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SHORT REPORTS

Functional characterization of tau class glutathione-*S*-transferase in rice to provide tolerance against sheath blight disease

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Abstract

Glutathione-*S*-transferase (*GST*) is an important defense gene that confers resistance against several abiotic and biotic stresses. The present study identifies a tau class GST in rice (*Oryza sativa* L.), *OsGSTU5* (*Os09g20220*), which provided tolerance against sheath blight (SB) disease, caused by a necrotrophic fungus, *Rhizoctonia solani* (RS). Overexpression and knockdown rice transgenic lines of *OsGSTU5* were generated and tested for the severity of infection during sheath blight disease. The results obtained after RS infection showed that the lesion cover area and hyphal penetration were more in knockdown line and lesser in the overexpression line. Analysis of reactive oxygen species (ROS) accumulation showed more spots of H_2O_2 and O^{2-} in knockdown lines compared to overexpressed lines. Later, RS transcript level was analyzed in RS-infected transgenic lines, which manifested that the knockdown line had higher RS transcripts in comparison to the control line and least RS transcripts were observed in the overexpressed line. In conclusion, rice transgenic lines overexpressing *OsGSTU5* were found to be more tolerant, while the knockdown lines were more prone to *Rhizoctonia* infection compared to control lines.

Keywords Glutathione-*S*-transferase · Rice · *Rhizoctonia solani* · Sheath blight disease

Worldwide, rice is a staple food and the major population in the Asian belt is dependent on rice for their daily diet intake. There are various biotic and abiotic entities present in the environment, which severely affects the rice production. Among various biotic stresses, *Rhizoctonia solani* (RS), a soil-borne necrotrophic fungal pathogen, significantly decreases rice productivity (Kouzai et al. 2018) by causing sheath blight (SB) disease. RS in its favorable humid condition, spreads well on a large surface area of leaf sheath and blade. The SB disease is destructive under both high humidity and temperature, and its resistance depends on the interaction between plant, pathogen as well as environmental factors (Zeng et al. 2017). The SB disease is epidemic in nature and two phases of its development are recognized in plants. One is upward (leaf sheath and blade) development and the other is horizontal (neighboring shoots) development (Willocquet et al. 2000).

GST is a vast group of defense proteins, which is classified as a "phase II detoxification" system (Edwards et al. 2000). Detoxification via GST is done by the incorporation of reduced glutathione on its target co-substrates such as xenobiotic compounds, foreign proteins etc. (Cummins et al. 2011). These glutathionylated xenobiotic compounds get sequestered into the plant vacuole for its further detoxification (Dixon and Edwards 2010), whereas glutathionylation of foreign protein leads to changes in its three-dimensional structure. These structural changes result in the declined functional activity of the foreign protein.

Plant *GST's* had been categorized into four classes named as phi, tau, lambda, and dehydroascorbate reductase; DHAR ((Edwards and Dixon 2005). Plant tau class GST is a multifunctional protein which provides resistance against different abiotic stresses such as heavy metal, drought (Srivastava et al. 2019; Tripathi et al. 2014) as well as biotic stresses like *Pseudomonas* and viruses (Cicero et al. 2017; Chen et al. 2013).

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One of the members of tau class GST, *OsGSTU5*, in rice was found to be upregulated during transcriptome profiling under different biotic stresses (Nino-Liu et al. 2006). The present study will aim to functionally characterize the role of rice tau class glutathione-*S*-transferase (*OsGSTU5*) under *Rhizoctonia solani* stress. The *OsGSTU5* was overexpressed and knockdown by artificial miRNA technology to generate transgenics. The transgenics were exposed to RS fungus to uncover the potential function of *OsGSTU5* during sheath blight disease.

Plant growth material

The full-length cDNA sequence of *OsGSTU5* (*Os09g20220*) was obtained from the rice genome annotation project. For overexpression of *OsGSTU5*, the *OsGSTU5* fragment was amplified from the cDNA of rice, Nipponbare (*O. sativa* L. ssp Japonica) and cloned in pTZ cloning vector followed by cloning in expression vector i.e., pIRS154. To generate knockdown *OsGSTU5* construct, an artificial miRNA technique was used and primers were synthesized from https://wmd3.weigelworld.org/cgi-bin/webapp.cgi website tool. The microRNA fragment of *OsGSTU5* was cloned to the pIRS vector. pIRS154 (control), knockdown, and overexpression *OsGSTU5* constructs were transformed to *Agrobacterium* strain EHA101 using freeze–thaw method (Wise et al. 2006) and transformed to rice, Nipponbare (*O. sativa* L. ssp Japonica) according to the protocol (Shri et al. 2013).

