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Brief Report

Insect growth regulators with hydrazide moiety inhibit strigolactone biosynthesis in rice

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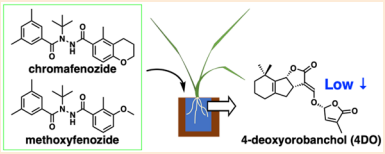
Supplementary material

Strigolactones (SLs) are carotenoid-derived plant hormones involved in several growth and developmental processes. Also, SLs are allelochemicals that induce the seed germination of root parasitic plants and the hyphal branching of arbuscular mycorrhizal fungi. In this study, to identify novel lead chemicals that inhibit SL biosynthesis, we evaluated the effect of agrochemicals on SL biosynthesis. We found that the diacylhydrazine insect growth regulator, chromafenozide, reduced the endogenous level of 4-deoxyorobanchol (4DO), a major SL in rice. Furthermore, treatment with the same class of insect growth regulator, methoxyfenozide, also resulted in the reduction of 4DO levels in rice root exudates. These results suggest that chromafenozide and methoxyfenozide are novel lead inhibitors of SL biosynthesis.



Keywords: strigolactone biosynthesis inhibitor, insect growth regulator, screening, rice.

Introduction

Strigolactones (SLs) are one group of plant hormones that control several developmental processes, such as the outgrowth of axillary shoots, stress tolerance, and leaf senescence.[1–3] In 1966, SLs were first isolated as germination stimulants of the root parasitic plant, *Striga lutea* Lour., from the root exudates of cotton (*Gossypium hirsutum* L.).[4] Subsequently, SLs have been found to induce hyphal branching in arbuscular mycorrhizal fungi that supply inorganic phosphate to plants.[5] Research on SLs is agriculturally important because SLs regulate these useful characteristics.

Root parasitic plants, such as broomrapes (*Phelipanche* and *Orobanche* spp.) and witchweeds (*Striga* spp.), infest staple crops in sub-Saharan Africa, the Middle East, and Asia.[4,6,7] Once attached to the host plant, root parasitic plants take up nutrients and water from the host. The damage caused by *Striga* in Africa, in particular, is severe and is estimated to account for the annual losses of US $7 billion.[6] Because infestation with the root parasitic plants is alleviated in SL-biosynthesis mutants,[2] SL-biosynthesis inhibitors could be useful in controlling root parasitic plants.

Genetic and biochemical analyses have revealed that SLs are biosynthesized from all-*trans*-β-carotene by several enzymes. Carlactone (CL), which is an important precursor in the SL-biosynthetic pathway, is converted from all-*trans*-β-carotene by a carotenoid isomerase (D27) and two carotenoid cleavage dioxygenases, CCD7 (MAX3 in *Arabidopsis*/RMS5 in pea/D17 in rice/DAD3 in petunia) and CCD8 (MAX4 in *Arabidopsis*/RMS1 in pea/D10 in rice/DAD1 in petunia).[8,9] Subsequently, CL is oxidized by CYP711A family enzymes of cytochrome P450 proteins (P450), although their enzymatic activities differ among plant species. In rice, among five CYP711As, Os900 (CYP711A2) and Os1400 (CYP711A3) participate in orobanchol biosynthesis.[10] Os900 catalyzes the conversion of CL to carlactonoic acid (CLA) and CLA to 4-deoxyorobanchol (4DO). In contrast, Os1400 converts CL to CLA and 4DO into orobanchol (see Supplementary Material, Fig. S1). In most plant species, except rice, the enzymatic activity of CYP711As only shows the conversion of CL to CLA.[11,12]

To date, some compounds have been reported to be SL-biosynthesis inhibitors. B2 and D6, hydroxamic acid derivatives, exhibit inhibitory activity against D27, CCD7, or CCD8.[13] We have previously reported that TIS108 and KK5 reduce the level of 4DO in rice, suppressing *Striga* germination.[14,15] In addition, by using these SL-biosynthesis inhibitors, some physi-

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ological roles of SLs have been uncovered in some plant species.[16–18] However, since suitable inhibitors of SL biosynthesis differ among plant species,[15,16] a novel lead chemical, whose structure is substantially different from those of the reported SL-biosynthesis inhibitors, is needed for SL research.

