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Isolation and characterization of altered root growth behavior and salinity tolerant mutants in rice

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Generation, screening and isolating mutants for any developmental and adaptive traits plays a major role in plant functional genomics research. Identification and exploitation of mutants possessing contrasting root growth behavior and salinity tolerance in rice will help us to identify key genes controlling these traits and in turn will be useful for manipulating abiotic stress tolerance through tilling and genetic engineering in rice. In this study, we have screened about 1500 mutants (M_2 generation) generated by treating an upland drought tolerant genotype Nagina 22 with Ethyl Methane Sulfonate (EMS), for their root growth behavior and salinity tolerance under hydroponic conditions. Six independent mutant lines possessing significantly shorter roots and three mutant lines exhibiting greater degree of salinity tolerance than the wild type plants were identified. The identified mutant lines were advanced to M_5 generation to allow the mutants to reach homozygosity, and the fixed mutants were confirmed for their phenotype. One mutant namely N22-C-241-5-6 was found to possess significantly shorter roots than wild type N22, and it was also noticed that the mutant was devoid of root cap. Among the three salinity tolerant mutant lines identified, N22-C-334-3 was found to possess a greater degree of tolerance upto 250 mM Nacl stress at germination stage. These identified mutant lines can be used for further physiological, biochemical and molecular biology experiments to identify candidate gene(s) controlling root growth behavior and salinity tolerance in rice.

Key words: Rice, mutation, EMS, altered rood growth and salinity tolerant mutant.

INTRODUCTION

Abiotic stresses such as drought and salinity are major yield limiting factors for upland and rainfed lowland rice ecosystems. Improving rice yield with no additional lands available for cultivation depends mainly on the development of drought and salinity resistant rice genotypes suitable for these marginal environments. Efforts through conventional breeding approaches are resulting in slow progress in developing drought and salinity resistant rice genotypes mainly due to the complexity of mechanisms controlling these traits.

Developing rice genotypes with the right combination of characters for specific drought and salinity prone environments therefore require an understanding of the physiological processes of the plant, environment and interaction

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Abbreviations: QTLs, Quantitative trait loci; ***rss2*,** rice salt senstive2; ***crl mutants*,** *crown rootless* mutant.

between them (O'Toole and Chang, 1979). In this context, biotechnology offers us a powerful means of manipulating drought and salinity tolerance in rice through various approaches. Among various biotechnological means, functional genomics helps us to generate knowledge on networks of stress perception, signal transduction and defensive responses.

Nguyen et al. (1997) suggested that drought resistance in rice depends mostly on one or more of the following components: ability of the roots to exploit deep soil moisture to meet evapotranspirational demand; moderated water use through reduced leaf area and shorter growth duration; capacity for osmotic adjustment and the control over non-stomatal water loss from leaves. Among the above traits, root system architecture that is, root length, volume and thickness determines the efficiency of water and nutrient absorption ability of a plant. Although rice is generally adapted to well water or irrigated environments, there is genetic variation for root traits. Some of the traditional upland cultivars are having deep roots whereas most of the cultivated semi-dwarf varieties are of shallow rooted types.

By exploiting this genetic variation, few studies have been carried out to identify quantitative trait loci (QTLs) controlling root traits, which need confirmation across the genetic background before putting them into use. Difficulty in precise phenotyping and tissue sampling of rice roots prevents the progress in generating knowledge on the gene(s) controlling root traits. Identification and characterization of suitable single gene root mutants possessing contrasting root traits will serve as near isogenic lines for functional genomics studies. In rice, only very few attempts have been made to isolate and characterize root system mutants.

