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Evaluation of activation tagged rice mutants for variability in response to *Meloidogyne graminicola* under challenged inoculation

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Abstract

Activation tagging is one of the most preferred strategies in functional genomics. The major advantage of the technique is the development of variability to a particular character(s) in a single genetic background based on the site of T-DNA integration. The understanding of resistance/ susceptibility mechanism portrayed by the mutants and identification of the genes responsible for the respective trait has always been a lucrative option for crop improvement. In rice, *Meloidogyne graminicola* has emerged as one of the major nematode pests and a main constraint for yield losses. The activation tagged rice mutants developed depicted a range of phenotypic variations with respect to panicles, height, flowering time, seed colour etc. Further, evaluation of the response of selected 16 activation tagged rice mutants to deliberate challenging with *M. graminicola* under pot culture studies showed a large variation in the ability of the nematode to infect these mutants. Among them, five mutants (lines 8, 9, 10, 11 and 15) portrayed a resistant response by producing least number of galls ranging from 1.5 to 2.6 galls per plant whereas wild type and other mutants were moderately susceptible (5 to 8.10 galls per plant) to *M. graminicola*. Corroborately, the multiplication factor of *M. graminicola* in resistant mutants was significantly less ranging from 0.64 to 1.71 as against 6.36 to 17.43 in wild type and other susceptible mutants due to reduction in total endoparasites and nematode fecundity. This variability towards the nematode challenge can be very useful in deciphering the molecular mechanisms of underlying responses, which can be exploited for breeding resistant rice varieties against this serious pest.

Keywords: *Meloidogyne graminicola*, rice, activation tagging, glyphosate, variability, screening, resistant, susceptible

Introduction

Rice (*Oryza sativa* L.) is the most cultivable and important cereal crop providing food to more than half of the world's population (Fageria 2007). Escalating the productivity of rice through minimizing yield losses occurring due to various biotic and abiotic stresses is the need of the hour. Among the various biotic stresses, plant parasitic nematodes are one of the key constraints for rice production. Rice root knot nematode (RRKN), *Meloidogyne graminicola* has attracted major attention due to its ability to infect and cause serious damage (Pokharel et al. 2007). *M. graminicola* forms characteristic hook like galls on the roots of the infected plant leading to axial swelling with above ground symptoms like patches of stunted plant growth and yellowing, similar to the symptoms occurring due to water scarcity or nutrient deficiency. In India, *M. graminicola* is reported to cause 17-30% yield losses due to poorly filled kernels (Jain et al. 2007). Therefore, minimizing losses and nematode management are of paramount importance with emphasis on seed borne solutions for long term durability (Lorenzana et al. 1998; Kandoth and Mitchum 2013). To combat nematode population, resistant source is one of the most economical and sustainable methods (Kandoth and Mitchum 2013). Though a resistant source against *M. graminicola* has been found in African rice (*Oryza glaberrima* and *O. longistaminata*) (Soriano et al. 1999), documentation of resistance and its mechanism against *M. graminicola* is insufficient in Asian rice except for a recent report where a rice cv. Abhishek was found to be resistant (Mhatre et al. 2015; 2017).

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Identification and characterization of novel source of resistance against *M. graminicola* in rice must be done under favourable environmental conditions required for this nematode (Tandingan et al. 1996). Functional genomics approaches provide options for the identification of novel/superior genes that would enormously reinforce the attempt to mitigate biotic/ abiotic stresses in rice. To facilitate this, activation tagging (AT) or T-DNA insertional mutagenesis has emerged as a powerful biotechnological tool by exploiting transgenic technology for the development of mutants (Manimaran et al. 2017). The strategy can be utilized for the generation of a large number of independent transformed lines even in a recalcitrant crop like rice. Further, the technology aids in the identification of variability for a particular trait and can thus be exploited to study the underlying mechanisms as well as identification of the responsible genes. In view of this, the present study demonstrates the utility of variability portrayed by a panel of T-DNA insertional mutants to deliberate challenging against *M. graminicola* under greenhouse conditions. The study could pave way for an in-depth understanding of the molecular mechanisms essential for both the susceptible and resistance response of the plants to this nematode.

