Identification of new abscisic acid receptor agonists using a wheat cell-free based drug screening system

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Supplementary Methods

Synthesis of JFA2 and QFA

For synthesis of 

\[ N-(1,2,3,4\text{-tetrahydro-2-oxo-6-quinolinyl})-4\text{-trifluoromethoxybenzenesulfonamide} \]

(QFA, quinoline and fluorine containing ABA receptor activator), 6-amino-3,4-dihydro-2(1H)-quinolinone, (20.5 mg, 0.126 mmol) was dissolved in dichloromethane (0.6 mL), and triethylamine (42.4 mg, 0.419 mmol) was added while stirring followed by dropwise addition of 4-trifluoromethoxybenzenesulfonyl chloride, (35.9 mg, 0.138 mmol). After stirring at room temperature overnight, water was added to the reaction mixture. The mixture was washed successively with water, 1N HCl aq. (twice), saturated sodium hydrogen carbonate solution and brine. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The crude product was dissolved in ethyl acetate and subjected to silica-gel column chromatography (hexane-ethyl acetate as the eluent) to afford the product as a colorless solid (18.2 mg, 37 %). \[^1\text{H} \text{NMR (400 MHz, CDCl}_3\text{)} \delta 7.85 - 7.77 (m, 2H), 7.33 - 7.25 (m, 2H), 6.73 (s, 1H), 6.70 (s, 1H), 6.63 (s, 1H), 3.88 - 3.80 (t, \text{J} = 6.2 \text{ Hz}, 2H), 2.79 (dd, \text{J} = 8.8, 6.1 \text{ Hz}, 2H), 2.69 (t, \text{J} = 6.2 \text{ Hz}, 2H), 2.61 (dd, \text{J} = 8.8, 6.1 \text{ Hz}, 2H), 1.90 (quint, \text{J} = 6.2 \text{ Hz}, 2H). \]

For synthesis of 

\[ N-(1,2,3,4\text{-tetrahydro-2-oxo-6-quinolinyl})-4\text{-trifluoromethoxybenzenesulfonamide} \]

(2,3,6,7-tetrahydro-3-oxo-1H,5H-benzo[jj]quinolizin-9-yl)-4-trifluoromethoxybenzenes
ulfonamide (JFA2, julolidine and fluorine containing ABA receptor activator 1), 9-amino-2,3,6,7-tetrahydro-1H,5H-benzo[ij]quinolizine-5-one (5.2 mg, 0.026 mmol) was dissolved in dichloromethane (0.1 mL), and triethylamine (3.8 mg, 0.038 mmol) was added while stirring followed by dropwise addition of 4-trifluoromethoxybenzenesulfonyl chloride (8.8 mg, 0.034 mmol). After stirring at room temperature for 10 h, water was added to the reaction mixture. The mixture was washed successively with water, 1N HCl aq. (twice), saturated sodium hydrogen carbonate solution, and brine. The organic layer was dried over anhydrous sodium sulfate and evaporated to dryness in vacuo. The crude product was dissolved in ethyl acetate and subjected to preparative TLC on silica-gel (hexane-ethyl acetate as the eluent) to afford the product as colorless solid (5.7 mg, 50 %). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.17 (s, 1H), 7.83 – 7.74 (m, 2H), 7.31-7,26 (m, 2H), 6.97 (s, 1H), 6.94 (br s, 1H), 6.83 (dd, $J$ = 8.4, 2.4 Hz, 1H), 6.65 (d, $J$ = 8.4 Hz, 1H), 2.91 (t, $J$ = 7.6 Hz, 2H), 2.61 (t, $J$ = 7.6 Hz, 2H).

Construction of the in vitro transcription templates

The genes of PYR1, PYL1, PYL2, PYL4-6, PYL8, and PYL11 were amplified by PCR from the RIKEN Arabidopsis full-length (RAFL) cDNA library. The genes of PYL3, PYL7, PYL9, PYL10, PYL12, and PYL13 were amplified by PCR using total cDNA prepared from mRNA of Col-0 plant as a template (described below).
The DNA fragments of the open-reading frame (ORF) were cloned in a pDONR221 vector using the gateway cloning system (Thermo Fisher Scientific). After confirming sequences, we generated these expression vectors by LR Clonase recombination with pEU-His-blz-GW vectors for in vitro transcription. Then, these regions of the gene containing ORF and tag sequence were amplified by PCR and used as transcription templates.

For preparation of in vitro transcription templates for FLAG-PP2CAs and biotinylated SnRK1.1, the genes of ABI1, ABI2, HAB1, HAI1, HAI2, AHG1, AHG3, and SnRK1.1 were amplified by the split-primer PCR method\(^1\) using the RAFL cDNA library as the template. For preparation of in vitro transcription templates for AGIA-tagged ABA receptors, gene regions of ABA receptors were amplified by the split-primer PCR method\(^1\) using the genes sub-cloned into pDONR 221 as templates. All primer sequences and plasmids used in this study are listed in supplementary Table 2 and Table 3.

