Note

Tiadinil, a Novel Class of Activator of Systemic Acquired Resistance, Induces Defense Gene Expression and Disease Resistance in Tobacco

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Systemic acquired resistance (SAR) is a potent innate immunity system in plants and effective against a broad range of pathogens. Tiadinil (TDL), *N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide, was recently developed to control rice blast disease without direct antimicrobial activity. To clarify the mode of action of TDL, effects of TDL on tobacco plants were investigated. TDL induced resistance against tobacco mosaic virus and tobacco wildfire disease (*Pseudomonas syringae* pv. *tabaci*) without antimicrobial activity in tobacco plants. TDL also induced the expression of SAR marker genes. These results indicated that TDL induced SAR in tobacco plants.

Keywords: systemic acquired resistance (SAR), tiadinil, tobacco, plant activator, *Pseudomonas syringae*.

INTRODUCTION

Systemic acquired resistance (SAR) is a defense mechanism induced after a local hypersensitive response to pathogens and plays an important role in defending plants from further attacks by various kinds of pathogens.^{1,2)} The mechanism of induction of SAR has been well-characterized in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* and a set of pathogenesis-related (PR) genes have been identified as SAR marker genes.³⁻⁶⁾ Chemicals capable of inducing SAR in plants have been reported; they are salicylic acid (SA), 2,6-dichloroisonicotinic acid (INA), benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), 3-allyloxy-1,2-benz[*d*]isothiazole-1,1-dioxide (probenazole; PBZ) and *N*-cyanomethyl-2-chloroisonicotinamide (NCI) (Fig. 1).^{4,6-12)} These chemicals exhibit several essential criteria of SAR inducers: they induce a broad range of disease resistance;

their effects are not due to their antibiotic activities; and they induce an SAR molecular marker, *PR* gene expression.^{4,13)} Some of these chemicals have been used in the field to control diseases such as rice blast. Despite extensive studies on the mechanism of SAR over the years, many parts of the SAR signaling pathway still remain to be clarified; for example, no targets of these chemicals have been determined thus far.

Tiadinil (TDL), *N*-(3-chloro-4-methylphenyl)-4-methyl-1,2,3-thiadiazole-5-carboxamide (Fig. 1), was recently developed by Nihon Nohyaku Co., Ltd. and registered in 2003 with MAFF as a novel class of agent (V-GET[®]) to control rice blast disease. To clarify the mode of action of TDL, we investigated whether TDL induces SAR using a tobacco-pathogen model system. We here report evidences that TDL induces SAR in tobacco.

MATERIALS AND METHODS

1. Chemicals

TDL was a gift from Nihon Nohyaku Co., Ltd. PBZ and BTH were provided by Meiji Seika and Syngenta Japan, respectively.

2. Plant Materials and Treatment

Nicotiana tabacum cv. Xanthi nc was grown in sterilized potting soil (Kureha, Japan) in pots (6 cm diameter × 9 cm) inside a growth chamber under a 16 : 8 light : dark regimen, at 22°C, with 60% humidity. Treatment with various concentrations of chemicals or water was performed by soil drenching of 5-week-

Fig. 1. Chemical structures of SAR inducers and tiadinil. SA, salicylic acid; PBZ, probenazole; BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester; NCI, *N*-cyanomethyl-2-chloroisonicotinamide; INA, 2,6-dichloroisonicotinic acid; TDL, tiadinil.

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old plants with 5–6 developed leaves.

3. Tobacco Pathogen Infection Assays

In tobacco mosaic virus (TMV) infection assay, 5 days after treatment, a challenge inoculation with TMV was performed. The three upper developed leaves of treated plants were dusted with carborundum (Mesh 400; Maruto co., Japan) and mechanically inoculated with TMV suspended in 10 mM phosphate buffer, pH 7.0. The inoculated plants were incubated at 22°C and lesions were measured 5 days later.

A challenge inoculation with *Pseudomonas syringae* pv. *tabaci* (*Pst*), a pathogen of wildfire of tobacco, was performed 5 days after treatment. *Pst* was cultured in nutrient broth for 1 day at 28°C, and a bacterial suspension was prepared in 10 mM MgCl₂ [2×10^5 colony-forming units (CFU)/ml]. Challenge inoculation was performed by infiltration of the bacterial suspension by gently pressing a 1 ml syringe without a needle.¹⁴⁾ Leaf disks (6 mm in diameter) were taken from the infiltrated part of the leaves at 4 days post-inoculation and three disks from each plant were combined and homogenized in 200 μ l of 10 mM MgCl₂. The population size of *Pst* was estimated from growth on nutrient broth agar plates after dilution. Three plants were used for each experiment, and three samples were prepared from each plant.

4. RNA Analysis

Tobacco plants were treated with various concentrations of TDL or water by the soil drench method, and the leaves were harvested 5 days after treatment. Total RNA was extracted from frozen leaf samples using TRIzol reagent (Life Technologies, Rockville, MD, USA) following the manufacturer's instructions. DNA fragments of the coding regions for tobacco *PR* genes were prepared as previously described.^{9,10)} ³²P-labeled cDNA probes were synthesized by the random priming of these fragments of acidic *PR-1a*, *PR-2* and *PR-5* genes.⁴⁾ 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 a u. v. linker (GS GENE LINKER, Bio-Rad, Hercules, CA, USA). 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 $2 \times$ SSC containing 0.1% SDS for 30 min at 68°C and then twice with $0.1 \times$ SSC containing 0.1% SDS for 15 min at 68°C. The detection was performed with a BAS2500 image analyzer (Fujifilm).

