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Finding new elicitors that induce resistance in rice to the white-backed planthopper *Sogatella furcifera*

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ABSTRACT

Herein we report a new way to identify chemical elicitors that induce resistance in rice to herbivores. Using this method, by quantifying the induction of chemicals for GUS activity in a specific screening system that we established previously, 5 candidate elicitors were selected from the 29 designed and synthesized phenoxyalkanoic acid derivatives. Bioassays confirmed that these candidate elicitors could induce plant defense and then repel feeding of white-backed planthopper *Sogatella furcifera*.

Chemical insecticides have a pivotal role in crop protection. However, the extensive application of these insecticides not only causes severe environmental and farm produce pollution but also damages the ecosystem and thus results in, for example, the resurgence of herbivores and the reduction in populations of the natural enemies of herbivores and biodiversity. Therefore, developing safe and effective methods to control insect pests is essential.[1,2]

To protect themselves from damage by invaders, plants in nature have evolved a series of defense mechanisms, including induced defenses that are activated by herbivore attack.[3–6] Studies on the mechanisms underlying herbivore-induced plant defense have revealed that many compounds with low molecular weight, such as herbivore-associated molecule patterns, jasmonic acid (JA), salicylic acid (SA) and ethylene, play an important role in this process. Exogenous application of these compounds or their synthetic analogues, defined as chemical elicitors, can induce plant defense responses and thus increase the resistance of plants to herbivores.[7,8] Since the chemical elicitors themselves are not toxic to herbivores or their natural enemies and decompose easily in nature, the development of safe plant protection agents based on chemical elicitors is highly desired.

Chemical genetics has been used widely to develop drugs and offers a powerful toolkit with which to discover chemical elicitors.[9,10] With the methods that chemical genetics makes available, we have established a specific high-throughput screening system, which contains a 1.55-kb promoter region of the S-linalool synthase gene (*LISp*), fused to a β-glucuronidase (GUS) reporter gene.[7] With this system, the potential chemical elicitors can be identified according to the GUS activity that they induce.[7] Thus far, we have identified an herbicide, 2,4-D (2,4-dichlorophenoxyacetic acid), that is a chemical elicitor and found that it has potential to help control the rice brown planthopper *Nilaparvata lugens* in the field.[7]

In order to develop new chemical elicitors that are safe protection agents for controlling insect pests, we designed and synthesized 29 phenoxyalkanoic acid derivatives and explored the relationship between their structures and the levels of GUS activity that they induced. Moreover, we investigated the effect of these potential elicitors on transcript levels of two defense-related genes in rice, a mitogen-activated protease kinase (MPK) gene, Os*WPK3*, and a linalool synthase gene, Os*LIS*, whose encoding enzyme catalyzes the production of a volatile monoterpenoid linalool; both of these represent early and late events, respectively, in the herbivore-induced plant defense responses.[11,12] Finally, we measured the effect of treatment with the potential elicitors on the resistance of rice to the white-backed planthopper *Sogatella furcifera*, one of the most important rice insect pests in Asia.[13,14] Here, we report our findings.

In this study, we used the high-throughput screening system[9,10] to evaluate the induction activities of 29 phenoxyalkanoic acid derivatives. GUS activity was quantified in the roots of transgenic

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rice plants that had been treated with one of the tested compounds at a concentration of 5 mg L^{-1}, using the same method as described in Xin et al.[7] The relative induction of GUS activity in the roots of plants treated with each compound compared to in the roots of the control (non-treated) plant is summarized in Tables 1 and 2.