Screening of transgenic plants

Total genomic DNA was isolated from the flag leaf of *OsGSTU5* rice transgenic plants (control, knockdown, and overexpressed) and checked for the *HPTII* marker gene via PCR amplification (Fig. 1d). The positive rice transgenic plants were also tested for an abundance of *OsGSTU5* transcripts via semiquantitative RT-PCR at T2 generation (Fig. 1e). For semiquantitative RT-PCR analysis, mRNA from positive rice transgenic plants was isolated via Qiagen RNeasy kit (Qiagen, USA) and 1 µg of RNA was utilized for cDNA synthesis via RevirtAid first-strand cDNA synthesis kit (Fermentas, Thermo Scientific). For analysis of *OsGSTU5* transcript abundance, 2 µl of each cDNA was used in each PCR amplification reaction. The target cDNA was also amplified with actin as an internal control. The primer sequence of *OsGSTU5* specific primer and actin has been provided in Table S1.

Rhizoctonia solani infection to rice seedling

To study the colonization of RS pathogen on control, knockdown, and overexpression lines, the transgenic seeds of the T2 generation were sterilized and plated on water agar plates for germination. Germinated seedlings were transferred on new water agar plates and infected with an 8 mm bit of the RS pathogen culture. Roots were harvested after 15 days post-infection (dpi) for qRT-PCR and microscopic studies. To stain fungal hyphae, roots were treated with 0.1% trypan blue (Srivastava et al. 2016). Pictures of intact stained root were taken at 10× magnification. The roots were mashed by gently tapping the cover slip to expose the internal tissues and pictures were taken at 20× magnification. Photos were taken on an Olympus U-CTR30-2 Microscope.

Real-time PCR

Total mRNA was isolated from each RS-infected transgenic line (T2 generation) via Qiagen RNeasy kit (Qiagen, USA) and cDNA was synthesized from RevirtAid first-strand cDNA synthesis kit (Fermentas, Thermo Scientific). For quantification of the sheath blight disease, *Rhizoctonia solani* transcripts were analyzed by qRT-PCR. For qRT-PCR analysis, gene-specific primers (*RsAROM*) of *Rhizoctonia solani* (Su'udi et al. 2013) and Fast SYBR Green Master Mix (ABI, USA) was used. To minimize the error, the actin gene was used as an internal control. Relative expression of different genes was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

Reactive oxygen species (ROS) analysis

Reactive oxygen species is an important stress marker and its production is alleviated under stress conditions. To analyze the ROS status, one set of transgenic lines was infected with RS, while the other set were non-infected. These two sets were analyzed for O^{2-} staining via NBT (Nitro Blue Tetrazolium) and H_2O_2 staining via DAB (3,3′-Diaminobenzidine) according to the protocol (Kidwai et al. 2019).

Chlorophyll content

Chlorophyll content was analyzed according to the given protocol (Rajalakshmi and Banu 2015).

Biochemical analysis

Protein was isolated according to the protocol (Rai et al. 2011). Superoxide dismutase (SOD) and peroxidase (PRX) activities were performed according to the protocol (Kidwai et al. 2019).

