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Short Communication

Expression analysis of defence related genes and histopathological changes in direct seeded rice genotypes (*Oryza sativa* L.) against root-knot nematode (*Meloidogyne graminicola*)

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Abstract

The root-knot nematodes, *Meloidogyne* spp. are one of the most important diseases causing organisms in direct seeded rice. In order to gain insight of differential response of susceptible and resistant rice cultivars upon nematode infection, expression of *OsEDS1, OsPAD4* and *OsWRKY* genes involved in plant innate immunity was investigated. The expression analysis showed that EDS1- and PAD4-mediated SA-upstream signaling triggered the induced defense at an the early stage of infection *i.e*., 2dpi and 6dpi in the resistant rice variety NDR-97, while it was negatively regulated in the susceptible genotype PB-1121. The histopathological studies of root galls of susceptible and resistant plants showed significant differences in the development of females, giant cells and egg production. The fecundity of the nematode was suppressed in the resistant cultivars.

Key words: Root-knot nematode, resistance, susceptibility, histopathology, rice

In South-East Asia, direct seeded rice (DSR) is mostly adopted in the dry season due to better control of water, but dry-season accounts for less than one-quarter of rice production. Around 23% of rice is grown as direct-seeded globally (Rao et al. 2007). Besides advantages, the productivity of DSR is hampered due to several biotic and abiotic factors (Mantelin et al. 2017).

Understanding the mechanism involved in resistance response holds tremendous potential for engineering novel plant resistance to nematodes (Kandoth and Mitchum 2013). In case of root-knot nematodes, the establishment of multinucleate giant cells is a major change inside the root system, but the resistant reaction involves the death of cells at or around these giant cells to suppress nematode development and reproduction (Curtis et al. 2013; Mhatre et al. 2015). But nematode also counteracts these resistance response genes by suppressing transcription of resistance regulator genes, such as *OsEDS1, OsPAD4* and *OsWRKY13* (SA signaling), in young galls (Kumari et al. 2016). Meager information is available with respect to these genes on direct seeded rice upon *M. graminicola* infection, thus in the current studies, a direct seeded rice cultivar was identified and relative expression of several defence genes were studied in susceptible and resistant cultivars of rice.

The population of *M. graminicola* used in the experiment was isolated from heavily infected rice roots. Nematodes were cultured on a susceptible rice cultivar, PB1121. The second stage juveniles (J2s) of *M. graminicola* were extracted using modified Baermann's funnel technique (Schindler 1961). The nematode suspension was collected and observed under binocular microscope.

The experiment was conducted in the Division of Nematology, ICAR-IARI, New Delhi during 2015-2016. The seeds of two cultivars were pre-soaked in petri dish overnight and then sown in 2.5 inch's plastic

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pots, each containing 150g sandy loam soil. Two week old plants were then inoculated with 450 J2s at 1 cm depth. The plants were uprooted at an interval of 2 and 6 days of post inoculation (DPI), the roots were gently washed to remove soil adhering to the roots, and were excised separately and stored at –80 °C until use and collected samples were labeled properly, were powdered using liquid nitrogen.

Expression analysis of resistance regulator genes

Total root RNA of rice plants was isolated using NucleoSpin RNA kit (MACHEREY-NAGEL (MN)). with addition of an on-column DNase I digestion. Extracted RNA was assessed for quality and quantity using Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Approximately 500 ng of the purified RNA was reverse transcribed to cDNA using cDNA synthesis Kit (Superscript VILO, Invitrogen). Further, cDNA was used for amplification of few candidate defence genes of rice *OsEDS1,* OsPAD4 and *OsWRKY13*.

The synthesized cDNA from all RNA samples were 10 times diluted with nuclease free water. Diluted cDNA was used as a template in RT-qPCR. MESA Blue qPCR MasterMix Plus for SYBR® Assay (Eurogentec) was used in RT-PCR. Reactions were performed in the eppendrof real p/ex^2 machine. Forty cycles were applied to reach optimal product amount and to generate the full sigmoid fluorescence trajectory. Two step amplification programme was constructed because annealing and extension temperature was same (60°C). At least two biological and three technical replicates were used for each of the samples. In order to determine the relative gene expression in different rice cultivars, mean Ct values were obtained and fold change values were calculated using 2–ΔΔCT method64. Data acquisition was performed during the extension step.

Primer selection and synthesizing

Primers for target gene and primers for internal control gene were selected based on studies of Nguyen et al. (2014), and synthesized by Eurofins. Os18SrRNA (GenBank: AF069218) and Os-actin (RAP-DB: Os03g0718100) usually suffices as internal control genes (Table 1).