**Chemical library screening**

For the AlphaScreen-based chemical library screening, we used a diverse set of 9,600 synthesized chemicals established by the Drug Discovery Initiative (The University of Tokyo, Tokyo, Japan). All compounds dissolved in DMSO were predispensed on a 384-well Optiplate (250 nL/well) with the concentration of 100 µM,
and then 15 µL of PYR1/ABI1 mixture containing AlphaScreen buffer, 1 µL of biotinylated PYR1, 1 µL of FLAG-ABI1, and 1 µM ABA (0.067% DMSO), or 0.067% DMSO were dispensed to each well by using a FlexDrop dispenser (PerkinElmer). These reaction mixtures contained DMSO at a 1.73 % final concentration. Each 384-well plate contained 16 negative control wells (containing biotinylated PYR1 and FLAG-DHFR) and 16 positive control wells (containing biotinylated PYR1, FLAG-ABI1, and 1 µM ABA). After incubation at 25 °C for 1 h, 10 µL of detection mixture were added to each well of the 384-well Optiplate by using a FlexDrop dispenser (PerkinElmer), followed by incubation at 25 °C for 1 h. The composition of the detection mixture was described above (see interaction analysis of ABA receptors and PP2CAs). The final concentration of each compound was 1 µM, ABA was 0.6 µM, and DMSO was 1% in the final solution. Luminescence was analyzed using the AlphaScreen detection program. Data were expressed as relative values normalized by the value of the AlphaScreen signal between biotinylated PYR1 and FLAG-ABI1 in the presence of ABA only.

Docking study of Arabidopsis ABA receptor, PYR1 with JFA1/JFA2/Qui

We used 3NJO2 as a template for the computational docking study of PYR1 with JFA1/JFA2 because we assumed that binding modes of JFA1/JFA2 were similar to that of an ABA agonist, pyrabactin with PYR1 regarding their sulfonamide. Additionally,
we used a homology model of PYR1-quinabactin complex constructed based on PYL2-quinabactin complex (4LA3) as JFA1/JFA2 is structurally similar to quinabactin than pyrabactin. Docking studies and homology modeling were performed by using Molecular Operating Environment (MOE) software package (MOE, 2014.09; Chemical Computing Group Inc., Montreal, QC, Canada) with the Amber10:EHT force field. During docking simulations, we used template forcing mode, targeting sulfonamide of pyrabactin or quinabactin, with the induced fit method, including water molecules. To confirm the results, we performed two types of induced fit modes, tether and free modes: side chains of receptor pocket are tethered with a constant weight and free to move, respectively. We chose the best models based on the GBVI/WSA dG score, which estimates the free energy of binding for the ligands, and summarized the results in supplementary Table 1.

Transcriptome sequencing (RNA-seq) analysis and gene ontology biological process enrichment analysis

For RNA-seq analysis, 20 day-old-plants, after germination on half-strength MS agar plate, were sprayed with chemical solutions containing 50 µM ABA, JFA1, or JFA2, and 0.04% Silwet L-77. After incubation for 5 h, above-ground part of plants were homogenized, and total RNA was extracted using TRI Reagent (Sigma). Total RNA was treated with DNase I (TaKaRa) for 30 min at 37 °C, and then purified using
the RNeasy mini kit (Qiagen). The concentration and quality of the RNA was verified with the Quantus Fluorometer (Promega) and Agilent 2100 Bioanalyzer, respectively. All the samples had RIN values over 7. Sequencing libraries were prepared according to the manufacturer’s instructions for the Agilent SureSelect Strand-Specific RNA Library Prep for Illumina. Briefly, poly-A RNA was purified from 300 ng total RNA per sample using oligo dT magnetic beads. The libraries were PCR amplified for 13 cycles and purified with AMPure XP beads. Sequencing of the libraries was conducted on the Illumina HiSeq1500 system performing single-end 50 bp reads.

Each read was assessed for quality using the FASTX Toolkit (v0.0.13) software. Base trimming was done from the 3’ end of each read to remove bases with quality below Q30 up to a minimum of 25 bp. Reads shorter than 25 bp were removed before further analysis. The reads were mapped with the TAIR10 genome using Strand NGS software (v2.6, Agilent Technologies). After DeSeq normalization, we analyzed the gene expression profile across the samples.

