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SHORT COMMUNICATION

Involvement of an Efflux Transporter in Prochloraz Resistance of *Fusarium fujikuroi* CF245 Causing Rice Bakanae Disease

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Abstract Rice bakanae disease pathogens resistant to conazole fungicide prochloraz have been reported in Korea. Understanding fungal response associated with the resistant is required for successful control of the disease. Investigation of prochloraz-resistant responses of the rice bakanae disease pathogen, *Fusarium fujikuroi*, showed significant growth inhibition of the resistant strain after sodium azide treatment suggested involvement of the ABC transporter in fungal prochloraz-resistant responses. Prochloraz degradation was accompanied by fungal growth, dependently on sodium azide treatment. Partial sequence analysis of the ABC transporter gene confirmed that the resistant strain possesses an efflux transporter with high sequence similarity to genes of conazole fungicide-resistant pathogens. These results suggest that the prochloraz-resistant responses of *F. fujikuroi* CF245 involve an efflux transporter.

Keywords fungicide resistance · plant pathogen · prochloraz · rice bakanae disease

Fusarium fujikuroi is a plant pathogen that causes rice bakanae disease. Disease symptoms of rice seeds infected by the pathogen include leave chlorosis, shoot overgrowth, and inhibition of seed germination. This disease has become a major agricultural problem in Korea, because it causes a significant reduction in rice productivity (Kim, 2000). The disease is not detectable at the seedling stage, but after transplantation becomes widespread and cannot be controlled effectively (Kim, 1981). Thus, successful control of the disease could be achieved at the seedling stage. One of the general methods to control the disease is disinfection of rice seeds by soaking them in fungicide solution for a few days (Park et al., 2003). Prochloraz (*N*-propyl-*N*-[2-(2,4,6-trichlorophenoxy) ethyl] imidazole-1-carboxamide) is a typical conazole fungicide that has been used to control rice bakanae disease in Korea since 1983. Recently, a number of cases of rice bakanae pathogens resistant to prochloraz have been reported in rice fields (Shin et al., 2008; Lee et al., 2010). Thus, understanding the resistant response of the fungal pathogen to prochloraz is required for successful control of the disease.

Rice bakanae disease pathogens examined were *F. fujikuroi* CF106 (CF106) and *F. fujikuroi* CF245 (CF245), as the sensitive and the resistant strains to prochloraz, respectively. They were kindly provided by the National Academy of Agricultural Science, Rural Development Administration of Korea. To investigate the effects of an electron transport chain inhibitor on fungal growth, the pathogens were grown for 4 days in triplicate flasks containing 100 mL of potato dextrose broth (PDB) medium supplemented with prochloraz or sodium azide or both chemicals. Sodium azide is an electron transport chain inhibitor generally used for microbial transport studies (Parkinson et al., 1995; Albertson et al., 1996). Prochloraz was added at levels of 0.05 mg/L for CF106 and 0.05 or 2.0 mg/L for CF245. Sodium azide was treated at a concentration of 10 μM, at which fungal growth was similar to the controls grown in PDB without prochloraz. The effects of sodium azide on fungal growth are presented in Table 1. Relative to the controls incubated without prochloraz, CF106 and CF245 showed

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Table 1 Growth of fungal pathogens after incubated with prochloraz, sodium azide or prochloraz plus sodium azide

Strain	Fungal growth after treatment (%)[a]				
	Prochloraz (mg/L)		Sodium azide	Sodium azide + Prochloraz (mg/L)	
	0.05	2.0		0.05	2.0
CF106	99.2±1.7	No growth	84.2±7.5	105.3±3.8	No growth
CF245	93.8±1.3	94.2±4.2	92.5±3.5	85.4±7.1	No growth

[a]The values are means ± SD of triplicate experiments. Growth of fungal pathogens was calculated based on the dry weight, relatively to the control pathogens incubated without the chemicals.

