimbatore; Punjab Agricultural University (PAU), Ludhiana and University of Agricultural Sciences (UAS), Bangalore. The primary objective of this effort was to generate and characterize a large set of EMS induced mutants of the upland rice variety Nagina22 for various morphological, developmental and yield related traits as well as for biotic and abiotic stress tolerance. The uniqueness of this effort are: (i) induction of mutation in the genetic background of an *aus* type upland variety, (ii) creation of allelic series by employing ethyl methane sulphonate (EMS) and (iii) phenotyping of the mutagenised lines for a number of traits. The functional genomics studies are limited by unavailability of genetically defined mutants in the country in rice, which is intended to be addressed by this effort.

Of the available options to generate mutants, chemical induction of mutations using EMS was chosen in this study. This was undertaken for two main reasons: (i) EMS mutagenesis gives high point mutation densities by base substitution with a low level of chromosome breaks and thus less of aneuploidy, sterility and dominant lethality (Rao 1977; Vidal *et al*. 1995; Bentley *et al*. 2000). Base substitutions either in the transcribed regions or in the regulatory elements of a gene might alter gene function leading to creation of a series of alleles of a gene. In contrast, insertional mutagenesis using T-DNA, transposons or retrotransposons create gene knockouts mostly with loss of function. Although gene isolation is easier using the insertion mutants, EMS offers the advantage of creating an allelic series and (ii) Large-scale phenotyping of the mutagenised lines would be required to identify mutants for traits of agricultural importance. This would require elaborate field experiments without any restrictions. Mutants created by T-DNA or transposons would be considered Genetically Modified (GM) and thus suffer from restrictions for field evaluation unlike the EMS mutants.

Nagina22 is an upland variety known to be an '*aus*' type that is different from both *indica* and *japonica* ecotypes (Garris *et al*. 2005). There are many characteristics that make this variety an attractive target for mutation induction: (i) Nagina22 has been found to have the largest response in terms of increased yield, biomass and harvest index (0.6) to increased CO_2 concentration at temperature 29/21^{o}C. Also, it was the only variety out of 17 tested, which produced seed at high temperature of 37/29^{o}C. It is thus tolerant to both high CO2 and heat (Ziska *et al*. 1996). High level of heat tolerance in Nagina 22 has been further confirmed recently (Jagadish *et al*. 2010), (ii) It is an upland variety that shows less spikelet sterility under moisture deficit stress (Selote and Chopra 2004; Rang *et al*. 2011). Its panicles maintain higher relative water content and turgor potential and lower H_2O_2 levels across the developmental stages under water stress than the susceptible genotypes (Selote and Chopra 2004), (iii) Nagina22 is known to display very high level of seed dormancy among the rice cultivars that were tested (Gu *et al*. 2003), (iv) Abortion of female gamete in *indica-japonica* crosses of rice does not allow

efficient use of wide genetic diversity existing between the two rice ecotypes. Genotypes possessing wide-compatibility genes can be crossed to both *indica* and *japonica* to obtain fertile F_1 hybrids. Nagina22 is one of the well-recognized wide compatible cultivars (Vijaya Kumar and Virmani 1988) and thus is useful in generating fertile *indica-japonica* hybrids and (v) It is reported to possess resistance to gall midge and white-backed plant hopper (Sidhu *et al*. 1979).

In terms of the effectiveness of the EMS dose applied, reduction in germination percentage was observed with increase in the EMS dose in M1 seeds. The treatment from 0.6 to 1.0 % was found to be effective at which 72-92% germination of EMS-induced Nagina22 mutants was observed. In contrast, drastic reduction of germination percentage was observed at 1.5% EMS dose. Therefore 0.6 to 1.0 % was considered the optimal level of EMS dosage for mutagen treatment and 0.8% was used for generation of mutant population in subsequent experiments. In contrast, more than 1.5% EMS has been found more effective in case of indica variety IR64 (Wu *et al*. 2005; Till *et al*. 2007). In *Arabidopsis*, EMS-induced mutants were generated in the genetic backgrounds of Columbia (Col) and *Landsberg erecta* (Ler). Frequency of induced mutations detected in Ler population was nearly twice higher than that of Col (Koornneef *et al*. 1982). These results suggest that the effectiveness of different doses of the chemical mutagen EMS in creating high frequency mutation varies according to genotypic background within a given plant species.