Rice is moderately sensitive to salinity (Akbar et al., 1972) and the most practical approach to overcome the problem of salinity is development of tolerant varieties. The basic requirements are genetic variability, screening techniques and understanding of genetics and physiological mechanism of tolerance. The progress in developing salinity tolerant rice genotypes through conventional approaches is limited due to non-availability of rich genetic diversity in rice germplasm for salinity tolerance. Some traditional cultivars and landraces of rice are more tolerant than many elite cultivars to salinity stress. However, they generally have poor agronomic traits, such as tall plant stature, photosensitivity, poor grain quality, and low yield which limit their exploitation in conventional breeding programs. In addition, polygenic nature of tolerance mechanisms slows down the rapid generation of knowledge on its genetic mapping and molecular basis (Bohnert and Jensen, 1996). Genetic analysis of salt sensitive rice mutant increase the Na^+ in rice salt senstive2 (*rss2*) controlled single recessive gene (Zhou et al., 2013). Therefore, isolation of single gene-mutants exhibiting contrasting degree of salinity tolerance will be an ideal material for functional genomics studies which will help us to manipulate salinity tolerance in rice through genetic engineering.

By keeping the above in mind, the present study was formulated with an aim of identifying single-gene mutants in rice possessing contrasting root morphology and salinity tolerance. Ethyl methane sulfonate (EMS)induced Nagina 22 mutants were screened under hydroponic conditions and putative independent mutants possessing significantly shorter roots and gain-of-function mutants for salinity tolerance were identified.

MATERIALS AND METHODS

Screening for mutants was performed using M_2 seedlings derived from single M_1 plants rice (*Oryza sativa L. cv. Nagina 22; indica* type) derived by treating the seeds with 0.8% EMS.

Screening for altered root growth behavior mutants

About 1500 M_2 lines were screened for identifying putative mutants possessing short roots and enhanced level of tolerance against salinity. Ten seeds per each M_2 line were germinated in a germination paper medium. Seven day old seedlings (seven seedlings per line) were transferred onto a styro-foam (with holes) floated on plastic container containing Yoshida nutrient solution. The pH of the nutrient solution (0.18 mM $(NH_4)_2SO4$, 0.27 mM, $MgSO_4.7H2O$, 0.09 mM KNO_3, 0.18 mM $Ca(NO_3)_2.4H_2O$, 0.09 mM KH_2PO_4, 0.04 mM K_2SO_4 and 0.02 mM $NaEDTAFe.3H_2O$) was adjusted to 4.5, and the solution was changed once in a week. Plants showing significantly shorter root than the wild-type Nagina 22 were selected on the 15 day after transplanting. Selected lines/plants were transferred to pots containing soil mixture and advanced to further generations. The selected lines were retested at M_5 stage for the uniformity in phenotype.

Screening for salinity mutants

In order to identify mutants exhibiting enhanced level of tolerance against salinity stress, the Yoshida nutrient solution was salinized on the 22^{nd} day after observing the root characters by adding NaCl up to required concentration (100 mM). The pH of the solution was adjusted to 4.5, and monitored daily. Putative gain-of-function mutants exhibiting enhanced tolerance against 100 mM NaCl stress was identified based on the development of leaf rolling, wilting and drying symptoms. Identified mutant lines were maintained under salt-free conditions up to M_5 generation and the mutants were again tested for their salinity tolerance along with the salinity tolerant rice genotype FL 478. The mutant lines were also tested for their ability to germinate under high salinity conditions by germinating 25 seeds (3 replications) of each mutant line in the presence of 100, 150, 200 and 250 mM Nacl in Petri dishes. Emergence of radicle and plumule was considered as a criterion for germination.

Statistical analysis

All data were analyzed by statistical software SAS 9.1 Versions. Where appropriate, Duncan's multiple range test (DMRT) at the 0.05 probability level was used to group means. Standard error were calculated by manually using the formula, SE= SD /√(N)); where, SD = standard deviation of mean; N= number of observation of sample.

