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SHORT COMMUNICATION

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Thienopyrimidine-type compounds protect *Arabidopsis* plants against the hemibiotrophic fungal pathogen *Colletotrichum higginsianum* and bacterial pathogen *Pseudomonas syringae* pv. *maculicola*

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ABSTRACT

Plant activators activate systemic acquired resistance-like defense responses or induced systemic resistance, and thus protect plants from pathogens. We screened a chemical library composed of structurally diverse small molecules. We isolated six plant immune-inducing thienopyrimidine-type compounds and their analogous compounds. It was observed that the core structure of thienopyrimidine plays a role in induced resistance in plants. Furthermore, we highlight the protective effect of thienopyrimidine-type compounds against both hemibiotrophic fungal pathogen, *Colletotrichum higginsianum*, and bacterial pathogen, *Pseudomonas syringae* pv. *maculicola*, in *Arabidopsis thaliana*. We suggest that thienopyrimidine-type compounds could be potential lead compounds as novel plant activators, and can be useful and effective agrochemicals against various plant diseases.

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Plants have evolved effective defense mechanisms against different types of diseases (fungal, bacterial, and viral) and pests. Plants respond to pathogen attacks by increasing their resistance. Diseases in plants occur rarely because many plants defend themselves against microbial pathogens by employing elaborate defense mechanisms, including both localized and systemic resistant responses. Systemic acquired resistance (SAR) is an inducible plant defense response to pathogen infection and is simultaneously activated in uninfected organs of the plant as well.[1] This results in enhanced resistance in the entire plant against further pathogen attacks. Accumulation of salicylic acid (SA), which is an endogenously synthesized signaling factor, is required for the induction of SAR.[2] Although defense responses are genetically controlled, artificial tools are also able to regulate them. Not only pathogen attacks but also chemicals, called plant activators, activate disease resistance in plants. Plant activators activate SAR-like defense responses or induced systemic resistance (ISR).[1,3] Consequently, various defense-related genes, including *Pathogenesis-Related* (*PR*) genes are expressed in the whole plant. For example plant activators, 2,6-dichloroisonicotinic acid (INA), benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), Imprimatin, *N*-cyanomethyl-2-chloroisonicotinamide (NCI), and probenazole (PBZ) and its derivative, benzisothiazole (BIT) induce SAR by stimulating the signal transduction pathway for SAR development.[4-9]

To identify the main compounds that function as plant activators, large-scale and high-throughput screening procedures using plant immune system were established.[6,10-13] These screenings enabled us to identify small molecules that protect plants against diseases. We previously developed a high-throughput screening procedure for identifying plant activators, employing a β-glucuronidase (GUS) histochemical staining assay. This method considered promoters of the *Arabidopsis thaliana* defense-related genes, *PR-1* as a marker for the SA-dependent signal transduction pathway, and *PR-4* and *PDF1.2* as markers for the ethylene (ET)/jasmonic acid (JA)-dependent signal transduction pathway.[14,15] In particular, this system could monitor the activation of SA- and ET/JA-induced resistance in *A. thaliana* plants. This system enabled us to perform 1,000 to 2,000 screenings per week per person, and was economical in terms of both time and space. Using this screening system, we previously reported that pyrimidine-type plant activator (PPA) induces plant defense programs by moderating reactive oxygen species.[16]

In the present study, we describe thienopyrimidine-type compounds, obtained by our screening system, protecting *A. thaliana* plants against the hemibiotrophic fungal pathogen, *Colletotrichum higginsianum*, and bacterial pathogen, *Pseudomonas syringae* pv. *maculicola*.

Using the previously established screening system, we screened a chemical library composed of structurally diverse small molecules. We isolated six plant immune-inducing thienopyrimidine-type compounds and their analogs (N2781, N2835, N2947, N2969, N2972, N2914C, N2914A1 to N2914A4) (Fig. 1).