So far, a total of 22,292 mutagenized lines have been generated, which are in different generations and stages of phenotypic evaluation. The average frequency of mutation having visible phenotype, estimated based on morphological variation or screening for salt tolerance of a subset of 1800 lines (Table 1), was 7.3%. The frequency of mutation for specific traits varied from 0.05 to 1.0%. In the popular rice variety, IR64 background, similar frequency of mutation has been reported by using 1.6% EMS (Wu *et al*. 2005). These frequencies are also similar to those observed in Arabidipsis. Thus, the data suggest effectiveness of a lower dose of EMS in inducing usable level of mutation and that the mutagenized population harbors a considerable level of genetic variability that can be further used after the mutants are subjected to appropriate phenotypic screens. In fact different subsets of lines have already been phenotyped for tolerance to moisture deficit stress and salinity, phosphorus use efficiency, herbicide tolerance, and resistance against bacterial leaf blight and blast diseases by different partner institutions. This has led to a total of 548 distinct and validated mutants that have been pooled and are being maintained in a "Mutant Garden" in the IARI farm. A wide range of variation has been observed for plant height, maturity, grain shape and size, leaf shape and size, panicle length and branching, spikelet density and grain number in the mutant population (Fig. 1, Table 2). Lines, which continue to yield new phenotypically distinct mutants in each generation, have also been observed. Interestingly, mutants with high level of tolerance to herbicide, drought, salinity and bacterial leaf blight have been identified in the limited screening undertaken for these traits so far (Fig. 2).

Phenotypically distinct mutants being grown in the mutant garden have been classified into several groups (Fig. 3). These include mutants for plant architecture, growth, yield contributing traits, and stress tolerance. The objective of this Garden is to maintain all the allelic variants of any gene and display the phenotypic effect of the mutation for comprehension particularly of the students. Having all mutants for a trait at one place makes comparison of effects of mutation easier and analysis of allelic relationships is greatly facilitated. Three hundred eighty one of the 548 mutants in the mutant garden have been characterized for 44 DUS characters and given in supplementary data. DUS characterization data will help in correct description of the mutants and their protection.

Most of the mutants exhibited changes for single trait as compared to Nagina22. However, some of the mutants depicted multiple changes which could be due to pleiotropy or mutation in multiple genes affecting different traits. EMS is known to produce

Table 1: Mutants exhibiting altered morphological traits in M3 generation in a subset of 1800 lines

Traits	Classification	No. of mutant progenies identified	Phenotypic value		Mutation frequency (%)
			N22	Mutants	
Albino	Chlorosis	1	Green	Albino plant	0.05
Flowering (days)	Early	3	71	60-66	0.16
	Late	3	71	88-90	0.16
Plant height (cm)	Tall	18	105	115-151	1.00
	Dwarf	12	105	42-67	0.66
Tillering habit (no. of tillers)	High	11	8	30-120	0.61
	Low	15	8	1—3	0.83
Panicle type (length in cm)	Long	9	21	26-31	0.50
	Short	10	21	6—9	0.55
	Dense	4	135	175-231	0.22
	Open type	2	Normal	Open	0.11
	Compact	2	Normal	Compact	0.11
Grain type	Short	12	Medium	Short	0.66
	Bold	3	Medium	Bold	0.16
	Long	10	Medium	Long	0.55
Grain colour	Purple	3	Straw	Purple	0.16
	Golden brown	1	Straw	Golden brown	0.05
Grassy mutant		2	Normal	Grassy tillers	0.11
Antherless mutant		1	Fertile	Sterile	0.05
Vivipary mutants		1	Normal	Viviparous	0.05
Nodal mutant	Nodal root formation	2	Normal	Nodal roots	0.11
Short root mutants		4	24.22 cm	8.87-10.78 cm	0.22
Salinity tolerant		4	Susceptible	Resistant	0.22
Total		133			7.30

mainly GC to AT transition (Till *et al*. 2007). Since the rice genome can tolerate a large number of point mutations, it is possible that each of the mutants carry multiple changes. This also implies that full genome coverage can be achieved with a relatively low number of EMS mutants. Selected mutants validated by progeny testing have been crossed with the parent Nagian22 and also with the popular rice variety IR64 to develop populations to study the inheritance pattern and map the target loci. Some of these populations have been also phenotyped. The mode of inheritance of traits such as BLB resistance, plant height, and seed size will be shortly determined. The mapping populations developed in the process will be used to map, fine map and clone the genes carrying mutations that influence trait expression.

