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Identifying markers associated with yield traits in Nagina22 rice mutants grown in low phosphorus field or in alternate wet/dry conditions

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Abstract

Mutants are powerful genetic resources in plant breeding and functional genomics studies. Sixty seven stable ethyl methane sulphonate (EMS) induced rice mutants and the wild type parent Nagina 22 (N22) were characterized for plant height, tiller number, panicle number and grain yield under normal, low P field and alternate wet and dry (AWD) conditions in the same season. They were also genotyped with 44 SSR markers and four *Pup1* (Phosphorus uptake1) gene specific markers. Genetic diversity was analysed by combining phenotype and marker data using Ward- MLM method. Single marker analysis showed significant association of four markers RM19696, RM263, RM3688 and RM1942 with grain yield in all three conditions. K-1, a *Pup1* gene specific marker was significantly associated with tiller number only under low P conditions. The average dissimilarity between mutants was 0.86 and cophenetic correlation coefficient was 0.74. Six mutants were selected as gain-of-function mutants as they showed significantly higher grain yield in all three conditions, compared with N22. The selected mutants are an important resource for gene discovery for enhanced tolerance to low P and water stress conditions and associated markers can be useful in marker assisted selection.

Keywords: AWD; low P; mutants; *Pup1* marker; tiller number.
Abbreviations: P _Phosphorus; AWD _alternate wetting and drying; SSR_Simple sequence repeats; N22_Nagina 22; EMS_ethyl methane sulfonic acid; SMA_Single marker analysis.

Introduction

Phosphorus (P) is an essential macronutrient required for plant growth. It is a significant component of nucleic acids and cell membranes and plays an important role in lipid metabolism. P is also required for photosynthesis and respiration processes. One third of the agricultural land in the world does not have adequate amount of P in the soil for optimum plant growth and development (MacDonald et al., 2011). Availability of P from soil to plant is limited due to its low mobility in nature and is often a limiting factor for crop yield. Application of rock phosphate, the major source of P fertilizers, is uncertain as it is non renewable resource and the world reserves of rock phosphate are likely to be exhausted (Wiel et al., 2016). Hence, P fertilizers are becoming expensive and thus lower the profit to farmers in low input rainfed agricultural systems. On the other hand, abundant use of P fertilizers derived from livestock manures, a common practice in intensive agriculture, affects environment in the form of eutrophication of freshwater habitats (Tiessen, 2008; Ashley et al., 2011). One approach suggested to tackle this problem of low or excess use of P in farming is the identification and development of genotypes with high phosphorous use efficiency (PUE) in low P soils without compromising yield (Aluwihare et al., 2016; Rose et al., 2010, 2011, 2012, 2015). More than PUE, it is the ability to tolerate P deficiency to different extent and yet give high yield which is more important. Tolerance to low P is a complex trait involving plant architectural, physiological, biochemical and molecular mechanisms that provide plasticity. Therefore, there is a necessity to identify and develop new genetic resources to explore diverse molecular mechanisms for P deficiency tolerance.

Rice is the major irrigated cereal crop and it requires two to three times more water than other crops such as wheat and maize (Bouman et al., 2007). It requires 700-1500 mm of water per cropping season under normal traditional irrigation practices (Bhuiyan, 1992). It is estimated that by 2025, 15 million ha of Asian rice production under irrigation will experience physical water scarcity as the per capita available water resources in Asia are expected to decline by 15-54 percent (Tuong and Bouman, 2003; Rahman and Bulbul, 2015). However more irrigation water is required to increase rice production to meet growing food demand. Hence, efficient water management practices are needed in rice cultivation by adopting water-saving irrigation technologies (WSI). Alternate wetting and drying (AWD) is one of the most commonly used practices of WSI technique. In this practice, irrigation water is applied to achieve alternate flooded and non-flooded soil condition. The first treatment of draining the field starts at 1 to 2 weeks after transplanting until the water level reaches 15 cm below the soil surface. The field is re-flooded to a ponded depth of around 5-10 cm. This irrigation scheme is followed throughout the cropping season except from 1 week before and 1 week after flowering (Siopongco, et al., 2013). AWD irrigation system reduced

