ACS central science

Research Article

Cite This: ACS Cent. Sci. 2018, 4, 230−234

Discovery of Shoot Branching Regulator Targeting Strigolactone Receptor DWARF14

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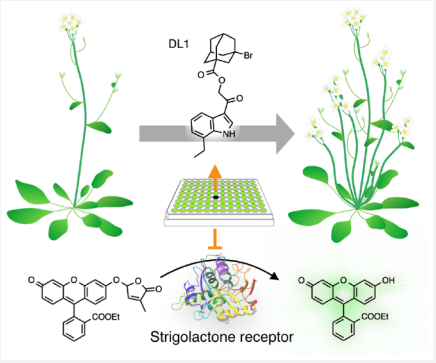
Supporting Information

ABSTRACT: DWARF14 (D14) is a strigolactone receptor that plays a central role in suppression of shoot branching, and hence is a potential target to increase crop productions and biomass. Recently, we reported a fluorescence turn-on probe, Yoshimulactone Green (YLG), which generates a strong fluorescence upon the hydrolysis by D14-type strigolactone receptors. Herein, we applied a YLG-based *in vitro* assay to a high-throughput chemical screening and identified a novel small molecule DL1 as a potent inhibitor of D14. DL1 competes with endogenous strigolactones, thereby increasing the number of shoot branching in a model plant *Arabidopsis* as well as in rice. Thus, DL1 is expected to be useful not only as a tool to understand the biological roles of D14 receptors in plant growth and development, but also as a potent agrochemical to improve the crop yield.



Regulating the number of branches in plants is critical for controlling plant architecture and efficient production of biomass.[1] Increased shoot branching has historically been a desirable trait because it is associated with high crop yields.[2] Substantial progress has been made in our understanding of the mechanism of how plants increase or suppress shoot branching in response to the environment. Genetic analysis revealed that carotenoid-derived signaling molecules regulate the shoot branching. This key molecule was identified as strigolactone,[3,4] which was initially discovered as a germination stimulant of the parasitic plant *Striga hermonthica*.[5] Strigolactone is a sesquiterpene lactone composed of a tricyclic lactone (ABC-ring) and a butenolide (D-ring), which are connected via an enol ether bond (Figure 1a).[6] Recent studies revealed that strigolactone also plays a significant role in symbiosis with arbuscular mycorrhizal fungi.[7] In *Arabidopsis thaliana*, *DWARF14 (AtD14)*[8] and *MORE AXILLARY GROWTH2 (AtMAX2)*[9] genes are essential in strigolactone-dependent suppression of axillary bud outgrowth. Loss-of-function in these signaling components results in an increased number of shoot branching. *D14* encodes a group of α/β-hydrolase fold proteins that bind to and hydrolyze strigolactones into an ABC-ring and D-ring (5-hydroxyl-3-methyl-butenolide) during the perception process.[10] *MAX2* encodes a F-box protein of ubiquitin E3 ligase. Previous studies including crystallographic analysis of strigolactone-induced complex of AtD14 and D3 (MAX2 orthologue in rice) demonstrated that these two proteins interact through strigolactone-dependent conformational change of AtD14.[11,12] This complex ubiquitinates specific downstream repressors including SMXL6, 7, and 8 for proteasomal degradation.[13−16] As a consequence, transcription factors including BRANCHED1 (BRC1) are upregulated to express a subset of strigolactone inducible genes.[17,18] It was revealed that BRC1 is expressed in developing bud, and its downregulation leads to bud outgrowth.[19]

As AtD14 plays a critical role in strigolactone-induced branching suppression, molecules that inhibit D14 should increase the number of branching. A small-molecule approach is beneficial for agriculture because it enables precise control of timing and degree of branch emergence. However, *in vivo* chemical screening with the branching phenotype is impractical due to long incubation time, growth space, and the amount of small molecules required for the assay. Recently, we reported a fluorogenic molecule, Yoshimulactone Green (YLG),[20] whose fluorescence emission is turned on by AtD14-dependent hydrolysis.[21] Because YLG shares the binding site of AtD14 with strigolactones, competition assays with YLG allowed us to estimate the binding affinity of natural and synthetic strigolactones to AtD14. Here, we utilized the YLG-based competition assay to high-throughput screening of ligands for AtD14 (Figure 1b).

