INTRODUCTION

Pyricularia is a phytopathogenic filamentous fungal genus that causes blast disease, mainly in monocotyledonous plants. Rice blast caused by Pyricularia oryzae (syn. Magnaporthe oryzae) is one of the most devastating plant diseases. Another species, Pyricularia grisea, a crabgrass pathogen, is closely related and morphologically similar to P. oryzae (Murata et al., 2014). When a Pyricularia spore adheres to the leaf surface, the spore sprouts germ tubes and forms an appressorium. The appressorium deposits melanin on the inner layer of the cell wall (Howard & Ferrari, 1989) and accumulates large amounts of glycerol via glycogen, which generates strong turgor pressure (Thines et al., 2000). Finally, P. oryzae uses the penetration peg generated at the bottom of the appressorium to penetrate the leaf surface (Howard et al., 1991). Various genes and signalling pathways are involved in this process, including the cAMP signalling...
pathway, mitogen-activated protein kinase signalling pathways, the autophagy pathway, and NADPH oxidase-mediated cytoskeleton dynamics (Ikeda et al., 2019; Wilson & Talbot, 2009). Although P. oryzae is known to be pathogenic to the leaves, ears, and other aboveground parts of plants, it can also infect the roots (Dufresne & Osbourn, 2001). The characteristic of a diseased root is browning. P. oryzae invades root cells via hyphopodia and pre-infection hyphae instead of the appressorium (Sesma & Osbourn, 2004; Tucker et al., 2010). Moreover, this infection process does not require the cAMP signalling pathway or melanin synthesis (Dufresne & Osbourn, 2001; Sesma & Osbourn, 2004). These different infection strategies represent significant structural and metabolic differences between aboveground and underground tissues.

The interaction between the host plant and P. oryzae is well studied. Host specificities are determined by the combinations of avirulence (Avr) genes of the pathogen and disease resistance (R) genes of the host plant. In rice (Oryza sativa), numerous Avr genes of P. oryzae and R genes of the plant have been identified, and these combinations explain the race–cultivar specificity (Li et al., 2019; Wang et al., 2016; Wu et al., 2015). Most Avr genes encode signal peptides and are thought to function as effectors; therefore, these race–cultivar specificities are called effector-triggered immunity. The P. oryzae population comprises various pathotypes with different host ranges, such as the Oryza, Triticum, Setaria, Lolium, and Eleusine pathotypes (Kato et al., 2000). These pathotype–genus specificities are robust and are thought to have been brought about by the co-evolution of host plants and pathogens (Kato et al., 2000; Snezhko et al., 2011). These specificities are also explained by the combination of Avr and R genes (Inoue et al., 2017). The robustness of these resistances seems to be stronger than that of race–cultivar specificity because of the pyramiding Avr–R combinations (Tosa & Chuma, 2014). In addition to these specificities, pathogen-associated molecular pattern-triggered immunity, which is recognized by pattern recognition receptors, may function in more distant host–pathogen combinations (Jones & Dangl, 2006).

In plants, various pathogenesis-related (PR) genes have been reported to be induced during P. oryzae infection (Jung et al., 2016; Manandhar et al., 1999). PR genes are thought to be induced on the recognition of the presence of pathogens, and are induced in both susceptible and resistant combinations, although PR gene expression is faster and higher in resistant combinations (Jung et al., 2016; Muhae-UD-Din et al., 2020). The expression analysis of PR genes appears to reflect the behaviour of pathogens in the host plant.

However, most of these reports were the results of leaf inoculation. Until now, it has been thought that similar host specificity is established in the leaves and in roots. For example, P. oryzae isolate Guy11 transformed with Avr1-CO39 could not colonize the rice cultivar CO39, suggesting that race–cultivar specificity functions in roots (Sesma & Osbourn, 2004). Some PR genes were induced in rice roots invaded by P. oryzae (Marcel et al., 2010). In addition, wild rice roots were more resistant to P. oryzae than cultivated rice roots, and the expression patterns of PR genes were different between the two rice lines (Tian et al., 2018). However, in our previous study, browning lesions were formed when Guy11, an Oryza pathotype isolate, was inoculated on wheat roots. This was an unexpected reaction because this combination was a pathotype–genus-specific resistance in leaves. It was also considered that browning might be associated with a resistance reaction rather than a compatible reaction. Therefore, we conducted an inoculation experiment to examine whether race–cultivar specificity and pathotype–genus specificity function in roots.