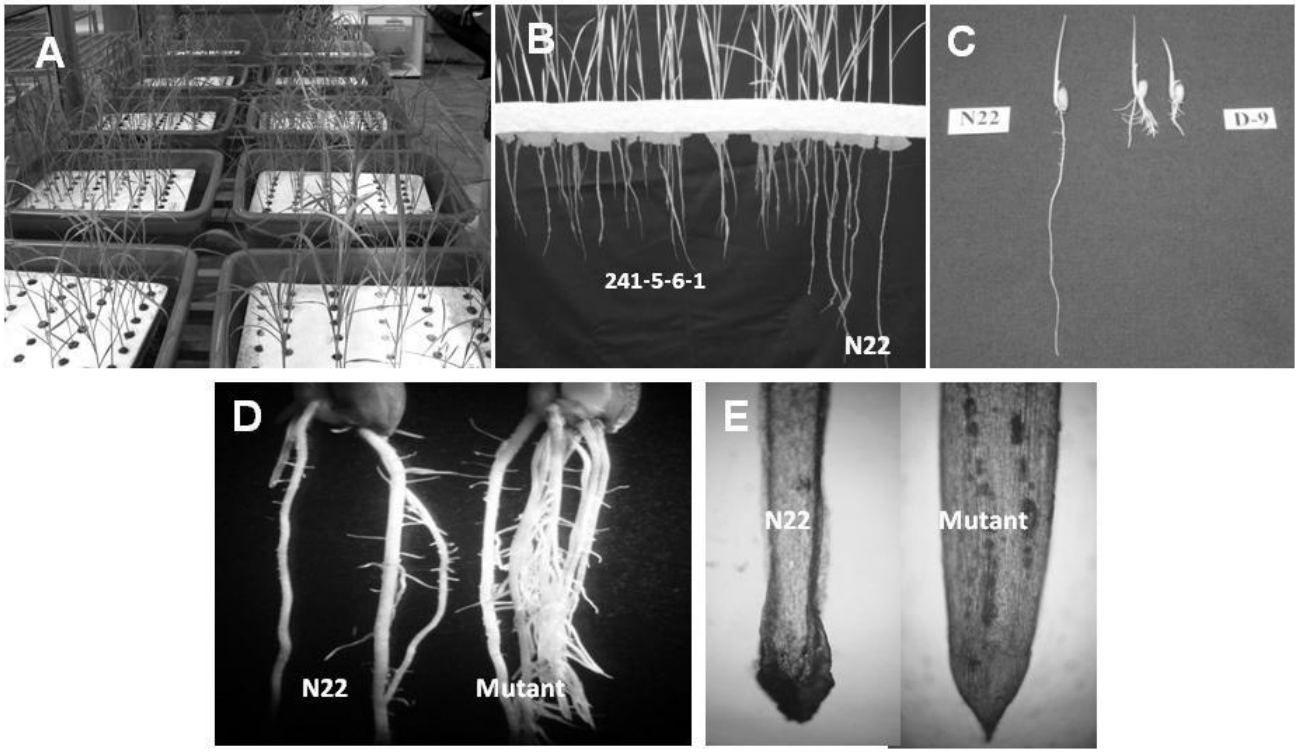

Figure 1. Identifying putative root mutants in EMS induced Nagina 22 mutant lines. A, Screening for root traits under hydroponic conditions; B, homozygous short root mutant (N22-C-241-5-6) showing significantly shorter root length than wild type Nagina 22; C, other two short root mutants; D, a mutant possessing more number of lateral roots than Nagina 22; E, putative mutant showing defect in the root cap.

Table 1. Root length and plant height of putative short root mutants.

Genotype	Root length (cm) (mean ± 2SE)	Plant height (cm) (mean ± 2SE)
Nagina 22	20.0 ± 1.14	99.8 ± 2.95
N22-C-241-5	9.6 ± 0.53	70.6 ± 6.52
N22-C-241-5-6-1	8.5 ± 0.30	61.6 ± 1.52
N22-D-8	10.1 ± 1.06	76.2 ± 2.80
N22-D-9	8.8 ± 0.83	70.4 ± 2.65
N22-D-105	8.0 ± 0.38	78.4 ± 3.25
N22-D-106	7.9 ± 0.45	79.0 ± 3.50

Root length was measured at 22 days after germination. Plant height was measured at maturity. Each value is a mean of 7 plants.

