Chapter 11

Chemical Control of ABA Receptors to Enable Plant Protection Against Water Stress

Masanori Okamoto and Sean R. Cutler

Abstract

This chapter describes procedures to find small molecules that bind to abscisic acid (ABA) receptors and improve plant tolerance to water stress. Abscisic acid promotes the interaction between ABA receptors and protein phosphatase 2Cs (PP2Cs), which are negative regulators of ABA signaling. This receptor-mediated ABA-dependent inhibition of PP2C activity is required for ABA action in vivo. ABA agonists can be identified by high-throughput screening for molecules that promote agonist-induced ABA receptor–PP2C interactions using yeast two-hybrid assays. In addition to in vitro assays, an in vivo test to evaluate ABA agonist activity has been devised in which an ABA-inducible GUS reporter strain is used to evaluate the effect of each ABA agonist. The effects of ABA agonists can also be determined using thermal imaging analyses and a water loss assay of stomata. Finally, the ability of each ABA agonist to confer drought stress tolerance upon plants grown in soil is determined. These methods can be used to identify various ABA agonists that protect plants against water stress.

Key words Abscisic acid, Abscisic acid-responsive reporter transgenic plant, Agonist, Arabidopsis, Biosensor, Chemical screening, Drought stress, Thermal imaging camera, Yeast two-hybrid system

1 Introduction

The plant hormone abscisic acid (ABA) regulates drought stress tolerance, stomatal closure, and seed dormancy. The action of ABA is controlled by both endogenous ABA levels and ABA signal transduction [1, 2]. Identifying compounds that control ABA metabolism and signaling is useful for understanding hormone action at the molecular level. Additionally, such compounds may be suitable for the development of plant growth regulators to control the physiological action of ABA. Conventional plant growth regulators have been generated by organic synthesis. The identification of ABA metabolic and signaling components allows for the discovery of compounds targeting specific factors from small-molecule libraries using molecular biology methods. New chemistry methods have led to the generation of vast numbers of

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small molecules suitable for high-throughput screening. Consequently, several new compounds affecting ABA action have been reported recently [3–7].

In plants, ABA is perceived by pyrabactin resistance1/PYR1-like (PYR/PYL) receptors, which belong to the START protein family [6, 8]. On ABA binding, the ABA-PYR/PYL complex binds to and inhibits group-A protein phosphatase 2Cs (PP2Cs) including HAB1, ABI1, and ABI2 (Fig. 1) [6, 9, 10]. Then, ABA-mediated PP2C inhibition leads to the activation of SNF1-related protein kinase subfamily 2s (SnRK2s), and the SnRK2s phosphorylate downstream target proteins including basic leucine zipper (bZIP) transcription factors and slow anion channel 1 (SLAC1) [9, 11–14]. Thus, a small molecule that promotes the interaction between the ABA receptor and PP2C functions as an ABA agonist. To find ABA agonists, an ABA sensor to detect ABA-like small molecules has been created using the auxotrophic yeast two-hybrid system expressing both the ABA receptor and PP2C (Fig. 2) [5]. This ABA sensor enables high-throughput screening of large numbers of small molecules. Together with in vitro screening, it is possible to search efficiently for compounds that induce ABA action in plants using an ABA-responsive GUS reporter in transgenic *Arabidopsis* plants to monitor the ABA response. Finally, nondestructive measurement of transpirational water loss from leaves by a thermal imaging camera and a drought stress test of soil-grown plants can reveal whether the identified ABA agonist truly confers drought stress tolerance. The approach described here can be used to find novel ABA agonists in various chemical libraries using ABA receptors from different plant species.

2 Materials

2.1 Chemical Library

1. 96-Well polypropylene plates.
2. Dimethyl sulfoxide (DMSO).
3. Aluminum sealing tape for 96-well microplates.
4. (+)-Abscisic acid (ABA).
5. Chemical library (12,000 compounds (Life Chemicals Inc.), diversity collection picked by University of California, Riverside; structure data for the libraries screened can be downloaded from http://chemminedb.ucr.edu/intro/data-sources/).

2.2 Yeast Two-Hybrid System

1. Yeast (*Saccharomyces cerevisiae*) MAV99 [15] and Y190 strains.
2. pACT and pBD GAL4 plasmids.
3. YPD medium: 20 g Peptone, 10 g yeast extract, 20 g glucose, pH 6.5, and final volume adjusted to 1 L. Autoclaved and stored at 4 °C.