To examine race–cultivar specificity, we used the rice cultivar Lijiangxintuanheigu (LTH) and LTH monogenic lines (Tsunematsu et al., 2000) carrying R genes (Pik-p, Pish, Piz, and Piz-t) that confer resistance against the isolate Guy11. To examine pathotype–genus specificity, we used five isolates belonging to P. oryzae (Oryza isolate Guy11, Setaria isolate GFS1-7-2, Eleusine isolate MZ5-1-6, and Triticum isolate Br48) and P. grisea (Digitaria isolate Dig4-1), and three host gramineous genera (O. sativa ‘CO39’, Triticum aestivum ‘Norin 4’, and Hordeum vulgare ‘Nigrate’).

We found that the roots of gramineous plants were vulnerable to Pyricularia species infection, although the roots induced resistance responses. We also found that Pyricularia species produced some secretion molecules involved in root growth inhibition.

## RESULTS

### 2.1 Race–cultivar specificity: Brown lesions formed in the roots of LTH and the monogenic lines

To evaluate race–cultivar specificity, we used the Oryza pathotype isolate Guy11 and LTH monogenic lines. The Japanese race code for Guy11 is 126.4 (Hayashi, 2005). We selected the LTH monogenic lines IRBLsh-Fu (Pish), IRBLz-Fu (Piz), IRBLz-T (Piz-t), and IRBL-Kp-K60 (Pik-p), which are presumed to be resistant to Guy11. In the leaf inoculation experiment, the disease indices of the LTH monogenic lines were significantly lower than those of the original cultivar LTH (Figure 1a,b). In contrast, all inoculated roots exhibited strong browning (Figure 1c,d). The cross-sections of Guy11-inoculated roots showed that numerous infection hyphae had spread to root cortex cells, and there was no discernible difference in the colonisation of hyphae between LTH and the monogenic lines (Figure 1e). These results suggest that root browning is associated with a susceptible reaction.

### 2.2 Race–cultivar specificity: R and PR genes were expressed in the roots of LTH and the monogenic lines

As the resistance reaction did not function in roots, it was possible that the R gene or the downstream PR genes were not functioning. The expression of two R genes, Pish and Pik-p, was analysed. Both genes were constitutively expressed in the leaves and roots, and their expression increased after Guy11 inoculation (Figure S1). To further explore the variation in the expression of PR genes, we
selected six genes from rice: OsPR-1a (serine carboxypeptidase-like protein, acidic), OsPR-1b (serine carboxypeptidase-like protein, basic), OsCht-3 (chitinase), OsPBZ1 (probenazole-induced protein), RSoSPR-10 (root-specific PR-10), and OsLTP110 (lipid transfer protein). The expression of OsPR-1a, OsPR-1b, OsPBZ1, and RSoSPR-10 increased after Guy11 inoculation in both the leaves and roots (Figures 2 and S2). R gene-dependent variations were observed in the expression of OsCht-3 in roots, that is, monogenic lines carrying Pish, Piz, or Pik-p induced OsCht-3 expression in the roots, whereas LTH and a monogenic line carrying Piz-t did not induce OsCht-3 expression in the roots (Figure 2c,d). The expression of OsLTP110 in leaves was significantly induced at 2 days after inoculation (DAI), but there were no significant differences after the inoculation of roots (Figure 2e,f). However, the relative expression level of OsLTP110 in the roots was higher than that in the leaves (Figure 2e,f).