We screened 800 compounds from an in-house developed chemical library as below. YLG (1 μM) was incubated with

Received: November 17, 2017
Published: February 7, 2018

DOI: 10.1021/acscentsci.7b00554
ACS Cent. Sci. 2018, 4, 230−234

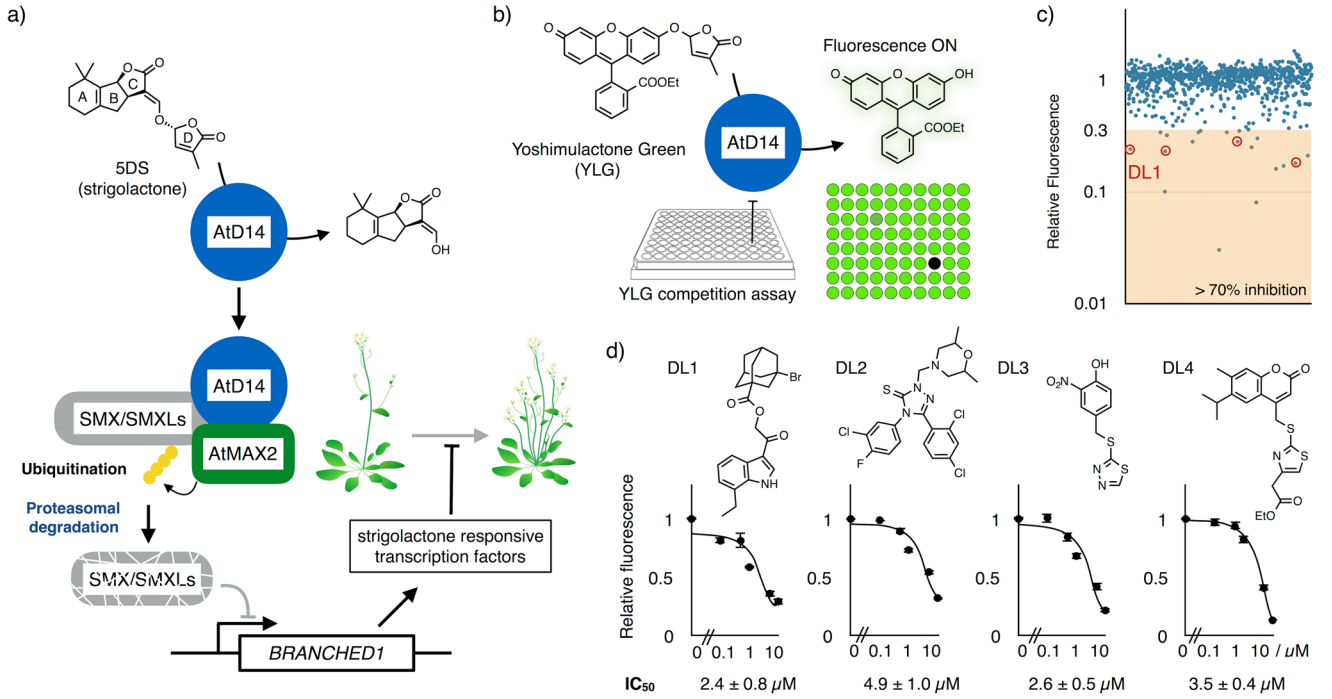


Figure 1. Yoshimulactone-based chemical screening of D14 ligands. (a) Strigolactone signaling that suppresses shoot branching. (b) Schematic workflow of high-throughput screening for AtD14-ligand. (c) AtD14-binding activity of 800 compounds examined by YLG competition assay. The fluorescence intensities were measured after the incubation of YLG (1 μM) with AtD14 in the presence of library compounds (10 μM). Vertical axis indicates relative fluorescence normalized by no ligand control. Red circles indicate the four compounds that passed the second screening. (d) Chemical structure of the hit compounds and their inhibitory activity for D14-mediated YLG hydrolysis. Error bar indicates SE (n = 3 biological replicates).

