# Discovery of shoot branching regulator targeting strigolactone receptor DWARF14

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1. General

Unless otherwise noted, all reactants or reagents including dry solvents were obtained from commercial suppliers and used as received. Unless otherwise noted, all reactions were performed with reagent-grade solvents under air. All work-up and purification procedures were carried out with reagent-grade solvents in air. Analytical thin-layer chromatography (TLC) was performed using E. Merck silica gel 60 F_{254} precoated plates (0.25 mm). The developed chromatogram was analyzed by UV lamp (254 nm). Flash column chromatography was performed with KANTO silica gel 60N (0.04-0.1 mm). Preparative thin-layer chromatography (PTLC) was performed using Wakogel B5-F silica coated plate (0.75 mm) prepared in our laboratory. The high-resolution mass spectra were conducted on Thermo Fisher Scientific™ Exactive™. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL JNM-ECA-400 (^{1}H 400 MHz, ^{13}C 100 MHz). Chemical shifts for ^{1}H NMR are expressed in parts per million (ppm) relative to tetramethylsilane (δ 0.00 ppm) or residual peak of CDCl_{3} (δ 7.26 ppm). Chemical shift for ^{13}C NMR are expressed in ppm relative to CDCl_{3} (δ 77.0 ppm). Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad signal), coupling constant (Hz), and integration.
2. Synthesis of DL1

![Synthesis of DL1 diagram]

**Synthesis of 1**
To a solution of 7-ethylindole (200 mg, 1.37 mmol) and pyridine (140 mg, 1.3 equiv.) in toluene (3.0 mL, 0.45 M), chloroacetyl chloride (205 mg, 1.3 equiv.) was slowly added under argon at 0 °C. After stirring at 60 °C for 1 h, the reaction mixture was diluted with H₂O. The solution was extracted with CHCl₃ three times. The combined organic phase was washed with H₂O and brine. The organic layer was dried over Na₂SO₄, filtrated and concentrated in vacuo. The residue was purified by PTLC (CH₂Cl₂) to afford the desired product 1 as a white solid (94 mg, 31%).

**1H NMR (400 MHz, CDCl₃)** δ 8.75 (br, 1H), 8.21 (d, J = 7.9 Hz, 1H), 7.98 (d, J = 3.1 Hz, 1H), 7.29 (t, J = 7.6 Hz, 1H), 7.16 (d, J = 7.3 Hz, 1H), 4.56 (s, 2H), 2.90 (q, J = 7.5 Hz, 2H), 1.37 (t, J = 7.6 Hz, 3H);

**13C NMR (100 MHz, CDCl₃)** δ 186.6, 135.1, 131.2, 126.9, 125.3, 123.5, 122.8, 120.0, 115.7, 46.1, 23.9, 14.0; HRMS (ESI) m/z calcd for C₁₂H₁₃ClNO₂ [M+H]+: 222.0680, found 222.0680.

**Synthesis of DL1**
To a solution of 1 (160 mg, 0.72 mmol) and 3-Bromoadamantane-1-carboxylic acid (280 mg, 1.5 equiv.) in DMF (2.0 mL, 0.36 M), K₂CO₃ (200 mg, 2.0 equiv.) was added. After stirring at room temperature for 12 h, the reaction mixture was diluted with H₂O. The solution was extracted with EtOAc three times. The combined organic phase was washed with H₂O and sat. NaHCO₃ aq. The organic layer was dried over Na₂SO₄, filtrated and concentrated in vacuo. The residue was purified by silica-gel chromatography (20% EtOAc in hexane) to afford DL1 as a colorless crystal (290 mg, 90%).