irrigation water input by up to 38% without reduction in yield with large scale adoption in Philippines, Vietnam and Bangladesh (Lampayan et al., 2015). Zhi and Cui (2001) reported that irrigation water use was reduced by 7-25% with AWD technique and Rahman and Bulbul (2015) showed that AWD saved 24-28% of irrigation water compared to continuous flooding in Bangladesh. AWD irrigation approach in India can reduce water use by about 40-70% compared to the traditional practice of continuous submergence, without significant yield loss (Singh et al., 1996, Rejesus et al., 2010).

Genetic variation produced by natural and artificial mutations is the basis of selection in crop breeding. Use of chemically induced mutants such as ethyl methane sulfonic acid (EMS) has helped to create useful genetic variations in the past (Bhat et al., 2007; Till et al., 2007; Henry et al., 2014; Mohapatra et al., 2014). It has been shown that mainly GC to AT transitions were induced by EMS in Arabidopsis, maize and wheat, (Till et al., 2003; Till et al., 2004; Slade, 2005). But in rice, it was reported that 70% mutations were GC to AT, 11% AT to GC, 4% GC to TA, and 15% AT to TA (Till et al., 2007). This was consistent with mutational spectrum of barley (Caldwell et al., 2004). Such mutations may in turn lead to other mutations if the initial mutations were in genes for DNA repair enzymes, or genes that help suppress transposon activation, for example.

A large number of rice mutant lines have been produced and studied with the aim of gene discovery for important traits and assigning functions to genes (Mithra et al., 2016). The mutant lines exhibiting significantly higher grain yield or higher tolerance to nutrient deficiency or abiotic stresses or other traits can be used for rice breeding by conventional backcrossing along with molecular marker-assisted selection (Jiang and Ramachandran, 2010; Sikora et al., 2011). EMS induced rice mutants were used to investigate the complex mechanism involved in water and salt stress tolerance (Huang et al., 2009; Zhou et al., 2013). The morphological, physiological and proteomic characterization of some EMS induced mutants showed more tolerance to abiotic stresses for example salt and heat in contrast to their respective wild type lines (Ghaffari et al., 2014; Poli et al., 2013; Nakhoda et al., 2012; Mithra et al., 2016). However, there are no previous reports of screening rice mutants for tolerance to low P at field level and also under AWD conditions.

Assessing genetic diversity is important in breeding programs to know the available genetic variability. Genetic diversity has been comprehensively analysed in rice, wheat, maize, barley and soybean. Simple sequence repeats (SSR) or microsatellite markers have been widely used due to their co-dominant nature and repeatability. SSR markers were also used to identify molecular diversity induced through EMS mutations in various crops for example in ground nut (Goswami et al., 2013), in mung bean (Singh et al., 2012) and in chick pea (Khan et al., 2010).

Genetic diversity in rice is usually estimated using either quantitative data such as plant height, number of tillers and panicles, days to flowering, days to maturity and yield traits or molecular marker data such as SSR markers and ISSR markers individually (Prasanth et al., 2016; Nachimuthu et al., 2015 ; Turki et al., 2015;). Both phenotype and molecular data were used to determine genetic diversity and group genotypes using Ward-MLM method in corn (Franco et al., 2005; Ortiz et al., 2008), wheat (Geleta and Heinrich 2012), tomato (Gonçalves et al., 2009), beans (Barbé et al., 2010), capsicum (Sudré et al., 2010), banana (Pestana et al., 2011; Reis et al., 2015) and cassava (Oliveira et al., 2015).