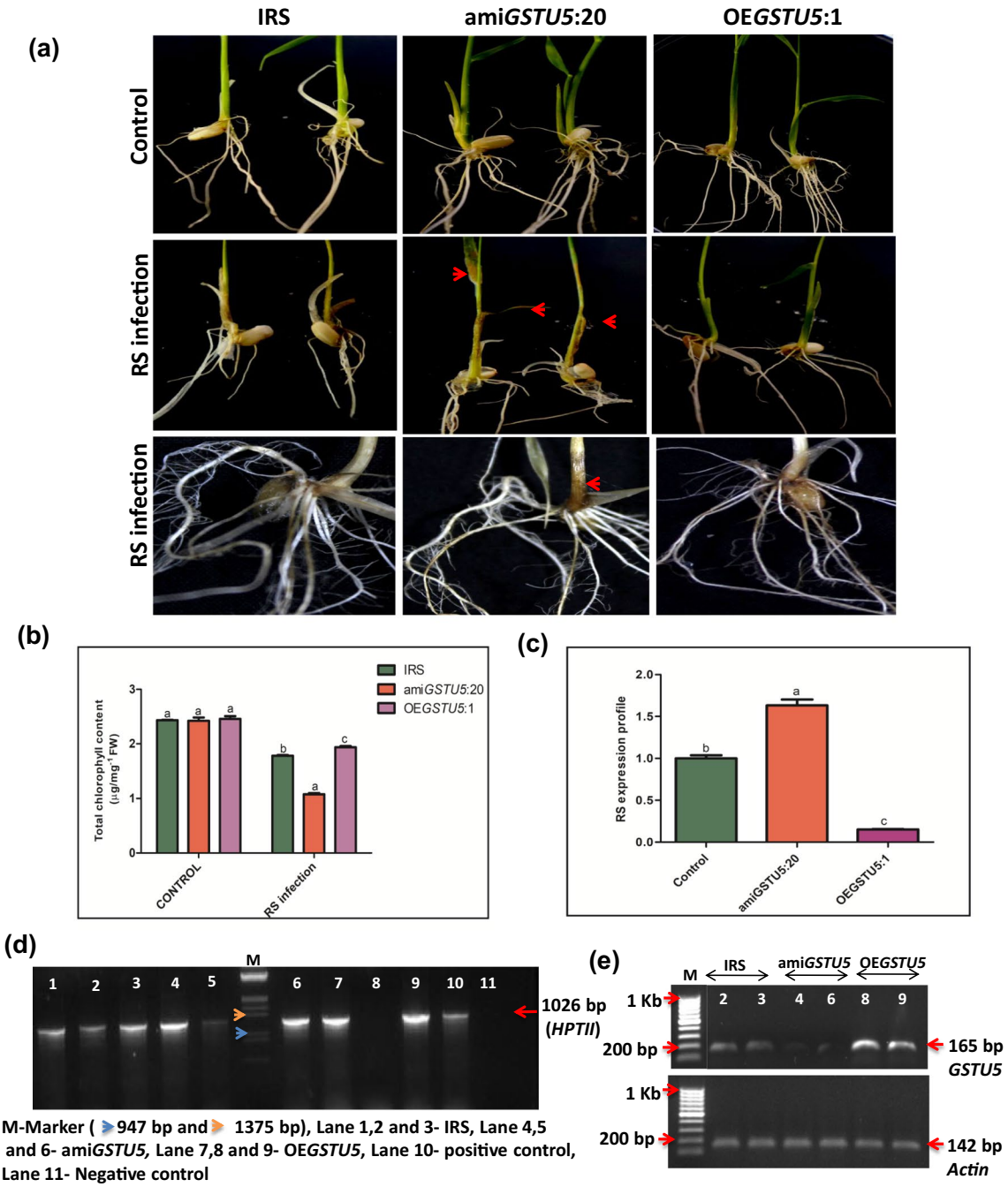

Fig. 1 Effect of *Rhizoctonia solani* (RS) infection on T2 transgenic seedlings. **a** Seedlings of IRS, knockdown and overexpressed *OsGSTU5* under control and RS infection condition, **b** Analysis of chlorophyll content in control and RS-infected seedlings. **c** Analysis of RS transcript via qRT-PCR. **d** Gel image of positive rice transgenics with *HPTII* marker gene. **e** Semiquantitative RT-PCR showing the level of expression of a gene on the basis of band intensity. The symbols above the bars indicate the significant difference at $P < 0.05$ according to Duncan's multiple range test (DMRT)

Greenhouse experiment

Rhizoctonia solani was inoculated to the *OsGSTU5* transgenic rice plants by placing RS sclerotia, wrapped in moist cotton (Srivastava et al. 2016). The inoculation was done after 45 days of transplantation at maximum tillering stage. To maintain humidity during RS infection, infected plants were covered with polythene bags for 48 h (Marshall and Rush 1980; Zheng et al. 2013). The difference in lesions were visible among all transgenic plants. The severity of SB disease depends on several internal and external factors such as plant vigor, soil condition, etc. One of the important

internal factors during SB disease is the defense response of the plant. *GST* serves as an important defense gene that protects the plants during biotic stress. One of the marker genes from *Arabidopsis thaliana*, *GSTF8*, has been identified as an early defense-related gene (Thatcher et al. 2015). Rioux et al. (2011) identified a glutathione-*S*-transferase kappa 1 gene in rice which detoxifies the toxic compounds released by RS pathogen during infection (Rioux et al. 2011).

