Analysis of Asian Rice (*Oryza sativa*) Genotypes Reveals a New Source of Resistance to the Root-Knot Nematode *Meloidogyne javanica* and the Root-Lesion Nematode *Pratylenchus zeae*

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ABSTRACT

The sedentary root-knot nematodes, *Meloidogyne* spp., and the migratory root-lesion nematodes, *Pratylenchus* spp., cause significant yield losses, particularly in aerobic and upland rice production systems. Recently, the Asian rice *Oryza sativa* accessions LD 24 and Khao Pahk Maw (KPM) were shown to be highly resistant to *M. graminicola*. In this study, we have analyzed the responses and broadness of resistance of these two rice genotypes to another root-knot nematode *M. javanica* and a root-lesion nematode *P. zeae*. The penetration as well as post-penetration development and reproduction of nematodes were compared including known susceptible and resistant genotypes. Our results indicate that the

genotype KPM confers strong resistance to both *M. javanica* and *P. zeae*, while LD 24 was resistant to *M. javanica* and susceptible to *P. zeae*. Detailed observations revealed that similar numbers of *M. javanica* or *P. zeae* penetrated the resistant and susceptible hosts during early infection stages. However, the development and reproduction of both nematodes were arrested or reduced in resistant genotypes, implying that resistance occurs at the post-penetration stage.

Keywords: broad-spectrum resistance, genetics and resistance, nematology, pre-infection, post-infection, nematode reproduction factor

Rice is one of the leading cereals in the world. More than half of the world's populations consume rice as a staple food predominantly in subtropical and tropical regions (Bridge et al. 2005; Seck et al. 2012). Human consumption of rice is very high, exceeding 100 kg per capita annually in many Asian countries and some African countries (Seck et al. 2012). There are two cultivated species of rice: *Oryza sativa* L. and *O. glaberrima* Steudel. While *O. sativa* is known as Asian rice grown all over the world, *O. glaberrima* is the indigenous African rice species grown on a small scale in West Africa (Seck et al. 2012; Sweeney and McCouch 2007). Rice is a very adaptable crop grown in a range of agroecological zones, for instance, from river deltas to mountainous regions. Rice cultivation is categorized based on hydrological features including irrigated, deep-water or floating, rain-fed lowland and rain-fed upland systems. Irrigated systems dominate in Asia, while rain-fed systems in Africa (Seck et al. 2012).

Plant-parasitic nematodes can infect both the aboveground and belowground parts of rice at different growth stages resulting in significant yield losses (Bridge et al. 2005). The sedentary root-knot nematodes (*Meloidogyne* spp.) are the most damaging nematode species infecting rice (De Waele and Elsen 2007). While *M. graminicola* causes yield losses in irrigated, upland, lowland, and deep-water rice, *M. javanica* causes damage in upland and irrigated systems (Bridge et al. 2005). On the other hand, the migratory root-lesion nematode *Pratylenchus zeae* has been found to cause substantial damage to rice in the upland system (Bridge et al. 2005). Both the root-knot and root-lesion nematodes have a wide distribution and broad host range (Bridge et al. 2005). Since the last two decades, aerobic rice cultivation has been practiced by

the farmers in many rice-growing areas particularly in Southeast Asia due to freshwater scarcity (Bouman et al. 2006; De Waele and Elsen 2007). The root-knot nematode *M. graminicola* has emerged as one of the major biotic causal agents that resulted in remarkable yield losses in the aerobic rice production systems (De Waele and Elsen 2007; Kreye et al. 2009).

The *Meloidogyne* spp. are obligate biotrophic, sedentary root endoparasites. Under suitable conditions, the infective second-stage juveniles (J2) hatch from eggs and search the host roots, and then penetrate the roots by mechanical pressure created by repeated stylet thrusting and secretions that help breakdown the plant cell wall. The J2 induces a feeding site that consists of five to eight metabolically highly active and multinucleated cells known as giant cells, enclosed in a gall. After feeding and successive moltings, the J2 develops into an adult male or female. The females are pear-shaped and sedentary and reproduce by parthenogenesis. The males are migratory and vermiform, but rarely occur. *M. javanica* lays eggs on the surface of the root in a gelatinous matrix, while *M. graminicola* lays eggs inside the gall (Jones et al. 2013; Kyndt et al. 2014). *M. graminicola* completes its life cycle in about 2 to 3 weeks, while *M. javanica* takes about 3 to 4 weeks (Khan et al. 2006; Kyndt et al. 2014).