Induced resistance against pathogen-attack and chemicals is associated with the expression of defense-related marker genes,

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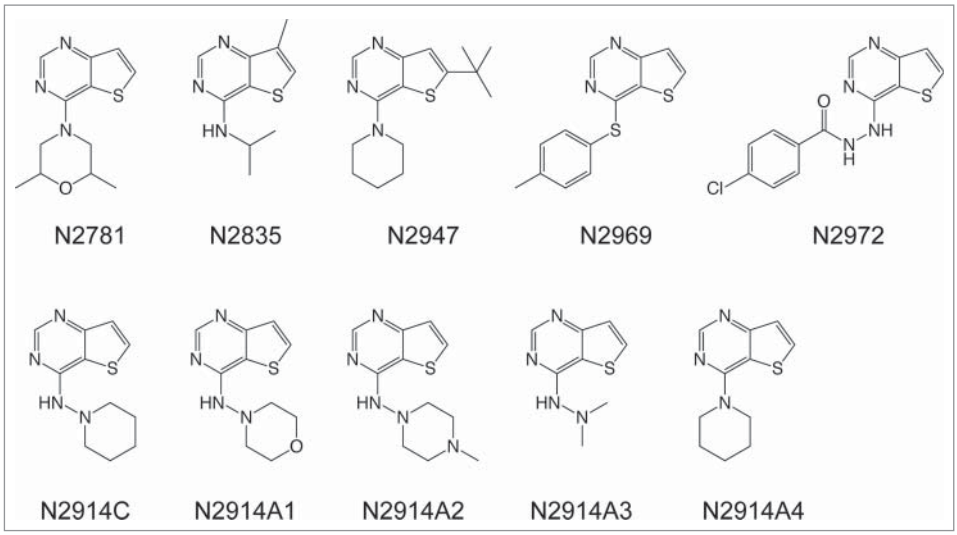

Figure 1. Molecular structure of plant immune-inducing thienopyrimidine-type compounds and its analogous compounds.

SA-associated *A. thaliana PR-1* gene,[2] and the JA/ET-dependent *PDF1.2* gene.[17] To determine whether these thienopyrimidine-type compounds function as activators of induced resistance, we investigated the transcription profiles of *PR-1* and *PDF1.2* mRNA in *A. thaliana* plants (Col-0 accession) treated with these compounds by quantitative real time-polymerase chain reaction (qRT-PCR). The *A. thaliana* plants were grown in a mixture consisting of Soil-mix (Sakata Seed Corp.), expanded vermiculite (1.5 to 2 mm granules), and pearlite (2.5 to 3.5 mm granules) in a 2:1:1 ratio for 28 days in a growth