**1H NMR (400 MHz, CDCl₃)** δ 8.97 (br, 1H), 8.11 (d, J = 7.9 Hz, 1H), 7.98 (d, J = 3.1 Hz, 1H), 7.29 (t, J = 7.6 Hz, 1H), 7.12 (d, J = 7.3 Hz, 1H), 5.15 (s, 2H), 2.85 (q, J = 7.7 Hz, 2H), 2.61 (s, 2H), 2.38-2.28 (m, 4H), 2.24 (s, 2H), 2.08-1.97 (m, 4H), 1.72 (s, 2H), 1.34 (t, J = 7.6 Hz, 3H); **13C NMR (100 MHz, CDCl₃)** δ 187.6, 175.4, 135.0, 130.8, 127.3, 124.9, 123.2, 122.5, 119.4, 114.6, 65.9, 63.6, 49.5, 48.0, 44.9, 37.1, 34.3, 31.6, 23.7, 13.9; Three peaks are overlapping.; HRMS (ESI) m/z calcd for C₂₃H₂₇BrNO₃ [M+H]+: 444.1169, found 444.1169.
3. $^1$H and $^{13}$C NMR Spectra of 1 and DL1

$^1$H NMR of 1

$^{13}$C NMR of 1
$^1$H NMR of DL1

$^{13}$C NMR of DL1
Figure S1. Result for the 2nd screening. The AtD14-binding activity of 18 hit compounds were retested by a competition assay using YLG. The fluorescence intensity after the incubation of YLG (1 µM) with AtD14 (10 µg/mL) in the presence of 18 hit compounds (10 µM) were normalized to no ligand control. Red line indicates 70% inhibition on YLG hydrolysis. Error bar indicates SE (n = 3 biological replicates).
Figure S2. LC-MS based analysis of inhibition of AtD14-mediated 5DS hydrolysis by DL1. (a) Scheme of the inhibition of AtD14-mediated 5DS hydrolysis by DL1. (b) The mass intensity of 5DS (331.1540, ESI positive) and ABC-ring (233.1660, ESI negative) were measured after incubating 5DS (0.1 µM) with AtD14 in the presence of DL1 at indicated concentration for 40 min. Error bar indicates SE (n = 2 biological replicates). (c) The mass intensity of DL1 (442.1023, ESI negative) was measured after incubating DL1 (20 µM) with AtD14 for 0, 20, 40 and 60 min. Error bar indicates SE (n = 2 biological replicates).
Figure S3. Strigolactone-dependent germination of Arabidopsis seed. a) Freshly harvested Arabidopsis seeds were incubated in the presence of racemic-GR24 or DL1 under continuous light at 26 °C. After incubation for 36 hours, germinating seeds were counted. Error bar indicates SE (n = 3 biological replicates). b) Arabidopsis seeds treated with racemic-GR24 and DL1. The red arrowheads indicate radicle emergences. Scale = 1 mm.
Figure S4. DL1-enhanced shoot branching in *Arabidopsis*. 30-day-old seedlings of the wild-type *Arabidopsis* treated with DL1. Numerals indicate the number of primary rosette branches.
Figure S5. DL1-enhanced shoot branching in rice. 40-day-old seedlings of rice treated with indicating concentrations of DL1. Numerals indicate the number of shoot branching.
Figure S6. Shoot dry weight of 40-day-old rice treated with DL1. After rice seedlings were incubated in the presence of indicated concentration of DL1 for 40 days, dry weight of their shoot was measured. Values are mean ± SD (n = 9 biological replicates).
5. Supplementary Material and Methods

Plant materials
The *Arabidopsis thaliana* Columbia (Col) accession was used as wild type. The nipponbare (lowland-adapted *japonica*) was used in rice branching assay. 35S::AtD14-GFP plasmid was constructed through LR recombination (Thermo Fisher Scientific) with pGWB5 and used for transformation into *atd14-1* by floral dip method (ref. 1).

