Effects of Colonization of a Bacterial Endophyte, Azospirillum sp. B510, on Disease Resistance in Rice

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Agriculturally important grasses contain numerous diazotrophic bacteria, the interactions of which are speculated to have some other benefits to the host plants. In this study, we analyzed the effects of a bacterial endophyte, Azospirillum sp. B510, on disease resistance in host rice plants. Rice plants (Oryza sativa cv. Nipponbare) were inoculated with B510 to enhance resistance against diseases caused by the virulent rice blast fungus Magnaporthe oryzae and by the virulent bacterial pathogen Xanthomonas oryzae. In the rice plants, neither salicylic acid (SA) accumulation nor expression of pathogenesis-related (PR) genes was induced by interaction with this bacterium, except for slight induction of PBZ1. These results indicate the possibility that strain B510 is able to induce disease resistance in rice by activating a novel type of resistance mechanism independent of SA-mediated defense signaling.

Key words: endophyte; Azospirillum; disease resistance; rice; pathogenesis-related genes

Since diseases caused by microorganisms are inevitable and constitute a serious stress to plants, plants have evolved a unique self-protection system to survive. Responses to pathogenic attack are complex and involve the activation of a large number of genes encoding diverse proteins, many of which are believed to function in defense. The primary response in this self-protection system involves specific pathogen recognition and rapid induction of localized host cell death.1) The secondary response is to develop induced resistance to protect the plant’s body from further attack by the pathogen.2) As a form of induced resistance activated by pathogens, systemic acquired resistance induced through the salicylic acid (SA)-mediated signaling pathway has been well characterized in rice as well as in Arabidopsis.3,4) Recent studies have indicated that nonpathogenic microorganisms such as actinobacterium and rhizobium enhance defense signaling in plants. Wheat-derived Streptomyces sp. strain EN27, possessing antimicrobial activity against wheat fungal pathogens in vitro and in planta, can activate defense signaling pathways in Arabidopsis against Erwinia carotovora sub sp. carotovora.5) One kind of plant growth-promoting rhizobacteria (PGPR), Bradyrhizobium sp. strain ORS278, not only promoted the growth of Arabidopsis and rice but also enhanced disease resistance in Arabidopsis.6) Pyocyanin secreted by Pseudomonas aeruginosa 7NSK2 induced resistance in rice against rice blast disease caused by Magnaporthe oryzae, but enhanced susceptibility to sheath blight caused by Rhizoctonia solani.7) Among these forms of induced systemic resistance (ISR) due to nonpathogenic microorganisms, resistance induced by non-pathogenic rhizobacteria Pseudomonas fluorescense WCS417r has been well studied. It is effective in Arabidopsis, radish, tomato, bean, and carnation.8–11) Recently, in rice ISR induced by Serratia plymuthica IC1270 was identified, but the detailed mechanism including activation of defense-related genes remained to be clarified.12)

Azospirillum, free-living nitrogen fixing rhizobacteria found in close association with plant roots and stems, is a well-studied genus of plant growth promoting bacteria (PGPB) that have beneficial effects on plants, such as increasing crop yield.13,14) A recent study indicated that tomato plants inoculated with Azospirillum brasilense showed enhanced disease resistance against a virulent bacterial pathogen, Pseudomonas syringae pv. tomato.15)

Azospirillum sp. B510 was isolated from stems of O. sativa cv. Nipponbare. It was motile and showed pectinase and cellulase activities.14) Although the nitrogen fixation activity of Azospirillum sp. B510 was detected in vitro, the nitrogen contribution of this strain to plant growth was very low (less than 1%) (Isawa et al., unpublished data). Thus, a question arises as to the significance of the existence of non-diazotrophic bacteria inside plants. To clarify the significance of endophytic bacterial colonization of rice, we analyzed the effects of a bacterial endophyte, Azospirillum sp. B510, on the

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Abbreviations: ISR, induced systemic resistance; PGPB, plant growth promoting bacteria; PR, pathogenesis related; SA, salicylic acid; SAR, systemic acquired resistance
development of rice blast disease caused by *Magnaporthe oryzae* and of rice bacterial blight disease caused by the *Xanthomonas oryzae* pv. *oryzae*.