RESULTS

Isolation of altered root growth behavior mutants

Isolation of short root mutants

In order to identify rice plants showing abnormalities in morphogenesis and growth of root system, 1500 M_2 mutant lines (seven seedlings per line) were germinated in papers and transferred onto nutrient solution filled trays and maintained hydroponically (Figure 1A). A total of 12 M_2 lines were found to possess significantly shorter roots than the wild type. Out of the 12 lines, six of them had uniformly shorter roots than the wild type and they did not exhibit segregation for root length. In all the six mutant lines, root length was more than 50% less than the wild type (Table 1 and Figure 1B). In the remaining six mutant lines, single plants possessing significantly shorter roots that were 50% shorter than those of the wild type were identified (Figure 1C). These M_3 lines were found to show segregation for root length and are being forwarded to next generations to fix the mutation. Further studies on these identified mutants helped us to identify putative

Table 2. Observations on number of lateral roots present in seminal root and crown roots of wild type Nagina 22 and putative short root mutants.

Genotype	Number of lateral roots in seminal roots			Number of lateral roots in crown roots		
	5 DAS	10 DAS	15 DAS	5 DAS	10 DAS	15 DAS
N22	38.4^{a}	220.4^{a}	326.6^{a}	10.4^{a}	46.0^{a}	90.4^{b}
N22-C-241-5-6-1	4.0^{c}	29.0^{d}	51.6^{e}	2.0^{c}	4.8^{b}	63.6^{bc}
N22-C-12-1	36^{a}	56.4^{d}	92.6^{de}	3.8^{bc}	25.6^{ab}	96.0^{b}
N22-C-241-5	17.2^{b}	154.4^{b}	$182.^{bc}$	3.2^{bc}	35.8^{a}	84.0^{b}
N22-D-8	38^{a}	184.4^{ab}	217.4^{b}	7.2^{ab}	45.0^{a}	93.4^{b}
N22-D-9	3^{c}	149.0^{bc}	180.6^{bc}	4.8^{bc}	46.2^{a}	122.0^{b}
N22-D-105	0.0^{c}	0.0^{e}	108.8^{d}	0.0^{c}	0.0^{c}	222.0^{a}
N22-D-106	15.8^{c}	107.0^{c}	157.6^{c}	2.2^{c}	24.0^{ab}	73.2^{b}

Means followed by same letter are not significantly different at the 5% level by Duncan's Multiple Range Test. Plants were germinated and transferred onto hydroponic culturing and observations were made on 5, 10 and 15 DAS. Each values is mean of 8 plants.

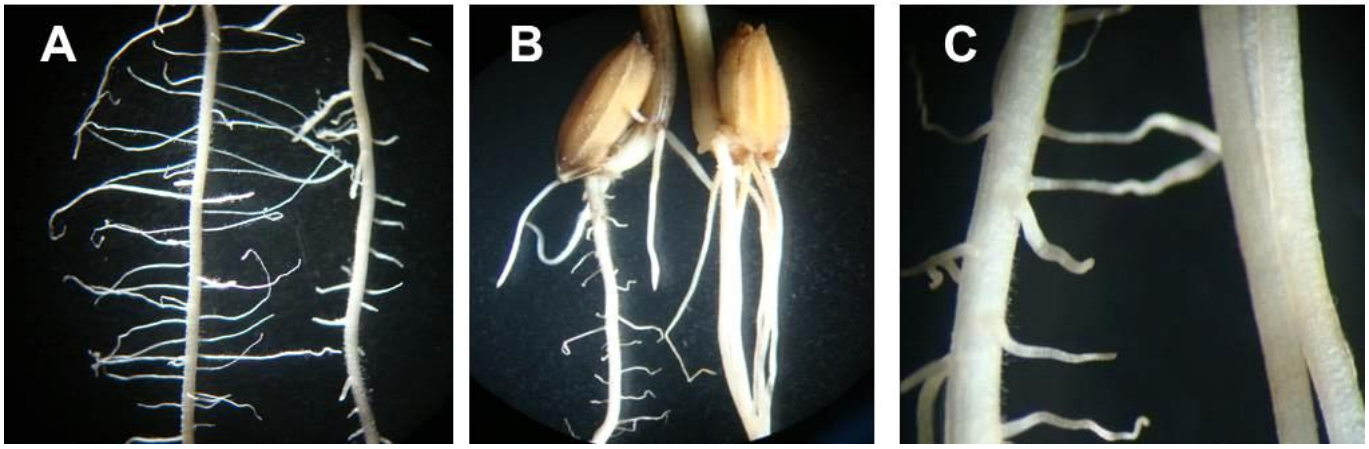

Figure 2. Mutants exhibiting variation in lateral root morphology. A, Mutant (N22-C-241-5-6) possessing significantly lesser number of lateral roots than the wild type Nagina 22; B, mutant (N22-D-105) showing absence of lateral roots at 7 DAS; C, close-up view of lateral root-less mutant.