Nagina 22 (N22) is a deep-rooted, drought and heat tolerant upland aus variety widely used as a donor for heat tolerance (Markandeya et al., 2007; Jagadish et al., 2008). In India, 20,000 stable EMS induced mutant lines of N22 have been developed as a national resource for functional genomic studies in rice (Mohapatra et al., 2014, Mithra et al., 2016). The objective of the present work was 1) to evaluate EMS induced N22 mutants for phenotypic variation under three field environments - normal irrigated, low P soil and AWD conditions, 2) to study genetic diversity using phenotype and SSR data using Ward-MLM method and 3) to identify markers associated with plant height, tiller number, panicle number and yield per plant under normal, low P and AWD conditions in field.

Results

Field experiments

Normal vs low P conditions

ANOVA results showed statistically significant effects on all four traits (plant height, tiller number, number of panicles and yield/plant) among mutants, between normal and low P conditions and interaction between mutants and treatments (Table1). Descriptive statistics and absolute mean values of three replications for the four traits in each genotype under normal conditions and low P conditions during wet season 2012 are shown in Supplementary Table 1. All traits were normally distributed with absolute values of skew and kurtosis of <1.0 in the mutant population. A highly positive correlation among panicle number, tiller number and yield per plant was observed under both low P and AWD treatments (Fig. 1).

In low P, plant height was significantly more in only three mutants, NH404 (8.7%), NH418 (15.3%) and NH685 (19.5%) when compared with normal condition. NH101 was the tallest among all mutants in both normal (101 cm) and low P (85 cm) fields. Both number of tillers and panicles decreased in low P in all mutants and N22. However, the percent decrease was significantly less in 46 mutants for tiller number and 37 mutants for panicle number compared to N22. Similarly, there was significant reduction in yield per plant under low P condition in all mutants when compared with normal conditions. However, when compared with N22, the percent reduction in yield per plant was significantly less in 26 mutants and significantly more in 11 mutants. NH377 had the least percent reduction in yield per plant (38.24%). In addition, ten mutants either did not flower (eg. NH101) or flowered but did not set seed under low P (Supplementary Table 1).

The mean yield per plant in N22 was 13.84 g in normal and 1.61 g in low P conditions thus showing 88% reduction. Three mutants (NH669, NH686 and NH787) exhibited maximum yield per plant in both normal (22-27 g) and low P treatments (8.2- 8.9 g), with 63-68% reduction in yield due to low P stress (Supplementary Table 1).

Normal vs AWD conditions

ANOVA revealed statistically significant differences in all traits among mutants, between normal and AWD conditions and also due to interaction between mutants and treatments (Table1). Absolute mean values of three replications for all four morphological traits of each genotype under normal conditions and AWD conditions in wet season 2012 are shown in Supplementary Table 1. The correlations were similar to that observed in normal vs low P conditions (Fig. 1). Plant height reduced up to 30% in AWD in all lines

Table 1. Analyses of variance for N22 mutants under normal Vs low P and AWD conditions.

		Normal Vs low P conditions				Normal Vs AWD conditions			
Source	DF	Plant height (cm)	Tiller number	Panicle number	Yield per plant (g)	Plant height (cm)	Tiller number	Panicle number	Yield per plant (g)
Genotype	67	23.09***	7.55***	11.8***	40.77***	89.73***	17.4***	23.53***	83.7***
Treatment	1	797.03***	1514.21***	1728.23***	11381.1***	946.18***	582.68***	370.86***	445.79***
Genotype*Treatment	67	16.71***	5.79***	7.43***	18.86***	6.12***	2.32***	2.3***	7.27***
Error	272								
Grand mean	-	73.304	11.324	10.235	9.1076	74.547	13.299	12.623	14.003
CV	-	5.6	17.66	18.22	13.16	3.96	11.81	12.06	9.82