The root-lesion nematodes (*Pratylenchus* spp.) are migratory root endoparasites. Under favorable conditions, the J2 of *Pratylenchus* spp. hatch from eggs, find the host roots, and then penetrate at any site of the root. After entering the roots, the J2 nematodes start feeding on epidermis, cortex, and endodermis and successively molt into J3, J4, and reproductive adult stages. Unlike the root-knot nematodes, all stages of *Pratylenchus* are vermiform, migratory and can infect the host root. The *Pratylenchus* spp. feed by puncturing the root cells and sucking the cell contents resulting in a series of dead cells, which culminates into visible necrotic lesions. The females lay eggs inside the roots or in the adjacent soil. The life cycle of *P. zeae* takes about 3 weeks at 30°C (Jones et al. 2013; Olowe and Corbett 1976).

Currently, the options to control root-knot and root-lesion nematode infection in rice are limited. Crop rotation, flooding, and the use of nematicides are generally practiced to manage

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plant-parasitic nematodes in the infested rice fields, but each of these practices has a number of drawbacks (Bridge et al. 2005). In this context, the identification and use of rice genotypes resistant to these harmful nematodes would offer an excellent and durable alternative strategy to minimize the yield losses. Plant resistance to nematodes has been described as the ability of a host genotype to restrict or prevent nematode reproduction (Cook and Evans 1987; Trudgill 1991). Initially, complete resistance to the root-knot nematodes was only found in African rice (*O. glaberrima*) but not in Asian rice (*O. sativa*) accessions. Resistance to *P. zeae* has recently been found in Africa in the locally adapted *O. sativa* cultivar Supa (Nzogela et al. 2020b; Pili et al. 2016).

Recently, we have identified two *O. sativa* accessions, LD 24 and Khao Pahk Maw (KPM), that have complete resistance to *M. graminicola* (Dimpka et al. 2016; Lahari et al. 2019a). These findings provide great potential for improving nematode resistance in high yielding and commercial *O. sativa* cultivars. Here, we investigated the broadness of resistance of LD 24 and KPM using

M. javanica and *P. zeae*. The penetration, post-penetration development, and reproduction of the nematodes were studied to gain insights into the timing of resistance responses.

MATERIALS AND METHODS

Plant materials and growth conditions. Four rice genotypes were used in this study: Nipponbare (*O. sativa* subsp. *temperate japonica*), LD 24 (*O. sativa* subsp. *indica*), KPM (*O. sativa* subsp. *aus*), and TOG5674 (*O. glaberrima*). The genotypes Nipponbare and TOG5674 were used as susceptible and resistant reference genotypes, respectively. The seeds were first germinated in petri dishes containing moist tissue paper at 30°C for 4 days in the dark. Each germinated seedling was planted into a specially designed polyvinylchloride (PVC) tube containing fine sand and absorbent polymer, the SAP medium (Sannier et al. 1999). Then the seedlings were grown in a growth chamber under controlled environmental conditions (26°C/24°C, day/night temperatures; 70% relative humidity; 12 h/12 h, light/dark

Fig. 1. Penetration and gall formation of the root-knot nematode *Meloidogyne javanica* in different rice genotypes at 1 and 3 days after inoculation (DAI). Total numbers of **A**, galls and **B**, nematodes inside galls per plant. **C**, Representative pictures of galls observed under stereomicroscope after staining with acid fuchsin at 3 DAI. No statistical differences ($P > 0.05$) were obtained. Each bar represents mean $\pm$ standard error ($n = 8$). nd = not detected. Bars = 0.2 mm.

Fig. 2. Development of the root-knot nematode *Meloidogyne javanica* in different rice genotypes at 24 days after inoculation. Total numbers of **A**, galls, **B**, nematodes, and **C**, egg-laying females, and **D**, percentages of the developmental stages of the observed nematodes within galls per plant. **E**, Representative pictures of the three developmental stages of *M. javanica* observed under stereomicroscope after staining with acid fuchsin. Different letters indicate means that are statistically different ($P < 0.05$). Bars represent mean $\pm$ standard error ($n = 8$). J(s) = juvenile(s), YF(s) = young female(s), and ELF(s) = egg-laying female(s). Bars = 0.2 mm.

cycle) and were fertilized with Hoagland's solution as described by Lahari et al. (2019b).

Nematode cultures. The *M. javanica* culture was obtained from INRA, France, and the culture was maintained using the susceptible tomato cultivar Moneymaker in 2-liter plastic pots containing potting soil (Structural, Kaprijke, Belgium). The *P. zae* strain was originally isolated from a rice field in Tanzania. A pure *P. zae* culture was obtained by inoculation of a single gravid female nematode on a carrot disc and then subcultured and maintained on carrot discs at 27°C as described by Kagoda et al. (2010).