Functional genomics of abiotic stress tolerance in rice will be greatly facilitated by the use of mutants generated by this National Initiative. Since drought tolerance is a highly challenging trait to deal with, a systematic effort is being made to identify the right kind of mutants to work with. Different batches of the mutagenized lines are first exposed to moisture deficit stress in the field/rain-out shelter and under laboratory conditions using Polyethylene glycol

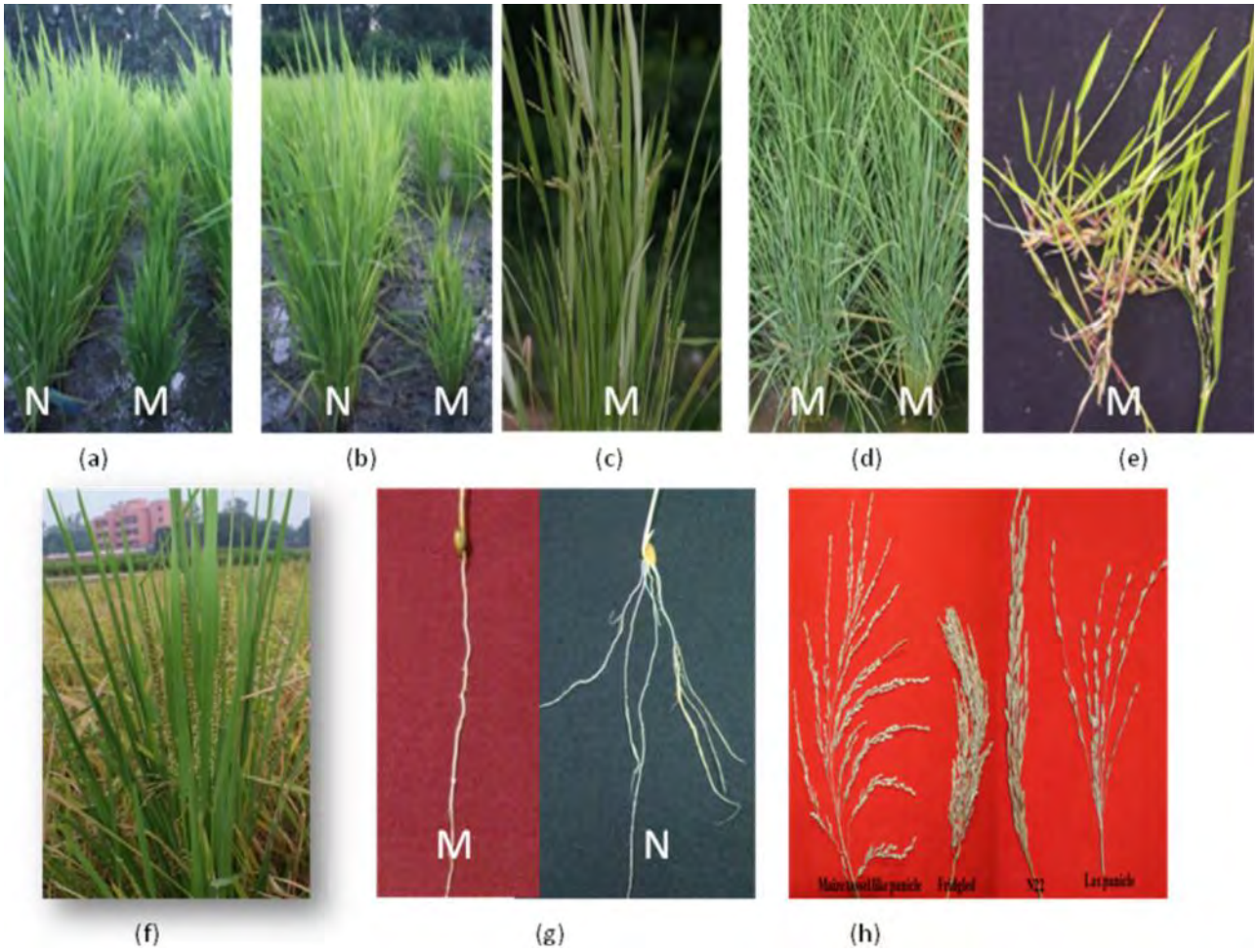

Fig. 1: Representative variation for different traits in the mutagenized population of Nagina 22. N – Nagina22; M – Mutant. a - dwarf mutant; b - dwarf mutant; c – Chlorophyll mutant striata; d – Narrow leaf mutant; e – Mutant with shoot regeneration from panicle parts; f – bacterial leaf blight resistant mutant; g – Mutant with a single crown root; h – A representative variation in panicle architecture in Nagina22 panicle mutants

Table 2: Mean and range of variation in M3 generation derived from a representative set of 381 phenotypically different M2 plants