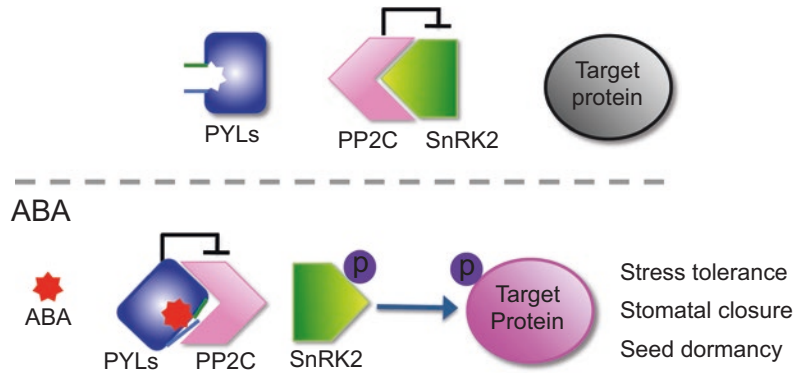

Fig. 1 ABA signaling pathway in higher plants

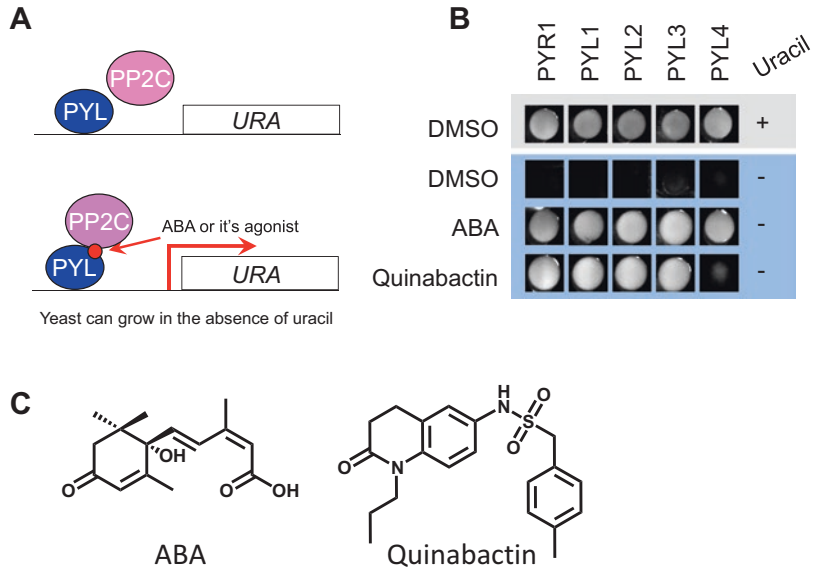

Fig. 2 Yeast two-hybrid assay for high-throughput screening. (**a**) Diagram of yeast two-hybrid system using uracil auxotrophic strain. In the absence of uracil in the medium, this yeast system can grow in the presence of ABA or its agonist. (**b**) Uracil auxotrophic yeast two-hybrid assays on SD medium in the absence of uracil, but containing DMSO, ABA, and quinabactin (Quina). (**c**) Chemical structures of ABA and quinabactin (synthetic ABA agonist)

4. Lithium acetate (1 M): Autoclaved and stored at 4 °C.
5. Polyethylene glycol 4000 (PEG 4000): Dissolved in water to 50% (w/v), filter-sterilized, and stored at room temperature (RT).
6. Salmon sperm DNA (2 mg/mL): Dissolved in water, boiled for 10 min, and kept on ice until use.
7. Transformation solution: 240 μL 50% w/v PEG, 36 μL 1 M LiOAc, 25 μL 2 mg/mL salmon sperm DNA.

8. SD plates: 182.2 g D-sorbitol, 20 g glucose, 6.7 g nitrogen base without amino acids, 20 g agar, pH adjusted to 5.8, and final volume adjusted to 1 L. To make SD -Leu/-Trp/-Ura plates, 620 mg DO supplement -Leu/-Trp/-Ura (630426, Clontech) is added to 1 L SD medium. To make SD -Leu/-Trp plates, 640 mg DO supplement -Leu/-Trp (630417, Clontech) is added to 1 L SD medium. Media used to make SD plates are autoclaved. In the chemical screening, the medium contains 0.75% (w/v) agar.
9. Agar (0.5%, w/v): Agar is mixed with water, then melted in a microwave oven, and kept at 60 °C until use.
10. Chloroform.
11. Z buffer: 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.25 g $MgSO_4 \cdot 7H_2O$, pH adjusted to 7.0, and final volume adjusted to 1 L. Buffer is autoclaved and stored at RT. Before use, 0.5% (w/v) agar is added to Z buffer and melted in a microwave oven before adding 0.27% (v/v) β-ME and 1.67% X-gal (20 mg/mL).
12. 2-Mercaptoethanol (β-ME).
13. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (20 mg/mL): Dissolved in *N*,*N*-dimethylformamide (DMF) and stored at −20 °C until use.