Nematode infection experiments. Three-month-old *M. javanica*-infected tomato roots were removed from pots and carefully washed with tap water to remove the soil. The egg masses were picked from the root surface and placed onto a 70 µm sieve. Additionally, the infected roots with galls were cut into very small pieces and placed onto a 200 µm sieve having moist tissue paper. Both the egg masses and cut roots were incubated for 3 days at room temperature. Then the infective J2 were extracted using the modified Baermann's method (Luc et al. 2005). Each 14-day-old rice seedling was inoculated with approximately 200 J2. On the other hand, *P. zae* were collected from the cultures by washing nematodes out from the carrot discs. The mixed vermiform stages were counted and each 14-day-old rice plant was inoculated with approximately 300 nematodes. The nematode infection experiments were performed thrice, including at least eight individual plants per genotype in each experiment.

Nematode penetration, development, and reproduction assays. The infected root samples were collected at early time points, 1 and 3 days after inoculation (DAI), to study nematode penetration and at 24 DAI to study nematode development and reproduction. The inoculated rice seedlings were carefully removed from the SAP medium, the whole root systems were gently washed with tap water, and the root and shoot lengths were measured. Then the galls and nematodes were visualized by boiling the infected roots in 0.8% acetic acid and 0.013% acid fuchsin solution for 3 min. After that, the roots were washed with running tap water and incubated in acidified glycerol for at least 7 days. The penetration of *M. javanica* was analyzed by counting the total numbers of nematodes and galls, while the penetration of *P. zae* was evaluated by counting the total number of nematodes inside each root system under a stereomicroscope (Leica S8 APO, Leica microsystems, Germany) at 1 and 3 DAI. For *M. javanica* at 1 and 3 DAI, the swollen root tips containing nematodes were counted as galls. To assess the development and reproduction of *M. javanica*, the galls were dissected using a sharp needle and the developmental stages were counted under the microscope at 24 DAI. The swollen female nematodes laying eggs on the root surface were counted as egg-laying females (ELFs), and this criterion was used as a measure of nematode reproduction. The tested rice genotypes were classified as resistant if the number of ELFs was significantly lower than the susceptible check and similar/lower than the resistance check. The reproduction of *P. zae* was analyzed by counting total number of nematodes and calculating the reproduction factor, $RF = Pf/Pi$ (final nematode population/initial nematode population) values at 24 DAI. The rice genotypes were classified based on RF values as resistant (if $RF < 1$) or susceptible (if $RF > 1$) (Pili et al. 2016).

Statistical analysis. All data were analyzed using R version 3.5.1. Normality of data and homogeneity of variances were checked using Shapiro-Wilk and Levene's test, respectively. If testing assumptions were not met, data were log transformed. Data were then analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to compare differences among the treatment groups.

RESULTS

Infection of rice genotypes LD24 and KPM with *M. javanica*. At 1 DAI, no gall was found in LD 24, KPM, and TOG5674, and

Fig. 3. Penetration and reproduction of the root-lesion nematode *Pratylenchus zae* in different rice genotypes at 1, 3, and 24 days after inoculation. Different letters indicate means that are statistically different ($P < 0.05$). Each bar represents mean $\pm$ standard error ($n = 8$).

TABLE 1. Shoot and root lengths of different rice genotypes at 1, 3, and 24 days after inoculation (DAI) with *Meloidogyne javanica*^a

Rice genotypes	1 DAI		3 DAI		24 DAI	
	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)	Shoot length (cm)	Root length (cm)
Nipponbare	20.43 $\pm$ 2.69 b	12.23 $\pm$ 1.29 a	28.04 $\pm$ 2.23 b	14.23 $\pm$ 1.49 a	54.47 $\pm$ 2.13 b	18.40 $\pm$ 0.24 a
LD 24	38.86 $\pm$ 0.46 a	13.25 $\pm$ 0.38 a	43.55 $\pm$ 0.58 a	14.90 $\pm$ 0.40 a	65.95 $\pm$ 1.03 a	17.45 $\pm$ 0.40 a
KPM	42.43 $\pm$ 0.94 a	13.63 $\pm$ 1.02 a	44.29 $\pm$ 0.70 a	13.94 $\pm$ 0.44 a	67.95 $\pm$ 1.29 a	17.94 $\pm$ 0.47 a
TOG5674	40.49 $\pm$ 0.80 a	13.63 $\pm$ 0.35 a	43.40 $\pm$ 1.26 a	14.35 $\pm$ 0.70 a	67.14 $\pm$ 1.36 a	18.50 $\pm$ 0.31 a

^a Data represent means $\pm$ standard error ($n = 8$). Time-point data for shoot or root lengths were analyzed by one-way analysis of variance followed by Tukey's post-hoc test. Means in the same column followed by different letters are statistically different ($P < 0.05$).