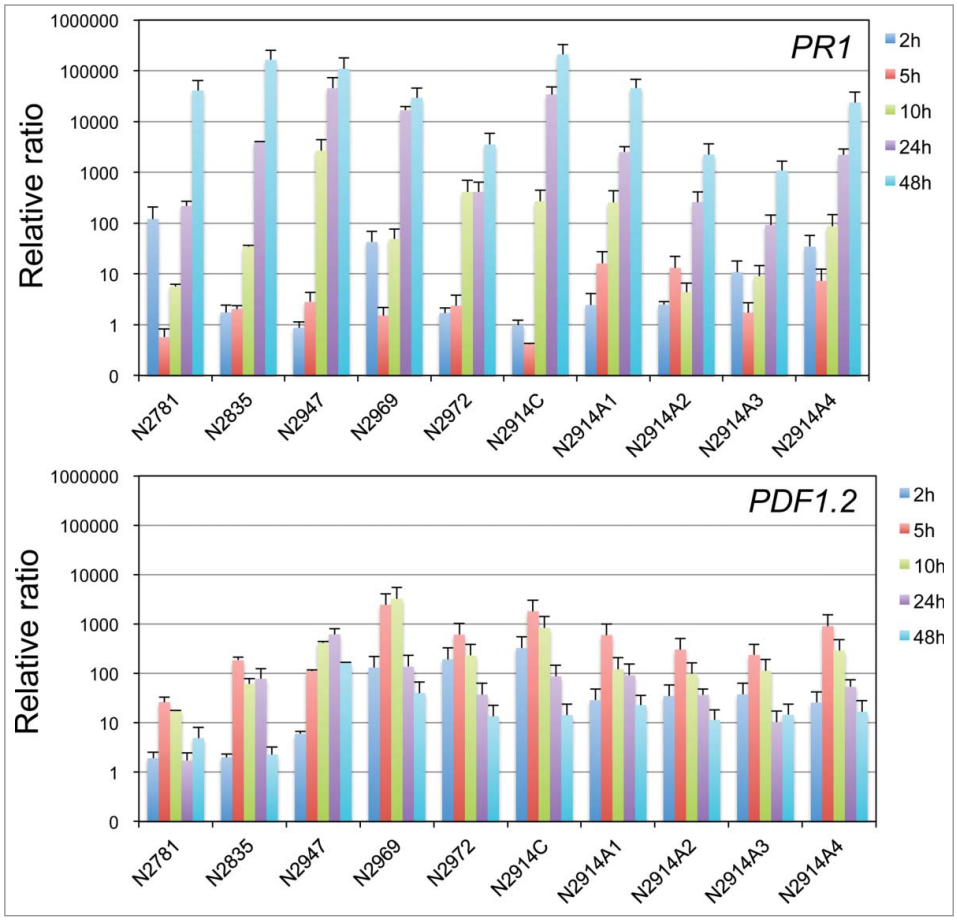

Figure 2. Expression of defense-related genes after treatment with TPA. Twenty eight day-old *A. thaliana* Col-0 plants were foliar-sprayed with 0.08 mM TPAs. The leaves were collected 2, 5, 10 24, and 48 h after treatment, and total RNA was isolated. The transcription levels of *PR-1* and *PDF1.2* mRNA were monitored by qRT-PCR analysis. The transcription levels of these genes were normalized against that of housekeeping gene, *CBP20*. The nucleotide sequences of the gene-specific primers for each gene were described previously.[18] The relative expression ratios are shown as fold induction relative to the expression level at 0 h. Bars indicate the standard error (SE). The experiment was repeated at least twice with similar results.

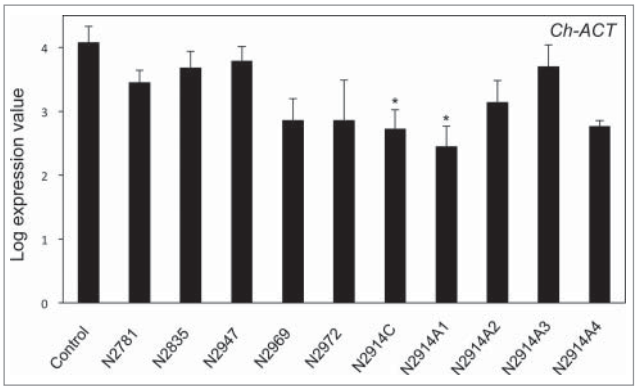

Figure 3. Effect of TPA application on *C. higginsianum* in *Arabidopsis* plants. Twenty eight day-old *A. thaliana* Col-0 plants were foliar-sprayed with 0.08 mM TPAs or water (control), 2 days prior to spray inoculation with a spore suspension (5×10^5 spores mL^{-1}) of *C. higginsianum* Saccardo isolates (MAFF305635). The leaves were harvested 5 days after the inoculation and total RNA was isolated. Fungal growth was monitored by quantifying *C. higginsianum actin* (*Ch-ACT*) mRNA using qRT-PCR as described previously.[19] Bars indicate the SE. The asterisk indicates a significant difference compared with the control (Dunnett's method,[21] $P < 0.05$). The experiment was repeated at least twice with similar results.

