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Identification of *Oryza glaberrima* as a potential resistance source to rice root-knot nematode, *Meloidogyne graminicola*

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Abstract

The root-knot nematodes (RKN) (*Meloidogyne graminicola*) are a devastating threat to rice worldwide. The cultivated germplasm is either susceptible or moderately resistant to rice RKN. Therefore, there is a need to identify resistance sources against *M. graminicola* as an eco-friendly management strategy. The present study evaluated the host response of *Oryza sativa* genotypes comprising basmati, non-basmati improved varieties, their advanced breeding lines (83) and *Oryza glaberrima* accessions (42) against *M. graminicola* in the nematode-infested plot for two consecutive years. All *O. sativa* genotypes exhibited susceptible responses, while *O. glaberrima* accessions showed variable levels of resistance. Three of the *O. glaberrima* accessions (IRGC102196, IRGC102538 and IRGC102557) were highly resistant. *M. graminicola* significantly affected plant growth parameters in susceptible genotypes compared to resistant *O. glaberrima* accessions. The results were supported by histopathological studies that showed apparent giant cell formation in PR121 while penetration and development of *M. graminicola* juveniles were low in the *O. glaberrima* acc. IRGC102196. *In silico* analysis indicated that none of the reported nematode resistance genes from different crops had homology with the rice genome. The two anti-nematode genes (*Oryzacystatin-I* and *Oryzacystatin-II*) from *O. sativa* japonica revealed homology with *O. sativa* cv. PR121 and *O. glaberrima* acc. IRGC102206. Comparative analysis of these genes between PR121 and *O. glaberrima* acc. IRGC102206 resulted in the identification of SNPs/InDels that could be associated with nematode resistance. The identified SNPs/InDels could be validated, and further molecular studies are needed to provide insights into the resistance mechanism against rice RKN.

Introduction

The rice root-knot nematode, *Meloidogyne graminicola*, Golden and Birchfield, is one of the most damaging nematode species. It has emerged as a serious threat to rice production due to its worldwide distribution, particularly in Southeast Asia, which constitutes 90% of total rice production (Rusinque *et al.*, 2021). This endoparasitic sedentary nematode is prevalent in almost all rice cropping systems, including lowland, upland, irrigated and deepwater (Pokharel *et al.,* 2010; Mantelin *et al.*, 2017). It causes considerable damage to rice root systems in nurseries and significant yield loss in the field ranging from 70 to 80% in lowland and upland rice cultivation (Patil and Gaur, 2014; Dimkpa *et al.,* 2016; Galeng-Lawilao *et al.,* 2018).

Rice RKN infective second-stage juveniles (J2) penetrate the root system and develop nematode-specific feeding sites. These feeding sites are multinucleated giant cells (GC) formed through endomitosis and cellular hypertrophy and are often referred to as 'metabolic sinks' due to the diversion of host nutrition to the nematode. Further, terminal hook-shaped galls are formed on roots due to the division of the pericycle cells which are also the direct representation of nematode infestation and susceptibility of rice plants to RKN (Ralmi *et al.*, 2016). Among the physiological processes that have often been considered responsible for the reduction of the photosynthetic activity in nematode-infested plants are decreased chlorophyll content (Nagesh and Dhawan, 1988), changes in stomatic conductance (Saeed *et al.,* 1998) and photochemical limitations (Schans and Arntzen, 1991). This results in a lower photosynthetic rate and ultimately the lower translocation of photosynthates lead to stunting, chlorosis, loss of vigour and yield losses (Swain and Prasad, 1988; Wenting and Deliang, 2007; Chen *et al.*, 2022).

M. graminicola is well-adapted to the wide variety of agro-ecosystems in which rice is cultivated. Today, the impact of water scarcity has shifted focus to water-saving rice cultivation

systems such as aerobic rice. Consequently, the agronomic practices in aerobic, non-puddling and non-flooding or non-saturated fields conditions have led to an increase in nematode infestations (Ravindra *et al.*, 2017). The options to control *M. graminicola* are limited. De-registration of frontline nematicides and their hazardous impact on health and the environment has shifted the focus to other management strategies (De-Waele *et al.*, 2013; Dimkpa *et al.*, 2016). The low-cost, environmentally beneficial and sustainable approach is the use of resistant cultivars. Most of the Asian rice germplasm screened so far is susceptible to RKN, and only a few of them were found to be resistant (Dimkpa *et al.*, 2016; Subudhi *et al.*, 2017). Therefore, it is a pressing need to identify nematode resistant sources from wide range of germplasm which could be deployed in breeding programs for its management. Natural plant resistance to RKN had already been reported from *O. glaberrima* and *O. longistaminata* (Soriano *et al.*, 1999) through pot experiments. One accession (WL02) of *O. longistaminata* and three accessions of *O. glaberrima* (TOG7235, TOG5674 and TOG5675) were resistant to RKN (Soriano *et al.*, 1999). As the study was limited to only three accessions of *O. glaberrima*, so there is need to explore more accessions for resistance. Despite the identification of *O. glaberrima* (West Africa cultivar) as resistant source for RKN, it is not economically important due to low yielding in comparison to Asian rice cultivars. Therefore, it is essential to introgress RKN resistance from West African cultivars into economically important and high yielding Asian rice cultivars through backcross breeding. Introgression of RKN resistance into Asian cultivars has been initiated but was not successful so far, because progenies from interspecific crosses did not express same resistance level as that of *O. glaberrima* (Plowright *et al.*, 1999). Sexual incompatibility and hybrid sterility further limits the efforts of combining *M. graminicola* resistance and yielding potential from these two different rice species. However, fertility can be achieved through continual backcrossing for fewer generations (Jones *et al.*, 1997, Cabasan *et al.*, 2018).