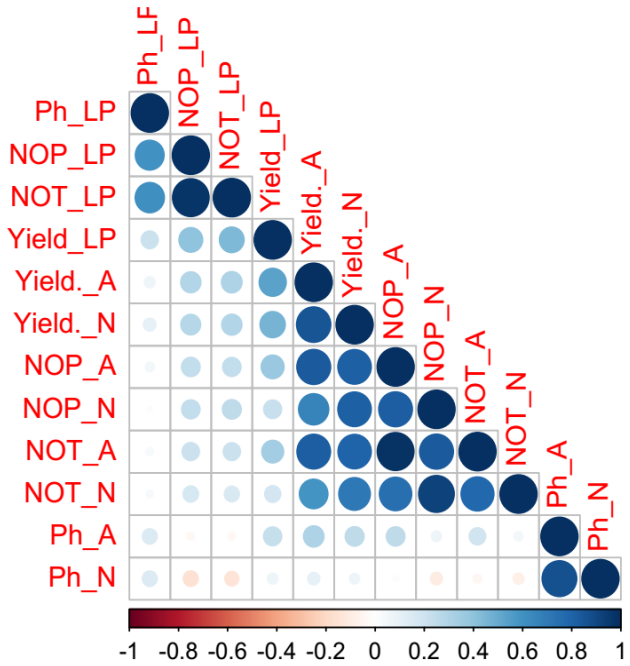

Fig 1. Pearson correlation among traits under normal, low P and AWD irrigation.Ph: Plant height; NOT: Number of tillers; NOP: Number of panicles; Yield: Yield per plant; N: Normal; LP: Low P soil; A: AWD irrigation. The bar below the picture represents Pearson correlation value, Blue circles indicate positive correlation and red circles indicate negative correlation

Table 2. Significant results of single marker analysis of N22 mutants with F value under normal, low P and AWD irrigation.

Trait			Normal	Low P field	AWD
	Marker	Chromosome	F	F	F
Plant height	RM541	6	10.29**	-	13.27***
	RM304	10	3.15*	-	-
	RM1920	2	5.80***	-	-
	RM224	11	-	2.41*	3.49*
	RM449	1	-	-	-
Number of tillers	RM19697	6	3.64**	-	7.28***
	RM263	2	6.31**	-	4.5**
	RM3688	2	12.2***	-	20.75***
	RM1942	2	5.59**	-	11.68***
	RM8007	7	4.81*	-	
	K-1 (*pup* gene specific marker)	12	-	3.77*	-
	RM205	9	-	-	4.24**
	RM260	12	-	-	3.21*
Number of panicles	RM19697	6	4.41**	-	7.34***
	RM263	2	6.03**	-	4.63**
	RM3688	2	12.71***	-	18.88***
	RM1942	2	7.34**	-	13.21***
	RM205	9	-	-	3.18*
	RM260	12	-	-	3.41*
Yield per plant	RM19697	6	5.68***	4.48**	7.14***
	RM263	2	5.97**	3.98*	3.93*
	RM3688	2	10.8**	12.68***	14.66***
	RM1942	2	5.74**	7.73***	7.5***
	RM242	9	3.16*	-	-
	RM1	1	3.98*	-	4.04*
	RM260	12	-	3.47*	-
	RM205	9	-	-	4.24**

***Significant at 0.001 level of probability; ** Significant at 0.01 level of probability; * Significant at 0.05 level of probability.
Note: Markers information is available at http://www.gramene.org/markers/microsat.

Fig 2. Dendrogram of 67 N22 rice mutants and N22 based on quantitative data (yield related traits in three environments) and 48 markers data and using the UPGMA method in DARwin

except NH8 which showed a 2% increase but it was not significant. Similarly, number of tillers and panicles reduced upto 61% in all lines in AWD except in NH125 and NH444 which showed a non significant increase compared to normal irrigation. Yield per plant also decreased in AWD in all mutants except in three mutants which showed a non-significant increase. NH221 showed the maximum yield reduction of 71.24% and NH123 the least (1.1%) compared to 19.22% in N22.