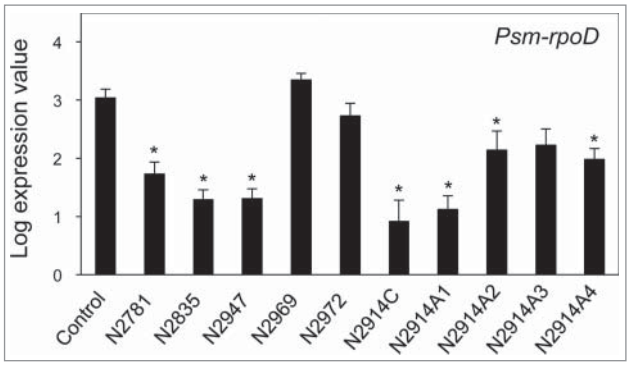

Figure 4. Effects of TPA application on *P. syringae* pv. *maculicola* in *Arabidopsis* plants. Thirty five-day-old *A. thaliana* Col-0 plants were foliar-sprayed with 0.08 mM TPAs or water (control), 2 days prior to spray inoculation with a bacterial suspension (10^8 cfu mL^{-1}) of *P. syringae* pv. *maculicola* (MAFF302783Rif4). The leaves were harvested 3 days after the inoculation and total RNA was isolated. Bacterial growth was monitored by quantifying *P. syringae* pv. *maculicola-rpoD* (*Ps-rpoD*) mRNA using qRT-PCR as described previously.[20] Bars indicate the SE. The asterisk indicates a significant difference compared with the control (Dunnett's method,[21] $P < 0.05$). The experiment was repeated at least twice with similar results.

chamber at 22 °C under a 12-h light/ 12-h dark cycle. The *A. thaliana* plants were treated with 0.08 mM thienopyrimidine-type compounds using foliar sprays, and total RNA was then extracted from the leaves at 2, 5, 10, 24, and 48 h. These compounds strongly induced *PR-1* mRNA expression in *A. thaliana* plants over a period (Fig. 2). The mRNA expression of *PDF1.2* rapidly increased, reaching a peak at 5 to 10 h in *A. thaliana* plants foliar-sprayed with the compounds compared with that of *PR-1* gene. The timing of induction of the SA- and JA/ET-dependent representative marker genes differed in *A. thaliana* plants foliar-sprayed with the compounds and yeast cell wall extract.[18] In addition, the transcription levels of *PR-1* mRNA were much higher in *A. thaliana* plants foliar-sprayed with the compounds than those of *PDF1.2*. These results showed that thienopyrimidine-type compounds function as activators of induced resistance and, therefore, were named as thienopyrimidine-type plant activator (TPA).

A. thaliana plants were pre-treated with 0.08 mM TPAs using foliar sprays, and then inoculated with a spore suspension (5×10^5 spores ml^{-1} in distilled water) of *C. higginsianum*. The inoculated plants were then placed in a growth chamber at 22 °C under a 12-h light/12-h dark cycle, and maintained at 100% relative humidity.[19] The control plants were treated only with distilled water. Our results showed that treatments with N2914C, and N2914A1 effectively protected *A. thaliana* leaves against anthracnose, a group of fungal diseases, commonly affecting the developing shoots and leaves, when compared with the control (Fig. 3). The N2969, N2972, N2914A2, and N2914A4 treatment showed moderate reduction in disease incidence (Fig. 3).

To determine whether TPA protects *A. thaliana* against bacterial pathogen, the plants were sprayed with 0.08 mM TPAs, 2 days before inoculation with *P. syringae* pv. *maculicola* (*Psm*).[20] The treatment with 0.08 mM TPAs, N2781, N2835, N2947, N2914C, N2914A1, N2914A2, and N2914A4 controlled the bacterial infection and growth in the leaves (Fig. 4). Consequently, TPA protected the plants against bacterial leaf spot caused by the pathogen. The TPA N2914C-treated plants contained three hundred-fold lower bacterial titers than that in the control plants. In addition, this concentration of TPA did not cause any phytotoxicity, i.e, inhibition of plant growth, reduction in yield, or leaf burn.

Thus, in this study, we showed that the core structure of thienopyrimidine plays a role in induced resistance in plants. Furthermore, we indicated the protective effect of TPA against hemibiotrophic fungal as well as against bacterial pathogens in *A. thaliana* plants. We suggest that TPA could be a significant potential lead compound as a novel plant activator, and a useful agrochemical against various plant diseases.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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