TABLE 2. Reproduction of *Pratylenchus zeae* on different rice genotypes at 24 days after inoculation with 300 nematodes^z

Rice genotypes	Number of nematodes (Pf)	Reproduction factor (RF) values	Host status
Nipponbare	1,032.63 ± 87.78	3.44	S
LD 24	617.63 ± 42.95	2.06	S
KPM	175.00 ± 19.54	0.58	R
TOG5674	220.75 ± 25.10	0.74	R

^z RF = Pf/Pi. RF = reproduction factor, Pf = final population, Pi = initial population (300 mixed stages nematodes). RF > 1 = S (susceptible) or RF < 1 = R (resistant). Data represent means ± standard error ($n = 8$).

only a few Nipponbare plants showed up to two to three galls per plant. However, at 3 DAI, galls were found in all genotypes and the average numbers were statistically similar (Fig. 1A and C; ANOVA, $P = 0.232$). Moreover, the numbers of J2 were not significantly different among all genotypes both at 1 DAI (ANOVA, $P = 0.287$) and 3 DAI (ANOVA, $P = 0.129$) (Fig. 1B).

At 24 DAI, the number of galls was significantly higher in Nipponbare compared with LD 24 and KPM (Fig. 2A; ANOVA, $P < 0.001$). Interestingly, the total numbers of nematodes as well as egg-laying females were significantly higher in Nipponbare compared with all other genotypes (Fig. 2B and C; ANOVA, $P < 0.001$). In Nipponbare, around 33.05% of the total nematodes developed into ELFs. In contrast, only 18.64, 12.14, and 13.23% of the total nematodes were ELFs in LD 24, KPM, and TOG5674, respectively (Fig. 2D and E).

The shoot lengths of the genotypes LD 24, KPM, TOG5674 were similar, but they were taller than Nipponbare at all time points (Table 1; ANOVA, $P < 0.001$). However, the root lengths were comparable among all genotypes (Table 1; ANOVA, $P > 0.05$).

Infection of rice genotypes LD24 and KPM with *P. zeae*.

The number of nematodes per plant was similar in Nipponbare, LD 24, KPM, and TOG5674 at both 1 DAI (ANOVA, $P = 0.904$) and 3 DAI (ANOVA, $P = 0.333$) (Fig. 3). At 24 DAI, the total nematode numbers were similar between KPM and TOG5674 (175.00 ± 19.5 and 220.75 ± 25.10 , respectively), but were significantly lower than those in LD 24 (617.63 ± 42.95) and Nipponbare ($1,032.63 \pm 87.78$) (Table 2, Fig. 3). The RF values were 3.44, 2.06, 0.58, and 0.74 for Nipponbare, LD 24, KPM, and TOG5674, respectively (Table 2). These results indicate that the genotypes Nipponbare and LD 24 are susceptible (RF > 1) while genotypes KPM and TOG5674 are resistant (RF < 1) to *P. zeae*.

DISCUSSION

Several screening studies have been performed to search for natural resistance in rice against the root-knot nematode species *M. graminicola*. Genotypes with good resistance to *M. graminicola* have been found in wild rice *O. longistaminata* (Brar et al. 1999) and in domesticated African rice *O. glaberrima* (Cabasan et al. 2012; Coyne et al. 1999; Brar et al. 1999). In Asian rice *O. sativa*, some genotypes have been reported to be resistant to *M. graminicola* (Jena and Rao 1996; Prasad et al. 2006; Sharma-Poudyal et al. 2004; Yik and Birchfield 1979); however, according to Bridge et al. (2005), only a few of these genotypes are truly resistant and the majority are in fact susceptible to *M. graminicola*. Recently, it has been found that two Asian rice (*O. sativa*) genotypes LD 24 (an *indica* cultivar, from Sri Lanka) and KPM (an *aus* cultivar, from Thailand) confer strong resistance to *M. graminicola* (Dimkpa et al. 2016; Lahari et al. 2019a). In this study, we have analyzed the responses of LD 24 and KPM to *M. javanica* and *P. zeae*. *O. sativa* Nipponbare had been reported to be susceptible to *M. graminicola* and *P. zeae* (Lahari et al. 2019a; Pili et al. 2016), and in a preliminary infection experiment, we also found it to be susceptible to *M. javanica*. The African rice (*O. glaberrima*) genotypes are generally resistant to different types of nematodes (Coyne et al. 1999). Studies have shown that *O. glaberrima*

genotype TOG5674 confers resistance to *M. graminicola* (Cabasan et al. 2012; Dimkpa et al. 2016). Here, we have demonstrated that TOG5674 is also resistant to *M. javanica* and *P. zeae*.