mutants for other root growth traits also. One of the six short root mutants was found to possess significantly more number of lateral roots then the wild type (Figure 1D). A putative root mutant showing an abnormality in root cap structure was also identified (Figure 1E). This mutant was found to be devoid of root cap when compared to the wild type which had a well-developed root cap at the same growth stage.

Isolation of mutants showing variation in number of lateral roots

Number of lateral roots present in both seminal root and crown roots were observed at different growth stages of seedlings. One of the short root mutants namely, N22-C-241-5-6-1 was found to possess significantly lesser number of lateral roots in both seminal and crown roots than wild type Nagina 22 (Table 2). Moreover, it was observed that the length of lateral roots was found to be significantly shorter than the wild type Nagina 22 (Figure 2A). Another short root mutant N22-D-105 was found to possess no lateral roots at 7 DAS (Figure 2B and C). But interestingly, it was observed that this mutant started developing large number of lateral roots after 10 DAS (Table 2). At 15 DAS, this mutant N22-D-105 was found to possess significantly greater number of lateral roots in both seminal and crown roots (Table 2). All the mutants were found to possess significantly lesser number of lateral roots in the seminal root.

Isolation of mutants showing variation in number of crown roots

All the seven short mutants were characterized for the number of crown roots along with the wild type Nagina 22. The wild type Nagina 22 was found to possess a

Table 3. Observations on number of crown roots per plant.

Genotype	Number of crown root		
	5 DAS	10 DAS	15 DAS
N22	2.2^{bcd}	3.2^{b}	4.4^{b}
N22-C-241-5-6-1	0.0^{d}	1.4^{c}	3.0^{c}
N22-C-12-1	2.8^{bc}	3.4^{b}	4.6^{b}
N22-D-7	0.0^{d}	0.3^{c}	0.8^{d}
N22-D-8	4.0^{a}	3.2^{b}	3.6^{c}
N22-D-9	0.0^{d}	0.0^{d}	0.0^{e}
N22-D-105	3.2^{ab}	6.4^{a}	7.0^{a}
N22-D-106	1.8^{c}	3.0^{b}	3.2^{c}

Plants were germinated and transferred onto hydroponic culturing and observations were made on 5, 10 and 15 DAS. Each values is mean of eight plants. Means followed by same letter are not significantly different at the 5% level by Duncan's multiple range test.

average of five crown roots at around 10 -15 DAS. The present study resulted in the identification of few interesting mutants showing variation for number of crown roots. A mutant N22-D-105 was found to possess significantly greater number of crown roots (7) than the wild type (4.4) at 15 DAS (Table 3). This mutant was found to exhibit another interesting developmental variation for the growth of lateral roots also as described in the previous section. Another mutant namely N22-D-9 was found to possess no crown roots even up to 15 DAS (Table 3). This mutant was designated as *crown rootless* mutant (*crl mutants)*. The mutant N22-D-7 was found to possess significantly lesser number of crown roots than the wild type Nagina 22 (Table 3).