Reactive oxygen species (ROS) generation is an indicator of plant resistance reactions (Levine et al., 1994). We evaluated ROS generation in roots by 3,3′-diaminobenzidine (DAB) staining. Although it was difficult to strictly evaluate the positive DAB reaction in the browning lesion at the inoculation site, it was confirmed that ROS were generated; however, no difference was observed between LTH and the monogenic line IRBLzt-T (Piz-t) (Figure S3).

2.3 | Pathotype–genus specificity: Various pathotypes caused different disease symptoms in the roots of gramineous plants

To investigate pathotype–genus specificity, four P. oryzae isolates, Oryza pathotype Guy11, Setaria pathotype GFSI1-7-2, Eleusine pathotype MZ5-1-6, and Triticum pathotype Br48, and P. grisea...
Digitaria pathotype Dig4-1 were inoculated on the leaves and roots of rice cultivar CO39. In CO39 leaves, only Guy11 induced disease lesions after inoculation (Figure 3a). However, browning was observed in the roots inoculated with all isolates (Figure 3b). We evaluated the pixel intensity of browning and found that Dig4-1 caused the strongest browning of the roots (Figure 3b). Dig4-1 also inhibited crown root branching in CO39 cells (Figure 3c). In addition, we found that root growth was significantly inhibited by inoculation with Br48 and Dig4-1 (Figure 3d). To clarify the generality of these effects in rice, we inoculated the roots of LTH and IRBLzt-T (Piz-t) with Guy11, Br48, and Dig4-1. We observed similar root growth inhibition upon inoculation with Br48 and Dig4-1 as well as crown root branching inhibition upon inoculation with Dig4-1 (Figures S4 and S5).

We also inoculated the leaves and roots of wheat cultivar Norin4 and barley cultivar Nigrate with Pyricularia isolates. Only isolate Br48 induced lesions in wheat leaves (Figures 4a and 4b). Inoculation with all Pyricularia isolates resulted in lesions, but the severity was variable in barley leaves (Figures 4b and S6). All combinations induced browning in wheat and barley roots, but the colour indices were lower than that in rice (Figure 4c, d). Root growth inhibition was
also observed in wheat and barley inoculated with Br48 and Dig4-1 (Figure 4e,f). However, root branching inhibition, which was found in rice inoculated with Dig4-1, was not observed in wheat and barley roots (Figure 4g,h).

2.4 | Pathotype–genus specificity: Infection hyphae of various pathotypes differentially colonized the roots of gramineous plants

Root sections inoculated with *Pyricularia* isolates were observed under a microscope. The infection hyphae were extended in root cells in all combinations tested (Figure 5a,e). However, the localization of infection hyphae in rice root tissues differed depending on the pathotype. We divided the rice root sections into an outer layer (epidermis cells and sclerenchyma cells) and a middle layer (cortex cells). The infection hyphae of Br48 and Dig4-1 were localized in the outer layer, but those of the other pathotypes were evenly distributed (Figure 5b). Furthermore, after inoculation with Br48 or Dig4-1, the average number of infection hyphae per infected rice root cell was higher than after inoculation with the other isolates, suggesting that the hyphal mass per single cell was larger than that of the other isolates (Figure 5c). Interestingly, many Guy11 hyphae extended to the intercellular space rather than inside root cells (Figures 5d and S7). This characteristic differed from that of the other isolates. In contrast to rice roots, in wheat and barley roots inoculated with Guy11, Br48, or Dig4-1 the sclerenchyma layer was not developed (Figure 5e); all isolates invaded internal cortical cells in these roots (Figure 5e). The average number of infection hyphae per infected root cell in wheat and barley did not differ between the isolates tested (Figure 5f,g). However, the infection hyphae of Guy11 were preferentially localized in the intercellular space of wheat and barley roots compared to those of the other isolates (Figure 5h,i).