The present study reveals the role of tau class GST, *OsGSTU5*, in rice during SB disease. Earlier, the comparative transcriptome analysis has shown that the *OsGSTU5* gets upregulated under different biotic stresses (Zhou et al. 2010). To investigate the biological role of *OsGSTU5* during SB disease in rice, the transgenic lines with overexpressed and knockdown *OsGSTU5* constructs have been generated and confirmed via semiquantitative RT-PCR (Fig. 1e). The T2 transgenic rice plants (control, knockdown, and overexpressed) were infected with *Rhizoctonia solani* fungal pathogen. The severity of RS infection was analyzed at seedling and greenhouse grown stages of T2 rice transgenic plants. We observed that knockdown seedlings were infected severely (Fig. 1a, arrowhead) in comparison to control (Fig. 1a) and the least infection was observed in overexpressed transgenic lines (Fig. 1a). In case of greenhouse grown rice transgenic plants, a brown lesion was observed on infected plants. More lesion was identified on knockdown line (Fig. 2b) while, overexpressed line has had the least lesion (Fig. 2b) in comparison to the control transgenic line (Fig. 2b). To analyze the transcript profile of *Rhizoctonia solani* fungus in infected rice transgenic lines, we performed a real-time analysis of RS transcripts and found that knockdown line have had 1.5-folds higher transcripts in comparison to control while overexpressed rice transgenic lines were showing sixfold lesser RS transcripts in comparison to control line (Fig. 1c). Earlier it has been reported that the branched hyphal tips of RS fungus get aggregated at the site of infection in plants (Łaźniewska et al. 2012). To analyze the aggregation of RS hyphae in RS-infected rice transgenic lines (control, overexpressed, and knockdown), the microscopic study of infected roots has been performed. The hyphal colonization was analyzed at the surface (Fig. 3a–c) and at the intercellular spaces of transgenic roots (Fig. 3d–f). Profuse fungal colonization was observed in knockdown rice transgenic line which successively decreases in control and overexpressed line. The generation of reactive oxygen species (ROS) has a negative impact on plant growth and development. The *OsGSTU5* transgenic lines were analyzed for ROS abundance under stressed (with RS) and non-stressed (without RS) conditions. The non-stressed *OsGSTU5* transgenic lines have had no significant difference when stained with NBT (deep blue color) and DAB (brown color) (Fig. 2a). The staining reflects the superoxide and hydrogen peroxide abundance in plants, which increases during the stress condition. Under RS-infected condition, the knockdown lines have had a higher accumulation of superoxides in comparison to control and least accumulation was observed in the overexpressed line (Fig. 2a, panel 1, 2). The accumulation of hydrogen peroxide was also following a similar pattern (Fig. 2a, panel 3, 4). The transgenic lines were also analyzed for chlorophyll content under stressed and non-stressed conditions. It was identified that there was a significant decrease in chlorophyll a and total chlorophyll content in knockdown lines in comparison to control and overexpressed transgenic lines (Fig. 1b). Among antioxidant enzyme activities, no significant difference in SOD and PRX activities was observed under the control condition. But when RS infection was given, an increase in the SOD and PRX activities was observed in the overexpressed line followed by control and the lowest activity was observed in knockdown *OsGSTU5* lines (Fig. 2c, d).

In the present study, we hypothesize that the *OsGSTU5* is a potent defense gene in rice that provide tolerance against SB disease. The *OsGSTU5* overexpression line has had better tolerance against SB disease. In contrast, the *OsGSTU5* knockdown lines found to be more susceptible to RS pathogen and thus for SB disease.

Despite having abundant knowledge about the sheath blight disease, there is a knowledge gap regarding host response during this host–pathogen interaction (Zheng et al. 2013; Basu et al. 2016). The identified *OsGSTU5* confers resistance during *Rhizoctonia* infection. In future, the molecular mode of *OsGSTU5*-mediated resistance during *Rhizoctonia* infection will be studied in detail. Protein–protein interaction assays and molecular analysis will allow to identify the effector/virulence protein of *Rhizoctonia* which is important for sheath blight disease.