2.3 Protein Expression

1. *Escherichia coli* (*E. coli*) BL21(DE3) pLysS and DH5α strains.
2. Protein expression plasmids (PEX4T-1 and pET28).
3. Antibiotics (kanamycin and ampicillin): Dissolved in water at 100 mg/mL, filter-sterilized, and stored at −20 °C.
4. LB medium: 5 g Tryptone, 10 g yeast extract, 10 g NaCl, pH adjusted to 7.0, and final volume adjusted to 1 L. Autoclaved and stored at 4 °C. To make LB agar plates, 15 g agar is added to 1 L LB medium before autoclaving.
5. TB medium: 12 g Tryptone, 12 g yeast extract, 4 mL glycerol, 12.54 g K_2HPO_4, 2.31 g KH_2PO_4, 6 g NaCl. Final volume is adjusted to 1 L. Autoclaved and stored at 4 °C.
6. 10× TBS buffer: 80 g NaCl, 2 g KCl, 30 g Tris(hydroxymethyl) aminomethane (Tris), pH adjusted to 7.4, and final volume adjusted to 1 L. Autoclaved and stored at RT. Before use, 10× TBS buffer is diluted ten times with water.
7. $MnCl_2 \cdot 4H_2O$ (1 M): Dissolved in water at 1 M, filter-sterilized, and stored at RT. This reagent should not be stored for a long time. A pale pink color is important for a successful experiment.
8. Isopropyl-beta-D-thiogalactoside (IPTG): Dissolved in water at 1 M, filter-sterilized, and stored at −20 °C.

9. Reduced L-glutathione: Final concentration of 20 mM concentration in 50 mM Tris–HCl, pH 8.0. Stored at 4 °C until use.
10. Buffer A (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0): 20 mL of 500 mM NaH_2PO_4, 20 mL of 3 M NaCl, pH adjusted to 8.0, and final volume adjusted to 200 mL.
11. Imidazole: Dissolved in water at 1 M and pH adjusted to 8.0. Autoclaved and stored at RT.
12. Nickel-charged resin (Ni-NTA Agarose, 30210 Qiagen).
13. Bradford protein assay (Quick Start™ Bradford, Bio-Rad).
14. Immobilized glutathione resin (Pierce Glutathione Agarose, 16100, Thermo Fisher Scientific).
15. Dialysis tubing cellulose membrane (Spectra/Por 3, Spectrum Labs).
16. Polypropylene columns (5 mL, 34964; 1 mL, 34924, Qiagen).
17. 10× Reaction buffer for PP2C enzyme assay: 33 mL 1 M Tris-acetic acid pH 7.9, 66 mL 1 M potassium acetate. Final volume adjusted to 1 L. Diluted two times with water to make 5× PP2C reaction buffer.
18. 4-Methylumbelliferyl phosphate (4-MP): Dissolved in water at 1 M and stored at −20 °C.
19. *p*-Nitrophenyl phosphate (*p*NPP): Dissolved in 5× PP2C reaction buffer at 125 mM and stored at 4 °C. This reagent should not be stored for a long time.

2.4 GUS Expression Analysis

1. MS Gelzan plates: ½ Murashige and Skoog basal salt mixture (MS) (M5524-10 L, Sigma), 0.5% (w/v) sucrose, 0.35% (w/v) Gelzan™ CM (G1910-250G, Sigma). Autoclaved and poured into plates.
2. MS solution: ½ MS and 0.5% (w/v) sucrose. Autoclaved and stored at RT.
3. Sodium phosphate buffer (200 mM, pH 7.0): 38 mL 200 mM NaH_2PO_4 mixed with 62 mL 200 mM Na_2HPO_4. Autoclaved and stored at RT.
4. 5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide cyclohexylammonium salt (X-gluc): Dissolved in DMF at 50 mM and stored at −20 °C.
5. GUS staining buffer: 50 mM Sodium phosphate buffer, pH 7.0, 0.05% (v/v) Tween-20, 2.5 mM $K_4[Fe(CN)_6] \cdot 3H_2O$, 2.5 mM $K_3[Fe(CN)_6]$, and 1 mM X-gluc.
6. 70% Ethanol.

2.5 Expression Analysis of ABA-Responsive Genes

1. Microcentrifuge tubes (2 and 1.5 mL sizes).
2. Round stainless steel beads (2 and 5 mm sizes).
3. RNA isolation kit (PureLink™ Plant RNA Reagent, 12322012, Thermo Fisher Scientific).