Material and methods

Plant material

A selected panel of 30 activation tagged mutants developed in the rice accession JBT 36/14 and their wild type (WT) were procured from Department of Crop Physiology, UAS GKVK, Bengaluru.

Screening on glyphosate for confirmation of transgenic nature

In order to ascertain their transgenic nature, the resistance of the mutants to glyphosate due to the presence of an EPSPS gene in the T-DNA was exploited. For this, seeds of the selected mutants and WT were soaked in water overnight and placed on germination paper in a Petri dish at 30^{o} C. Two to three days old seedlings were used for glyphosate screening under greenhouse conditions. The seedlings were transferred to a bowl containing 500 g quartz sand. The bowls were further drenched with an optimized 8 ppm concentration of glyphosate solution until saturation (Hatzade et al. 2019) and untreated WT was drenched with water. Weight of the bowl was measured and loss of water on each day was compensated by supplementing with water and later scored for growth and recovery. Greenhouse grown plants were continuously observed for phenotypic variations during the entire crop growth period.

Establishment and maintenance of M. graminicola *culture for the study*

Pure culture of *M. graminicola* was isolated and multiplied on the susceptible rice cv. Pusa Basmati 1121 under optimum conditions (Kumari et al. 2016). Juveniles were extracted following a modified Baermann funnel technique (Schindler 1961) from matured galls which were brownish in colour having females and eggs as confirmed by microscopic observation and used for various experiments.

Assessment of the response of the mutants to deliberate challenging against M. graminicola

Seeds of selected mutants including WT were utilized for the nematode bioassay. The seeds were surface sterilized with 70% ethanol followed by rinsing with distilled water three to four times. Sterilized seeds were placed on germination paper in a Petri dish incubated in growth chamber maintained at 27-30p C. Three to four days old germinated seedlings were transplanted in small trays filled with approximately 150 g autoclaved soil with ten replicates for each mutant and maintained under controlled greenhouse conditions (27-30^{o} C, 60% RH with 16 h of light and 8 h of dark) where the nematode life cycle can be completed within 20 days. Eleven days post transplantation, pure culture of *M. graminicola* juveniles (J2) extracted as above was inoculated in the rhizosphere of each seedling @ 2 J2/g of soil. After 20 days of nematode inoculation (Mhatre et al. 2015; Kumari et al. 2016), rice plants were uprooted, gently washed with water and roots were stained with acid fuchsin (Byrd et al. 1983). Parameters *viz.* number of galls, total endoparasites, egg masses and average eggs/egg mass were counted by dissecting stained roots under binocular microscope. Nematode multiplication factor [(number of egg masses ×number of eggs/egg mass) ÷ nematode inoculum level] was also calculated.

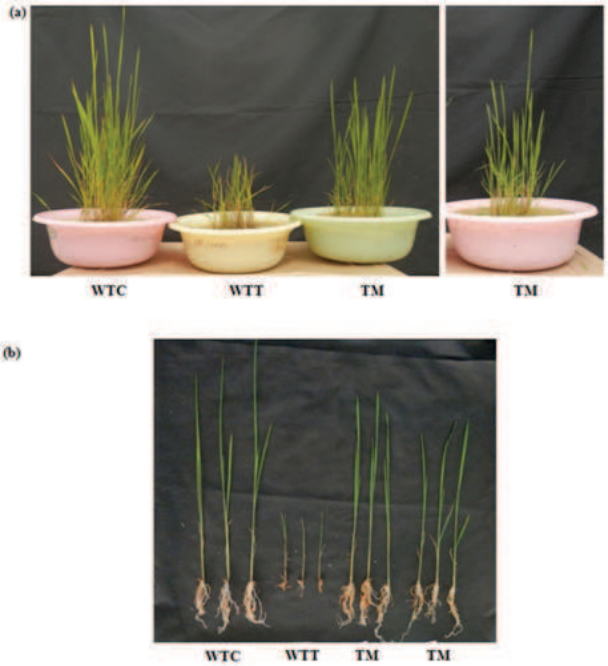
To evaluate the resistant/susceptibility of these mutants gall index was calculated according to Pederson and Windham (1989) with small modification. Root galling was calculated based on a scale 0 to 5, where level 0 = no galls, level 1 = 1-2, level 2 = 3-10, level 3 = 11-20, level 4 = 21-30, level 5$\geq$30 galls per root system. The gall index (GI) was calculated using following formula:

GI = $\Sigma(S_i \times N_i)/(N \times 5) \times 100$. Where, S_i was root galling scale of 0, 1, 2, 3, 4, 5. N_i was number of plants in each root galling scale. N was the total number of evaluated plants. GI was used to score resistance/susceptibility as follows: immune (I) GI=0; highly resistant (HR) $0.1 \leq GI \leq 5.0$; resistant (R) $5.1 \leq GI \leq 25.0$; moderately susceptible (MS) $25.1 \leq GI \leq 50.0$; susceptible (S) $50.0 \leq GI \leq 75.0$; highly susceptible (HS) GI>75.0 (Zhan et al. 2018).

Data of bioassay experiments was subjected to one way analysis of variance (ANOVA) and completely randomized design (CRD) with statistical significance that was determined at P = 0.05, P = 0.01 and F value. Values of the mean of total replications in each treatment were taken for statistical analysis.

Results

A panel of 30 activation tagged rice mutants that were earlier selected in the presence of glyphosate and confirmed for T-DNA integration was used in the present study (data not shown). However, T-DNA integration was authenticated in the mutants by screening in the presence of 8 ppm glyphosate. The results demonstrated continued growth of the selected mutants in the presence of glyphosate whilst the wild type plants portrayed stagnated growth (Fig. 1a, b). The mutant seedlings showing normal growth in the presence of glyphosate were further confirmed for the transgenic nature by PCR analysis (Fig. 1c).



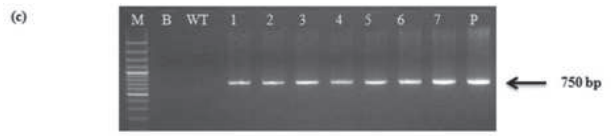
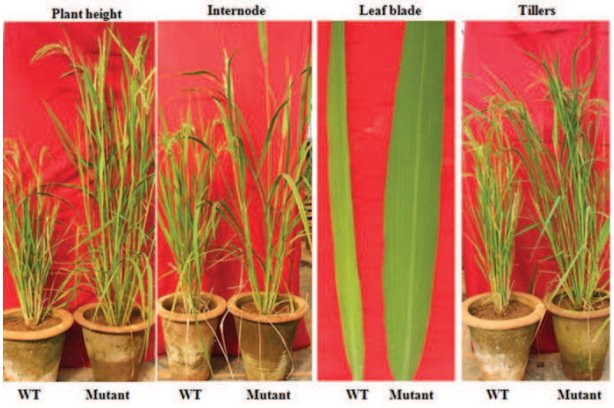


Fig. 1. Glyphosate screening of activation tagged mutants in quartz sand (representation) (a) variation in shoot and root length of mutants *vis a vis* WT upon screening on glyphosate (b) (WTC = wild type control, WTT = wild type treated, TM = treated mutant). PCR analysis of the selected activation tagged rice mutants for the amplification of 750 bp *nptII* gene fragment (c) **(Lane M = Gene Ruler 100 bp plus DNA Ladder (Thermo scientific), Lane B = blank, Lane WT = wild type, Lanes 1-7 = activation tagged mutants line 5, line 8, line 9, line 10, line 11, line 15 and line 27, Lane P = plasmid)**