Isolation of gain-of-function mutants for salinity tolerance

In order to screen plants showing altered response against salinity stress, 1500 M_2 mutant lines (seven seedlings per line) were germinated in papers and transferred onto nutrient solution filled trays and maintained hydroponically. Salinity stress was imposed to 25-day's old seedlings by adding NaCl to 100 mM concentration and tolerance level of the mutants was assessed based on the development of wilting and drying symptoms. The wild-type Nagina 22 was found to be completely dried within seven days after imposition of salinity stress and most of the mutant lines were also found to be susceptible. Three single plants from three independent mutant lines namely N22- SPS-5, N22-334-3, and N22-293-1, exhibited the very high degree of tolerance against salinity stress. All the three mutants were found to remain green even after 15 days of salinity stress when all other mutants and wild type plants were dried completely. These three single plants were transferred onto pots and maintained under greenhouse conditions normally. Seeds were collected from all the three mutant plants and forwarded to further generations till M5 stage. Fixed mutants were tested for their degree of salinity tolerance at both germination and vegetative stage. The germination of wild type Nagina 22 was found to be affected significantly beyond 100 mM Nacl stress. Of the three mutants, N22-334-3 mutant exhibited very high degree of salinity tolerance and it was found to germinate even at 250 mM NaCl stress (Figures 3 and 4). During vegetative stage, all the three mutants were noticed to exhibit tolerance against salinity stress same as that of tolerant check FL 478 (Figure 5).

DISCUSSION

Mutants for root morphological traits have been reported in crops like maize (Miller and Moore, 1990), barley (Tagiliani et al*.,* 1986) and Arabidopsis (Benfey et al*.,* 1993). However only few mutants exhibiting altered root growth behaviour in rice have been reported (Ichii and Ishikawa, 1997; Wang et al*.,* 2006). Reduction in root length was found to be associated with reduction in plant height as in the case of semi-dwarfing genes. But there are reports where reduction in root length was independent of shoot length (Ichii and Ishikawa, 1997). In this study, it was observed that the plant height of the six homozygous short root mutants was not affected signify-cantly by the reduced root length at seedling stage. But there was a reduction in the final plant height at maturity stage. Thorough analysis of the anatomical differences in roots of the mutants and genetic complementation analysis may help us to categorize the mutants before getting into functional genomics studies. The six homozygous short root mutants identified in this work showed approximately 50% reduction in root length over the wild type and exhibited segregation for root length. Several researchers have identified and characterized rice mutants with reduced seminal root length (Liang and Ichii, 1996; Ichii and Ishikawa, 1997; Inukai et al*.,* 2001). Mutants possessing uniform short roots were further confirmed by growing in large numbers along with Nagina 22. In the present study, one of the short root mutant, N22-C-241-5-6-1 was found to have significantly fewer lateral roots in both seminal and crown roots than the wild type Nagina 22 (Table 1). Moreover, it was observed that the length of lateral roots was found to be significantly shorter than the wild type Nagina 22. Isolation of lateral rootless (*lrt*) rice mutants were done recently by Wang et al. (2006) rice (Oryza sativa L. cv. Nipponbare). In our study, the six mutants were found to possess significantly less number of lateral roots in the seminal root. The developmental mechanisms of lateral root formation in rice isolation of mutants exhibiting either loss-of-function or gain-of-function property in develop-ment of lateral root formation is important. Debi et al*.* (2003) reported the isolation of a novel lateral root mutant that is specifically affected in lateral root elongation and root hair formation.