Host resistance to nematodes can act at different stages of the infection process, for instance, (i) preinfection by reducing nematode attraction and/or penetration into the host plant, (ii) postinfection by restricting migration, development, and reproduction of nematodes, and (iii) both the pre- and postinfection processes. A preinfection mechanism is mostly characterized by pre-existing physical barriers, which hamper nematode penetration. This mechanism of resistance has been found in the African rice (*O. glaberrima*) genotypes with resistance to *M. graminicola* infection (Cabasan et al. 2012). Dimkpa et al. (2016) also showed that the Asian rice (*O. sativa*) genotypes LD 24 and KPM confer resistance to *M. graminicola* at a very early stage of infection by reducing nematode penetration and gall formation in comparison with the susceptible genotypes. In Africa, two locally adapted *O. sativa* rice genotypes Supa and Komboka have been found to be partially resistant to *M. graminicola* and *M. javanica* by preventing nematode penetration at the early stage of infection (Nzogela et al. 2020a). In contrast, we found no significant difference in *M. javanica* penetration among all the four tested genotypes Nipponbare, LD 24, KPM, and TOG5674.

After successful penetration, the nematodes developed into the reproductive stage (egg-laying females) in all genotypes but to a different magnitude. The genotypes KPM and TOG5674 strongly, while the genotype LD 24 moderately suppressed the development and reproduction of nematodes compared with the susceptible Nipponbare. In agreement with our observations, other studies also found reduced nematode development and reproduction in (partially) resistant rice genotypes upon *M. graminicola* or *M. javanica* infection (Cabasan et al. 2012; Dimkpa et al. 2016; Nzogela et al. 2020a).

To investigate the broadness of resistance, the responses of LD 24 and KPM along with Nipponbare and TOG5674 were analyzed against infection with *P. zeae*. The penetration efficiencies of *P. zeae* were not significantly different in Nipponbare, LD 24, KPM, and TOG5674, suggesting that the ability to attract these nematodes is similar among all tested genotypes. In agreement with our observations, other studies have also found no difference in nematodes penetrating resistant and susceptible plants, for instance, wheat infected by *P. thornei* (Linsell et al. 2014).

The analysis of post-penetration nematode development or reproduction of *P. zeae* reveals that the genotypes Nipponbare and LD 24 supported a high level of nematode reproduction, suggesting these genotypes are susceptible to *P. zeae* infection. In contrast, KPM and TOG5674 were found to be resistant to *P. zeae* as the number of nematodes did not increase beyond the initial inoculum level throughout the infection period. In agreement with our study, restriction in nematode reproduction was also found on wheat resistant to *P. thornei* (Linsell et al. 2014) and rice resistant to *Ditylenchus angustus* (Khanam et al. 2018).

In this study, we showed that the Asian rice (*O. sativa*) genotype KPM, which is resistant to *M. graminicola* (Dimkpa et al. 2016; Lahari et al. 2019a), also confers strong resistance to *M. javanica* and *P. zeae*. It appears that KPM has resistance to root-infecting nematodes of different lifestyles, which mainly acts at postinfection level by suppressing the development and reproduction of nematodes. On the other hand, the Asian rice (*O. sativa*) genotype LD 24 shows resistance to *M. javanica* but susceptibility to *P. zeae*. However, both LD 24 and KPM were found to be equally resistant to *M. graminicola* (Dimkpa et al. 2016). For a genetic analysis of the resistance, these cultivars have been crossed with the susceptible cultivar Vialone Nano (*O. sativa*, a temperate japonica cultivar) resulting in progenies segregating for the resistance to *M. graminicola* infection. Analysis of the segregating F₂ population using quantitative trait loci-seq predicts the same locus on rice chromosome 11 to be responsible for *M. graminicola* resistance in both cultivars (Lahari et al. 2019a). Here, the responses of LD 24

and KPM to *P. zeae* appeared different, indicating that the underlying resistance mechanisms might be different from the root-knot nematodes. The broad resistance of the KPM genotype to both root-knot and root-lesion nematodes is an interesting observation. Future research should focus on understanding the underlying molecular mechanism of such a broad resistance against parasitic nematodes. Further studies are also required to test the resistance under field conditions. These findings would be helpful to the breeders developing a breeding program to transfer the nematode resistance trait into the commercial *O. sativa* cultivars.

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