Besides the phenotypic evaluations, an insight into the genetic analysis or molecular approaches enables us to ascertain the number of genes involved in resistance. It has been documented that resistance to rice RKN is not monogenic but multigenic in nature (Prasad *et al.*, 2006; Dimkpa *et al.*, 2016; Galeng-Lawilao *et al.*, 2018). Several natural host resistance genes and proteinase/protease inhibitor genes have been associated with nematode resistance, and other plant species could share similar resistance genes (Ali *et al.*, 2017). The availability of whole-genome rice sequence information will lead to identifying the ortholog sequences known from other crop systems like cowpea, potato, maize and tomato for nematode host resistance genes (Hepher and Atkinson, 1992; Paal *et al.*, 2004; Roderick *et al.*, 2012; El-Sappah *et al.*, 2019). It will provide information about a gene sequence's structure and function that could be associated with nematode resistance. The identification of genetic variation in terms of SNPs/InDels through *in silico* approach could be used for functional validation. In plants, the preponderance of traits of interest is linked with SNPs and thought to bring individual variation, community diversity and the evolution of species (Shirasawa *et al.*, 2013). Thus, the present study was conceded to (i) screen potential sources of RKN resistance among *O. sativa* genotypes and *O. glaberrima* accessions and (ii) study histological variations between resistant and susceptible genotypes, along with *in silico* studies using bioinformatics tools to identify SNPs/InDels for resistance.

Materials and methods

Experimental materials

Eighty-three genotypes of *O. sativa* and forty-two accessions of *O. glaberrima* were evaluated along with three susceptible checks (Pusa1121, PR116 and PR121) in nematode infested soil during the summer seasons of 2017 and 2018. Also, all the genotypes were planted in non-infested soil to measure plant growth parameters. The *O. glaberrima* accessions were procured from International Rice Research Institute, Manila, Philippines, and maintained at the School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana. All 128 genotypes were raised in a randomized complete block design in three replications. Each genotype was planted in triplicate with a plant to plant distance of 15 cm and 50 cm from row to row. Standard agronomical practices were followed for raising the crop.

Preparation of RKN infested plot

The nematode infested plot was established during 2015–16 using roots of RKN infested rice seedlings, and soil collected from fields of Punjab Agricultural University, Ludhiana as well as different regions of Punjab (Ludhiana, Faridkot and Sangrur). The population of *M. graminicola* from the Punjab region used in the experiment was previously identified and the identity of the nematode was confirmed by the method described by Yik and Birchfield (1979). A raised bed was prepared with nematode infested soil and infested seedlings which were directly transplanted on the bed for rapid spreading of *M. graminicola* infestation uniformly throughout the plot. After infested seedlings reached maturity, shoots were separated from roots. Infested roots were chopped into small pieces and mixed in the upper layer of soil. The plot was kept saturated but not flooded to maximize the reproduction or RKN. During the growth period of seedlings, RKN completes their life cycles and helps in increasing the soil nematode population density. Later on, the nematode population density was maintained throughout the year by sowing PR121 during the summer season and an alternative host (wheat) in the winter season.

Experimental design and assessment of nematode population in soil

M. graminicola infested plot (30×8 m) was sub-divided into ten micro plots, and the size of each micro plot consisted of 3×5 m (Online Supplementary Fig. S1). The micro plots were separated from each other by 1 m. An earthen levee was constructed around infested plot to prevent the spread of nematode to a non-infested plot, which was maintained at a buffer space of 10 m from the infested plot. Six composite soil samples from each microplot were taken to determine the initial soil nematode population density. The soil nematode population was determined using Cobb's sieving and decanting method (Cobb, 1918). The average initial population density of 10 different micro plots was assured uniform throughout the nematode infested plot by a continuous infestation of nematode infested soil/root samples in the infested plot before initiating the screening experiment.

Screening and rating

All genotypes were raised in nursery beds and transplanted after 25 days of sowing in the nematode infested plot with an average

initial population density of 1 J2/cc soil. The nematode infested plot was not flooded but kept saturated during the experiment to maximize the penetration of juveniles inside the roots. Standard agronomical practices were followed for the growth of seedlings. Nine plants of each entry from three replications were uprooted carefully after sixty days of infestation, and roots were washed immediately to count the number of galls per total root system. The incidence of root-knot disease was measured by a root gall index scale of 1–5 (Gaur et al., 2001). The rating was done 1 for 0–1 gall (highly resistant), 2 for 1–10 galls (resistant), 3 for 11–30 galls (moderately resistant), 4 for 30–100 galls (susceptible) and 5 for >100 galls (highly susceptible). The final soil nematode population density was assessed from 250 cc soil collected with a soil auger at a depth of 35 cm of three plants of each genotype from three replications. The reproduction factor (Rf) was calculated by dividing the final population density (Pf) by the initial population density (Pi) of juveniles.

Effect of nematode infestation on plant growth parameters

Data on different growth performance factors like plant height (cm) and root length (cm) from both uninfected and nematode infested plots were recorded among six O. glaberrima resistant accessions and susceptible checks (Pusa1121, PR116 and PR121) in three replicates over two years. The height of each plant was measured from the ground surface to the tip of the longest leaf. Root length was measured from the ground surface to the longest root.

Histopathological studies

The nematode infested roots of susceptible and resistant plants were stained with acid fuschin and were observed under a stereoscope microscope to check the presence of nematode/egg masses inside the gall. Subsequently, M. graminicola infested roots of PR121 and O. glaberrima acc. IRGC102196 was collected after 30 days of transplanting. The roots were first gently washed in running tap water to remove adhered soil. Root galls were hand sectioned by placing samples in potato pith. The samples were then mounted on DPX with a coverslip. The slides were observed under a microscope equipped with a digital camera and computer imaging system using software NIS Elements F 30 at 4X and 10X. 4-5 root segments (1–2 cm long) of both genotypes were selected and excised for scanning electron microscopic analysis. The cross and longitudinal sections (4–5 μm long) were further cut from root segments and fixed in 2.5% (v/v) glutaraldehyde at 40°C overnight. After draining the fixative, the samples were treated with 0.1 M $CaCO_3$ buffer with an interval of 15 min each three times. Later on, samples were treated with 1% Osmium tetroxide (OsO_4) for 1–2 h at 40°C. Again three washings were given with 0.1 M $CaCO_3$ after draining OsO_4. The samples were then dehydrated in ethanol concentrations from 30% to 100% v/v (30, 50, 70, 80, 90 and 100%). The samples were later dried at a critical point and placed in vacuum desiccators overnight. A coating of carbon on each sample was done with the help of a sputter coater; these samples were then observed under a scanning electron microscope (SEM).