For histological investigation, paraffin embedding method was followed as per the procedure described by Ruzin (1999). The following steps were followed for histological studies of root galls of resistant (cv. NDR-97) and highly susceptible (cv. PB-1121) rice genotypes to *M. graminicola*: selection of tissue or root gall; fixation; dehydration; infiltration; embedding; sectioning; staining; mounting and microscopic observations. The galls from both resistant and susceptible plants (7 and 21 DPI) were processed as per the procedure (Johansen, 1940; Mhatre et al, 2015). The morphological observations of surface area, equilibrium diameter and mean intensity of female and giant cells from ten sections each of rice cvs, PB-1121 and NDR-97 were measured using NIS Element Image analyzer software through Nikon Eclipse 80i compound microscope.

Histopathological data were subjected to analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) test for mean comparison. Statistical significance was determined by P = 0.05. Statistical analyses were performed using the SAS 9.0® software.

In order to gain insight of differential response of susceptible and resistant rice cultivars upon nematode infection, expression of genes involved in plant innate immunity was investigated. The infected root tissues at 2 and 6 dpi of both susceptible cv., PB-1121 and the resistant cv. NDR97 were used in quantitative real-time PCR (qRT-PCR) based expression profiling. At 6dpi, the mRNA

Table 1. Primers used for reverse transcription-quantitative polymerase chain reaction gene expression studies

Name of primers	Sequence of primers (5' 3')	Reference
OsEDS1(F)	CAGGAGAGGCAGTGTTAATCAG	
OsEDS1(R)	GCAAGCGGAGTAAGTGGTATG	
OsPAD4 (F)	TCAGAGGCAAGGCAGTAGTG	
OsPAD4 (R)	ACCGCTCACGCAGGATAG	NguyÅn *et al*. (2014)
OsWRKY13(F)	GCCAGCGGAGAACGAATC	
OsWRKY13(R)	CTCCTCCTGCTTCACAACC	
Os-actin (F)	CTCTCAGCACATTCCAGCAG	
Os-actin (R)	AGGAGGACGGCGATAACAG	
Os18SrRNA (F)	CGCGCAAATTACCCAATCCTGACA	
Os18SrRNA (R)	TCCCGAAGGCCAACGTAAATAGGA	

levels of *OsEDS1, OsPAD4*and *OsWRKY13* were found significantly repressed as compared to 2dpi in the susceptible variety PB-1121 (Fig. 1). By contrast, temporal expression of *OsEDS1* and *OsPAD4* was significantly upregulated in 2dpi and 6dpi of *M. graminicola*-infected root of resistant variety NDR-97 as compared to their corresponding mock-inoculated tissues. However, *OsWRKY13* significantly down regulated 2dpi but, it was significantly upregulated 6dpi in the resistant cultivar, NDR97 (Fig. 1).

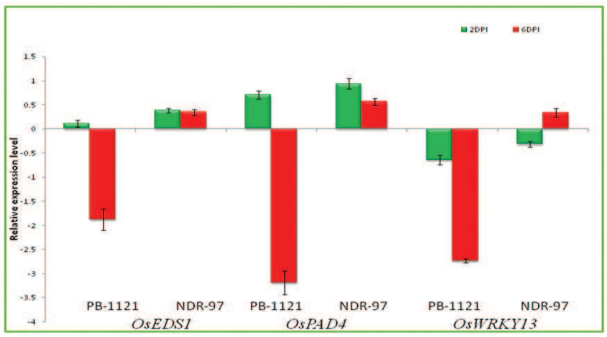

Fig. 1. Differential expression patterns of defence-related genes in the root tissue of susceptible and resistant cultivars of rice infected with *Meloidogyne graminicola*