Table 2 Prochloraz degradation by fungal pathogen after incubated with or without sodium azide

Strain	Prochloraz degradation (%)[a]	
	without sodium azide	With sodium azide
CF245	65.3±5.9	12.2±8.0

[a]The values are means ± SD of triplicate experiments.

approximately 99.2 and 93.8% growth, respectively, when incubated with 0.05 mg/L prochloraz. Growth of CF106 and CF245 decreased to about 84.2 and 92.5%, respectively, when incubated with sodium azide. In the presence of sodium azide, the growth values of CF106 and CF245 were approximately 105.3 and 85.4%, respectively, when incubated with 0.05 mg/L prochloraz. These observations suggest that sodium azide did not affect the fungal growth when incubated with 0.05 mg/L prochloraz. Growth of strain CF245 was about 94.2% relative to the controls when incubated with 2.0 mg/L prochloraz, whereas no growth of strain CF106 was observed. The growth of strain CF245 was significantly inhibited when incubated with 2.0 mg/L prochloraz in the presence of sodium azide. These results suggested the involvement of an efflux transporter system to allow the resistant strain CF245 to grow in the presence of prochloraz at 2.0 mg/L.

During fungal growth, prochloraz degradation was investigated in fungal cultures grown in triplicate flasks containing 100 mL of PDB medium supplemented with 2.0 mg/L prochloraz for 4 day. The cultures were centrifuged and filtered with 2 μm-sized membrane filters. The filtrates were subjected to quadruple time-of-flight mass spectrometry (Q-TOF MS, Germany) analysis for detection of prochloraz remaining in the cultures. Mass spectrometry analysis detected approximately 35% of treated prochloraz in the cultures after incubation with 2.0 mg/L prochloraz in the absence of sodium azide (Table 2), suggesting approximately 65% degradation of prochloraz during the growth. Approximately 12% degradation of prochloraz was observed after incubation in the presence of sodium azide. Sodium azide treatment revealed a correlation between prochloraz degradation and fungal growth. Significant growth inhibition of resistant CF245 in the presence of sodium azide and 2.0 mg/L prochloraz, could consequently result in low degradation of prochloraz (Tables 1 and 2).

To further investigate whether the resistant strain possesses an efflux transporter, ABC transporter genes were investigated by a modified method of previous study (Moslem et al., 2010). Genomic DNA of CF245 was isolated from fungal cultures grown in PDB medium described above and subjected to polymerase chain reaction (PCR). PCR was performed using MG96+Thermal Cycler (Longene Scientific Instruments Co., China) with a pair of primers. Primers were designed on the basis of the conserved sequences related to fungal ABC transporter from the GenBank database: forward 5'-TACGTTCAGCAACAGGATCT-3' and reverse 5'-CATCCATTCTGCAGGGTT-3'. PCR products were analyzed on 1% (w/v) agarose gel and purified with *AccuPrep* purification kit (Bioneer, Korea) following the manufacturer's instruction. The purified products were cloned into *Escherichia coli* JM109 with Promega *p*GEM-T Easy vector System, and the genes in the plasmid were sequenced by 3730XL DNA Analyzer (Applied Biosystems, USA). The sequences were compared to other ABC transporter genes available in the GenBank database. Partial sequence analyses of the cloned PCR products (500 bp) showed about 70–76% similarities to those of *PMR1*, *BcatrD*, *atr2*, and *atrE* (Fig. 1), the genes that contribute to ABC efflux transporter of other conazole fungicides (Nakaune et al., 1998; Hayashi et al., 2002). These observations indicated that the resistant strain possess an efflux pump related to transport of prochloraz. The transporter gene has been deposited in the GenBank database with accession number JQ346074.

The mechanisms associated with fungicide resistance can be demonstrated in terms of point mutations and modifications in the genes that encode fungicide target-enzymes (Délye et al., 1997; Leroux et al., 2007), reduced accumulation of fungicide in cells through ABC efflux transporter (Hayashi et al., 2002), and detoxification and degradation of fungicides, resulting in reduced levels in fungal cells (Kapteyn et al., 1992; Miguez et al., 2004). In the present study, the resistant responses of strain CF245 were determined by reducing accumulation of prochloraz in cells through an efflux transporter and degrading of the fungicide for detoxification, thus providing valuable insights for understanding the resistance mechanisms of rice bakanae pathogens against prochloraz.