Plant culture and treatment
For plant assays using *Arabidopsis*, the seeds were sterilized, stored in the dark at 4 °C for a few days, transferred to 0.5 x Murashige and Skoog (MS) culture medium (0.5% [w/v] MES, 1% [w/v] sucrose, pH 5.7 with KOH and 0.8% [w/v] agar) and grown under a 16 h/8 h light/dark cycle at 22 °C. For *Arabidopsis* branching assays, the wild-type of *Arabidopsis* were treated with various concentrations of DL1 and grown on media. After the incubation for 30 days, the number of primary rosette branches at least 0.5 cm long was counted. For *AtD14-GFP* observation, 28-week-old seedlings of the wild-type and 35S::AtD14-GFP/atd14-1 were transferred to 0.5 x MS culture medium containing 0.2% DMSO (mock), DL1 (20 µM or 50 µM) or/and (+)-GR24 (1 µM). After 24 hours, GFP fluorescence at hypocotyl and root was observed using Zeiss Axio Imager A2 and Nikon ECLIPSE Ti2. Note that the shoot was cut off before the measurement.

For rice branching assays, the rice seeds were sterilized after the seed coats were removed. The sterilized seeds were transferred to MS culture medium (0.1% myo-inositol, 3% sucrose, pH 5.8 and 0.8% [w/v] agar, 0.1% DMSO) with or without DL1, and grown under continuous light with a light intensity of 88.4 µmol·m⁻²·s⁻¹ at 30 °C. After 40 days incubation, the number of shoot branching at least 0.5 cm long was counted. The samples separated from the roots were dried at room temperature for 1 day and their dry weight were measured.

RNA extraction and RT-PCR.
10-day-old seedlings were transferred to 0.5 x MS culture medium containing 0.2% DMSO (mock), 20 µM DL1 or 1 µM (+)-GR24. After 24 hours, total RNAs were extracted and purified using ReliaPrep™ RNA Tissue Miniprep system (PROMEGA). Each RNA sample was prepared from a pool of 40 mg whole seedlings. For RT-PCR, reverse transcription was carried out using ReverTra Ace (TOYOBO) according to the manufacturer’s instructions. RT-PCR was performed using the Power SYBER Green PCR Master Mix and StepOne Real-Time PCR system (Applied Biosystems). For gene-specific amplifications of the *STH7* and *BRC1* transcripts, the following primer sets were used: for *STH7*, 5’-CATCTCCGGTTCTCTCATTTCC-3’ (forward) and 5’-CATCTTCATGCTTCC-3’ (reverse); for BRC1, 5’-CCAGTGATTAACCACCATCG-3’ (forward) and 5’-TGATGAGGTCTCTTTGTTT-3’ (reverse). Relative quantification was carried out using the comparative cycle threshold method, and the *ACT2* gene transcripts, which are amplified with the primers 5’-TCCCTCAGCATTCCCAGCAGAT-3’ (forward) and 5’-AACGATTCTTGAGCTC-3’ (reverse), were used as an internal control. The relative expression levels of the target genes were compared with the mock-treated seedlings.

Preparation of recombinant proteins
The procedure for the purification of His-tagged proteins was described previously (ref. 2). Aliquots of purified recombinant AtD14 and AtHTL were kept at −80 °C until use.

YLG-based assays
*In vitro* YLG hydrolysis assays were conducted using 1 µg of recombinant AtD14 in 100 µL of the reaction buffer (100 mM HEPES, 150 mM NaCl, pH 7.0) with 0.2% or 1.1% DMSO on a 96-well black plate (Greiner). After YLG was incubated with recombinant AtD14 for 1 hour, the fluorescence intensity was measured by spectraMax i3 (Molecular Devices) at excitation by 480 nm and detection.
by 520 nm. For high-throughput screening, 10 µM of library compounds (1% DMSO) were co-incubated with 1 µM of YLG. The high-throughput experiments were supported by using automated liquid handling system (Sciclone ALH3000, Perkin Elmer). 800 library compounds were selected from the ITbM chemical library (20101 compounds, 1 mM DMSO solution, specially prepared reagent for biochemical research, Nakalai-tesque, Code: 13445-45) purchased from ChemDiv, Inc (San Diego, CA, USA) and Enamine, Inc (Kiev, Ukraine). For dose-dependent inhibition assays, DL1 and other hit compounds at the range between 0.1 to 10 µM were treated with 1 µM YLG in the presence of recombinant AtD14. IC₅₀ values were calculated by a curve-fitting program in Kaleidagraph software. For Lineweaver-Burk plot analysis, AtD14-mediated hydrolysis of YLG at the range between 0.1 to 40 µM was conducted in the presence or absence of DL1. Kₘ and Vₘₐₓ values were calculated by using the linear least square method.