In silico analysis of nematode resistance genes

A total of 14 cloned nematode resistance genes from different crops were selected (Online Supplementary Table S1) to identify the orthologs in O. glaberrima acc. IRGC102206. The sequences of nematode resistance genes were retrieved through the NCBI database (www.ncbi.nlm.nih.gov). The FASTA sequence files were BLAST against Oryza sativa Nipponbare reference genome of rice on RGAP (Rice Genome Annotation Project) (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml). Genome sequences of the O. sativa indica group and O. glaberrima were retrieved from Ensembl Plants (https://plants.ensembl.org/species) for comparative analysis. The nucleotide sequences of Oryzacystatin-1 and Oryzacystatin-2 genes were retrieved from whole-genome resequencing data of O. sativa cv. PR121 and O. glaberrima acc. IRGC102206 (our unpublished data) to confirm sequence variations. Both genes were aligned separately between PR121 and O. glaberrima acc. IRGC102206 through CLUSTAL software using the CodonCode Aligner tool (https://www.codoncode.com/).

Statistical analysis

The average initial nematode population density from each microplot, final soil nematode population density, reproduction factor, root gall index and growth parameters were analysed through analysis of variance using a generalized linear model (GLM) of SAS software version 9.4 (SAS Institute, Cary, NC). The average root gall index of each genotype was compared using Tukey's host significant difference (Tukey's HSD) test.

Results

Host response to M. graminicola

The initial nematode population density was an average of 1 J2/gram of soil throughout the nematode infested plot. There was no significant difference in the initial soil nematode population density (F9, 47 = 1.04; P > 0.426) among the micro plots. On confirmation of the uniform distribution of nematode population density in the infested plot, the screening of rice germplasm was done. The germplasm screened for their response to RKN in the present studies were categorized into three groups viz. O. sativa basmati genotypes, O. sativa non-basmati genotypes and O. glaberrima accessions. These three groups showed significant variations in their susceptibility to M. graminicola. Amongst the three groups, root gall numbers and final soil nematode population were higher in O. sativa genotypes than in O. glaberrima accessions. Fifteen basmati genotypes were highly susceptible, while 13 were rated as susceptible and five as moderately susceptible based on the root gall index (Table 1). Similarly, forty-one and nine non-basmati genotypes were classified as highly susceptible and susceptible, respectively (Table 2). The reproduction factor in all O. sativa genotypes was >1.5, indicating that these genotypes reproduced nematode populations. Root gall index was also >3 in all the susceptible genotypes. Root galling was found throughout the root system along with characteristic terminal hook-shaped galls at the root apex as observed in susceptible control PR121 (Fig. 1a).

O. glaberrima accessions exhibited very few galls (Figs 1b and 1c), and the reproduction factor was <1, indicating that they did not favour the build-up of nematode populations. Three O. glaberrima accessions (IRGC102196, IRGC102538 and IRGC102557) were highly resistant and promising for utilization in nematode resistance breeding. Thirty-three and six O. glaberrima accessions were resistant and moderately resistant,

Table 1. Average soil nematode population, reproduction factor (Rf), root gall index and the response of *O. sativa* genotypes (Basmati) for the consecutive two years against *M. graminicola*

S. No.	Genotypes	Soil nematode population/250cc soil	Reproduction factor (Rf)	Root gall index (RGI)	Reaction
1.	RYT – Bas – 72	482.50 ± 12.7	1.93 ± 0.1	$2.90 ± 0.1^{nopqrs}$	MS
2.	RYT – Bas – 47	509.50 ± 7.8	2.04 ± 0.0	$3.25 ± 0.0^{n}$	S
3.	PB – 3	582.00 ± 14.7	2.33 ± 0.1	$4.25 ± 0.0^{defghijk}$	HS
4.	Bas – 1	473.50 ± 5.3	1.89 ± 0.0	$3.00 ± 0.0^{nopqr}$	MS
5.	RYT – BT – 22	488.50 ± 17.6	1.95 ± 0.1	$3.09 ± 0.0^{nopq}$	S
6.	BT – 2–10	570.00 ± 24.5	2.28 ± 0.1	$4.15 ± 0.0^{ghijk}$	HS
7.	Bas – 61	514.00 ± 11.4	2.06 ± 0.0	$3.30 ± 0.1^{mn}$	S
8.	PB – 144	631.50 ± 1.2	2.53 ± 0.0	$4.15 ± 0.0^{ghijk}$	HS
9.	RYT – BT – 1–2	456.50 ± 19.2	1.83 ± 0.1	$2.97 ± 0.0^{nopqr}$	MS
10.	BT – 2–5	520.00 ± 16.3	2.08 ± 0.1	$3.25 ± 0.0^{n}$	S
11.	BT – 2–7	443.50 ± 19.2	1.77 ± 0.1	$3.09 ± 0.0^{nopq}$	S
12.	BT – 2–11	648.50 ± 15.1	2.59 ± 0.1	$4.29 ± 0.0^{cdefghijk}$	HS
13.	BT – 2–12	452.50 ± 11.8	1.81 ± 0.0	$3.00 ± 0.0^{nopqr}$	MS
14.	Bas – 386	490.00 ± 8.2	1.96 ± 0.0	$3.30 ± 0.1^{mn}$	S
15.	BT – 2–8	469.00 ± 25.3	1.88 ± 0.1	$3.34 ± 0.1^{lmn}$	S
16.	BT – 2–14	570.00 ± 24.5	2.28 ± 0.1	$4.14 ± 0.1^{ghijk}$	HS
17.	BT – 2–1	485.50 ± 15.1	1.94 ± 0.1	$3.09 ± 0.0^{nopq}$	S
18.	BT – 2–4	450.50 ± 14.3	1.80 ± 0.1	$2.92 ± 0.0^{nopqrs}$	MS
19.	Bas – 76	616.50 ± 13.5	2.47 ± 0.1	$4.25 ± 0.0^{defghijk}$	HS
20.	Bas – 56	490.00 ± 8.2	1.96 ± 0.0	$3.15 ± 0.0^{nop}$	S
21.	Bas – 80	579.00 ± 17.1	2.32 ± 0.1	$4.20 ± 0.1^{efghijk}$	HS
22.	PB – 2	559.50 ± 33.1	2.24 ± 0.1	$4.09 ± 0.0^{hijk}$	HS
23.	Bas – 67	648.50 ± 15.1	2.59 ± 0.1	$4.42 ± 0.1^{abcdefghij}$	HS
24.	Bas – 71	624.00 ± 19.6	2.50 ± 0.1	$4.09 ± 0.0^{hijk}$	HS
25.	Bas – 36	505.00 ± 4.1	2.02 ± 0.0	$3.24 ± 0.0^{n}$	S
26.	Bas – 26	589.50 ± 8.6	2.36 ± 0.0	$4.14 ± 0.1^{ghijk}$	HS
27.	BT – 2–13	564.00 ± 29.4	2.26 ± 0.1	$4.02 ± 0.1^{hijk}$	S
28.	BT – 2–9	606.00 ± 22.0	2.42 ± 0.1	$4.17 ± 0.0^{fghijk}$	HS
29.	Bas – 45	490.00 ± 8.2	1.96 ± 0.0	$3.12 ± 0.0^{nop}$	S
30.	BT – 2–6	594.00 ± 4.9	2.38 ± 0.0	$4.09 ± 0.0^{hijk}$	HS
31.	PB – 1509	646.50 ± 11.0	2.59 ± 0.0	$4.25 ± 0.0^{defghijk}$	HS
32.	Bas – 50	638.00 ± 23.7	2.55 ± 0.1	$4.19 ± 0.1^{fghijk}$	HS
33.	Bas – 57	482.50 ± 14.3	1.93 ± 0.1	$3.29 ± 0.0^{mn}$	S
	Pusa1121 (check)	488.00 ± 26.0	1.90 ± 0.1	$3.20 ± 0.1^{n}$	S