Genetic diversity and molecular analysis

Forty four SSR markers and 4 *Pup1* specific markers amplified 95 alleles in all. Dissimilarity matrices were prepared separately with genotyping data and morphological data in three environments. The correlation between these two distance matrices was very low (r = 0.0199) and the genetic variability was analysed by combining both genotyping and morphological data using Ward-MLM method. The distance among all mutants ranged from 0.17 to 1.96 with average distance of 0.861 and cophenetic correlation coefficient was 0.739 ($p<0.01$). As per pseudo-F and pseudo-t^2 criteria, the optimum number of groups was five. Among the five clusters, cluster I had 14 mutants, cluster II had 12 mutants, cluster III had 14 mutants, cluster IV had N22 and 14 mutants and cluster V had 13 mutants (Fig. 2). The closest mutants were NH664 and NH427 with 0.17 genetic distance and the most dissimilar were two tall and stay green mutants NH363 and NH162 with genetic distance 1.96. These results revealed clear genetic variability for 4 traits among N22 mutants in the three conditions.

Single marker analysis

F values and significant marker-trait associations between 48 markers and four traits in normal, low P and AWD are listed in Table 2.

Marker trait associations in normal conditions

Two loci RM541 and RM1920 on chromosome 2 and RM304 on chromosome 10 were significantly associated with plant height under normal conditions. The two tall mutants NH363 and NH101 showed a different allele compared to N22 allele at locus RM1920. Four markers RM263, RM3688 and RM1942 on chromosome 2, and RM19697 on chromosome 6 were significantly associated with number of tillers, number of panicles and yield per plant. In addition, RM8007 was associated with only number of tillers and RM242 and RM1 with only yield per plant. The three mutants NH719, NH733 and NH686 which showed significantly higher yield per plant shared the same allele at loci RM19697, RM242, RM1, RM263, RM3688, RM1942 and RM8007.

Marker trait associations in low P conditions

RM224 was significantly associated with plant height and the allele at this locus was similar in 44 mutants whose height was significantly more in control condition than in low P. Only one marker K-1 out of the four *Pup1* specific markers was significantly associated with number of tillers and showed a clearly different allele in mutants with high tiller number in low P. Five loci RM19697, RM263, RM3688, RM1942 and RM260 were significantly associated with yield per plant. The alleles at RM19697, RM263 and RM260, were similar in 17 mutants including six mutants with higher grain yield per plant in low P.

Marker trait associations in AWD conditions

Two markers RM541 and RM449 were significantly associated with plant height and the allele in notably tall mutants in AWD was different from N22 allele. Five markers RM19697, RM263, RM3688, RM1942 and RM205 were significantly associated with number of tillers and panicles and yield per plant. In addition, RM260 was linked to number of tillers and panicles and RM1 to yield per plant. Alleles at loci RM19697, RM1 and RM205 were similar in 17 mutants group showing higher tiller and panicle number under AWD and different from corresponding N22 allele. In all, 15 markers out of 48 were associated with plant height, number of tillers, number of panicles and yield per plant based on single marker analysis. In the current study, four markers RM19697 (chromosome 6), RM263 (chromosome 2), RM3688 (chromosome 2) and RM1942 (chromosome 2) were associated significantly with yield per plant in all the three conditions. These markers were also associated with tiller number and panicle number under normal and AWD conditions but not in low P. In low P, only K-1 (*Pup1* gene) was associated with tiller number.