RESULTS AND DISCUSSION

We used the tobacco plant to assess the ability of TDL to enhance resistance against infection by pathogens, because tobacco is a suitable model for estimating the capability of chemicals to induce disease resistance.^{4,15)}

N. tabacum cv. Xanthi nc possesses the *N* gene, which confers resistance to TMV,¹⁶⁾ consequently, its defense response to TMV infection results in a necrotic lesion. Since SA and SAR

activators enhance this resistance and reduce the size of lesions,^{2,4,9,10,15)} the effect of TDL on TMV infection was examined. Treatment with TDL reduced the average lesion size compared to water-treated control plants in a dose-dependent manner, indicating that TDL enhanced *N* gene-mediated disease resistance (Fig. 2AB). The numbers of TMV lesions appeared on the leaves were not significantly different between the TDL-treated and control plants.

Next, we assessed the effect of TDL on the interaction between tobacco plants and a virulent bacterial pathogen, *P. syringae* pv. *tabaci* (*Pst*). *N. tabacum* cv. Xanthi nc does not have a resistance gene specific to *Pst* and is susceptible to *Pst*. Susceptibility was estimated by measuring bacterial growth in leaf tissues after challenge inoculation. Treatment with TDL at 1 mg/pot by soil drenching reduced bacterial growth in the infected tissues relative to the water-treated control plants (Fig. 3B), resulting in a reduction in disease symptoms (Fig. 3A). TDL did not show any direct antimicrobial activity in liquid culture at concentrations of up to 1 mg/ml (Table 1). These results

Fig. 2. Induction of resistance in tobacco against TMV by TDL. Tobacco plants were treated with 1 or 2.5 mg/pot of TDL or water by the soil drench method, 5 days prior to TMV inoculation. (A) Photograph was taken 5 days post-inoculation. (B) Average size of lesions was measured 5 days after the TMV inoculation. Each experiment was performed with three plants, three leaves of which were inoculated with TMV. Values are shown as the mean $\pm$ SD. The experiment was repeated three times with similar results.

(A)

(B)

Fig. 3. Induction of resistance against bacterial pathogen by TDL.

Five-week-old tobacco plants were treated with water (control) or 1 mg/pot of TDL by the soil drench method 5 days prior to inoculation with pathogen (*Pst*). (A) Symptoms of tobacco wild-fire disease on tobacco leaves. The photograph was taken 7 days post-inoculation. Arrows indicate the inoculated areas. (B) Growth of *Pst* in tobacco leaf tissues. The population of *Pst* in the sample was estimated 4 days after inoculation by growth on nutrient broth agar plates. Each experiment was performed with three plants and three samples were prepared from each plant by homogenizing leaf disks in 10 mM MgCl₂.

indicated that TDL induced resistance in tobacco plants against *Pst*. Since the TDL-treated tobacco plants exhibited resistance against two different types of pathogens, we conclude that, in tobacco, TDL induces SAR-like disease resistance.

Some PR proteins coordinately expressed in tobacco leaves during the induction and maintenance of SAR are also expressed during SAR induced by chemical activators such as SA, PBZ and BTH.^{2,9)} The expression of acidic *PR-1* (*PR-1a*), encoding one of these PR proteins, is a useful molecular marker for SAR and its expression in TDL-treated plants was examined.

Table 1. Effect of TDL on growth of *P. syringae* pv. *tabaci*

TDR concentration (μg/ml)	Bacterial growth ^{a)} (% Cont.)
1000	98
400	97
200	103
100	100
10	100
5	98

^{a)} *P. syringae* pv. *tabaci* was precultured for 1 day and aliquots (40 μl) were inoculated to new nutrient broth cultures (2 ml each) containing TDL. The culture was performed with shaking for 8 hr at 28°C and the OD₆₀₀ was measured.

Fig. 4. Northern blot analysis of SAR marker gene expression. Plants were treated with water, 5 mg/pot of PBZ, 1 mg/pot of BTH, or 1 mg/pot of TDL by the soil drench method. Leaf samples were collected 5 days after treatment. Each lane was loaded with 4 μg of total RNA. rRNA was used as an internal control for gel loading and transfer.

Northern blot analysis indicated that the transcript for acidic *PR-1* accumulated in leaves of tobacco treated with TDL by soil drenching (Fig. 4). The accumulation of transcripts for acidic *PR-2* (β-1,3-glucanase) and *PR-5* (thaumatin-like) was also detected in the leaves of TDL-treated plants (Fig. 4). TDL (at approximately 0.15 mM in the pots) was more effective than PBZ (at approximately 0.9 mM in the pots) but less effective than BTH (at approximately 0.19 mM in the pots) in inducing the expression of *PR* genes (Fig. 4). By contrast, no transcript for *PR-1* and *PR-2* and a very small amount of transcript for *PR-5* were detected in the leaves of water-treated control plants (Fig. 4). These results indicate that TDL is effective in inducing defense gene expression in tobacco.

In this paper, we demonstrate that TDL induces SAR-like disease resistance in tobacco without antimicrobial activity. During the resistance induction, TDL itself evokes *PR* gene expression in tobacco. Therefore, TDL, fulfilling all the criteria of an SAR inducer, is a novel class of chemical that induces SAR in tobacco. We speculate that TDL induces resistance in rice plants

against rice blast disease similarly to PBZ and BTH, however, the mechanism involved remains to be clarified.^{17,18)} In *Arabidopsis*, NPR1 is a key regulator of SAR, which functions downstream of SA in the signal transduction for SAR induction.^{19–21)} Since TDL exhibits all the necessary criteria for an SAR inducer, as reported here, it would be interesting to examine whether TDL requires NPR1 to induce SAR. Furthermore, there are various *Arabidopsis* mutants with defects in the SAR signaling pathway. Therefore, the mechanism of TDL-induced resistance could be clarified using *Arabidopsis*, and is currently under investigation.

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