Different lowercase letters indicate significant differences among basmati genotypes as determined by Tukey's honest significant difference (Tukey's HSD) test ($P < 0.05$). HS, S and MS stand for highly susceptible, susceptible and moderately susceptible, respectively.

respectively (Table 3). Statistical significant differences were observed for final soil nematode population density, reproduction factor and root gall index among the genotypes (Online Supplementary Table S2). The resistant accessions were also evaluated in the pots filled with nematode infested soil @ 1 J2 per cc soil. The reactions of the *O. glaberrima* accessions were similar to those in the nematode infested plot (data not presented).

Staining and teasing the mature gall of susceptible check PR121 showed adult female, egg masses (Online Supplementary Fig. S2a) and juveniles released from root galls (Online Supplementary Fig. S2b). Only a few juveniles penetrated *O. glaberrima,* and no egg masses were seen inside the roots after 60 days of nematode infestation (Online Supplementary Fig. S2d). However, many juveniles were observed in the roots of *O. sativa* cv. PR121 (Online Supplementary Fig. S2c). The average soil

Table 2. Average soil nematode population, reproduction factor (Rf), root gall index and the response of *O. sativa* genotypes (non-basmati) for the consecutive two years against *M. graminicola*

S. No.	Genotypes	Soil nematode population/250cc soil	Reproduction factor (Rf)	Root gall index (RGI)	Reaction
1.	14T – 62283	780.50 ± 11.8	3.06 ± 0.0	4.67 ± 0.1^{abcdefg}	HS
2.	62284	845.50 ± 10.2	3.41 ± 0.1	4.87 ± 0.1^{ab}	HS
3.	62290	570.00 ± 24.5	2.40 ± 0.0	4.05 ± 0.0^{hijk}	HS
4.	62291	806.50 ± 21.6	3.47 ± 0.0	4.97 ± 0.0^{a}	HS
5.	62296	579.00 ± 17.1	2.34 ± 0.1	4.04 ± 0.0^{hijk}	HS
6.	62347	826.00 ± 5.7	3.36 ± 0.0	4.97 ± 0.0^{a}	HS
7.	62348	564.00 ± 4.1	2.19 ± 0.0	3.97 ± 0.0^{jik}	S
8.	62349	633.00 ± 26.9	2.77 ± 0.0	4.22 ± 0.1^{efghijk}	HS
9.	62350	779.50 ± 16.7	3.25 ± 0.0	4.80 ± 0.0^{abcd}	HS
10.	62363	585.00 ± 12.2	2.50 ± 0.0	4.19 ± 0.1^{fghijk}	HS
11.	62385	831.50 ± 17.6	3.49 ± 0.0	4.97 ± 0.0^{a}	HS
12.	62386	772.50 ± 6.1	3.01 ± 0.0	4.77 ± 0.0^{abcde}	HS
13.	62392	646.50 ± 11.0	2.43 ± 0.0	4.35 ± 0.1^{bcdefghijk}	HS
14.	62650	811.00 ± 18.0	3.46 ± 0.0	4.92 ± 0.0^{ab}	HS
15.	62635	793.00 ± 13.9	3.07 ± 0.0	4.74 ± 0.1^{abcdef}	HS
16.	62665	570.00 ± 24.5	2.60 ± 0.0	4.14 ± 0.1^{ghijk}	HS
17.	62677	536.50 ± 2.9	2.07 ± 0.0	3.85 ± 0.0^{jklm}	S
18.	62678	756.50 ± 19.2	2.49 ± 0.1	4.97 ± 0.0^{a}	HS
19.	62693	826.00 ± 5.7	3.44 ± 0.0	4.89 ± 0.0^{ab}	HS
20.	62702	579.00 ± 17.1	2.54 ± 0.0	3.90 ± 0.1^{ijkl}	S
21.	62705	572.50 ± 5.3	2.43 ± 0.1	3.84 ± 0.0^{klm}	S
22.	62717	579.00 ± 17.1	2.35 ± 0.1	4.14 ± 0.1^{hijklmnop}	HS
23.	62718	841.00 ± 6.5	3.47 ± 0.1	4.97 ± 0.0^{a}	HS
24.	62723	851.50 ± 15.1	3.21 ± 0.0	4.83 ± 0.0^{abc}	HS
25.	62728	805.00 ± 4.1	3.30 ± 0.0	4.90 ± 0.1^{ab}	HS
26.	62729	782.00 ± 13.1	3.00 ± 0.0	4.74 ± 0.1^{abcdef}	HS
27.	62731	443.00 ± 18.8	1.93 ± 0.0	3.14 ± 0.1^{nop}	S
28.	62736	585.00 ± 12.2	2.50 ± 0.0	4.15 ± 0.0^{ghijk}	HS
29.	62738	826.00 ± 5.7	3.41 ± 0.0	4.94 ± 0.1^{a}	HS
30.	62752	836.50 ± 2.9	3.25 ± 0.0	4.97 ± 0.0^{a}	HS
31.	62753	646.50 ± 11.0	2.51 ± 0.0	4.17 ± 0.0^{fghijk}	HS
32.	62727	473.50 ± 5.3	1.93 ± 0.0	3.15 ± 0.0^{nop}	S
33.	62761	809.50 ± 7.8	3.13 ± 0.0	4.85 ± 0.0^{abc}	HS
34.	62779	851.50 ± 15.1	3.33 ± 0.0	4.93 ± 0.1^{a}	HS
35.	62780	592.50 ± 6.1	2.29 ± 0.1	4.17 ± 0.0^{fghijk}	HS
36.	62781	616.50 ± 13.5	2.63 ± 0.0	4.25 ± 0.0^{defghijk}	HS
37.	63143	725.00 ± 20.4	2.74 ± 0.0	4.45 ± 0.0^{abcdefghi}	HS
38.	63144	749.00 ± 13.1	2.88 ± 0.0	4.57 ± 0.0^{abcdefgh}	HS
39.	63160	536.50 ± 2.9	2.07 ± 0.0	3.85 ± 0.0^{jklm}	S
40.	63161	585.00 ± 12.2	2.31 ± 0.1	4.09 ± 0.0^{hijk}	HS
41.	63190	851.50 ± 15.1	2.77 ± 0.2	4.93 ± 0.1^{a}	HS
42.	63191	545.50 ± 10.2	2.05 ± 0.0	4.92 ± 0.0^{ab}	HS