**Materials and Methods**

*Plant growth conditions and endophyte inoculation.* Rice (*Oryza sativa* cv. Nipponbare) was grown in sterilized potting soil (Kureha, Tokyo) in plastic pots (2.5 cm × 2.5 cm × 4 cm) in a greenhouse at 25 °C during the day and at 19 °C during the night. *Azospirillum* sp. strain B510 was cultured in nutrient broth (Eiken Chemical, Tokyo) at 28 °C for 30 h, and a bacterial suspension was prepared in 10 mM MgCl2, 1 × 108 colony forming units (CFU)/ml. Two-week-old seedlings were transferred to a 50-ml polyethylene conical tube containing 20 ml of 0.5% agricultural fertilizer (Chayoda, Nissan Chemical, Tokyo), followed by treatment with the bacterial suspension by soil drenching. The endophyte-inoculated rice plants were cultivated continuously under the same conditions for 10 d before analysis.

*Construction of gus-tagged strain B510.* To investigate endophytic colonization in rice, mTNssgusA20(24) was used to label *Azospirillum* sp. strain B510. *E. coli* strain S17-1(17) harboring a plasmid containing mTNssgusA20 was used for conjugative transfer of the gus gene, encoding β-glucuronidase, to strain B510. Cell suspensions of *Azospirillum* sp. B510 and *E. coli* strain S17-1 were mixed and incubated on nutrient agar plates for 12 h at 30 °C. The cells were suspended in nutrient broth, and the transconjugant, gus-tagged *Azospirillum* sp. B510, was selected on nutrient agar containing streptomycin, spectinomycin, and polymyxin B (50 mg/ml each).

*GUS staining.* Plant samples were surface-sterilized with 70% ethanol, followed by rinsing with sterilized distilled water. GUS staining was performed as previously described.18)

*Rice pathogen infection assay.* In the rice blast assay, 5-leaf stage plants were sprayed with *M. oryzae* conidia suspension (1 × 106 spores/ml), kept under dark conditions at 100% humidity for 20 h, and then incubated for 4 d in a greenhouse at 25 °C. Necrotic spreading lesions appearing on the 5th leaves were counted. The protective value (PV) was calculated by the following equation: PV (%) = (Average lesion number of non-treated plants — average lesion number of treated plants)/average lesion number of non-treated plants × 100.

In the rice bacterial blight assay, plants were cut about 4 cm from the tip of the 5th leaf, sprayed with a cell suspension (105 CFU/ml) of *X. oryzae* pv. *oryzae*, and kept in a greenhouse. Lesion length was measured 14 d after inoculation.

*Measurement of salicylic acid level.* Fifth leaf tissues (approximately 0.5 g fresh weight) frozen in liquid nitrogen were ground with 10 ml of 90% methanol and then with 10 ml of 100% methanol. These two extracts were combined, and 2 ml of the resulting extract was dried at 40 °C. The SA and SAG (free SA and SA-glucoside) content was measured as previously described.19)

*Real-time PCR.* Total RNA was extracted using Sepasol-RNA I super reagent (Nacalai Tesque, Kyoto) and treated with DNAse I (Invitrogen, Carlsbad, CA), followed by phenol chloroform mixture purification, according to the manufacturer’s instructions. Total RNA (0.5 μg) was converted into cDNA with a PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan), according to the manufacturer’s instructions, yielding 10 μl of cDNA solution. Real-time PCR was performed using the GeneAmp SDS 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara Bio). The PCR reaction contained 1 × SYBR Premix Ex Taq, 0.2 μM of each primer, and the appropriate dilution of cDNA in a final volume of 24 μl. The following PCR program was used: initial denaturation at 94 °C for 10 s, then 40 cycles of 94 °C for 5 s and of 60 °C for 34 s. The expression level of each sample was normalized to ubiquitin (UBQ). The gene-specific primer pairs used for OsPR-4 were TGGGATCT-GGACTACGAGACC and GCAAGAGGCATAGCAACAT. The primer pairs previously described were used for other genes in the real-time PCR.4,20,21)