Among the tested chemicals derived from phenoxyacetic acid and 24 ring-substituted phenoxyacetic acid (Table 1), five compounds elicited GUS levels that were higher than those were found in the control. Moreover, levels of GUS activity in four monosubstituted phenoxyacetic acids—4-methylphenoxyacetic acid, 4-iodophenoxyacetic acid, 4-bromophenoxyacetic acid and 4-fluorophenoxyacid acid—were higher than the levels found in phenoxyacetic acid by 1.63-, 1.34-, 1.34- and 1.21-fold, respectively. Within the family of these monosubstituted phenoxyacetic acids, 2- or 3-position substitutions did not increase the level of GUS activity compared with level of phenoxyacetic acid (e.g., 2-methylphenoxyacetic acid < phenoxyacetic acid; 3-methylphenoxyacetic acid < phenoxyacetic acid). With the exception of 4-ethylphenoxyacetic acid, 4-*tert*-butylphenoxyacetic acid, 4-methoxyphenoxyacetic acid, 4-nitrophenoxyacetic acid and 4-acetamidophenoxyacetic acid, the 4-substituted halogphenoxyacetic acids and 4-methylphenoxyacetic acid induced higher levels of GUS activity than did phenoxyacetic acid. Substitution with strong electron-withdrawing characteristics, such as nitro group at 4-position, and di-substitution, reduced activity (e.g., 4-chloro-2-methylphenoxyacetic acid < phenoxyacetic acid < 4-methylphenoxyacetic acid).

The GUS activity levels induced by the substituted phenoxybutanoic acids, 1-naphthyloxypropanoic acid and phenoxyacetamide are listed in Table 2. After the length of the side chain between phenoxy moiety and carboxylic acid was increased, the levels of GUS activity diminished dramatically. This result suggests that an increase in the chain length does not improve the level of activity. In addition, phenoxyacetamide elicited lower levels of GUS activity than phenoxyacetic acid did, indicating that the carboxylic acid group is necessary for high activity.

From the GUS activity levels of the above phenoxyacetic acid derivatives, we can deduce that levels of GUS activity are increased when specific conditions are met: (1) Enhanced activity is limited to substitution at 4-position; Substitutions at 2- or 3-position decreased induction activity. (2) Substitution is sensitive to the induction of GUS activity, and the small methyl group, a fluorine atom, a bromine atom and an iodine atom enhance activity. (3) The length of the chain between phenoxy moiety and carboxylic acid is one of the important factors influencing the induction potency for GUS activity, and the methylene bridge enhances activity. (4) A carboxylic acid group is necessary and can increase activity dramatically.

To confirm whether screening out the phenoxyacetic acid derivatives can elicit defense responses, we investigated the expression levels of the two defense-related genes, Os*LIS* and Os*MPK3*, in the stems of rice plants whose roots were treated with one of the following compounds **1a**, **1p**, **1t**, and **1u** at a concentration of 5 mg L^{-1}. The result showed that all of the four compounds elicited increases in transcript levels of the two genes: levels of Os*MPK3* transcripts increased at 12 and 24 h after the start of treatment, whereas levels of *OsLIS* transcripts mainly increased 24 h after treatment (Fig. 1). This suggests that the screened chemical elicitors can profoundly induce defense responses in rice.

To further explore the effect of these chemical elicitor-induced defense responses on herbivore performance, we investigated the feeding preference of adult *S. furcifera* females in the lab for plants treated with one of these chemical elicitors and control plants. The results showed that *S. furcifera* female adults were more frequently found on control plants than on plants whose roots had been treated with one of the four chemical elicitors **1a**, **1p**, **1t**, and **1u** at a concentration of 5 mg L^{-1} (Fig. 2), suggesting the treated plants were repellent to the herbivore.

Moreover, the tested chemical elicitors themselves were not repellent to the herbivore: the number of female adults that stayed on cotton balls containing 500 μl of 30% sucrose solution was equal to those that stayed on cotton balls containing 500 μl of 5 mg L^{-1} the tested chemical elicitor in the solution (Fig. 3).Taken together, the data demonstrate that the repellent role of the elicitor-treated plants to the herbivore comes from the defense responses induced by the elicitors. Among the tested four chemical elicitors, **1p** induced the highest and longest-lasting level of repellency in the herbivore: it reached about 50% at 48 h after treatment (Fig. 2).