2.5 | Pathotype–genus specificity: PR gene expression in the leaves and roots of gramineous plants challenged by *Pyricularia* isolates

To evaluate the cellular response to the invasion of *Pyricularia* isolates, we analysed the expression patterns of PR genes in rice, wheat, and barley. In the leaves inoculated with Guy11, *OsPR-1a*
and OsPR-1b were induced up to 6 DAI; however, inoculation with the other isolates decreased OsPR-1a expression (Figure 6a,c). In the inoculated roots, OsPR-1a and OsPR-1b were induced by all isolate combinations (Figure 6b,d). Interestingly, the expression levels of OsPR-1b after inoculation with Br48 or Dig4-1 were higher than after inoculation with Guy11 (Figure 6d). We also evaluated PR-1 expression in wheat and barley. Inoculation with all combinations of Pyricularia isolates induced PR-1 genes in the leaves and roots (Figure S8).

2.6 | Culture filtrates of Br48 and Dig4-1 caused root growth inhibition

In the present study, Br48 and Dig4-1 inhibited root growth. As the root extends to the apical meristem, the site of inoculation might affect root growth inhibition. Therefore, we roughly divided the roots into the base and tip regions (Figure 7a). We separately inoculated these regions with mycelial plugs of Dig4-1, and found that inoculation of both regions resulted in strong browning and growth inhibition (Figure 7c,d). To test the possibility that some inhibitory factors were secreted by Dig4-1, we used a 0.22-μm filter membrane to avoid direct contact between the mycelia and roots (Figure 7b). Interestingly, strong growth inhibition of roots was observed in rice, wheat, and barley even without direct contact with the mycelia of Br48 or Dig4-1 (Figures 7c–e and S9). In addition, inoculation with Dig4-1 separated by a 0.22-μm filter membrane caused inhibition of crown root branching (Figure 7f). Although filter membrane inoculation with Dig4-1 and Br48 resulted in slight browning, the degree was substantially lower than that after inoculation by direct mycelial contact (Figures 7c and S9).

Next, we treated rice roots with culture filtrates of Br48 and Dig4-1 by filter paper inoculation (Figure 8a). The culture filtrates caused root growth inhibition (Figure 8b,c), supporting the idea that some secreted factors mediated root growth inhibition.

3 | DISCUSSION

In this study, we evaluated various combinations of Pyricularia isolates and their interactions with gramineous plants to better understand the tissue-specific infection strategies of Pyricularia. In the root inoculation, we used mycelial plugs instead of a spore suspension. Because it is rare for spores to reach the roots directly, root inoculation experiments were conducted assuming contact with hyphae extended from diseased residues in soil.

3.1 | Tissue-specific pathogenicity in Pyricularia

In the leaf inoculation experiment, it was confirmed that a resistance reaction was induced in the monogenic lines compared to that in the susceptible rice cultivar LTH. In a previous study, AvrPiz-t in Guy11 was not functional because a Pot3 insertion occurred in
the promoter region (Li et al., 2009). However, our Guy11 isolate in this study was confirmed by PCR to possess a Pot3 insertion and to express AvrPiz-t not only during appressorium formation on an artificial membrane but also during the infection process on rice leaves (Figure S10). Therefore, we treated the Piz-t-carrying LTH monogenic line with the Guy11 isolate as an avirulent strain.