Discussion

Four traits (plant height, number of tillers, number of panicles and yield per plant) showed significant differences among themselves and also between treatments of normal vs low P soils and normal vs AWD. This indicates the existence of variation in the mutants. Three mutants (NH669, NH686 and NH787) exhibited maximum yield per plant in both normal (22-27 g) and low P treatments (8.2- 8.9 g) hence were considered as the best mutants under both normal as well as in low P conditions. The reduction in yield due to P stress in these three mutants was 63-68%. Next to these three mutants, there were another set of three mutants (NH355, NH363 and NH719) which performed well as they showed 7g yield per plant under low P conditions with 70 - 73% reduction in yield when compared with that of normal condition. Though NH377 exhibited least percent reduction in yield per plant in low P stress but it did not give good absolute yield under both normal 7.88 g as well as low P (4.86 g). Hence, this mutant can be exploited for low P tolerance in genomics and breeding programme but may not be commercially important. Similarly, NH101 was the tallest mutant but it did not flower under low P and can be used to map genes for low P susceptibility as loss-of-function mutant for flowering in low P.

There are very few studies on identification or development of rice cultivars tolerant to low P conditions at field level as maintenance and preparation of a field with low phosphate needs much more attention than a normal field and also due to lack of proper screening methods, for traits that confer low P tolerance (Panigrahy et al., 2014). Aluwihare et al. (2016) selected low P tolerant rice genotypes by growing plants in pots filled with soil, collected from cultivated field without P fertilizer application for past 40 years.

There was significant reduction in trait values due to water stress under AWD in mutants or N22 when compared with normal flooded irrigation. However, a few mutants performed better than N22. Two mutants NH355 and NH787 showed highest yield per plant (25g) under AWD conditions. These two mutants also exhibited maximum yield per plant in normal field (27g) with only 6% reduction in yield due to water stress. The percent reduction in yield in N22 was 19.22%. There were three other mutants (NH363, NH686 and NH719) which were next as they exhibited better yield per

plant (23g) under AWD conditions with 11-15% reduction in yield (except in NH719, showed equal yield as in normal) when compared with that in normal conditions (23-27g). NH363 was an exceptional mutant having maximum plant height as well as high grain yield. Thus NH363 is a gain of function mutant for both plant height and grain yield in both normal and AWD conditions. NH221 and NH407 showed minimum yield per plant (3.27 g and 4.08 g) with 71% and 61% reduction respectively in AWD conditions when compared with normal irrigation condition. Alternate wet and dry treatment increased the grain filling rate, shortened grain filling period and enhanced whole plant senescence in rice (Zhang et al., 2012).

The quantitative data and SSR marker data revealed different patterns of dissimilarity among the matrices generated; hence, a combined analysis approach offers a better assessment of the real variability. The hierarchical dendrogram broadly clustered the rice genotypes into five major groups. This indicates a high level of genetic diversity in mutants. Wild type N22 had minimum dissimilarity with NH218 and maximum with NH355 (1.28). Out of six best mutants (gain of function) selected for grain yield and low P tolerance, four (NH355, NH787, NH686 and NH669) were in the same cluster with dissimilarity ranging from 0.27 to 0.42. Likewise, two tall mutants (NH363 and NH101) were in the same cluster II. This indicates the effectiveness of Ward-MLM method of combined data analysis of both quantitative and molecular data in grouping rice genotypes. The results also show the effectiveness of EMS in inducing higher yield in some mutant lines as compared to N22 both in normal and two adverse soil conditions. Thus, it appears from the phenotype that a large number of genome wide mutations with large effects on phenotype were created in these mutants even though only 48 loci were tested.