Character	N22	Min	Max	Average	Std. dev
Plant Height (cm)	147	58.40	176.60	127.51	22.31
No. of Tiller	14	5	86	11	6
Panicle length (cm)	24.0	11.40	30.60	22.09	3.11
Grain length (mm)	8.07	6.22	11.74	8.20	0.93
Grain width (mm)	2.75	1.97	5.71	2.70	0.35
Spikelet fertility (%)	94.96	4.5	99.1	75.5	34.5
1000gw (g)	18.68	6.06	27.32	17.73	3.71
Yield/plant (g)	18.01	4.6	29.71	10.71	3.58

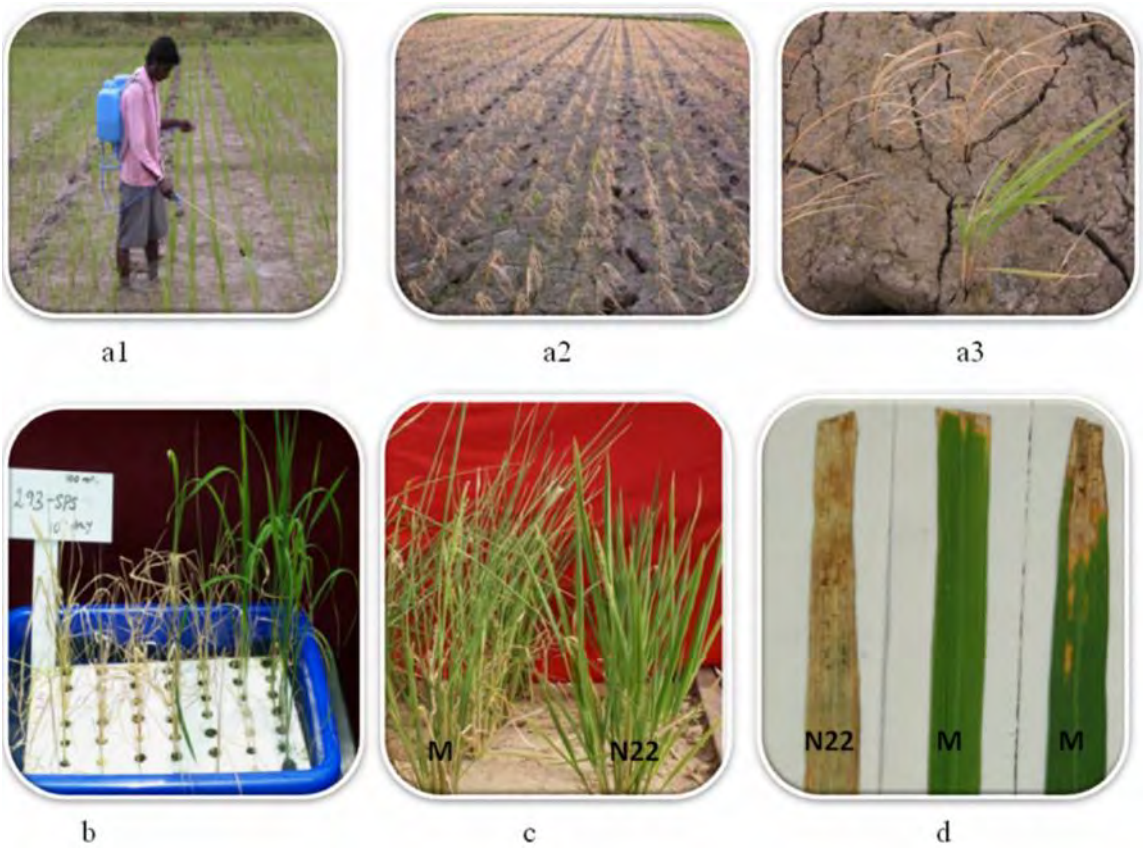

Fig. 2: Promising mutants identified by screening for different traits. a1: Herbicide spray on mutant lines grown in the field; a2: Field view after herbicide spray showing a few putative mutants a3: Mutant for herbicide tolerance; b: Screening for salinity tolerance showing three putative mutants surviving under 100mM Nacl stress; c: Mutant for drought tolerance and d: Mutant for Bacterial leaf blight tolerance (M: Mutant and N22: Nagina22)