Histopathology of plant roots

The histopathological studies of root galls of susceptible and resistant plants showed significant differences in the development of females as well as giant cells and egg production. In susceptible plant cv. PB-1121, the presence of well-developed group of giant cells was common in vascular region. The giant cells showed a typical multinucleate condition with dense cytoplasm, which is an indication of metabolically active nature of cells to act as a nutrient sink for nematode growth and its further development. In the same plant, the normal mature females and eggs were observed inside the cortical region of root which showed the completion of life cycle of nematode in cv. PB-1121. On contrary, in resistant cv. NDR-97, the giant cells were smaller and degenerated, the nematodes were immature or underdeveloped and in some sections it seems vacuolated. It seems that the growth and development of nematode was arrested in resistant plant because of host resistant response. The egg laying capacity of the nematode was suppressed as evident from the absence of the eggs in the cortical region in resistant cv. NDR-97. The present study is in agreement with the studies carried out by Mhatre et al. (2015). Besides, significant differences in nematode development and giant cells observations were made on differences in the surface area, equilibrium diameter and mean intensity of female and giant cells from cv. NDR-97 and PB-1121 using NIS-Element image analyzer software. The significant differences (Tukey's HSD, P=0.05) were observed in the area, diameter and intensity of females from both the varieties. The susceptible cv. PB-1121 had on an average a significantly larger mean female area (71896.8 μm^2) than the resistant cv. NDR-97 (14986.5 μm^2). The diameter of female in PB-1121 (259.5 μm) was significantly higher than NDR-97 at *P=0.05* (137.6 ìm) i.e. the size of females in NDR-97 was significantly reduced compared to PB-1121. The mean intensity of female was also found significantly larger in PB-1121 (145 μm) over NDR-97 (99.4 μm) which showed that the females from NDR-97 were starved of nutrition as compared to PB-1121 (Table 2). At *P=0.05,* the surface area and mean diameter of giant cells were not significantly different between the susceptible and resistant rice genotypes (Table 2). These observations showed that the resistance in cv. NDR-97 is post-infectional response wherein the nematodes are able to penetrate and develop specialized feeding sites or

Table 2. Morphological parameters of the females of infected *Meloidogyne graminicola*-resistant and susceptible rice genotypes, 21 DPI

Treatment	Female area (μm^2)	Diameter of female (μm)	Intensity of female	GC area (μm^2)	GC Diameter (μm)
Genotypes					
NDR-97 (R)	14986.5±1743.22[a]	137.6±14.968[a]	99.4±6.807[a]	7905.9±1020.48[a]	95.9±6.11[a]
PB-1121 (S)	71896.8±3767.09[b]	259.5±14.845[b]	145.4±19.266[b]	10465±1562.9[a]	114.0±6.62[a]
SE(d)	4150.87	21.08	20.75	1783.69	9.01
Tukey HSD	8725.3	44.31	43.62	NS	NS

Note: Values are the mean ± standard deviation. In a column, means followed by the same letter are not significantly different according to Tukey's honestly significant difference test (*P* = 0.01); R = resistant; HS=Highly susceptible and GC=Giant cell

giant cells but later they are unable to reproduce on the host. Similar responses were also observed in transplanted resistant plants (Mhatre et al. 2015).

The genes EDS1 and PAD4 were earlier known to be involved in SA signaling (NguyÅn et al. 2014). Based on the expression pattern observed, it can be deduced that the EDS1- and PAD4-mediated SA-upstream signaling would have triggered the induced defense against RKN at the early stage of infection i.e., 2pi and 6dpi in the resistant cv, NDR-97, while, it was negatively regulated in the susceptible cv. PB-1121. Therefore, considering the expression pattern of SA-related genes in the resistant and susceptible cultivars, it can be speculated that their exist role of SA in inducing systemic defense in rice upon RKN infection. The gene *OsWRKY13* was found upregulated at 6dpi in resistant cv. NDR-97 but was found downregulated at 2dpi and in susceptible cv. PB-1121, indicating its possible role in nematode resistance. The gene *OsWRKY13* was earlier reported to play a pivotal role in rice disease resistance (Qiu et al. 2007) which is involved in activation of salicylic acid (SA) synthesis-related genes and SA-responsive genes and the suppression of jasmonic acid (JA) synthesis-related genes and JA-responsive genes. Therefore, the major findings from this study can be extrapolated to understand the molecular mechanisms governing the resistance/susceptibility in direct seeded rice in response to *M. graminicola* infection.

The cell necrosis in the cortical region was earlier showed that there was delay in establishment of feeding site, growth and development of the J2s (Mhatre et al. 2015). The histopathological studies showed the presence of normal mature females and eggs inside the cortical region of root indicative of the completion of life cycle of root-knot nematode in cv. PB-1121. On contrary, in resistant cv. NDR-97, the giant cells were poorly developed, the nematodes were immature and small or did not develop normally, and in some sections it was vacuolated.

Author's Contribution

Conceptualization of research (P, AS, AKS); Designing of the experiments (P, AKS); Contribution of experimental material (AKS, RKE); Execution of field/ lab experiments and data collection (DKP, P, AS, AKS, RKE); Analysis of data and interpretation (DKP, P, RKE); Preparation of manuscript (DKP, P, RKE).

Declaration

The authors declare no conflict of interest.

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