Plants have different morphological, physiological and biochemical mechanisms such as root growth and architecture, the release of root exudates and associations with soil microorganisms (Wiel et al., 2016; Vandamme et al., 2016; White and Hammond, 2008). Extensive work has been carried out to map QTLs for P uptake and PUE and relevant QTLs have been identified in rice (Wissuwa et al., 1998; Wissuwa et al., 2002; Shimizu et al., 2004; Lang and Buu, 2006; Zhang et al., 2009, Gamuyao et al., 2012; Wiel et al., 2016). Phosphorus uptake 1 (*Pup1*) and phosphorus starvation tolerance 1 (*PsTOL1*) QTLs lead to efficient P uptake and larger root system under P deficient conditions to take up more phosphorus in Kasalath, an Indian aus variety (Wissuwa et al., 2002; Gamuyao et al., 2012). Tyagi et al. (2012) reported two rice genotypes LR 23 (with *Pup1*) and LR 26 (without *Pup1*) as tolerant to low P. Dkhar et al. (2014) studied these two genotypes further and revealed differential expression of two genes MO5 (LOC_Os02g29620) and MO7 (LOC_Os03g28920) involved in maintaining cellular homeostasis under low P conditions in these two low P tolerant rice genotypes. In LR 23, these two genes (MO5 and MO7) were down regulated where as in LR 26, they were up regulated. These results indicate there may be diverse mechanisms of P deficiency tolerance in different genotypes. N22 is also an aus variety known to have *Pup1* genes (Tyagi et al., 2012). Tiller number, number of roots, and shoot biomass per plant is associated with phosphorus deficiency tolerance (Raghothama, 1999; Wissuwa and Ae, 2001). A larger genetic variation for P uptake traits relative to P utilization has also been reported in wheat, maize, rice, sorghum and pearl millet (Jones et al., 1989; Wissuwa et al., 1998; Parentoniet al., 2010; Leiser et al., 2014; Gemenet et al., 2015).

In the current study, four markers RM19697 (chromosome 6), RM263 (chromosome 2), RM3688 (chromosome 2) and RM1942 (chromosome 2) were associated significantly with yield per plant in all the three conditions. These markers were also associated with tiller number and panicle number under normal and AWD conditions but not in low P. This shows that the genomic regions responsible for grain yield and number of tillers were different under AWD and low P stress. In low P, only K-1 marker of *Pup1* gene was associated with tiller number but not with yield. Thus both presence of K-1 or tiller number in low P can be taken as a surrogate for low P tolerance. The sequenced gene, *Pup1* in Kasalath rice variety was reported to provide tolerance to P deficiency under field conditions in Japan (Wissuwa et al., 1998; Wissuwa et al., 2002; Chin et al., 2010, 2011) and it was independently mapped on chromosome 12 by Ni et al. (1998). Introgression lines with *Pup1* region in different genetic backgrounds have the potential to significantly increase grain yield under P deficient field conditions (Chin et al., 2011). In the present study also, it is significant that K-1(one of the *Pup1* markers) was linked to tiller number only in low P conditions but not in normal and AWD conditions. This has not been shown previously. Moreover, at this locus, the allele group contributing for more number of tillers was present in the three highest yielding mutants (NH787, NH363 and NH686) under low P and also different from N22 allele type. In these three mutants, the allele for K-1, (associated with tiller number in low P) was different from that of N22. Increased tiller number under P deficiency is a very good indicator of P deficiency tolerance in rice (Wissuwa et al., 1998, 2002; Ni et al., 1998; Alam et al., 2009). At IIRR, we have been using tiller number as a surrogate for root traits in low P field. Wissuwa et al. (2002) reported that 80% of the variation for tiller number was explained by genetic variations at *Pup1* locus.

In the present study, RM263 was associated with yield per plant in all three treatments: normal, low P and AWD. The same marker was also associated with number of tillers and panicles in normal and AWD conditions. Luo et al. (2011) reported positive association of RM263 with 1,000 grain weight in the introgression lines of indica cultivar Guichao 2 and *O. rufipogon*. Marri et al. (2005) also reported RM263 flanking a major yield QTL *qyld2.2* and grain number QTL *gnp2.2* in interspecific BC_2 testcross progeny (IR58025A/ *O. rufipogon*// IR580325B/// IR58025B//// KMR3). They also reported RM242 and RM205 on chromosome 9 flanking QTLs for yield (*qtlp9.1*) and grain weight (*gw9.2*). However, in the present study RM205 was linked with yield per plant and number of panicles in AWD conditions only and RM 242 with yield per plant only in normal conditions. RM242 was used in marker-assisted back-crossing to significantly increase root length under both irrigated and drought stress in Kalinga III (an Indian upland rice variety) using Azucena (an upland *japonica* variety from Philippines) as donor parent.