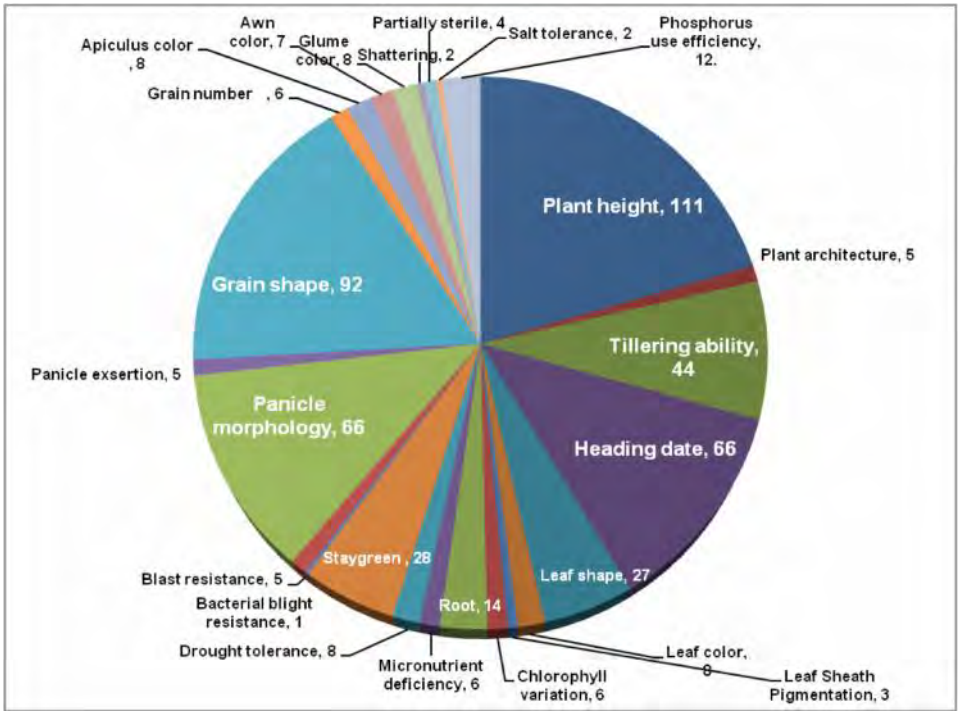

Fig. 3: Classification of the 548 EMS mutants currently maintained in the mutant garden under different traits

Table 3: Variation in root traits and $\Delta^{13}C$ (surrogate for water use efficiency) among 30 M5 lines short-listed based on earlier screening for drought tolerance under field conditions

Source	Root length (cm)	Root volume (cm^3)	Root wt (g. pl^{-1})	Stem weight (g. pl^{-1})	$\Delta^{13}C$	Specific leaf area (SLA)
Mean	28.59	21.32	16.20	20.03	19.76	62.84
Minimum	21.33	10.00	13.50	10.50	18.60	42.59
Maximum	36.50	45.00	30.33	43.75	20.63	86.55
N22	24.4	11.67	15.56	21.22	21.05	87.55

(PEG). The lines showing better or worse performance than Nagina22 are short listed for a detailed analysis. A summary of their root characteristics and water use efficiency under drought is given in Table 3. This has led to identification of a set of lines having higher level of drought tolerance than Nagian22. A loss of function mutant has also been isolated by rigorous screening of mutagenized lines in rain-out shelter. A subset of the drought tolerant lines is likely to be tolerant to high temperature (Jagadish *et al*. 2011).

Mutations in a gene are expected to result in either loss or gain of function brought about by qualitative or quantitative alteration in its expression. Such loss or gain of function mutations affecting any of the traits would allow use of both forward and reverse genetic approaches to identify the genes involved and also to understand pathways. In addition to the mutations in developmental pathways relevant to growth, maturity, plant architecture and yield, mutations influencing high temperature and drought tolerance possessed by Nagina22 are of special importance. These two are highly complex traits and need immediate attention to meet the challenges of climate change.

. Thus, the present National Initiative has succeeded in generating the first set of mutants in the background of upland rice variety Nagina22, which have been phenotyped for a number of traits. The mutants already identified will be further characterized, registered and the seeds stored at the National Bureau of Plant Genetic Resources (NBPGR), New Delhi for future use. However, this resource needs to be enlarged in size, which is already underway, so that valuable genes are targeted by mutagenesis multiple times in order to create a series of alleles. A detailed phenotypic characterization of these lines will be required to identify mutants and then use them in rice functional genomics. This will require elaborate efforts, considerable investment of time, liberal funding and involvement of scientists from diverse fields of specialization. Hence, it is time that new trait specific consortia are put in place to take advantage of the leads already obtained in this national effort.

4. Conclusion

The authors have developed national mutant resource for rice functional genomics through EMS mutagenesis of an upland rice genotype Nagina22. A number of useful mutants have been identified for various traits such as Phosphorus use efficiency tolerance to drought, salinity, herbicide spray and resistance to bacterial leaf blight. This resource is expected to serve as a base for finding useful genes, alleles and unraveling the functional genomics of this model crop.

5. Acknowledgement

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