(Continued)

Table 2. (*Continued.*)

S. No.	Genotypes	Soil nematode population/250cc soil	Reproduction factor (Rf)	Root gall index (RGI)	Reaction
43.	63200	564.00 ± 29.4	2.34 ± 0.1	4.97 ± 0.0^a	HS
44.	63202	851.50 ± 15.1	3.25 ± 0.0	4.25 ± 0.0^{defghijk}	HS
45.	63206	592.50 ± 6.1	2.37 ± 0.0	4.14 ± 0.1^{ghijk}	HS
46.	63212	765.00 ± 12.2	3.06 ± 0.0	4.19 ± 0.1^{fghijk}	HS
47.	63224	442.50 ± 18.4	1.77 ± 0.1	3.17 ± 0.0^{no}	S
48.	63251	910.00 ± 65.3	3.64 ± 0.3	4.97 ± 0.0^a	HS
49.	63268	625.00 ± 4.1	2.50 ± 0.0	4.85 ± 0.0^{abc}	HS
50.	63279	555.00 ± 44.9	2.22 ± 0.2	4.05 ± 0.1^{hijk}	S
	PR116 (check)	683.00 ± 30.0	2.30 ± 0.1	3.20 ± 0.1^{efg}	S

Different lowercase letters indicate significant differences among non-basmati genotypes as determined by Tukey's honest significant difference (Tukey's HSD) test ($P < 0.05$). HS and S stand for highly susceptible and susceptible, respectively.

nematode population per 250 cc soil and root gall index among susceptible checks, highly resistant, and resistant accessions of *O. glaberrima* are presented in Online Supplementary Fig. S3. Soil nematode population per 250 cc soil was higher in susceptible checks (⩾500) compared to *O. glaberrima* accessions. The soil nematode population was multiplied by >2 fold in susceptibles against <1 fold in *O. glaberrima* accessions.

Effect of *M. graminicola* on growth parameters

The present studies revealed that infestations of *M. graminicola* had a significant suppressive effect on plant height and root length of susceptible checks despite a nominal decrease observed in highly resistant *O. glaberrima* accessions (Online Supplementary Table S3 and Fig. 2). The average plant height of PR121 was 57.83 ± 0.13 cm in the nematode infested plot and 79.33 ± 0.27 cm in non-infested conditions showing a reduction of 27.1%. The average plant height of *O. glaberrima* accessions ranged from 86.0 ± 0.43–134.0 ± 0.50 cm in nematode infested plot and 88.0 ± 0.40–137.0 ± 0.48 under controlled conditions. Root length showed a maximum reduction of 6.4% in *O. glaberrima* accessions, while PR121 had a reduction of 29.4% in the nematode infested plot. The two accessions (IRGC102196 and IRGC102380) maintained their root length even in RKN infestation.

Histopathological analysis

The histopathological studies of root galls of susceptible check PR121 showed the presence of a group of enlarged giant cells (thickened cell walls) inside the vascular region of the root cortex (Online Supplementary Figs. S4a and S4b). On the other hand, the giant cells did not develop normally, and little necrosis was observed in the root cortex of resistant *O. glaberrrima* acc. IRGC102196 (Online Supplementary Fig. S4c). Cross and longitudinal sections of *M. graminicola* infested roots showed intact vascular bundle (Fig. 3a), and stele (Fig. 3b) in *O. glaberrima*,

Figure 1. Root-knot nematode infested rice plants showing typical galls on root system of susceptible check *O. sativa* cv. PR121 (a) *O. glaberrima* acc. IRGC102196 (b) and *O. glaberrima* acc. IRGC100983 (c).

Table 3. Average soil nematode population, reproduction factor (Rf), root gall index and the response of *O. glaberrima* accessions for the consecutive two years against *M. graminicola*