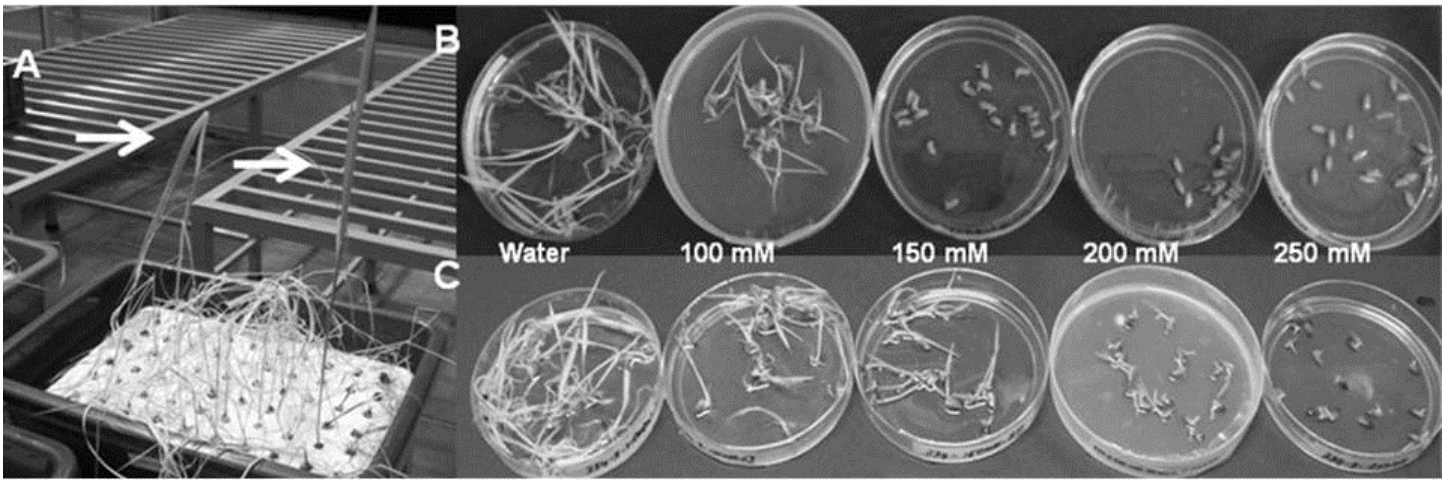

Figure 3. Identification of putative salinity tolerant mutants in rice. A, Survival of two independent mutants under salinity stress (two weeks under 100 mM NaCl stress); B, germination of wild type Nagina 22 at various levels of Nacl and it showed susceptibility at 150 mM Nacl stress; C, germination of a putative salinity tolerant mutant N22-C334-4 under various levels of NaCl which showed tolerance upto 250 mM NaCl stress.

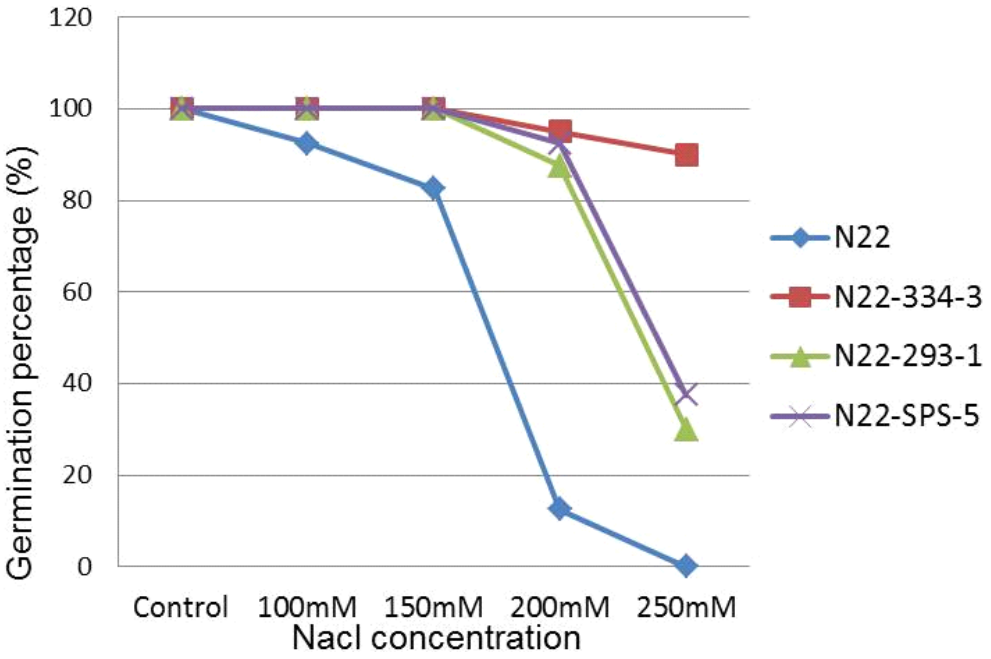

Figure 4. Observation on salinity tolerant mutants performance in germination percentage at 7 days after induced various levels of salinity stress. Each percentage value is mean of n = 75 from 3 replications.