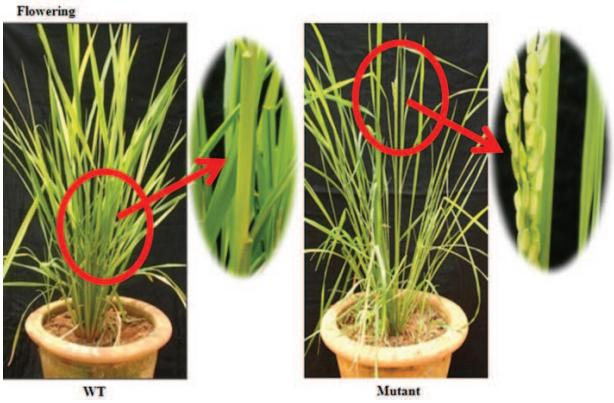


Fig. 2. Phenotypic variation in plant height, internode, leaf blade, tillering ability and flowering in mutants *vis-a-vis* wild type (WT = wild type)

Upon transfer of the mutants to the greenhouse, explicit phenotypic differences could be observed across the mutants *vis-a-vis* wild type. Variation was observed in plant height, internodal length and width

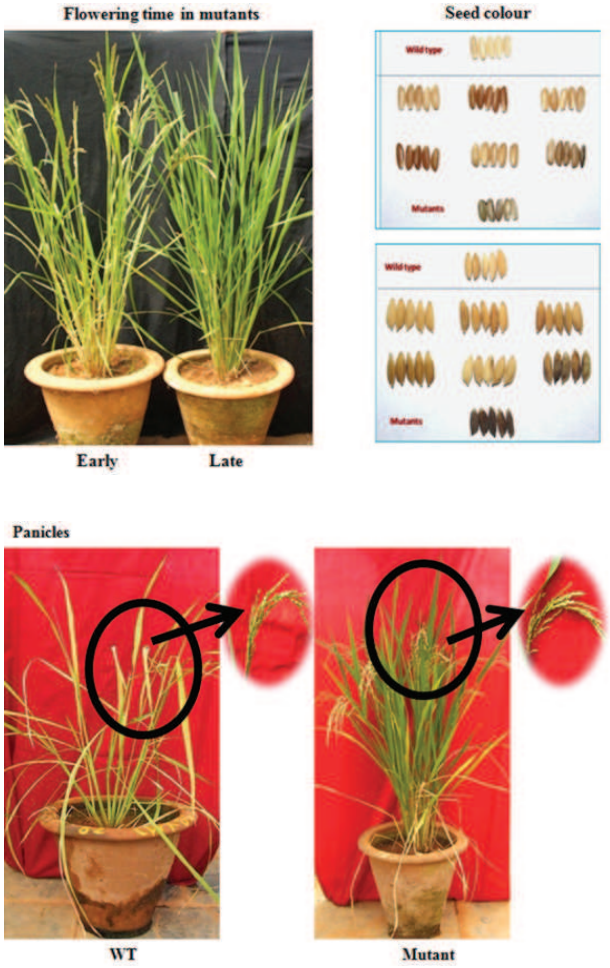


Fig. 3. Variation in the flowering time, seed colour, panicle emergence and numbers in mutants *vis-a-vis* wild type (WT = wild type)