**LC-MS based analysis**

10 µg of recombinant AtD14 was incubated with 0.1 µM of 5DS and DL1 at the range between 1 to 20 µM in 1 mL of the reaction buffer (20 mM Tris, 200 mM ammonium acetate, pH 7.0) with 0.2% DMSO. After the incubation for 40 min, the reaction mixture was analyzed with LC-MS. HPLC was carried out on a Dionex Ultimate 3000 HPLC system equipped with an autosampler (Thermo Fisher Scientific, San Jose, CA). The HPLC system was interfaced with an EXACTIVe Plus (Thermo Fisher Scientific) Fourier transfer mass spectrometer with an electrospray ionization source. Data acquisition and analysis were performed with Xcalibur software (version 2.2). A Cadenza CD-C18 column (12 nm, 3 µm, 3 × 150 mm) (Imtakt, Kyoto, Japan) was used. The chromatography mobile phases were solvent A (10 mM aqueous ammonium acetate (pH 7.0)) and solvent B (acetonitrile). The column was developed at a flow rate of 200 µl min⁻¹ with the following concentration gradient of acetonitrile: sustaining 1% B for 2 min, from 1% B to 95% B in 0.5 min, sustaining 95% B for 6.5 min, from 95% B to 1% B in 0.1 min, and finally re-equilibrating with 1% B for 7 min. The electrospray ionization source was operated in positive and negative ion mode. Filtering mass at 442.1023 (DL1, tolerance of 10 ppm: 442.0979-442.1067, ESI negative), 233.1660 (ABC-ring, tolerance of 10 ppm: 233.1137-233.1183, ESI negative) and 331.1540 (5DS, tolerance of 10 ppm: 331.1507-332.1573, ESI positive). By the methods retention time for small-molecules were as follows; DL1 at 12.7 min ABC-ring at 10.4 min and 5DS at 11.6 min.

**Intrinsic tryptophan fluorescence assay**

For investigating the affinity of small molecule to AtD14, 10 µM of recombinant AtD14 was incubated with small molecules at the range of 0 to 20 µM in 100 µL of the reaction buffer (10 mM HEPES, 150 mM NaCl, pH 7.0) with 0.1% DMSO on a 96-well black plate (Greiner). For investigating the affinity of small molecule to AtHTL, 10 µM of recombinant AtHTL was incubated with small molecules at the range of 0 to 250 µM in 100 µL of the reaction buffer (20 mM HEPES, 150 mM NaCl, 5% glycerol, pH 7.0) with 1% DMSO on a 96-well black plate (Greiner). After incubation at room temperature for 30 min, intrinsic fluorescence was measured at excitation by 288 nm and detection by 320-440 nm. The fluorescence intensity at 340 nm was plotted against the concentration of small molecules, and the dissociation constant (Kᵰ) was calculated by a curve-fitting program in Kaleidagraph software.

**Seed germination assay**

Freshly harvested seeds of *Arabidopsis* were suspended in distilled water and aliquoted in 48-well plates. racemic-GR24 and DL1 were added to the seeds (DMSO 0.2%). The seeds were incubated under continuous light at 26 °C. After the incubation for 36 hours, radicle emergences were counted under a dissecuting microscope.
6. References
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