Statistical analysis

Mean, standard error, and triplicate data were used for statistical evaluation using GraphPad Prism5. The statistical significance of differences between control and treated samples was tested by Duncan's multiple range test (DMRT) with a significant difference at $P < 0.05$.

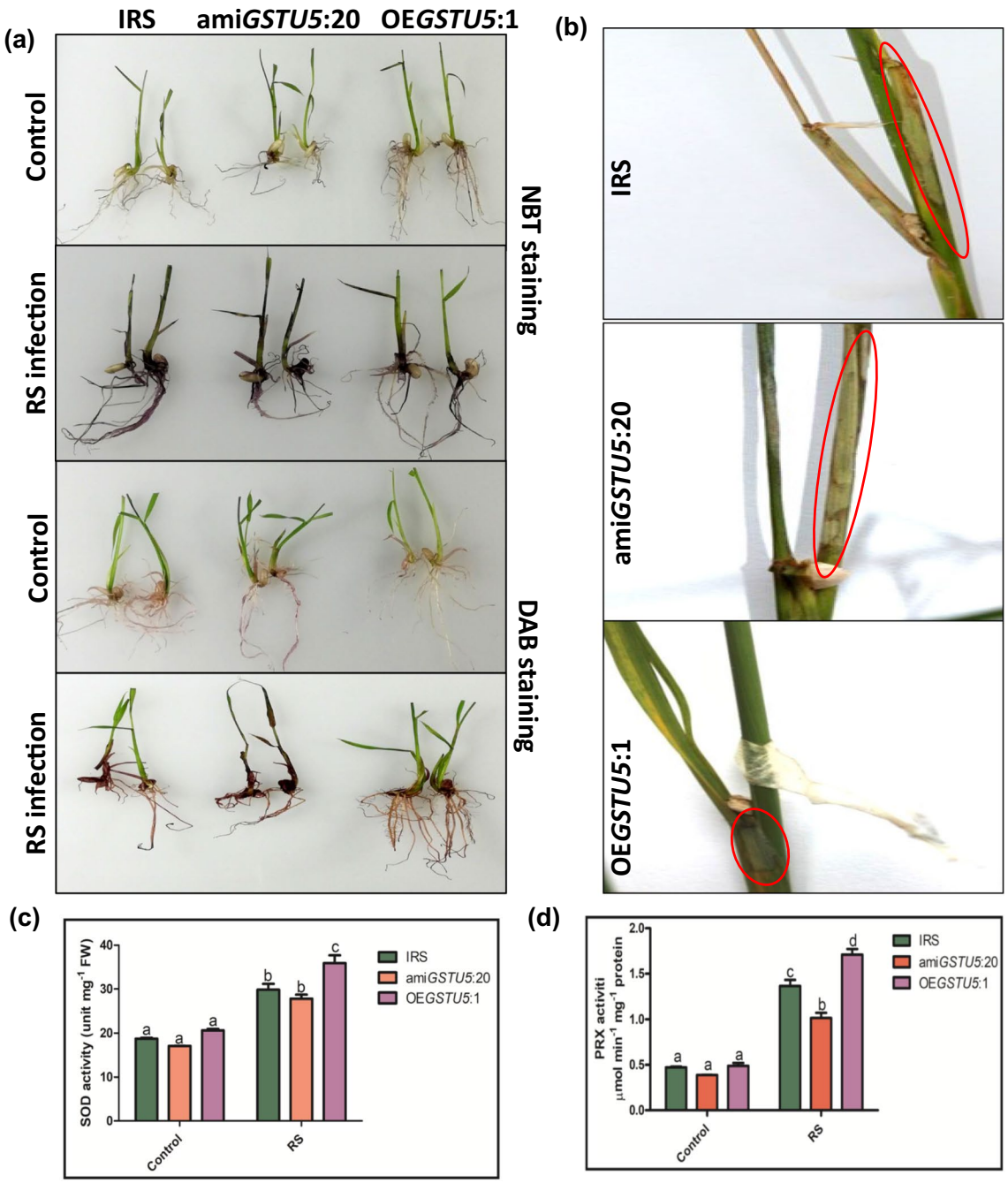

Fig. 2 **a** ROS staining. NBT staining for O^{2-} accumulation in control and RS-infected transgenic, DAB staining for H_2O_2 accumulation in control, and RS-infected transgenic lines. **b** The difference in lesion cover area of RS-infected transgenic plants marked by circled area. **c** SOD activity. **d** PRX activity. The symbols above the bars indicate the significant difference at $P<0.05$ according to Duncan's multiple range test (DMRT)