Chemicals such as pharmacological agents and agrochemicals can function as plant-growth regulators. For instance, spironolactone, a diuretic drug used in mammals, induces morphological changes in *Arabidopsis* by inhibiting brassinosteroid action.[19] Furthermore, the fungicides tebuconazole and triflumizole have been identified as SL-biosynthesis inhibitors.[20,21] Consequently, it is effective to screen pharmacological agents and agrochemicals—except plant growth regulators which affect plant morphology—for novel lead chemicals as SL-biosynthesis inhibitors.

20-hydroxyecdysone (20HE) is known as a molting hormone that regulates insect metamorphosis and development in most insects.[22] Because molting is an essential event for insects, several ecdysone agonists have been developed as insecticides. Especially, diacylhydrazine derivatives such as chromafenozide (CHR) and methoxyfenozide (MET) are commercially used as potent insecticides against lepidoptera insects (see Supplementary Material, Fig. S2). These chemicals bind to the ecdysone receptor complex and successively induce abnormal molting.

In this study, we evaluated the SL biosynthesis–inhibitory activity of six agrochemicals, shown in Fig. 1, to find a novel lead SL-biosynthesis inhibitor. CHR and MET, known to be molting-hormone agonists, were identified as effective SL-biosynthesis inhibitors.

Materials and methods

1. Plant material, chemicals, and growth conditions

We used Nipponbare species as the wild-type rice. Insecticides and insect growth regulators were purchased from Fujifilm Wako Chemicals (Osaka, Japan) and Sigma-Aldrich (St. Louis, MO, USA). Rice seedlings were grown as previously described.[2] Sterilized rice seeds with 2.5% sodium hypochlorite were incubated at 25°C in sterile water in the dark for 2 days. The germinated rice seeds were transferred into a phosphate-deficient hydroponic culture medium solidified with 0.7% agar and incubated at 25°C under fluorescent white light with a 14-hr light and 10-hr dark photoperiod for 7 days. Nine-day-old seedlings were transferred to a glass vial containing 12 mL of phosphate-deficient hydroponic culture media and grown under the same conditions for 6 days. Fifteen-day-old seedlings were transferred to a brown vial containing 12 mL of the same medium and 12 μL of the tested chemicals dissolved in DMSO, and it was incubated for 1 day. To analyze the 4DO levels, culture media and roots were collected.

2. Quantification of the 4DO level in rice root exudates and roots

We used 400 pg of deuterium-labeled 5-deoxystrigol (d_6-5DS) as the internal standard.[23] To measure 4DO in rice root exudates, we extracted the hydroponic culture medium with ethyl acetate twice. The organic phase was concentrated *in vacuo*. To measure 4DO in roots, we homogenized rice roots in ethyl acetate with d_6-5DS added, and the suspension was filtered. The filtrates were dried and dissolved in 10% acetone. The extracts were loaded onto Oasis HLB 3 cc (60 mg) extraction cartridges (Waters, Milford, MA, USA), washed with 10% acetone (6 mL), and eluted with acetone (6 mL). The solutions were concentrated *in vacuo* and dissolved in 1 mL of ethyl acetate : *n*-hexane (15 : 85). The SL-containing fractions were loaded onto Sep-Pak Vac 1 cc (100 mg) silica cartridges (Waters), washed with 2 mL of ethyl acetate : *n*-hexane (15 : 85), and eluted with 3 mL of ethyl acetate : *n*-hexane (35 : 65). The eluates were concentrated *in vacuo*.