The gene ontology biological process enrichment analysis was performed by the DAVID functional annotation clustering tool (http://david.abcc.ncifcrf.gov/home.jsp). Differentially expressed genes (1232 genes, log₂ fold change > 1 or < -1) responsive to both ABA and JFA2 treatments were annotated by the Arabidopsis Genome Initiative (AGI) locus codes, and were analyzed for gene ontology terms (counts ≥ 2 and a modified Fisher Exact p value, EASE score ≤
Supplementary References

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Supplementary Figure 1: Expression of recombinant ABA receptors and PP2CAs.
(a) Immunoblot analysis of AGIA-tagged PYR1 and PYL1-13. Expressed recombinant proteins were analyzed by immunoblotting anti-AGIA antibody. (b) Immunoblot analysis of recombinant N-terminal FLAG-tagged PP2CAs using anti-FLAG antibody. FLAG-HAB2 protein was not detected by immunoblot analysis. All recombinant proteins were expressed by a wheat cell-free system. The asterisk indicates a nonspecific band.
Supplementary Figure 2: Overview of interaction map of 14 ABA receptors and 7 PP2CAs. Interaction between 14 biotinylated ABA receptors and 7 FLAG-tagged PP2CAs in the absence (a) or presence of ABA (b). Blue lines, interactions in the absence of ABA; red lines, interactions in the presence of ABA; blue or red solid lines, interactions revealed in this study; blue or red dotted lines, interactions consistent with previous studies (BioGRID); grey lines, interactions has been reported in previous studies (BioGRID). HAB2 and HAI3 were not analyzed in this study.
Supplementary Figure 3: Overview of interaction map of ABA receptors.
Interaction between 14 biotinylated ABA receptors and 14 AGIA-tagged ABA receptors in the absence or presence of ABA. Blue lines, interactions in the absence of ABA; red lines, interactions in the presence of ABA; blue or red solid lines, interactions revealed in this study; blue or red dotted lines, interactions consistent with previous studies (BioGRID); grey dotted lines, interactions has been reported in previous studies (BioGRID);
Supplementary Figure 4: Chemical compounds screening based on a wheat cell-free system.

(a) Over-view of 9,600 chemical compounds screening based on analysis of protein-protein interaction (PPI) between biotinylated PYR1 (biotin-PYR1) and FLAG-ABI1 (a detailed method is described in the method). (b) Result of 9,600 chemical compounds screening. Data were expressed as relative values normalized by the value of the AlphaScreen signal between biotinylated PYR1 and FLAG-ABI1 in the presence of 1μM ABA only. The broken line indicates 15 % enhancement (red) or inhibition (blue). (c) Flow chart of ABA agonist or antagonist candidates validation to identify compounds.
Supplementary Figure 5: Stomatal closure test in intact-plant.

Four-week old plants were sprayed with chemical solutions containing with 25 μM chemical, ABA, JFA1, or JFA2, and 0.02% Silwet L-77. Then plants were incubated in a growth cabinet at 22 °C under a light condition. After incubation for 3, 6 and 24 hr, leaves were detached and treated with water containing with 1 μM rhodamine 6G (Sigma) for 2 min. Imaging of stomata was performed with a fluorescence microscope IX-73 (Olympus). Image analysis was performed using ImageJ software (https://imagej.nih.gov/ij/). The width and the length of the stomatal aperture were measured, and the stomatal aperture index was calculated by division of the aperture width through the length. Relative stomatal aperture index was expressed as a relative value with the DMSO-treatment as one. Stomatal aperture indexes are calculated from three independent experiments with 30 stomata per treatment. Error bars represent standard error. Statistically significant changes compared with mock control (DMSO) are indicated (*P < 0.05, **P < 0.01, two-tailed Student’s t-test)
Figure 3d

Supplementary Figure 1a

Supplementary Figure 1b

Supplementary Figure 6: Uncropped blot images.
**Supplementary Table 1.** The best scores of docking simulations of PYR1 with JFA1, JFA2, and Qui. HM designates “Homology Model”.

| Compounds | JFA1 | JFA2 | Qui |
|-----------|------|------|-----|
| Docking modes/Template | HM  | 3NJO | HM  | 3NJO | HM  |
| tether    | -9.6734 | -10.1663 | -11.0097 | -9.1457 | -12.4822 |
| free      | -12.8619 | -9.5284 | -11.9113 | -10.9391 | -13.6150 |
### Supplementary Table 2: List of PCR primers