**Figure 5** Microscopic observation of root cross-sections of rice, wheat, and barley inoculated with Pyricularia isolates. (a) Microscopy photographs of CO39 rice root sections inoculated with Guy11, Br48, or Dig4-1. Scale bars indicate 50 μm. The distribution ratio of invasive hyphae (IH) between the outer and inner layers (b), the average number of IH per infected root cell (c), and the distribution ratio of IH between intracellular and intercellular spaces (d) in rice roots inoculated with Guy11, GFS1-7-2, MZ5-1-6, Br48, or Dig4-1 isolates are shown. (e) Microscopy photographs of wheat and barley root sections inoculated with Guy11, Br48, or Dig4-1 isolates. Scale bars indicate 50 μm. The average number of IH per infected root cell of wheat (f) and barley (g) and the distribution ratio of IH between the intracellular and intercellular spaces of wheat (h) and barley (i) roots inoculated with Guy11, Br48, or Dig4-1 isolates are shown. Arrowheads in (a) and (e) indicate typical examples of IH. The bars represent standard deviations calculated from three biological replications. Different letters indicate significant differences between treatments (Tukey test, \( p < 0.05 \)). ns, no significant difference.
In contrast to leaf infection, the R genes in the monogenic lines had no obvious effect on resistance of rice roots to *P. oryzae*. In a previous study, R genes functioned not only in leaves but also in roots (Sesma & Osbourn, 2004). While we did not test the interaction between Avr1-CO30 and CO39 in rice as they did, we inoculated rice CO39 with *Setaria* pathotype GFSI1-7-2, which possesses Avr1-CO39 (Tosa et al., 2005); brown lesions were formed and mycelia invaded the roots. This result suggests that the resistance reaction between Avr1-CO39 and CO39 does not occur in roots. This difference may be explained by the inoculation method: in the previous study, mycelial plugs were placed under germinating seeds in vermiculite soil (Sesma & Osbourn, 2004), such that it is difficult for the mycelia to come into contact with the root as they grow. In the root inoculation experiment by Sesma and Osbourn (2004), infected hyphae were observed in the superficial cells of the roots in the section photographs of the resistant combination, and no inward extension was confirmed. Therefore, we prepared root sections and evaluated the degree of hyphal extension in root cells. We found no difference between the susceptible LTH cultivar and the resistant monogenic lines, that is, the hyphae extended to the inside of the cortical cells of the root. This indicated that the invasion of *P. oryzae* cannot be stopped by the R genes in the roots. Furthermore, we analyzed the pathogenicity of *Pyricularia* isolates in rice, barley, and wheat. In the leaf inoculation experiment, *Pyricularia* isolates belonging to different pathotypes exhibited genus-specific parasitism. However, we found that all *Pyricularia* isolates could infect roots of rice, barley, and wheat. This indicates that pathotype–genus-specific resistance against *Pyricularia* does not function in the roots of gramineous plants.

### 3.2 Plant defense responses to tissue-specific *Pyricularia* infection

In this study, we determined that host-specific resistance did not function in the roots, and the resistance reaction was not triggered. We evaluated R gene expression in the roots. Most R genes have been

**Figure 6** Relative expression levels of the *PR-1a* and *PR-1b* genes in the leaves and roots of rice cultivar CO39 inoculated with *Pyricularia* isolates at different stages as analysed by reverse transcription-quantitative PCR. Expression patterns of OsPR-1a in leaves (a) and roots (b) and of OsPR-1b in leaves (c) and roots (d). (a and c) Leaves at 2 days after inoculation (DAI), 4 DAI, and 6 DAI. White bars, control leaves (no inoculation); grey bars, leaves inoculated with Guy11; black bars, leaves inoculated with GFSI1-7-2; blue bars, leaves inoculated with MZ5-1-6; green bars, leaves inoculated with Br48; yellow bars, leaves inoculated with Dig4-1. (b and d) Roots at 3 DAI. Control is no treatment. The housekeeping gene OsActin was used as an internal standard. Relative gene expression was calculated using the comparative 2^ΔΔCt method. The bars represent standard deviations calculated from three biological replications. Different letters indicate significant differences between treatments (Tukey test, *p* < 0.05).
XIANG et al. reported that the expression of Pish was upregulated at 4 DAI. We also found that the expression of Pish and Pik-p in the roots was significantly increased by P. oryzae infection. These results suggest that Pish and Pik-p are induced by infection with P. oryzae, and the R genes appear to function in the roots as well as in the leaves.

Most PR genes (except OsLTP110) were induced in roots infected with P. oryzae, indicating that disease-resistant reactions were active. In the comparison of leaves at 0 DAI, the expression of most PR genes in LTH monogenic lines was higher than that in LTH; therefore, it was considered a factor that increased leaf resistance. It was notable that OsLTP110 was not induced by P. oryzae in the roots. In the control roots, the expression level of OsLTP110 was high; therefore, it does not explain why the roots were susceptible to any Pyricularia pathotypes.