Lateral rootless mutants such as *Lrt1* have been previously isolated in rice (Hao and Ichii, 1999). Crown rootless mutant is unable to initiate the crown root primordia and *crl* mutant have been isolated in previous studies in rice (Coudert et al., 2011; Kitomi et al., 2011). In our study, the mutant N22-D-9 was found to possess no crown roots even up to 15 DAS. This mutant was designated as *crl mutants*. The mutant N22-D-7 was found to possess significantly fewer crown roots than the wild type Nagina 22 (Table 3). Yao et al. (2003) reported such mutants showing extreme inhibition of elongation of seminal root, crown roots and lateral roots and altered root hair formation at the seedling stage or early growth stage.

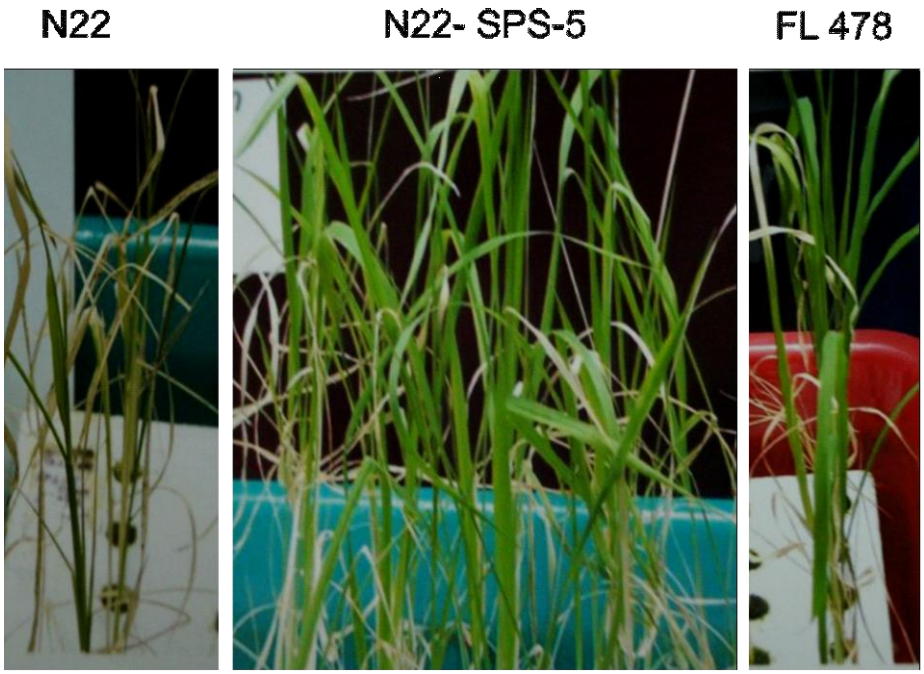

Figure 5. Confirmation of putative salinity tolerant mutants in rice. Confirmation of salinity tolerant ability in identified N22-SPS-5 mutant under salinity stress (two weeks under 100 mM NaCl stress); with Wild type N22; and check variety FL 478.

Genetically to improve the morphogenesis and function of roots in higher plants, it is important to collect mutants associated with various root growth traits. Availability of rice mutants exhibiting either loss-of-function or gain-of-function phenotype for salinity tolerance is limited especially in *indica* type (Miah et al., 1996; Sathish et al., 1997). In this study, among the 1500 mutants, three mutants exhibited significantly greater degree of tolerance to100 mM NaCl stress at seed ling stage. The level of tolerance was almost comparable to the tolerant check Fl 478, a derivative of the salinity tolerant tradi-tional cultivar Pokkali.

To improve rice genotypes for their tolerance against drought and salinity stresses, it is imperative to understand the physiological, genetic and molecular mechanisms underlying various components controlling these complex phenomena. In this context, it is important to collect various mutants associated with these traits contributing to drought and salinity tolerance in rice. There has been a long history of mutation breeding in the cereals with several important varieties resulting from selection of mutant phenotypes (Ahloowalia and Maluszynski, 2001). However, systematic development of mutant populations as genomic resource has only commenced recently. We used a large collection of EMS induced Nagina 22 mutants for identifying lines exhibiting contrasting behavior for root growth traits and salinity tolerance. As a result of our investigation, we have identified six homozygous mutants possessing significantly shorter roots than the wild type and three mutants exhibiting enhanced level of tolerance against salinity stress than the wild type. These mutants can now be used for further genetic, functional genomics and molecular marker studies.

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