S. No.	Accessions	Soil nematode population/250cc soil	Reproduction factor (Rf)	Root gall index (RGI)	Reaction
1.	IRGC102196	70.43 ± 1.0	0.28 ± 0.0	0.70 ± 0.1^{yz}	HR
2.	IRGC102538	92.53 ± 7.5	0.37 ± 0.0	0.70 ± 0.0^{yz}	HR
3.	IRGC102557	80.93 ± 3.6	0.32 ± 0.0	1.20 ± 0.2^{yz}	HR
4.	IRGC100854	170.58 ± 1.3	0.68 ± 0.0	1.80 ± 0.2^{vwxy}	R
5.	IRGC100983	166.50 ± 2.9	0.67 ± 0.0	1.85 ± 0.2^{vwxy}	R
6.	IRGC102206	166.50 ± 3.6	0.67 ± 0.0	2.15 ± 0.3^{qrstu}	R
7.	IRGC102226	169.53 ± 1.2	0.67 ± 0.0	1.50 ± 0.2^{xy}	R
8.	IRGC102263	169.72 ± 0.6	0.68 ± 0.0	1.80 ± 0.0^{vwxy}	R
9.	IRGC102336	168.68 ± 1.1	0.66 ± 0.0	2.10 ± 0.2^{tuvw}	R
10.	IRGC102356	178.10 ± 7.2	0.71 ± 0.0	2.05 ± 0.4^{stuv}	R
11.	IRGC102380	171.25 ± 2.1	0.69 ± 0.0	2.24 ± 0.5^{pqrst}	R
12.	IRGC102445	168.67 ± 1.9	0.67 ± 0.0	2.05 ± 0.3^{tuvwx}	R
13.	IRGC102500	172.13 ± 2.6	0.68 ± 0.0	1.85 ± 0.0^{vwxy}	R
14.	IRGC102512	170.27 ± 1.0	0.68 ± 0.0	1.60 ± 0.0^{wxy}	R
15.	IRGC102520	174.90 ± 4.0	0.70 ± 0.0	1.60 ± 0.1^{wxy}	R
16.	IRGC102532	166.92 ± 2.5	0.66 ± 0.0	1.80 ± 0.0^{vwxy}	R
17.	IRGC102542	167.85 ± 0.9	0.66 ± 0.0	2.00 ± 0.2^{uvwx}	R
18.	IRGC102550	169.53 ± 1.2	0.68 ± 0.0	1.65 ± 0.0^{wxy}	R
19.	IRGC102563	169.87 ± 0.7	0.68 ± 0.0	1.7 ± 0.0^{wxy}	R
20.	IRGC102600a	170.25 ± 1.0	0.68 ± 0.0	1.90 ± 0.2^{wxy}	R
21.	IRGC102600	173.23 ± 2.6	0.69 ± 0.0	1.85 ± 0.0^{vwxy}	R
22.	IRGC102615	177.25 ± 5.9	0.70 ± 0.0	1.60 ± 0.0^{vw}	R
23.	IRGC102925	169.53 ± 0.4	0.68 ± 0.0	1.85 ± 0.2^{vwxy}	R
24.	IRGC102980	164.53 ± 3.6	0.66 ± 0.0	1.75 ± 0.0^{wxy}	R
25.	IRGC103292	171.65 ± 0.5	0.69 ± 0.0	2.15 ± 0.3^{tuvw}	R
26.	IRGC103335	169.77 ± 1.0	0.67 ± 0.0	1.75 ± 0.0^{wxy}	R
27.	IRGC103383	168.55 ± 0.4	0.68 ± 0.0	2.00 ± 0.2^{uvwx}	R
28.	IRGC103445	167.85 ± 1.8	0.66 ± 0.0	2.10 ± 0.2^{tuvw}	R
29.	IRGC103530	162.57 ± 6.1	0.65 ± 0.0	1.90 ± 0.2^{vwxy}	R
30.	IRGC103750	170.52 ± 1.2	0.69 ± 0.0	1.50 ± 0.1^{xy}	R
31.	IRGC103930	168.35 ± 0.5	0.68 ± 0.0	1.65 ± 0.0^{wxy}	R
32.	IRGC103960	160.73 ± 6.7	0.64 ± 0.0	1.80 ± 0.0^{vwxy}	R
33.	IRGC103990	164.02 ± 4.9	0.66 ± 0.0	2.05 ± 0.2^{tuvwx}	R
34.	IRGC104020	163.13 ± 5.6	0.65 ± 0.0	1.85 ± 0.1^{vwxy}	R
35.	IRGC104033	172.17 ± 2.6	0.69 ± 0.0	1.70 ± 0.1^{wxy}	R
36.	IRGC104350	168.27 ± 1.4	0.68 ± 0.0	2.05 ± 0.3^{tuvwx}	R
37.	IRGC101800	214.40 ± 2.1	0.83 ± 0.0	1.95 ± 0.0^{uvwxy}	MR
38.	IRGC102277	221.58 ± 7.7	0.85 ± 0.0	2.50 ± 0.2^{rstu}	MR
39.	IRGC102489	216.43 ± 7.7	0.90 ± 0.0	2.50 ± 0.1^{rstu}	MR
40.	IRGC102544	235.32 ± 1.1	0.91 ± 0.0	2.00 ± 0.2^{uvwx}	MR
41.	IRGC103545	226.18 ± 1.5	0.88 ± 0.0	2.15 ± 0.1^{tuvw}	MR
42.	IRGC96717	235.07 ± 4.1	0.94 ± 0.0	2.60 ± 0.1^{opqrst}	MR

(Continued)

Table 3. (*Continued.*)

S. No.	Accessions	Soil nematode population/250cc soil	Reproduction factor (Rf)	Root gall index (RGI)	Reaction
	PR121 (check)	468.00 ± 26.0	2.30 ± 0.1	$3.20 ± 0.2^n$	S

Different lowercase letters indicate significant differences among *Oryza glaberrima* accessions as determined by Tukey's honest significant difference (Tukey's HSD) test ($P < 0.05$). HR, R, MR and S stand for highly resistant, resistant, moderately resistant and susceptible, respectively.

whereas necrosis in root cortex and hollow cross-section of mature galls in PR121 was observed (Figs 3c and 3d).

Identification of potential SNPs/InDels

Fourteen nematode resistance gene sequences from different crop plants (Online Supplementary Table S1) were aligned with *the O. sativa* Nipponbare reference genome of rice through the Basic local alignment search tool (BLAST). Only two of the genes *viz., Oryzacystatin-I* (NC_029256.1) on chromosome 1 and *Oryzacystatin-II* (NC_029260.1) on chromosome 5 of *the O. sativa* japonica group showed a significant length of sequence similarity whereas, others had only small regions i.e. <50 bases. Comparison of *Oryzacystatin-I* and *Oryzacystatin–II* genes between *O. sativa* indica group (ASM465v1) and *O. glaberrima* (GCA_000147395.1) retrieved from Ensembl Plants (https://plants.ensembl.org/species) showed the presence of one SNP