Table 1
Relative induction of GUS activity in rice by phenoxyacetic acid and ring-substituted phenoxyacetic acids

Compound	Substituent	Relative induction of GUS activity[a]
Control		**1.00**
2-Phenoxyacetic acid (**1a**)		**1.43****
Position 2 substitution		
2-Methylphenoxyacetic acid (**1b**)	CH_3	0.99
2-Isopropylphenoxyacetic acid (**1c**)	*iso*-Propyl	1.18
2-Methoxyphenoxyacetic acid (**1d**)	CH_3O	1.23
2-Fluorophenoxyacetic acid (**1e**)	F	0.87
2-Chlorophenoxyacetic acid (**1f**)	Cl	0.93
2-Bromophenoxyacetic acid (**1g**)	Br	0.67*
2-Iodophenoxyacetic acid (**1h**)	I	1.02
2-Nitrophenoxyacetic acid (**1i**)	NO_2	1.11
2-Acetamidophenoxyacetic acid (**1j**)	CH_3CONH	1.09
2-Acetylphenoxyacetic acid (**1k**)	CH_3CO	1.05
Position 3 substitution		
3-Methylphenoxyacetic acid (**1l**)	CH_3	0.69*
3-Methoxyphenoxyacetic acid (**1m**)	CH_3O	1.06
3-Chlorophenoxyacetic acid (**1n**)	Cl	0.88
3-Bromophenoxyacetic acid (**1o**)	Br	1.17
Position 4 substitution		
4-Methylphenoxyacetic acid (**1p**)	CH_3	**2.33****
4-Ethylphenoxyacetic acid (**1q**)	CH_3CH_2	0.62**
4-*tert*-Butylphenoxyacetic acid (**1r**)	*tert*-Butyl	1.19
4-Methoxyphenoxyacetic acid (**1s**)	CH_3O	0.95
4-Fluorophenoxyacetic acid (**1t**)	F	**1.72****
4-Bromophenoxyacetic acid (**1u**)	Br	**1.91****
4-Iodophenoxyacetic acid (**1v**)	I	**1.92****
4-Nitrophenoxyacetic acid (**1w**)	NO_2	0.94
4-Acetamidophenoxyacetic acid (**1x**)	CH_3CONH	1.00
Mixed halogen and alkyl substitutions		
4-Chloro-2-methylphenoxyacetic acid (**1y**)	2-CH_3-4-Cl	1.16

[a] GUS activity level induced by the tested compound (5 mg L^{-1}) divided by the level induced by the control 48 h after treatment. Asterisks indicate significant differences between treatments and controls (*P <0.05, **P <0.01, Student's *t*-tests).

Table 2
Relative induction of GUS activity in rice by ring-substituted phenoxybutanoic acids, naphthyloxypropanoic acid, and phenoxyacetamide

Compound	Relative induction of GUS activity*
Control	1.00
4-(2,4-Dichlorophenoxy)butanoic acid (**2a**)	0.92
4-(2,6-Diisopropylphenoxy)butanoic acid (**2b**)	0.98
2-(1-Naphthyloxy)propanoic acid (**2c**)	0.94
Phenoxyacetamide (**2d**)	1.06

* GUS activity level induced by the tested compound (5 mg L^{-1}) divided by the level induced by the control 48 h after treatment.

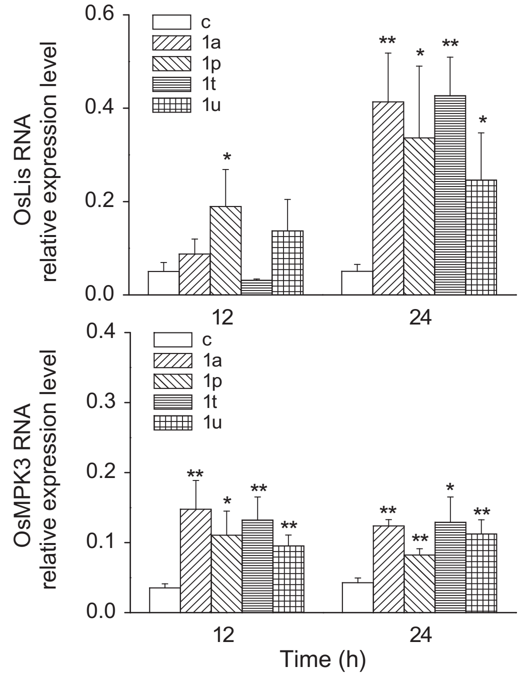

Figure 1. Mean expression levels (+SE, n = 5) of Os*Lis* (a) and Os*WPK3* (b) in stems of plants whose roots were treated with one of the four compounds, 2-phenoxyacetic acid (**1a**), 4-methylphenoxyacetic acid (**1p**), 4-fluorophenoxyacetic acid (**1t**), and 4-bromophenoxyacetic acid (**1u**) at a concentration of 5 mg L^{-1}. (C) Non-manipulated. Asterisks indicate significant differences between treatments and controls at each time point ($^*P$ <0.05, $^{**}P$ <0.01, Student's t-tests).