| Gene name | Primer name | Primer sequence | Notes | Use for |
|-----------|-------------|-----------------|-------|---------|
| R-ACTIN4  | F-ACTIN4    | TTCTGTCAGCCATTGACCAAAGAA |       |         |
| R-RD29A  | R-RD29A     | GGAGACTCATCAGTCACTTCCA | lower case letters indicate S1-linker sequence | cloning |
| R-RAB18  | F-RAB18     | GGCTGGGATATTCTATTTTATTCTTTTCTTG |       | cloning |
| AODA2306 | pDONR221 1stA4080 | CTAGGTCGGAGAAGCCGTTGG | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| SPu       | deSP6E02-Flag-S1 | TCAACTTCCTATTATGGACTACAAGGATGACGATGACAAAGCT | red color indicate AGIA tag epitope | cloning |
| S1-ABI1   | S1-ABI1     | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-ABI2   | S1-ABI2     | GTTACTGATCCCACCAAAGAAGA | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-AGIA   | S1-AGIA     | TCGACGTCAGACCCGTTTACTTAC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-AMCD   | S1-AMCD     | GGCTGGGATATTCTATTTTATTCTTTTCTTG |       | cloning |
| S1-AN5    | S1-AN5      | ATGGAGGAAGTATCTCCGG | lower case letters indicate S1-linker sequence | cloning |
| S1-ASG    | S1-ASG      | ATGGCGGATATTTGTTATG | lower case letters indicate S1-linker sequence | cloning |
| S1-ATH    | S1-ATH      | ATGGCTGGGATATTCTATTTTATTCTTTTCTTG |       | cloning |
| S1-BA2    | S1-BA2      | ATGGCTGGGATATTCTATTTTATTCTTTTCTTG |       | cloning |
| S1-BA3    | S1-BA3      | ATGGCTGGGATATTCTATTTTATTCTTTTCTTG |       | cloning |
| S1-BA7    | S1-BA7      | ATGGGAGCAAATCTACAGAA | lower case letters indicate S1-linker sequence | cloning |
| S1-BC1    | S1-BC1      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-BC2    | S1-BC2      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-BC3    | S1-BC3      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-BC4    | S1-BC4      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-BC5    | S1-BC5      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-BR1    | S1-BR1      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C1     | S1-C1       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C2     | S1-C2       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C3     | S1-C3       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C4     | S1-C4       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C5     | S1-C5       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C6     | S1-C6       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C7     | S1-C7       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C8     | S1-C8       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C9     | S1-C9       | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| S1-C10    | S1-C10      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |

**Split-primer PCR**

**Gene specific primers**

| AGI1     | S1-AH1      | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |
| AODA2306 | pDONR221 1stA4080 | CTAGGTCGGAGAAGCCGTTGG | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence | cloning and split-primer PCR |

**Universal primers**

| deSNP220-Flag-S1 | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence, green color indicate FLAG tag epitope | cloning and split-primer PCR |
| deSNP220-AGA-S1 | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence, red color indicate AGA tag epitope | cloning and split-primer PCR |

**RT-qPCR**

| AGI2300 | RT-qPCR | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence, green color indicate FLAG tag epitope | cloning and split-primer PCR |
| AGI2800 | RT-qPCR | TGCAGACCCGTTTACGTTGCAGTTC | lower case letters indicate T1-linker sequence, underline indicate S1-linker sequence, red color indicate AGA tag epitope | cloning and split-primer PCR |
| Gene name | Plasmid backbones | expressed proteins |
|----------|------------------|--------------------|
| PYL9     | pEU-His-bis-GW-STOP | His-biotin-PYL9    |
| PYL7     | pEU-His-bis-GW-STOP | His-biotin-PYL7    |
| PYL8     | pEU-His-bis-GW-STOP | His-biotin-PYL8    |
| PYL10    | pEU-His-bis-GW-STOP | His-biotin-PYL10   |
| PYL11    | pEU-His-bis-GW-STOP | His-biotin-PYL11   |
| PYL12    | pEU-His-bis-GW-STOP | His-biotin-PYL12   |
| PYL13    | pEU-His-bis-GW-STOP | His-biotin-PYL13   |
| PYL5     | pEU-His-bis-GW-STOP | His-biotin-PYL5    |
| PYL6     | pEU-His-bis-GW-STOP | His-biotin-PYL6    |
| PYL4     | pEU-His-bis-GW-STOP | His-biotin-PYL4    |
| PYR1     | pEU-His-bis-GW-STOP | His-biotin-PYR1    |
| PYL1     | pEU-His-bis-GW-STOP | His-biotin-PYL1    |
| PYL3     | pEU-His-bis-GW-STOP | His-biotin-PYL3    |
| PYL2     | pEU-His-bis-GW-STOP | His-biotin-PYL2    |
| PYL9     | pDONR221          | -                  |
| PYL7     | pDONR221          | -                  |
| PYL8     | pDONR221          | -                  |
| PYL10    | pDONR221          | -                  |
| PYL11    | pDONR221          | -                  |
| PYL12    | pDONR221          | -                  |
| PYL13    | pDONR221          | -                  |
| PYL5     | pDONR221          | -                  |
| PYL6     | pDONR221          | -                  |
| PYL4     | pDONR221          | -                  |
| PYR1     | pDONR221          | -                  |
| PYL1     | pDONR221          | -                  |
| PYL3     | pDONR221          | -                  |
| PYL2     | pDONR221          | -                  |