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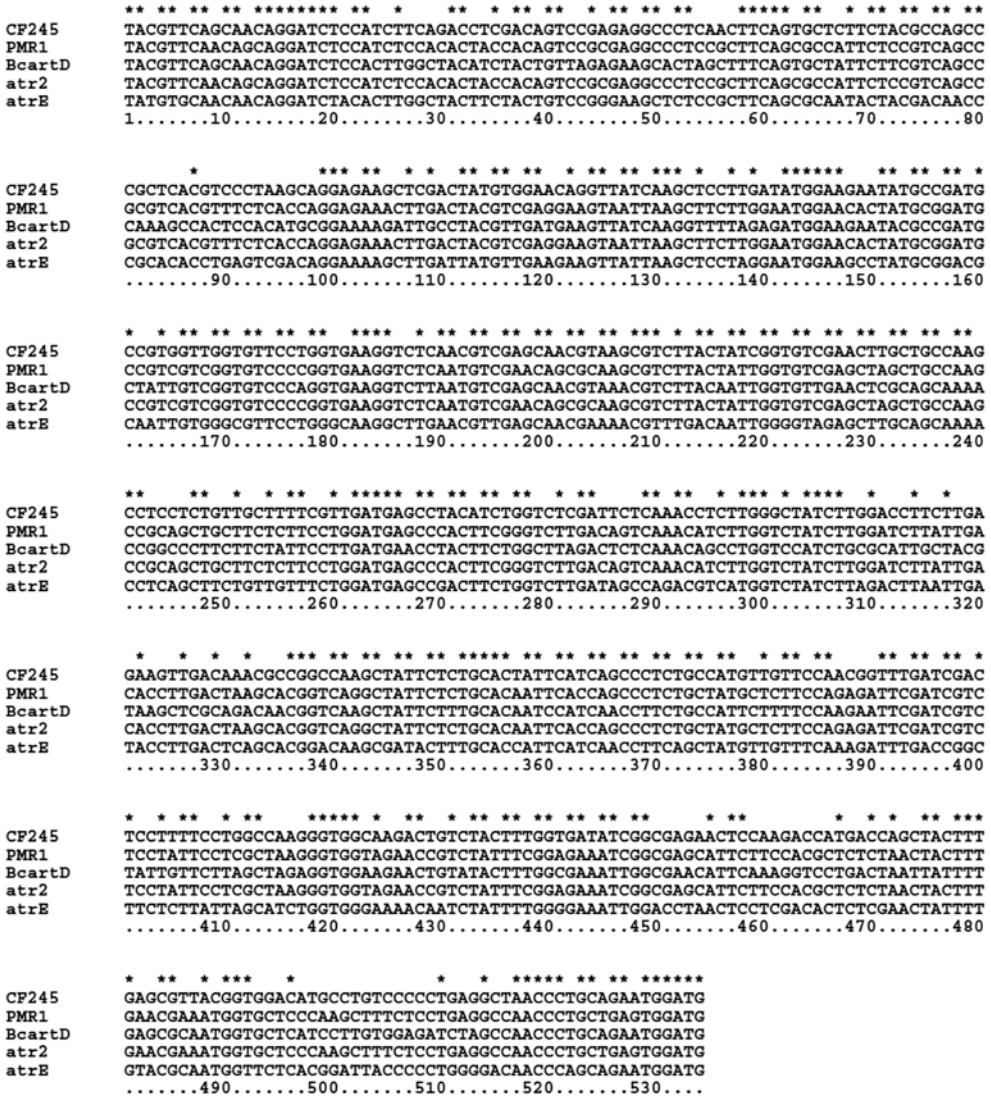

Fig. 1 Alignments of the ABC transporter nucleotide sequences of strain CF245 compared to other fungal ABC transporter genes involved in the efflux transport of conazole fungicides.

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