recombinant AtD14 (10 μg/mL) in the presence of library compound (10 μM). The difference in fluorescence intensity before and after the incubation is summarized in Figure 1c. In the first screening, 18 compounds exhibited >70% inhibition of AtD14-mediated YLG hydrolysis. The activities of these 18 compounds were retested in a second screening, and four compounds were validated as AtD14 ligands with >70% inhibitory activity at 10 μM (Figure 1d and Figure S1). We estimated the half maximal inhibitory concentration (IC_{50}) value from the dose−response curve of these compounds. Among the four compounds, DL1 displayed the highest inhibitory activity to AtD14 with the IC_{50} value of 2.4 ± 0.8 μM, which is comparable with the widely used synthetic strigolactone *racemic*-GR24 (4.9 ± 0.1 μM).[21−23] DL1 also inhibited the AtD14-mediated hydrolysis of the natural strigolactone (+)-5-deoxystrigol (5DS) with the IC_{50} value of 2.2 ± 0.2 μM (Figure S2). The intrinsic fluorescence of AtD14 was reduced by the addition of DL1 in a concentration-dependent manner, which further validates the binding of DL1 to AtD14 (Figure 2a). The dissociation constant (K_d) of DL1 to AtD14 was estimated as 3.4 ± 0.5 μM, whereas an inactive strigolactone analogue, carba-GR24, did not show a significant effect on the fluorescence (Figure 2b).[21] The binding of DL1 is specific to AtD14, as we did not observe binding to its closely related homologue, HYPOSENSITIVE TO LIGHT (AtHTL), which is involved in seed germination stimulated by wildfire smoke-derived karrikins (K_d > 250 μM).[24,25]

We evaluated inhibitory kinetics of DL1 on AtD14-mediated hydrolysis of strigolactone. No significant change of the maximal velocity (V_{max}) was observed in the Lineweaver−Burk plot, indicating that DL1 inhibits the hydrolytic activity of AtD14 in a competitive manner (Figure 2c). Moreover, we did not detect any hydrolytic product of DL1 after the incubation with AtD14 (Figure S2). Therefore, we concluded that DL1 reversibly binds to the active pocket of AtD14 and competitively inhibits its binding to strigolactones.

Next, we tested the activity of DL1 in planta. *BRANCHED1 (BRC1)*, a TCP transcription factor gene, and *STH7*, a double B-box domain transcription factor, are known as strigolactone-responsive genes.[9,17] We investigated whether the (+)-GR24-induced expression of *BRC1* and *STH7* could be inhibited by DL1. The results of real-time PCR experiments using 10-day-old seedlings of *Arabidopsis* are shown in Figure 3a. Consistent with previous reports, the expression of *BRC1* and *STH7* genes were increased by the treatment with (+)-GR24 for 24 h. The (+)-GR24-induced expression of *BRC1* and *STH7* were suppressed by DL1 (Figure 3a). Moreover, treatment of *Arabidopsis* with DL1 alone displayed a significant decrease in the expression level of *STH7*, indicating that the signaling of endogenous strigolactones was inhibited by DL1.