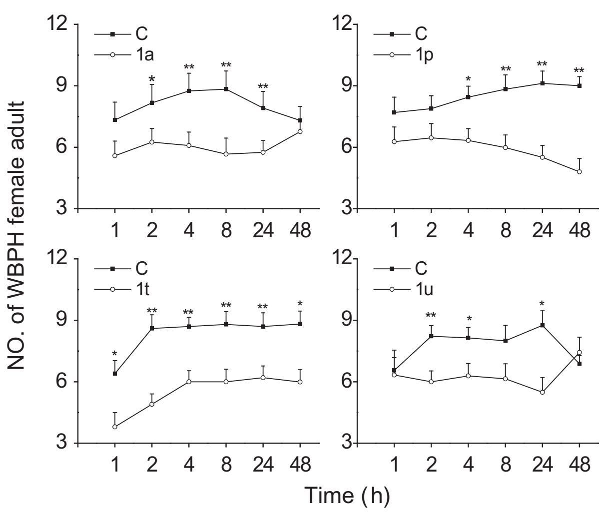

Figure 2. Mean number of female *Sogatella furcifera* adults (+SE, n = 10) on pairs of plants, a plant that had been treated with one of the four compounds, 2-phenoxyacetic acid (**1a**), 4-methylphenoxyacetic acid (**1p**), 4-fluorophenoxyacetic acid (**1t**), and 4-bromophenoxyacetic acid (**1u**) at a concentration of 5 mg L^{-1} for 12 h versus a control plant (C), 1–48 h after exposure. Asterisks indicate significant differences between treatments and controls at each time point ($^*P$ <0.05, $^{**}P$ <0.01, Student's t-tests).

In conclusion, among the compounds tested, only monosubstituted phenoxyacetic acids with a methyl group, a fluorine atom, a bromine atom or an iodine atom in the 4-ring position are more active in inducing GUS activity than phenoxyacetic acid is. We

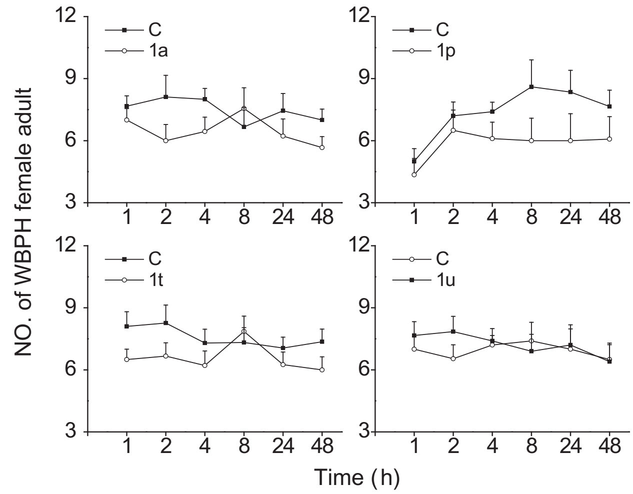

Figure 3. Mean number of female *Sogatella furcifera* adults (+SE, n = 10) on pairs of cotton balls, a cotton ball with 1 ml of one of the four compounds, 2-phenoxyacetic acid (**1a**), 4-methylphenoxyacetic acid (**1p**), 4-fluorophenoxyacetic acid (**1t**), and 4-bromophenoxyacetic acid (**1u**), at a concentration of 5 mg L^{-1} versus a cotton ball with 1 ml of the buffer (C), 1–48 h after exposure.

demonstrate that the ability of a chemical elicitor to induce GUS activity is correlated with its ability to induce resistance in rice to *S. furcifera*, suggesting the value of using the relative induction of GUS activity as a screening tool for identifying new chemical elicitors and developing insect control agents. The chemical elicitors screened out here could be exploited as induced insect repellants against *S. furcifera*. Future research should explore the effectiveness of these compounds for controlling *S. furcifera* in the field.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.10.041.

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