and eight SNPs, respectively, between these two species. Further, alignment of genic sequences between PR121 and *O. glaberrima* acc. IRGC102206 revealed 7 SNPs and 4 InDels (3–6 bp) for *the Oryzacystatin-I* gene (1610 bp). Only one SNP (G274C) was located in the first exon (127–354 bp), and the remaining 6 SNPs (C126G, A177G, C524T, T601C, C970T, T1057A) were present in the intronic region of *Oryzacystatin-I* gene. The identified SNPs included six transitions and 18 transversions (Online Supplementary Table S4). A 19 bp insertion (482–500 bp) and 58 bp deletion (989–1046) were detected within the introns of *the Oryzacystatin-I* gene in both PR121 and IRGC102206. The alignment of *the Oryzacystatin-II* gene (1517 bp) between PR121 and IRGC102206 showed 19 SNPs and 3 bp InDel (696–698 bp). Out of 19 SNPs, only three SNPs (T163C, G169A, C291T) were located in the first exon (127-387 bp). Similar to *Oryzacystatin-I*, a 16 bp insertion (525–540 bp) was identified in the intronic region of *Oryzacystatin-II* compared to
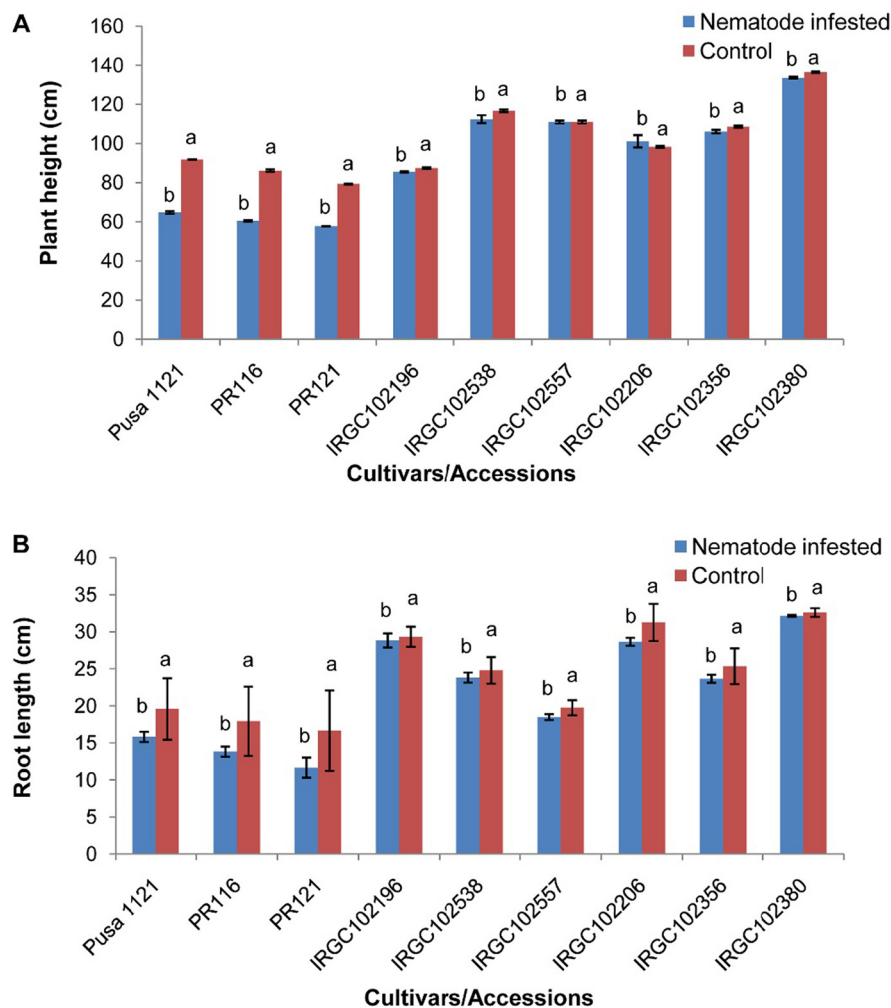

Figure 2. Effect of *M. graminicola* on plant height (a) and root length (b) of *O. sativa* cultivars (Pusa1121, PR116, PR121) and *O. glaberrima* accessions (IRGC102196, IRGC102538, IRGC102557, IRGC102206, IRGC102356, IRGC102380) in infested and non-infested conditions. Data are means of three replicates for two years. Error bars represent standard deviation. Different letters indicate significant differences between infested and non-infested conditions which were determined by Student's t-test.
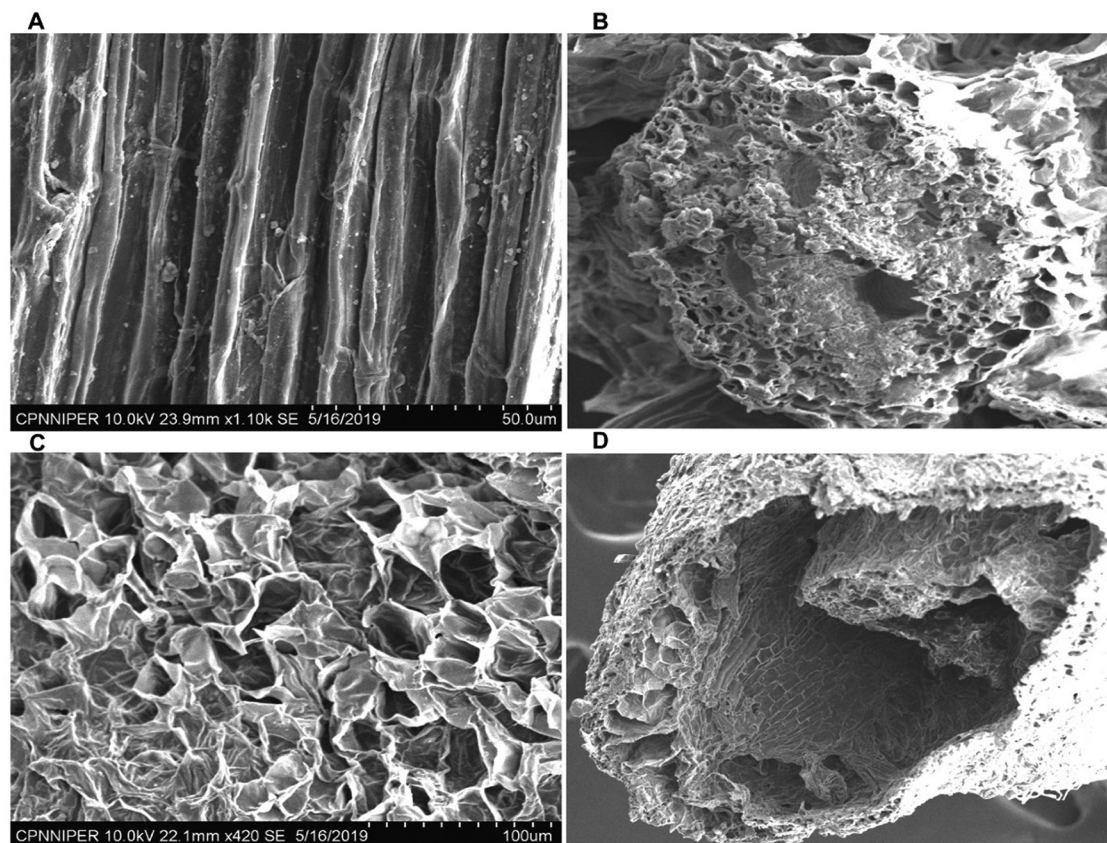
Figure 3. SEM analysis of infested roots from *O. glaberrima* acc. IRGC102196 shows an intact vascular bundle (a) and stele (b). SEM analysis of infested roots from *O. sativa* cv. PR121 showing necrotic tissue (c) and hollow mature root gall (d).