The extent of strigolactone signaling is suppressed by negative feedback regulation of AtD14. After activating downstream signal transduction, AtD14 protein is degraded through a MAX2-dependent proteasomal pathway.[26] We investigated whether DL1 inhibits the strigolactone-dependent AtD14 degradation using transgenic *Arabidopsis* plants expressing AtD14-GFP fusion protein. As reported previously, a striking reduction of GFP signal in hypocotyl was observed by the treatment with (+)-GR24 for 24 h (Figure 3b). The reduction of fluorescence was alleviated by DL1, indicating that DL1 inhibits strigolactone-dependent interaction of AtD14 with MAX2 within plant cell. Furthermore, the treatment of DL1 alone displayed brighter fluorescence than the no-treatment control, indicating that DL1 inhibits basal signaling induced by the endogenous strigolactones. This result was further validated at the root tissue (Figure 3c). Only a faint fluorescence was observed in a control experiment due to the degradation of AtD14-GFP induced by

DOI: 10.1021/acscentsci.7b00554
ACS Cent. Sci. 2018, 4, 230−234

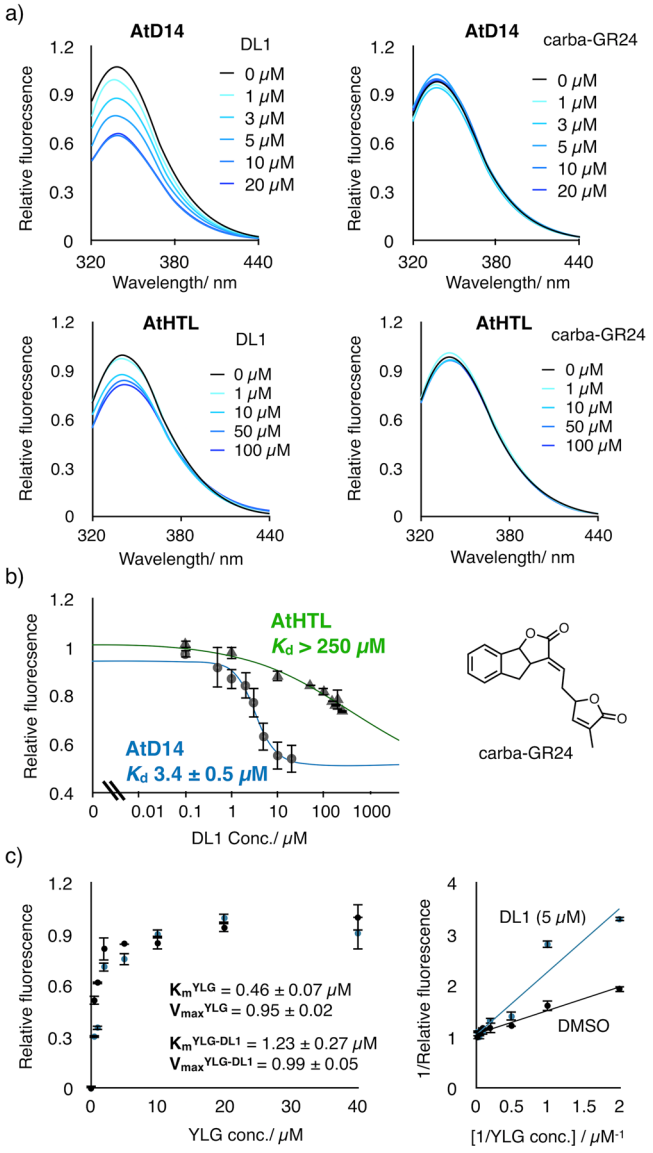


Figure 2. Biochemical analysis for the AtD14 inhibition by DL1. (a) Quenching of AtD14 or AtHTL intrinsic fluorescence by DL1. The intrinsic fluorescence of AtD14 and AtHTL was measured in the presence of DL1 or carba-GR24 at the indicated concentration (ex. 288 nm). (b) The relative fluorescence intensity of AtD14 (blue) and AtHTL (green) at 340 nm was plotted against DL1 concentrations. Error bar indicates SE (n = 3 biological replicates). (c) Michaelis–Menten (left) and Lineweaver–Burk plots (right) for the inhibition of AtD14 by DL1 (5 μM). K_m and V_{max} values were calculated by using the linear least-square method. Error bar indicates SE (n = 3 biological replicates).