Regarding pathotype-genus specificity, PR-1 genes (OsPR-1a, TaPR-1, and HvPR-1a) in all plant species tested were also induced after infection with Pyricularia isolates. Interestingly, the expression levels of TaPR-1 and HvPR-1a differed among inoculation with different isolates. These results imply that the recognition system of Pyricularia isolates in the roots may differ between rice, wheat, and barley. Based on the above results, PR genes were induced in both the leaves and roots. We also observed ROS generation at the infection site, suggesting that the resistance reaction is triggered. Nevertheless, induced resistance could not prevent the invasion of Pyricularia isolates, which succeeded in invading root cells and extending hyphae. This finding is thought to be due to the fact that the roots have fewer physical barriers (cuticle layer and wax layer) and are more susceptible to microorganism infection than the leaves. Therefore, it is considered that the spread of infection hyphae precedes the resistance reaction of the plant.

3.3 Infection strategies of Pyricularia isolates on roots

Root infections by P. oryzae have been reported to cause browning (Dufresne & Osbourn, 2001). In this study, we discovered that the isolates caused various degrees of disease in the roots, as indicated by the browning intensity, growth inhibition, and branching inhibition. These differences in infection modes may be reflected in the PR gene expression level such as OsPR-1b. These findings suggest that some Pyricularia isolates have evolved their characteristics in
terms of pathogenicity. In rice roots, the infection hyphae of Guy11 were adept at expanding into the intercellular space. This is similar to the intercellular hyphae of arbuscular mycorrhizal or endophytic fungi (Paszkowski et al., 2002; Tanaka et al., 2006). Additionally, the infection hyphae of isolates Br48 and Dig4-1 had a unique distribution, that is, the infection hyphae accumulated abundantly in root cells and clustered in the outer layer of sclerenchymatous cells. This was also similar to the discovery that some ericoid mycorrhizal fungi infect roots (Bergero et al., 2000; Perotto et al., 2002). Such differences in the localization of infection hyphae in rice roots were not observed in barley and wheat roots. Rice roots have a well-developed sclerenchymatous layer, whereas barley and wheat roots do not. It has been reported that exodermis and sclerenchymatous cells were observed only at the root–shoot junction in wheat (Ouyang et al., 2020), and barley does not form an exodermis in hydropic cells (Ranathunge et al., 2017).

FIGURE 8 Culture filtrates of Pyricularia isolates Br48 and Dig4-1 cause root inhibition. (a) Diagram of the extraction method of the culture filtrates. The roots of CO39 rice were inoculated with mycelial plugs of Guy11, Br48, or Dig4-1 in sterile distilled water (SDW). Three days after inoculation (invasion state), the cultures were filtered and used as culture filtrates. (b) Disease symptoms after culture filtrate inoculation. (c) The root growth length ratio of CO39 in the control compared to culture filtrate inoculation groups. Control is rice roots inoculated with a filter paper soaked in SDW. The bars represent standard deviations calculated from three biological replications. Different letters indicate significant differences between treatments (Tukey test, p < 0.05).

In our preliminary inoculation tests with filter membranes and culture filtrates, the production of secondary metabolites by Pyricularia isolates might have also played a role in root growth and crown root branching inhibition. Browning and root growth/branching inhibition were independent reactions. This result suggests that direct contact with, or invasion of, root cells is essential for browning.

Root growth occurs at the root apical meristem. Inoculation of any root part caused root growth inhibition, suggesting that these secondary metabolites inhibited the growth of distant root apical meristems. Pyricularasin H, a cytochalasan, is a pathogenicity factor in P. grisea that reduces resistance to avirulent Pyricularia isolates in crabgrass and inhibits root growth in barley (Meepagala et al., 2019; Tsurushima et al., 2005). Although pyrichalasin H is a candidate root growth inhibitor in P. grisea Dig4-1, P. oryzae Br48 has not been reported to produce this cytochalasan (Tsurushima et al., 2005). Multiple factors related to these substances are thought to be involved in the inhibition of root growth.

