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Note

Induction of Resistance against Rice Bacterial Leaf Blight by 3-Chloro-1-methyl-1*H*-pyrazole-5-carboxylic Acid

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A pyrazole derivative, 3-chloro-1-methyl-1*H*-pyrazole-5-carboxylic acid (CMPA), exhibited high anti-rice blast activity without any significant antimicrobial activity. To assess the mode of action of CMPA, its effects on rice bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* and the expression of a defense-related gene were examined. The treatment of CMPA reduced the disease symptoms in a dose-dependent manner, but CMPA did not exhibit any direct antibacterial activity against *X. oryzae* at concentrations up to 1 mg/ml. The treatment of CMPA induced the expression of *PBZ1*, a defense-related gene, which is evoked by several plant activators. This ability to induce *PBZ1* expression and enhance disease resistance without antimicrobial activity suggests that CMPA can activate systemic acquired resistance in rice as well as in tobacco.

Keywords: systemic acquired resistance, disease resistance, rice, rice bacterial leaf blight, probenazole.

INTRODUCTION

We previously reported that a novel pyrazole derivative, 3-chloro-1-methyl-1*H*-pyrazole-5-carboxylic acid (CMPA), exhibited high anti-rice blast activity at 0.05 mg/pot *in vivo*.[1] However, CMPA did not have any significant effects on the hyphal growth, spore germination, and appressorium formation of *Pyricularia oryzae*.[1] These results suggest that the anti-rice blast activity of CMPA was caused by its effect on the rice plants and not on *P. oryzae*. On the other hand, CMPA induced the expression of pathogenesis related (*PR*) genes and a broad range of disease resistance without exhibiting antibacterial activity in tobacco, indicating that CMPA acts as an inducer of systemic acquired resistance (SAR) in tobacco[2] like probenazole (PBZ; 3-allyloxy-1,2-benz[*d*]isothiazole 1,1-dioxide),[3–5] acibenzolar-*S*-methyl (BTH; benzo[1,2,3]thiadiazole-7-carbothionic acid *S*-methyl ester),[6,7] *N*-cyanomethyl-2-chloroisonicotinamide (NCI),[8–10] and tiadinil (TDL).[11] In tobacco, CMPA does not require the accumulation of salicylic acid (SA) to induce SAR,[2] having a similar mode of action to BTH and NCI but not PBZ, however, the mechanism of SAR in rice plants, including the requirement of SA, remains to be clarified. In this paper, to elucidate in more detail the mode of action of CMPA in rice, we investigated the effect of CMPA on rice bacterial leaf blight. Furthermore, the expression of *PBZ1*,[12,13] a defense-related gene, in the CMPA-treated rice was examined.

MATERIALS AND METHODS

1. Chemicals

CMPA was synthesized in Chemical Research Laboratories of Nissan Chemical Industries, Ltd.[1] PBZ, BTH and NCI were obtained from Meiji Seika Kaisha, LTD, Syngenta Co., and Nippon Kayaku Co., Ltd, respectively.

2. Plant Materials and Bacterial Preparations

Rice seeds (*Oryza sativa* L. cv. Nipponbare) were sown on diluvial soil in pots (80 cm^3) and grown in a greenhouse at 25°C. *Xanthomonas oryzae* pv. *oryzae* race 003 was cultured on a YPDA (yeast extract 10 g, polypeptone 10 g, dextrose 20 g, agar 15 g/liter; pH 7.0) medium. The bacterial suspension was prepared in sterile distilled water to provide the proper density.

3. Rice Bacterial Leaf Blight Inoculation Assay

Rice seedlings at the 3-leaf stage were pretreated with CMPA at various concentrations. PBZ, BTH and NCI were used as reference compounds. Each chemical was applied as an emulsified solution with acetone using a soil-drenching method. Challenge inoculation with *X. oryzae* was performed 5 days after the pretreatment with chemicals. Plants were cut at about 4 cm from the tip of the 4th leaf, sprayed with a cell suspension (10^9 colony-forming units (CFU) ml^{-1}) of *X. oryzae*, and kept in a greenhouse under the following conditions: 24°C and 70% humidity for 10 hr without light; 29°C and 70% humidity for 14 hr with light. The length of the bleached part of infected leaves was measured 12 days after the inoculation.

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4. In vitro Activity of CMPA against X. oryzae

CMPA was dissolved in sterile distilled water to provide a concentration of 0.02–1 mg/ml and used for antimicrobial assays. The assay plate (9 cm in diameter) was prepared by overlaying YPDA medium (5 ml, 0.5% (w/v) agar) containing *ca*. 10^7 CFU of *X. oryzae* on YPDA medium (10 ml). A paper disk (8 mm in diameter) containing 10μl of CMPA solution was placed on the assay plate. Antibacterial activity was evaluated by measuring the diameter of the halo that appeared around the disk after incubation at 28°C for 48 hr.

5. PBZ1 Gene Expression in Rice Plants Treated with CMPA

Rice seedlings at the 3-leaf stage were pretreated with chemicals at various concentrations by the soil-drenching method, and the 4th leaves were harvested 5 days after application. Total RNA was extracted from frozen leaf tissue samples by using TRIzol reagent (Life Technologies, Rockville, MD, USA) following the manufacturer's instructions. ^{32}P-Labeled *PBZ1* cDNA probe was synthesized as previously described.[14] Total RNA samples were subjected to 1.2% agarose-1.1% formaldehyde gel electrophoresis and transferred to a nylon membrane (Hybond N^+, Amersham, Buckinghamshire, UK). After the transfer, RNA was cross-linked to the membrane using an UV linker (GS GENE LINKER, Bio-Rad, Hercules, CA, USA). Hybridization and washing were performed as described by Church and Gilbert (1984).[15] Prehybridization and hybridization were performed at 68°C for 1 hr or longer and 8 hr or longer, respectively. The membrane was washed twice with 2×SSC containing 0.1% SDS for 30 min at 68°C and then washed twice with 0.1×SSC containing 0.1% SDS for 15 min at 68°C The detection was performed with a BAS2500 image analyzer (Fujifilm).