Discussion

the Nipponbare reference gene. Comparative sequence analysis showed more transitions (15) than transversions (7) in *the Oryzacystatin-II* gene. The identified SNPs/InDels may be associated with the functionality of proteins that might be responsible for conferring resistance in *O. glaberrima*. It could be explored further to have insights into predicting the role of these genes.

Discussion

The present study has great significance for breeding direct-seeded rice as ground-level water is going down, and today's scenario lays the foundation of agriculture for aerobic rice. In aerobic rice systems, the crop is grown in nonpuddled, non-flooded fields like an upland crop (unsaturated condition) with adequate inputs and supplementary irrigation when rainfall is insufficient (Priyanka *et al.*, 2012). Aerobic rice is more vulnerable to RKN due to increased water stress (Das *et al.*, 2011). Hence, identifying resistant sources could be valuable for exploiting them in breeding programs of direct-seeded rice. The absence of complete resistance against *M. graminicola* has been emphasized in earlier studies conducted by different researchers (Plowright *et al.*, 1999; Soriano *et al.*, 1999; Shrestha *et al.*, 2007; Dimkpa *et al.*, 2016; Subudhi *et al.*, 2017). The present study revealed the differential response of *O. sativa* and *O. glaberrima* genotypes upon *M. graminicola* infestation. All *O. sativa* genotypes were highly susceptible with a reproduction factor >1.5 except for five basmati genotypes which showed a moderately susceptible reaction.

However, the accessions of *O. glaberrima* displayed higher levels of resistance, amongst which three were highly resistant.

The underlying resistance mechanism of *O. glaberrima* was reported earlier by Petitot *et al.* (2017) through microscopic observation of infested roots and histological analysis of galls in *O. glaberrima* acc. TOG5681. Penetration and development of *M. graminicola* juveniles were limited in the resistant acc. TOG5681 as compared to susceptible *O. sativa* 'Nipponbare' rice. The giant cells showed degeneration in the resistant genotype from 15 days of post inoculation (dpi) onwards (Petitot *et al.*, 2017). The collapse of giant cells and degeneration has also been found in *O. glaberrima* before J2 developed into adults (Cabasan *et al.*, 2014). The incompatible interaction between *M. graminicola* and roots of *O. glaberrima* might be due to a hypersensitive response that blocks giant cell initiation and its expansion or impair the function of giant cells acting as active transfer cells. Production of phenolic compounds by plants might be associated with the degradation of giant cells and nematode collapsing (Petitot *et al.*, 2017).

We also observed a higher reduction in shoot and root growth of *O. sativa* genotypes compared to *O. glaberrima* accessions. Higher galling in *O. sativa* might have disrupted the proper supply of micronutrients from roots to shoots, which has compensated to an extent in resistant genotypes (Dangal *et al.*, 2009; Pandey *et al.*, 2016). The negative correlation of root gall index and soil nematode population density with plant height and root length of *O. sativa* genotypes and *O. glaberrima* accessions was observed. Previous studies also showed a negative and

significant correlation between galling with different agronomic traits of plants (Galeng-Lawilao *et al.,* 2018). The results showed that a higher gall index decreased shoot and root growth upon *M. graminicola* infestation.

The comparative mapping of nematode resistance genes from other crop species showed very low homology with the rice genome, indicating that there might be different resistance genes present in *O. glaberrima* against *M. graminicola*. The *in silico* analysis of *Oryzacystatin* (Cysteine proteinase inhibitors) genes between *O. sativa* and *O. glaberrima* revealed genetic differences for SNPs/InDels that could be putative defensin candidate genes involved in RKN resistance. The expression of serine or cysteine proteinase inhibitors as transgenes has been exploited for resistance to insect pests (Singh *et al.,* 2020). However, its use as anti-nematode gene has also been investigated (Ali *et al.,* 2017). So far, *Oryzacystatin* genes have not been explored against *M. graminicola*. Efforts have been made to understand the genetic variation for these two genes present between *O. sativa* and *O. glaberrima*. The SNPs identified between *O. sativa* and *O. glaberrima* for these two genes in the present study could be validated for association with nematode resistance in wet-lab experiments. Also, allele mining could be done for these two genes in all the accessions of *O. glaberrima* to detect favourable haplotypes associated with nematode resistance. It would provide a sight to understand the mechanism of RKN resistance as cross-talk exists between different genes.

The present study holds significance in the identification of three highly resistant donors viz., IRGC102196, IRGC102538 and IRGC102557, for their use against the damaging pathogen of rice *M. graminicola*. Despite *O. glaberrima* as a potential resistant source of RKN, its widespread use is limited by its lower yield potential. Therefore, advanced backcross progenies have been developed through interspecific crosses to introgress RKN resistance from African rice species to high-yielding *O. sativa* cultivars. These backcross progenies are being screened for resistance to the RKN and simultaneously for their use in molecular mapping of genetic loci responsible for *M. graminicola* resistance to gain sight in crop improvement programs.

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