Dig4-1 inhibits crown root branching in rice. Crown root branching occurs in the crown, a meristem on the lowest node of the stem. Interestingly, isolate Dig4-1 affected both the root tip and crown. Crown root branching in rice is regulated by auxin and cytokinin signalling (Kitomi et al., 2011). The auxin pathway may be perturbed by infection with the Dig4-1 isolate.

These results suggest that secondary metabolites may have different effects on underground plant parts. These results indicate that the infection strategies of Pyricularia spp. in plant roots may be complex and diverse.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant materials

Seeds were placed in culture dishes (with filter paper), soaked in water, and left overnight for germination. Rice (cvs LTH and CO39) and LTH monogenic lines (Tsunematsu et al., 2000) were grown in seedling soil (King soil; Kasanen Industrial Co., Ltd.) for 12–14 days in a plant growth chamber at 26°C. Fertilization by adding 0.015% (NH4)2SO4 was performed 7 days after sowing. Wheat (cv. Norin 4) and barley (cv. Nigrate) were grown in vermiculite for 7 days in a plant growth chamber at 23°C. The artificial light/dark cycle in the plant growth chamber was 12/12 h.

Seeds were placed on a filter paper in culture dishes and grown for 3–4 days. Roots that were elongated to 2–3 cm were used in the experiments.
4.2 | Inoculation methods

The isolates used in this study were Guy11 (Oryza pathotype), GFSI7-2 (Setaria pathotype), MZ5-1-6 (Eleusine pathotype), Br48 (Triticum pathotype), and DiG4-1 (Digitaria pathotype).

4.2.1 | Leaf

The Pyricularia isolates were grown on oatmeal agar for 7 days (25°C), after which the aerial hyphae were removed and placed under black light blue (BLB) lamp for 5–7 days to form spores. SDW was then added to the spore-producing medium, and the fungal surfaces were rubbed with a spatula. The spore suspension was filtered using a Kimwipe S-200 (Cresia Corp.). The plant leaves were spray-inoculated with spore suspension (10^5 spores/ml supplemented with 0.01% Tween-20), and the inoculated plants were incubated under humid conditions in the dark for 24 h and then moved to the plant growth chamber.

4.2.2 | Root

The Pyricularia isolates were grown on potato dextrose agar (PDA) for 7 days (25°C), and then the roots of the plants were directly inoculated with mycelial plugs (approximately 1 cm^2). The inoculated roots were incubated in a filter paper in culture dishes (9 cm diameter), which were maintained under moist conditions supplemented with a 0.22-μm pore filter membrane.

4.2.3 | Inoculation of roots with culture filtrates

Mycelia of Pyricularia isolates (grown on PDA) were placed at the bottom of a 300-ml conical flask containing 50 ml SDW. Next, three to five rice roots were placed on the mycelia. After 3 days of incubation, the aqueous solution was collected and filtered through a 0.22-μm pore filter. A sterilized filter paper (1 cm^2) was placed over the root, and 100 μl of the culture filtrates was dripped over the filter paper to moisten it.

4.3 | Root section observation

The inoculated roots were cut into 5-mm pieces and fixed with 2.5% glutaraldehyde (Nisshin EM) in 0.1 M phosphate buffer (pH 7.4) at 25°C for 1 h. The pieces were rinsed with phosphate buffer three times at 10-min intervals and then fixed with 1% buffered osmium tetroxide (Nisshin EM) at 25°C for 1 h. The samples were briefly rinsed with SDW and then immediately dehydrated in an ethanol series (70%, 90%, and three times 100%). The samples were immersed in an intermediate solvent, propylene oxide (Nisshin EM), for 10 min and in a mixture (1:1, vol/vol) of propylene oxide and Spurr’s resin (Polysciences) for 6 h at 25°C and then placed in pure Spurr’s resin at 4°C for 3 days. After that, the specimens were embedded in a plain embedding plate and polymerized at 70°C for 24 h.