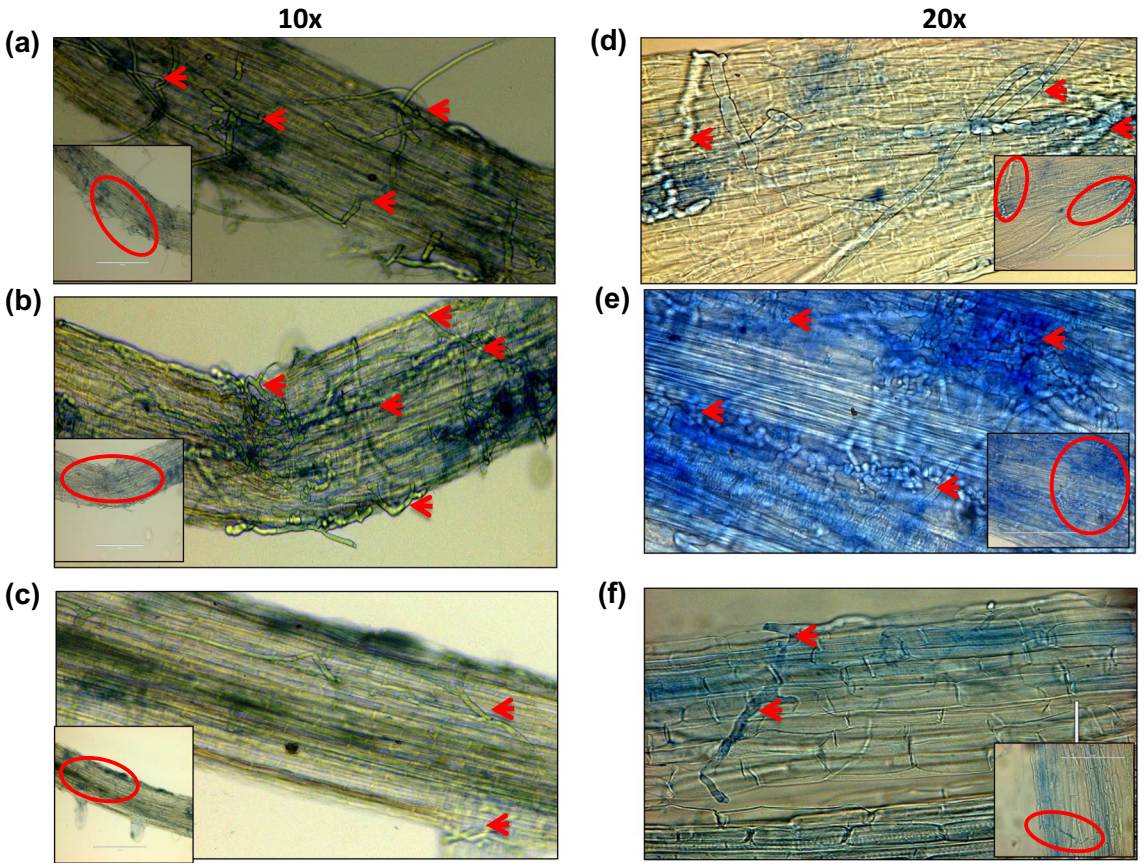

Fig. 3 Differential colonization of rice roots by *Rhizoctonia solani* in T2 transgenic plants. **a**, **d** Control (IRS), **b**, **e** knockdown *OsGSTU5* and **c**, **f** overexpressed *OsGSTU5*. Hyphae colonizing on the root surface (**a**–**c**) and intercellular colonization (**d**–**f**). **a**–**c** 10× magnification, bar size 400 µm, **c**–**f** 20× magnification, bar size 200 µm

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Author contributions DC planned, supervised the experiments, and also reviewed the manuscript. MT performed the experiments, executed data analysis, and wrote the manuscript. AKM supervised during the work. SS and PCS helped during the experimental work.

Compliance with ethical standards

Conflict of interest The authors do not have any conflict of interest.

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