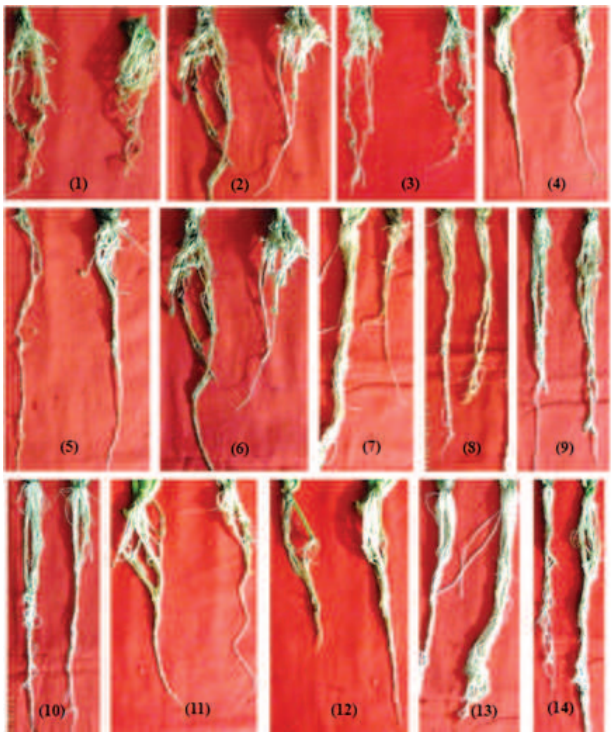


Fig. 4. Root phenotype of wild type and activation tagged rice mutants upon infection of *M. graminicola* at 20 days post inoculation (1 = wild type, 2 = line 3, 3 = line 4, 4 = line 5, 5 = line 6, 6 = line 7, 7 = line 8, 8 = line 9, 9 = line 10, 10 = line 11, 11 = line 12, 12 = line 13, 13 = line 14, 14 = line 15)

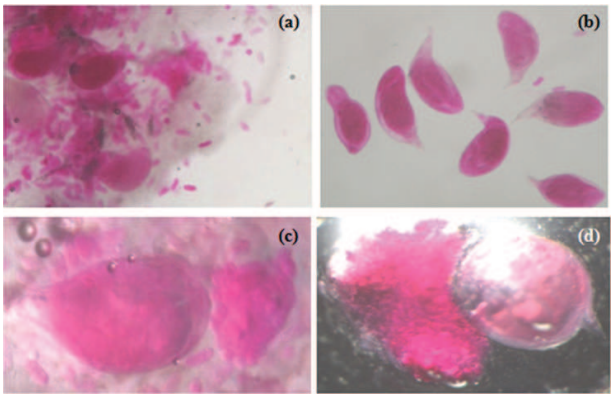


Fig. 5. Comparison of total endoparasites (a-resistant line, b-susceptible line) and number of eggs (c-resistant line, d-susceptible line) produced by *M. graminicola* in activation tagged rice mutants as depicted by acid fuchsin staining of roots after completion of nematode life cycle

of leaf blade of mutants when compared to WT (Fig. 2). Further, some of the mutants differed in the number of tillers as well as flowering time (Fig. 2 and 3). Additionally, distinct seed colour was also noticed in some mutants compared to WT. Thus the phenotypic variation exhibited by the mutants prompted us to assess their variability in response to the nematode, *M. graminicola.*

Deliberate challenging of the plants with the nematodes depicted distinct variation in the response of the mutants and WT (Fig. 4). Some of the mutants were observed to produce significantly less ($P<0.05$) number of galls [line 8 (2.6), line 9 (2), line 10 (1.7), line 11 (1.5) and line 15 (2.5)] while others showed increased number of galls [line 3 (6), line 4 (5.6), line 5 (5.3), line 6 (5), line 7 (6.1), line 12 (6), line 13 (6.5), line 14 (6), line 27 (5.7) and line 31 (5.1) as against 8.1 in WT] (Table 1). The mutants 8, 9, 10, 11 and 15

Table 1. Response of activation tagged rice mutants to the deliberate challenging by *Meloidogyne graminicola* under greenhouse conditions (value of number of galls/plant is the mean of ten replicates; figure in parentheses indicates $\sqrt{X+0.5}$ transformed value; values are significant at $P < 0.05$ and $P < 0.01$)