The dried concentrates were dissolved in deionized water : acetonitrile (1 : 1) and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using a Triple TOF 5600 system (SCIEX), as previously described.[24]

Results and discussion

To identify novel lead chemicals for SL-biosynthesis inhibitors among insecticides and insect growth regulators (see Supplementary Material, Fig. S3), we first measured the level of SL in the root exudates of two-week-old rice seedlings from each treatment group, because the levels of SL in rice root exudates show a good correlation with those in rice roots.[25] In this study, we analyzed the levels of 4DO, a major SL in rice, by LC-MS/MS. The detection of SL levels becomes easy *via* the upregulation of SL-biosynthetic gene expression when rice seedlings are grown in phosphate-deficient culture media.[26] Thus, we estimated the effects of chemicals on 4DO levels under conditions of phosphate deficiency. Of the tested chemicals, 10 μM CHR treatment significantly reduced the level of 4DO in root exudates as compared with the control (Fig. 1). CHR is commercially used as an insect molting-hormone agonist with a hydrazone moiety.[27] To check whether the insect molting hormone and its agonist show the inhibitory activity of SL biosynthesis, we performed the same

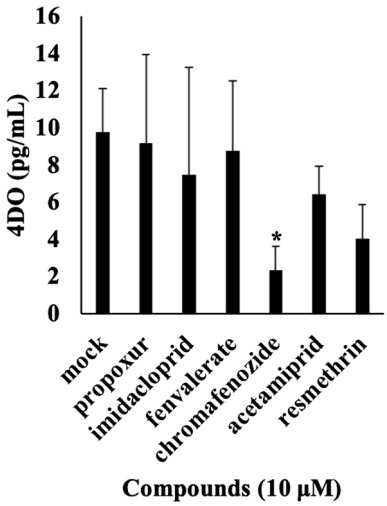

Fig. 1. The effect of insecticides (10 μM) on 4-deoxyorobanchol (4DO) levels in rice root exudates. The data are the mean ± S.D. (n=4). * Denotes a statistically significant difference from the 4DO level in no-application of chemicals plants (control) (Dunnett's test; $0.01<p<0.05$).

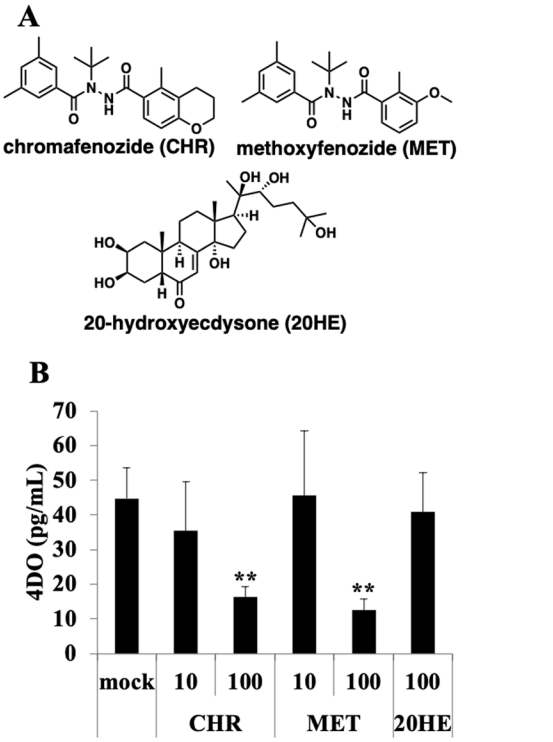

Fig. 2. The effect of the insect molting hormone on 4DO levels in rice root exudates. (A) The structure of the insect molting hormone and analogs. (B) 4DO levels in rice root exudates of 10 and 100 μM compound-treated rice. The data are the mean±S.D. (n=4). ** Denotes a statistically significant difference from the 4DO level in control plants (Dunnett's test; $p<0.01$).