Materials and methods

Plant materials

A set of 67 EMS induced Nagina 22 (N22) mutant lines of M_6 generation and their parent N22 (wild type) were used as experimental material. Panicle to row method was followed to advance generation from M_2 to M_6.

Field experiment

The plants were grown during wet season 2012 at fields of Indian Institute of Rice Research (IIRR), Hyderabad (latitude and longitude: 17°22′31″N and 78°28′27 E) under the following three environments; a) normal plot (15 mg kg^{-1} Olsen P) using normal irrigation practices b) low P plot (1.80-2.00 mg kg^{-1} Olsen P) using normal irrigation practices c) AWD practice in normal soil. The low P plot was developed by not giving any P fertilizers for the last 27 years, but supplied with 100 kg N ha^{-1} and 60 kg K ha^{-1} each season (Panigrahy et al., 2014). Plants were grown in 3 replications of 2 lines of each genotype and 22 plants per line with spacing of 20 cm × 20 cm in each treatment. Irrigation management practices in AWD treatment were followed as described by Siopongco et al., 2013. At physiological maturity, the following morphological traits were studied viz., plant height (length of the tallest tiller upto tip of panicle in cm), number of tillers, number of panicles (number of panicles with seeds exceeding 15%), and yield/plant (mean weight of filled seeds from 22 plants).

Genotyping

Genomic DNA was extracted from leaves of 67 N22 mutant lines and N22 using Cetyl Trimethyl Ammonium Bromide extraction buffer. For genotyping, polymerase chain reaction (PCR) was done with 44 SSR markers from RM series (McCouch et al., 2002) and four *Pup1* gene specific markers (Chin et al., 2011). The amplified fragments were evaluated as present (1) and absent (0) bands.

Genetic diversity and marker trait association analysis

General statistics, Anova, correlation and significant analysis comparing with normal and stress conditions and also between mutants and parent N22 for phenotyping data were performed using Statistix 8.1.

Genetic diversity in mutants was evaluated considering phenotyping data from three tested treatments (normal, low P and AWD) and genotyping data of 44 SSR markers and four *Pup1* gene specific markers (95 polymorphic bands) using CLUSTER and Ward's MLM procedure in SAS program. Gower algorithm was used (Gower, 1971) to obtain the distance matrix for the use of the Ward grouping method. The ideal number of groups was decided according to the pseudo-F and pseudo-t^2 criteria (SAS Institute 2011). Hierarchical cluster analysis was performed using Ward minimum variance method and cophenetic correlation coefficient (Fit critera) using DARwin 5 software (web: http://darwin.cirad.fr/darwin), based on the distance matrix by the Gower algorithm. Dendrogram was constructed to estimate number of clusters.

Single marker analysis (SMA) was done using MINITAB V14.0 (Minitab Inc., State College, PA, USA) to find out association between each marker and each trait in the three environments.

Conclusion

Six mutants (NH787, NH686, NH669, NH363, NH355 and NH719) were identified as the best mutants based on their yield under all three conditions normal, low P and AWD and considered as gain of function mutants for low P tolerance. On the other hand, NH101 was identified as a loss of function mutant for flowering and yield under low P. Mutants were delineated into five groups. The pair NH664-NH427 were the closest and NH363-NH162 were the most dissimilar. In all, 15 markers out of 48 were associated with plant height, number of tillers, number of panicles and yield per plant based on single marker analysis. Significantly, *Pup1* gene specific marker, K-1 was associated with tiller number but only in low P conditions. The selected mutants and associated markers are a good genetic resource to breed for tolerance to low P and AWD conditions and also for discovering genes related to these desirable traits.

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