S.No.	Mutants/ WT	No. of galls/ plant	Percentage reduction of galls over control (WT)	MF	Percentage reduction of MF over control (WT)	Gall index (GI)	Susceptible(S) Resistant(R)
1	WT	8.10(2.93)	0.00	17.43(4.22)	0.00	40	MS
2	L3	6.00(2.55)	25.93	11.48(3.45)	34.13	40	MS
3	L4	5.60(2.47)	30.86	10.88(3.33)	37.57	40	MS
4	L5	5.30(2.40)	34.57	11.50(3.44)	34.02	40	MS
5	L6	5.00(2.34)	38.27	8.94(3.04)	48.7	40	MS
6	L7	6.10(2.57)	24.69	11.22(3.43)	35.62	40	MS
7	L8	2.60(1.75)	67.90	1.28(1.33)	92.65	20	R
8	L9	2.00(1.57)	75.31	0.71(1.14)	95.92	20	R
9	L10	1.70(1.47)	79.01	0.89(1.12)	94.89	20	R
10	L11	1.50(1.40)	81.48	0.64(1.09)	96.32	20	R
11	L12	6.00(2.55)	25.93	10.43(3.30)	40.16	40	MS
12	L13	6.50(2.64)	19.75	10.30(3.29)	40.9	40	MS
13	L14	6.00(2.54)	25.93	10.77(3.35)	38.2	40	MS
14	L15	2.50(1.72)	69.14	1.71(1.52)	90.18	20	R
15	L27	5.70(2.49)	29.63	6.79(2.72)	61.04	40	MS
16	L31	5.10(2.36)	37.04	6.36(2.60)	63.51	40	MS
	F	113.64		2212.57			
	CD(p=0.05)	0.13		0.06			
	CD(p=0.01)	0.17		0.09			

(WT = wild type, L = line, MF = multiplication factor, MS = moderately susceptible, R = resistant)

showed resistance, with a root gall index of 20 while other mutant lines and WT exhibited moderate susceptibility with a gall index of 40 (Table 1). However, the percentage reduction of galls was greater in the resistant mutants when compared to other susceptible mutants and WT (Table 1). In view of this, mutant lines 8, 9, 10, 11 and 15 were grouped as resistant and others as moderately susceptible. The resistant mutants (lines 8, 9, 10, 11 and 15) also showed reduced number of endoparasites developing inside the roots when compared to susceptible mutants and WT (Fig. 5 and 6a). Other related parameters like egg masses (Fig. 6b) as well as average eggs/egg mass were seen to be corroborating (Fig. 5 and 6c) with rest of the parameters. Most significantly, multiplication factor (MF) of *M. graminicola* derived from other parameters was found to be very less in mutant lines 8, 9, 10, 11 and 15 (1.28, 0.71, 0.89, 0.64 and 1.71 respectively) while it was observed to be greatest in WT (17.43). However, the MF of other mutants was found to be varying but higher than that in the resistant mutants (Table 1). Most importantly, the percentage reduction of MF of *M. graminicola* was greater in resistant mutants when compared to other mutants and WT.

Discussion

In the present study, a panel of 30 activation tagged rice mutants with conspicuous variation in the phenotype were exploited (data not shown). These mutants were earlier developed by *in planta* transformation method and identified by glyphosate screening. Distinct phenotypic variation was seen across the mutants in the form of plant height, tillering, flowering, seed colour, etc. This variation in the