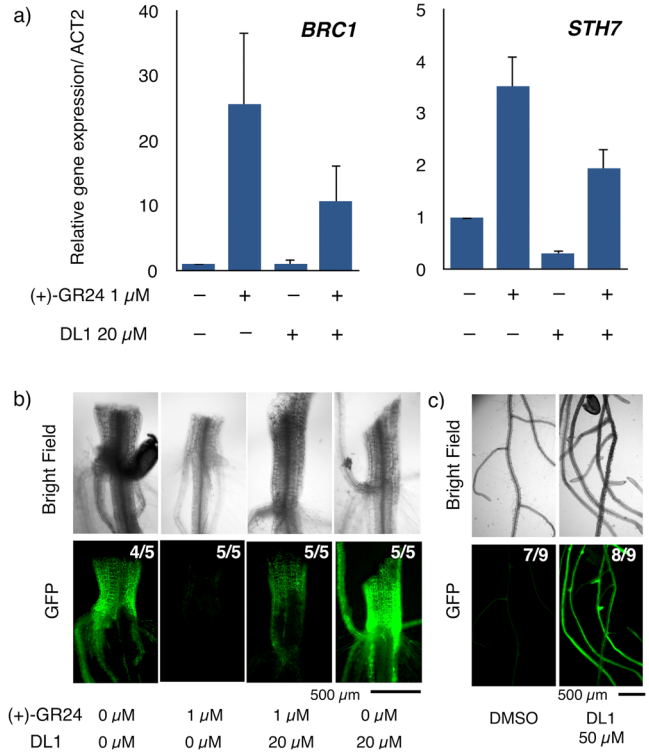


Figure 3. Inhibition of strigolactone signaling by DL1. (a) qRT-PCR analysis for the expression level of strigolactone-responsive genes, *BRC1* and *STH7*. The 10-day-old wild-type *Arabidopsis* seedlings were treated with 1 μM (+)-GR24 and 20 μM DL1 for 24 h. The relative values are normalized to *ACT2*. The value means ± SE (n = 3 biological replicates). (b, c) Bright field (top) and fluorescence (bottom) images of the hypocotyl (b) and root (c) in 2-week-old *35S::AtD14-GFP/atd14−1* seedlings treated with 1 μM (+)-GR24 and indicated concentration of DL1 for 24 h. Numerals at the upper right indicate the number of plants showing the figure phenotype per total number of plants examined.

endogenous strigolactones. In marked contrast, DL1-treated plants showed bright fluorescence in the root. These results imply that the DL1 inhibits strigolactone-dependent AtD14 degradation by competing with endogenous strigolactones.

Since DL1 was shown to inhibit the perception of endogenous strigolactones by AtD14, DL1 is expected to work as a shoot-branching enhancer. To investigate the effect of DL1 on shoot branching, *Arabidopsis* seedlings were grown in the presence of DL1 for one month. As expected, DL1-treated *Arabidopsis* displayed an increase in the number of branches (Figure 4a,b and Figure S4). In contrast, DL1 did not affect the AtHTL-dependent signaling. Karrikins as well as non-natural stereoisomers of GR24 were previously reported to stimulate seed germination through the binding to AtHTL in *Arabidopsis*.[27] DL1 neither induced nor inhibited the seed germination induced by *racemic*-GR24, which contains non-natural stereoisomer (Figure S3). Taken together, DL1 is a selective inhibitor of AtD14 and increases the number of shoot branching in a model plant *Arabidopsis* by competing with endogenous strigolactones.