The root blocks were cut using a Porter-Blum MT-1 ultramicrotome (Ivan Sorvall) and a diamond knife (Diatome) into approximately 300-nm-thick sections. Each section was then transferred to a water droplet on a glass slide using a platinum loop. The water on the glass slide was evaporated on a hot plate maintained at approximately 200°C, and toluidine blue (0.5% toluidine blue, 0.5% borax) stain was added. The sample was heated again for 30 s and then washed with SDW to remove excess stain. Finally, the glass slides were observed under a light microscope (Biorevo BZ-9000; Keyence).

4.4 | Gene expression analysis

Frozen leaf or root samples were ground using a mortar and pestle with liquid nitrogen to obtain a powder. The powders were suspended in Sepasol-RNA I Super G (Nacalai Tesque Inc.), and total RNA was extracted according to the manufacturer’s instructions. The extracted total RNA was stored at −80°C.

One microgram of the total RNA was reverse-transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo) to obtain cDNA. The reaction mixtures were incubated at 37°C for 15 min, 50°C for 5 min, and 98°C for 5 min, and finally maintained at −20°C.

Quantitative PCR (qPCR) analysis was conducted using KOD SYBR qPCR Mix (Toyobo) (20 μl reaction volume containing 0.2 μM of each primer and 1 μl of cDNA) on a thermal cycler Dice Real-Time System model TP850 (TaKaRa). The primers used in this study are listed in Table S1. The primers were designed using Primer3plus (v. 2.4.2; [https://primer3plus.com/cgi-bin/dev/primer3plus.cgi](https://primer3plus.com/cgi-bin/dev/primer3plus.cgi)) and verified using Primer-BLAST in NCBI. The target gene information involved in this study was provided by the NCBI search database ([https://www.ncbi.nlm.nih.gov/](https://www.ncbi.nlm.nih.gov/)). Housekeeping genes for rice (OsActin: Os11g0163100) (Kano et al., 2010), wheat (TaCDCP: Ta54227) (Takumi et al., 2017), and barley ( HvActin: AY145451.1) were used as internal standards. Relative expression levels were calculated using the 2^−ΔΔCT method. Three biological replicates were analysed independently.

4.5 | Analysis of ROS generation in roots

Rice roots were cut into 1–2 cm lengths and dipped in 1 mg/ml DAB dissolved in 50 mM Tris-HCl (pH 7.5). The specimens were decompressed using a vacuum pump and the reagent was infiltrated into the roots and incubated for 2h. The specimens of whole roots and cross-sections were observed under a light microscope (Biorevo BZ-9000).
4.6 Disease evaluation

In the leaf inoculation experiment, the disease index was determined as follows: 5 (disease symptoms appeared on the entire leaf at 6 DAI), 4 (70% lesion area of index 5), 3 (50% of index 5), 2 (30% of index 5), 1 (10% of index 5, including resistant brown lesions), and 0 (no symptoms).

Disease symptoms on the roots were transformed into data for evaluation, which were divided into colour index, root branching number, and root growth ratio. Three independent biological replicates were used for each experiment.

For objective inoculation, we used the pixel intensity to digitize the degree of browning. Photographs of the roots were converted to monochromatic images and then inverted, so the browning part of the root was lighter and the healthy part of the root was darker. We used a colour sampler tool to read the greyscale of the browning parts (G_{b}) and normal parts (G_{n}) and then determined the browning degree by calculating \( \frac{G_{b}}{G_{n}} \). The number of crown root branches was defined as the number of crown roots at 3 DAI minus that at 0 DAI:

\[
\text{Growth} = \text{root length (3 DAI)} - \text{root length (0 DAI)}.
\]

The root growth ratio was calculated by dividing the root growth in the fungal treatment group (Growth_{f}) by that in the control group (Growth_{c}):

\[
\text{Root growth ratio} = \frac{\text{Growth}_{f}}{\text{Growth}_{c}}.
\]

4.7 Data analysis

Data from each experiment were analysed by one-way analysis of variance using IBM SPSS (v. 28.0). Multiple comparisons were performed using Tukey's test (p < 0.05).

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DATA AVAILABILITY STATEMENT

The data that support the finding of this study are available from the corresponding author upon reasonable request.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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