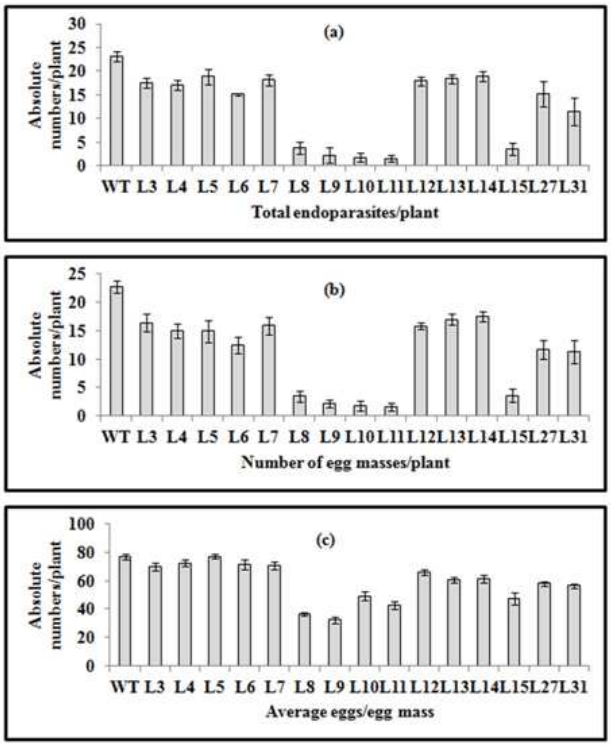


Fig. 6. Depiction of variability in total endoparasites (a), number of egg masses (b), average eggs/egg mass (c) produced by *M. graminicola* in mutants compared to wild type at 20 days post inoculation (WT = wild type, L3 = line 3, L4 = line 4, L5 = line 5, L6 = line 6, L7 = line 7, L8 = line 8, L9 = line 9, L10 = line 10, L11 = line 11, L12 = line 12, L13 = line 13, L14 = line 14, L15 = line 15, L27 = line 27, L31 = line 31)

phenotype enforced us to look for variability amongst selected mutant population for the response to nematode infection.

Resistance/susceptibility can be assessed by quantifying the population densities of nematode in the plant roots, while tolerance/sensitivity can be assessed by the effect of nematode population on plant growth, yield attributing traits or yield (Bos and Parlevliet 1995). Various criteria like egg mass index (Winstead and Sasser 1956; Bouquet et al. 1976), gall index (Dropkin 1954; Rohde 1960; Rohde 1965; Zhan et al. 2018), portion of root necrosis on seedlings, development of galls, failure of nematode life cycle upon reaching maturity, etc. have been used to determine the host resistance/susceptibility against root knot nematodes (Brodie et al. 1960). Penetration, migration, development and reproduction of nematode have been observed to be suppressed or delayed in resistant host (Trudgill 1991; Cabasan et al. 2012). Devaraja et al. (2018) observed significant differences among the susceptible and resistant rice genotypes in the development of females, giant cells and egg production. However in the present study, emphasis was given on number of galls (gall index) with other disease scoring parameters and the derived multiplication factor for designation of mutants either as resistant or susceptible.

Categorical assessment of the mutants to deliberate challenging of *M. graminicola* under soil conditions in the greenhouse depicted a large variation in their response. Five mutants (lines 8, 9, 10, 11 and 15) showed a resistant response by producing least number of galls ranging from 1.5 to 2.6 galls per plant whereas wild type and other mutants were moderately susceptible (5 to 8.10 galls per plant) to *M. graminicola*. Likewise, the multiplication factor of *M. graminicola* in resistant mutants was significantly less ranging from 0.64 to 1.71 as against 6.36 to 17.43 in wild type and other susceptible mutants. On the susceptible wild type and mutants, nematodes were found to invade, feed and complete the life cycle. However, in the resistant mutants, though the attacking nematodes were able to penetrate but with reduced reproductive potential.

M. graminicola generally completes its life cycle within 15-20 days in soil under optimum conditions (Cabasan et al. 2012; Nguyen et al. 2014). In the present study, due to the maintenance of optimum conditions for plant growth that enabled the completion of nematode life cycle by 20 days, the challenged plants could be uprooted within that time. Normally, the female *M. graminicola* lays eggs in the region of the cortex within the root system and eggs that hatch in the next season infect the new root tissues of rice (Pankaj et al. 2012). It is known that most of *O. sativa* genotypes were found to be susceptible to *M. graminicola* with very few identified as resistant (Bridge et al. 1990; Mhatre et al. 2015, 2017). Hence the present study is significant in terms of the successful depiction of variability and identification of resistant and susceptible response in the same genetic background. This demonstrates the importance of insertional mutagenesis in creating variability to a particular trait. The genes responsible for the resistant phenotype can be deployed through either transgenesis or molecular breeding in crop improvement programmes for not only rice but other crop species. Further, the panel of rice activation tagged mutants could also be exploited for various useful traits.

Authors' contribution

Conceptualization of research (UR, RS); Designing of the experiments (UR, RS, BH); Contribution of experimental materials (UM); Execution of field/lab experiments and data collection (BH, RS); Analysis of data and interpretation (BH, RS, UR); Preparation of manuscript (BH, RS, UR).

Declaration

The authors declare no conflict of interest.

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