**Results**

*Endophytic colonization of Azospirillum sp. B510.* To examine the endophytic behavior of *Azospirillum* sp. B510, the gus-tagged isolate was inoculated on germinated seedlings of *O. sativa* cv. Nipponbare. A GUS assay of the plant tissues after surface-sterilization revealed blue coloration with X-Gluc, suggesting entry into and colonization of the gus-tagged isolate in the stem (Fig. 1). No blue color appeared in the non-inoculated control rice seedlings (data not shown). Re-isolation of the gus-tagged isolate confirmed colonization in the stems and roots but not in the leaves of the inoculated rice plants, whereas the bacterium was not obtained from non-inoculated control plants (data not shown). This indicates that *Azospirillum* sp. B510 entered and colonized the stems and roots, whereas the population in the roots was not high enough to be detected by GUS staining.

*Induction of disease resistance in rice by Azospirillum sp. B510.* Rice blast disease, caused by the hemibiotrophic fungus *Magnaporthe oryzae*, is one of the most economically important diseases. It must be controlled in Asian countries. Various varieties of resistant cultivars created through classic breeding methods have resulted in the appearance of many races classified by pathogenicity to cultivars. *O. sativa* cv. Nipponbare is compatible with *M. oryzae* race 007, and the interaction produces spreading necrotic lesions. We used this model to assess the effects of bacterial endophyte *Azospirillum* sp. B510 on disease resistance in rice plants. The leaves of plants treated with 1 × 108 CFU/ml of *Azospirillum* sp. B510 exhibited sparse lesions, in contrast to the many spreading lesions on the leaves of the control plants (Fig. 2A). Treatment with 1 × 106 CFU/ml of *Azospirillum* sp. B510 cells resulted in over 50% protection from pathogen infection at 5 d after treatment (Fig. 2B), without any visible morphological changes in the leaf blades due to *Azospirillum* treatment (data not shown). Treatment with 1 × 106 CFU/ml *Azospirillum* required a longer period to induce the same level of disease resistance (Fig. 2B). No defense-inducing activity was detected when plants were treated with boiled bacterial cells (data not shown). The culture broth of this bacterium did not exhibit any antimicrobial activity against *M. oryzae* (data not shown). These results suggest that colonization of a certain number of living cells of *Azospirillum* sp. B510 is required for induction of disease resistance in rice plants.

Next, the effect of *Azospirillum* sp. B510 treatment on rice bacterial blight disease, caused by *X. oryzae* pv. *oryzae*, was tested. Treating *O. sativa* cv. Nipponbare plants with an *Azospirillum* sp. B510 bacterial suspension (1 × 108 CFU/ml) by soil drenching reduced the disease symptoms caused by infection with the virulent pathogen *X. oryzae* pv. *oryzae* race 003. By 14 d post-infection of *X. oryzae*, the infected leaves of the control plants exhibited severe bleaching, however, those of *Azospirillum*-treated plants showed bleaching in smaller
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areas (Fig. 3). The culture broth of this bacterium did not exhibit any antimicrobial activity against X. oryzae (data not shown). Thus Azospirillum sp. B510-induced resistance in rice plants was effective not only against fungal disease but also against bacterial disease, indicating that the plant-endophyte interaction activated the innate immune systems of the host plants.

Expression of defense-related genes in Azospirillum-colonized plants

It has been reported that the expression of a set of defense-related genes is induced during the development and maintenance of induced resistance.4 22) To determine the physiological changes in Azospirillum sp. B510-induced disease resistance, we analyzed the expression of pathogenesis-related genes (OsPR-1a, OsPR-1b, OsPR-2, OsPR-4, and PBZ1) and defense-related WRKY45 by quantitative real-time PCR (Fig. 4). All the genes tested were responsive to blast fungus infection. Among the SA-dependent signal transduction-related genes, OsPR-1a, OsPR-1b, and PBZ1 were expressed in Azospirillum-treated plants as compared with the water-treated control plants, whereas OsPR-1b and PBZ1 were not influenced by bacterial colonization. The Jasmonic acid responsive OsPR-4 gene was also slightly suppressed in Azospirillum-treated plants. Expression of OsPR-2 was not influenced by treatment of Azospirillum. Contrary to the other genes tested, the expression levels of PBZ1 in Azospirillum-treated plants were 2 times higher than in the control plants. These results suggest that colonization of the bacterial endophyte Azospirillum sp. B510 has at least partially negative effects on the expression of defense-related genes.