RESULTS AND DISCUSSION

CMPA induced *PR* gene expression and a broad range of disease resistance without antibacterial activity in tobacco.[2] CMPA also exhibited an anti-rice blast effect without direct antifungal activity.[1] To determine whether CMPA induces disease resistance in rice and protects against various pathogens, the effect of the treatment on rice bacterial leaf blight was examined. Treating *O. sativa* cv. Nipponbare plants with CMPA (0.05, 0.5, 5 mg/pot) reduced disease symptoms caused by infection with the virulent pathogen *X. oryzae* pv. oryzae race 003. The effect of CMPA was dose-dependent, with 5 mg/pot resulting in about 99.5% protection from the pathogen (Fig. 1). CMPA exhibited a slightly higher level of protection against this disease than PBZ and BTH, which is similar to the protective efficacy against rice blast disease.[1] However, CMPA did not exhibit any direct antibacterial activity against *X. oryzae* at concentraions up to 1 mg/ml (data not shown). Thus, in addition to the anti-rice blast effect reported previously,[1] CMPA provided resistance against a disease caused by a bacterial pathogen, without showing direct antibacterial activity. These findings suggest that CMPA acts on the rice plants to induce resistance against diseases.

In tobacco, some PR proteins are coordinately expressed during the induction and maintenance of SAR.[16] CMPA induced the expression of *PR1*, *2* and *5* genes without the accumulation of SA in tobacco plants.[2] To determine whether CMPA induces the expression of defense-related genes in rice, the expression of *PBZ1*, which is induced by PBZ treatment, was examined. Northern blot analysis indicated that transcripts for *PBZ1* moderately accumulated in rice leaves treated with CMPA in a dose dependent manner as well as in PBZ-, BTH- or NCI-treated plants (Fig. 2). In contrast, no transcript for *PBZ1* was detected in the leaves of the water-treated control plants. The ability of CMPA to induce the *PBZ1* gene expression and enhance disease resistance in the absence of antimicrobial activity strongly supports that CMPA can activate a disease resistance system like systemic acquired resistance in rice plants. CMPA induced a lower level of accumulation of the *PBZ1* transcript than did PBZ or NCI (Fig. 2), whereas these chemicals exhibited the same level of induction of disease resistance (Fig. 1), suggesting that the mode

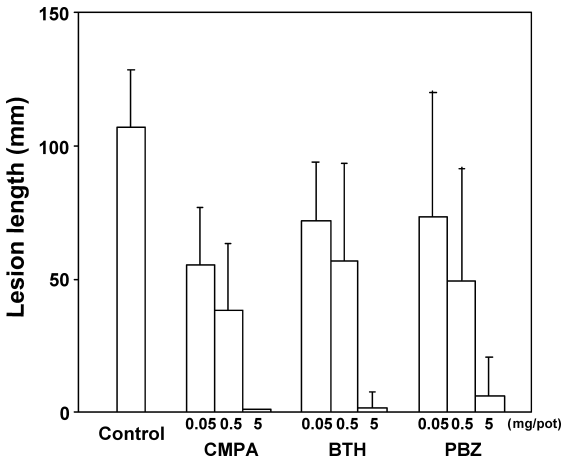

Fig. 1. Effect of CMPA treatment on rice bacterial leaf blight. Plants were treated with 0.05, 0.5 or 5 mg/pot CMPA by soil drench application 7 days prior to an inoculation with *Xanthomonas oryzae* pv. *oryzae*. BTH and PBZ were used as positive controls. Each experiment was performed with 3 pots, each containing 7 plants. The length of the lesion on the 4th leaf was measured 7 days after inoculation. Values are shown as the mean±SD. The experiment was repeated three times with similar results. Statistical analysis (ANOVA) indicates significant differences between the control and chemical-treated groups ($P<0.01$).

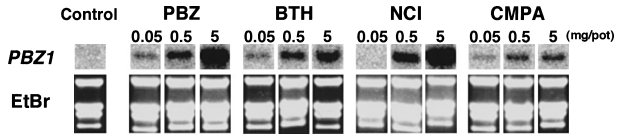

Fig. 2. Induction of *PBZ1* gene expression in rice plants by CMPA. Plants were treated with PBZ, BTH, NCI or CMPA (0.05, 0.5 or 5 mg/pot) or water (control) by soil drench application. The 4th leaves of rice plants in each pot were collected 5 days after treatment and used for RNA extraction. Each lane was loaded with 5 μg of total RNA. Equal loading was confirmed by ethidium bromide (EtBr) staining.

of action of CMPA is different from that of PBZ or NCI. This paper demonstrates that CMPA activates the innate immunity system of rice plants like PBZ. At present, the exact mode of action of CMPA to induce disease resistance in rice is unknown, because the mechanisms of rice SAR have not been determined yet. However, it was demonstrated in tobacco plants that CMPA activated the SAR signaling pathway by stimulating a point downstream of SA accumulation. Understanding the detailed mechanism of CMPA-induced disease resistance will help to clarify SAR in rice, which is under investigation.

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