Crystallographic studies revealed that the binding pocket of rice D14 protein (OsD14) shows high similarity to that in AtD14, indicating that DL1 might inhibit OsD14 as well.[28] To test the idea, we exposed rice plants to DL1 for 40 days. The DL1-treated rice displayed an increased tiller number, indicating that DL1 inhibited the endogenous strigolactone signaling in rice as well as in *Arabidopsis* (Figure 4c,d and Figure S5). Moreover, the 40 days' exposure with DL1 did not induce apparent toxicity nor growth inhibition on the rice plants as the DL1 treatment did not significantly change shoot dry weight (Figure S6). Our study shows that DL1 can be directly applied to crop species.

In conclusion, we have harnessed the YLG-based competition assay for a high-throughput screening of AtD14 ligands. This screening enabled identification of DL1 as the first AtD14-selective inhibitor that induces shoot branching in *Arabidopsis* and rice. Because the structure of strigolactone-binding pocket in D14 is conserved in angiosperm species,[8] DL1 may induce shoot branching in these plant species. DL1 can be a potent agrochemical to improve the crop yield as well as a tool to

DOI: 10.1021/acscentsci.7b00554
ACS Cent. Sci. 2018, 4, 230–234

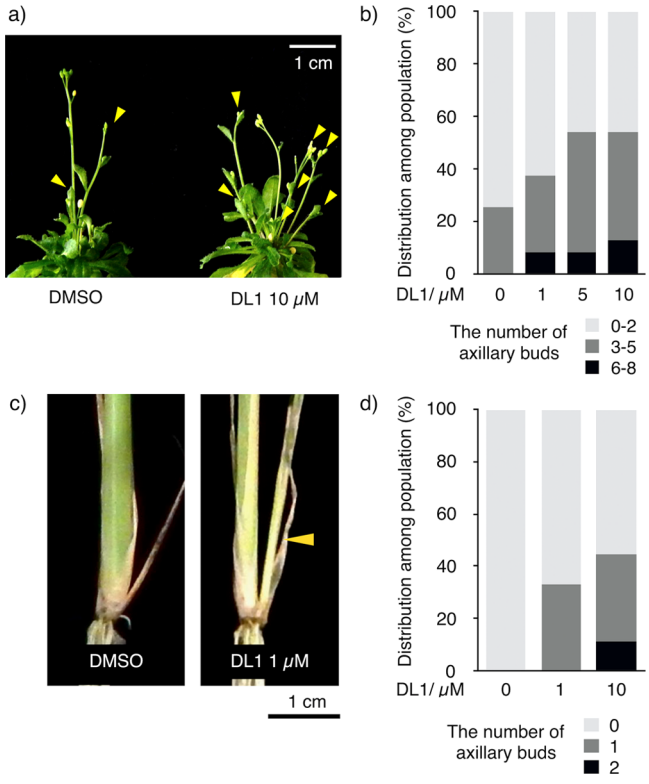


Figure 4. DL1-enhanced shoot branching in *Arabidopsis* and rice. (a) 30-day-old wild-type *Arabidopsis* plants treated with DMSO or 10 μM DL1. The yellow arrowheads indicate axillary buds. (b) Distribution diagram of plants with primary rosette branches at least 0.5 cm long (n = 24). (c) 40-day-old rice (nipponbare) treated with DMSO or 1 μM DL1. The yellow arrowhead indicates axillary bud. (d) Distribution diagram of plants with tillering (n = 9).

understand the biological roles of D14 receptors in plant growth and development. Feasibility studies of DL1 such as soil persistence and human toxicity are now ongoing.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00554.

Synthesis of DL1; NMR spectrum; Supplementary Figures S1−S5; Materials and methods (plant culture and treatment, RT-PCR analysis, protein purification, YLG-based assay, LC-MS based analysis, intrinsic fluorescence assay, seed germination assay (PDF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Tsuyoshi Nakagawa for providing pGWB605. This work was funded by a Grant-in-Aid for Scientific Research on Innovative Areas "Chemistry for Multimolecular Crowding Biosystems" (JSPS KAKENHI Grant No. JP17H06350) and PRESTO (Japan Science and Technology Agency, JPMJPR15Q9).

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