Salicylic acid levels in Azospirillum-colonized plants

A recent report indicated that SA dependent signaling is involved in the induced resistance of rice plants,4 but our gene expression analysis indicated that SA is not involved in this resistance. Furthermore, the suppression of OsPR-1a expression suggested downregulation of the SA-mediated signaling pathway by Azospirillum colonization (Fig. 4). To examine this possibility, the effect of bacterial colonization on endogenous SA accumulation was examined. The levels of free and total SA in the Azospirillum sp. B510-treated plants were not different from those in the non-treated control plants (Fig. 5).
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47% protection against rice blast disease. 7,12,23) Azospirillum IC1270 have been reported to exhibit 35 to 70% protection against bacterial blight disease. ISRs induced by endophyte colonization has no effect on the SA-dependent signaling pathway, but suppresses OsPR-1a expression by unknown mechanism.

**Discussion**

In this study, we found that colonization of the bacterial endophyte Azospirillum sp. B510 induced disease resistance in rice plants against rice blast disease and bacterial blight disease. ISRs induced by P. fluorescens WCS374r, P. aeruginosa 7NSK2, and Serratia plymuthica IC1270 have been reported to exhibit 35 to 47% protection against rice blast disease. 7,12,23) As shown in Fig. 2, endophytic colonization of rice by Azospirillum sp. B510 induced similar levels of protection against rice blast disease. This suggests that rice ISR induced by nonpathogenic microorganisms is moderate compared to SAR induced by pathogens. Recent study has indicated that A. brasilense Sp245 produces phenylacetic acid, an indole-3-acetic acid (IAA)-like molecule with antimicrobial activity, using the key enzyme for IAA biosynthesis. 24) However, from Azospirillum, a PGPB, apart from some bacteriocins and siderophores, no other antimicrobial substance has not been identified to date. 25-27) On the other hand, tomato plants treated with A. brasilense induced disease resistance against virulent bacterial pathogen, Pseudomonas syringae pv. tomato. 25) Azospirillum sp. B510 induced disease resistance in rice against rice blast and bacterial blight without the production of any antimicrobial substance against M. oryzae or X. oryzae, suggesting that biological interaction between rice cells and bacterial cells activates the innate immunity system of the host plant.

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Treatment with Azospirillum sp. B510 did not have a positive effect on the expression of the defense-related genes tested (Fig. 4), except for slight induction of PBZ1. It has been reported that infection of M. oryzae and treatment with probenazole (PBZ), an SAR inducer, induced expression of PBZ1, and that induced resistance by the SAR inducer accompanies the expression of defense-related genes such as OsPR-1a and OsPR-1b as well as PBZ1. 4,28) On the other hand, another form of induced resistance, brassinosteroid-mediated disease resistance (BDR), has been identified in tobacco and rice. 29) BDR does not accompany the induction of PR genes in tobacco or of PBZ1 in rice. P. fluorescens WCS347r produced pseudobacin-type siderophores and induced ISR in rice, in which PBZ1 and PR-1b were not induced. The expression of OsPR-1a was suppressed by the colonization with Azospirillum sp. B510, which phenomenon has not been observed in the development of other induced resistances. Thus, the different expression patterns of defense-related genes from other known induced resistances suggests the possibility that colonization with Azospirillum sp. B510 induces a novel type of induced resistance, although the detailed mechanism remains to be clarified. We speculate that priming of the defense response by bacterial colonization plays an important role in resistance against pathogens, as observed for other induced resistances. Since most plants contain a variety of endophytic bacteria, 30) this kind of resistance mechanism may be a common immune system among plants. To determine the importance of plant-endophyte interactions, clarification of the induction mechanism and the priming effect of bacterial endophyte-mediated induced resistance is important, and is under investigation.

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