assay using CHR, MET, and 20HE. In this assay, CHR and MET displayed statistically significant reductions in 4DO levels in root exudates at a concentration of 100 μM, while 20HE treatment did not affect the level of 4DO (Fig. 2). These results suggest that the chemical structure of the molting-hormone agonists, rather than their molting-hormone activity, is important for the reduction of 4DO levels in root exudates. Next, we analyzed the endogenous 4DO levels in CHR-treated roots. CHR treatment reduced endogenous 4DO levels in roots in a dose-dependent manner (1–

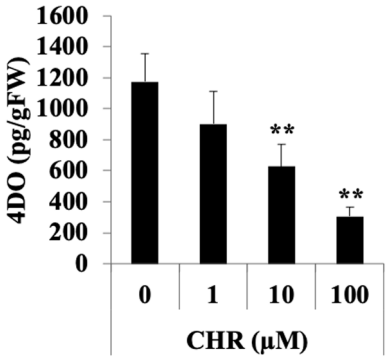

Fig. 3. The effect of chromafenozide on strigolactone biosynthesis. 4DO levels of chromafenozide-treated rice in roots. The data are the mean±S.D. (n=3). ** Denotes a statistically significant difference from the 4DO level in control plants (Dunnett's test; $p<0.01$).

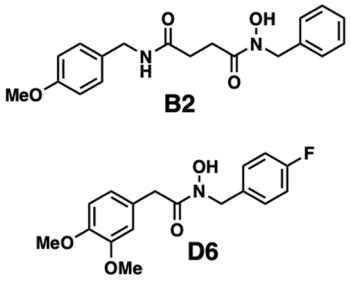

Fig. 4. The structures of B2 and D6.

100 μM) (Fig. 3). These results suggest that CHR and MET are novel lead chemicals for SL-biosynthesis inhibitors in rice.

We evaluated the inhibitory activity of SL biosynthesis on six agrochemicals and found that CHR inhibited the 4DO biosynthesis in rice when the compound was applied at concentrations ranging from 10 to 100 μM. CHR is an insect growth regulator that induces abnormal molting as an ecdysone receptor agonist.[28] Although root exudates of CHR- and MET-treated rice showed a reduction in 4DO levels, 20HE-treated rice root exudates did not. These results indicate that the inhibitory activity of SL biosynthesis, as shown by CHR and MET, is not related to the molting-hormone activity. In addition, as compared to the potent insecticidal activities (around 0.1 to 1 μM),[29] the inhibitory activities of SL biosynthesis were very weak, suggesting the importance of studying the structural activity relationship to develop specific SL-biosynthesis inhibitors. To date, it has been reported that B2 and D6 with hydroxamic acid and amide bonds showed inhibitory activity of SL biosynthesis. The target protein of B2 is D27, while D6 inhibits the enzymatic activities of CCD7 and CCD8.[13,30] The structures of B2 and D6 consisted of two benzene rings bridged by an amide bond (Fig. 4). The structures of CHR and MET also have two benzene rings bridged by an amide bond. Therefore, CHR and MET may inhibit SL biosynthesis by inhibiting the enzymatic activities of D27, CCD7, or CCD8. As B2 and D6 inhibit the activities of target enzymes at concentrations of 10 to 100 μM *in vitro*, the inhibitory activities of SL biosynthesis in CHR and MET might be comparable to those in B2 and D6. To understand the target enzymes of CHR and MET in detail, we need to assess the effects of CHR and MET on D27, CCD7, CCD8, and CYP711A through inhibitory assay of these enzymes in the near future.

In this study, we found that CHR and MET, known as molting-hormone agonists, inhibit SL biosynthesis in rice. This is the first observation in which molting-hormone agonists inhibit SL production. SL-biosynthesis inhibitors can control the endogenous levels of SLs in developmental stages and tissues. Although further structure–activity relationship studies are needed to develop specific and potent SL-biosynthesis inhibitors, CHR- and MET-derivatives will play an important role in analyzing the SL function and controlling the damage of root parasitic plants.

Acknowledgements

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Electronic supplementary materials

The online version of this article contains supplementary materials (Supplementary Figs. S1–S3).

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