4. Reverse transcription kit (QuantiTect Rev. Transcription Kit, 205311, Qiagen).
5. Real-time PCR master mix (Maxima SYBR Green qPCR Master Mix, K0221, Thermo Fisher Scientific).
6. Real-time PCR System.

2.6 Physiological Analysis of Arabidopsis

1. MS agar medium: ½ MS, 0.5% (w/v) sucrose, 0.75% (w/v) agar. Autoclaved and used for experiments in multi-well plates.
2. Soil (1:1 mixture of vermiculite:Promix BX).
3. Thermal imaging camera (FLIR T420 or Avio R300SR).

3 Methods

3.1 Chemical Preparation

1. Prepare working chemical plates at 2.5 mM concentration by diluting stock chemical libraries with DMSO. Seal prepared plates with aluminum tape and keep in the dark or at −20 °C until use in chemical screening test (*see* **Note 1**).

3.2 Preparation of Yeast Two-Hybrid System for HTS

1. Clone protein phosphatase 2C (AtHAB1) and ABA receptor (AtPYR1, AtPYL1-4) genes into pACT and pBD GAL4 plasmids, respectively (*see* **Note 2**).
2. Culture MAV99 strain up to OD_{600} = 1.0 in YPD medium.
3. Centrifuge 1 mL MAV99 culture liquid at 10,000 *g* for 1 min, discard supernatant, and suspend pellet in 0.1 M lithium acetate (LiOAc).
4. After recentrifuging, discard supernatant, suspend cells in ~300 transformation solution, and add 50 μL plasmid solutions containing approx. 1 μg pACT and pBD GAL4, respectively.
5. After adding transformation solution, thoroughly vortex mixture for 1 min until the yeast pellet is completely dissolved. Incubate mixture at 30 °C for 30 min, and then at 42 °C for 20 min.
6. Centrifuge yeast at 10,000 *g* for 1 min, discard supernatant, and spread transformed yeast suspension onto SD -Leu/-Trp plates. Incubate the plates at 30 °C for 4 days.
7. Pick up a colony, and suspend the cells in sterilized water. Prepare SD -Leu/-Trp/-Ura plates. Drop the yeast solution onto SD -Leu/-Trp plate, SD -Leu/-Trp/-Ura plate, and SD-Leu/-Trp/-Ura + 25 μM ABA plate. Check the ABA-dependent growth of yeast cells (*see* Fig. 2 and **Note 3**).
8. Suspend yeast cells in sterilized water, drop 5 μL yeast solution onto SD -Leu, -Trp plates, and incubate the plates at 30 °C for 4 days. Keep the plates at 4 °C until use in the chemical screening test (*see* **Note 4**).

3.3 Chemical Screens Using Yeast Expressing ABA Receptor and PP2C

1. Drop 1 μL 2.5 mM chemical solution into 96-well plate. Add 1 μL 1 mM ABA into an empty well as the positive control (*see* **Note 5**).
2. Keep the SD -Leu/-Trp/-Ura 0.75% (w/v) agar medium at 60 °C until use, and then add 100 μL agar medium into each well using a multichannel electronic pipette (*see* **Note 6**).
3. Suspend a previously prepared MAV99 yeast colony in sterilized water to OD_{600} = 0.2. Drop 2 μL yeast solution in the center of the each well using a multichannel electronic pipette.
4. Dry the yeast solution, and incubate the plate at 30 °C for several days; ABA agonists allow yeast growth on SD -Leu/-Trp/-Ura medium (*see* **Note 7**).
5. Perform the second screening by using the X-gal reporter system with Y190 yeast strain expressing pACT-HAB1 and pBD GAL4-ABA receptors. The preparation and transformation of the Y190 strain are described in Subheadings 3.2–3.6 above (*see* **Note 8**).
6. Prepare SD -Leu/-Trp plates containing 25 μM chemical of interest (*see* **Note 9**).
7. Drop 2 μL Y190 yeast solution onto SD -Leu/-Trp agar medium, dry yeast solution, and incubate plate at 30 °C for 4 days.
8. Protect the plastic partition and the wall of the plate by painting with melted 0.5% (w/v) agar. Then, apply chloroform to break the yeast cells, and incubate for 10 min. Remove chloroform by pipetting, and allow the remaining solution to evaporate in a fume hood (*see* **Note 10**).
9. Apply agar–Z buffer to the surface of yeast cells on the SD-Leu/-Trp agar plate. After the top agar has solidified completely, incubate the plate at 30 °C for several days, and then check blue staining (*see* **Note 11**).

3.4 Biochemical Characterization of ABA Agonists (See Note 12)

1. Clone HAB1 and ABA receptor genes into the pGEX 4T-1 and pET28 plasmids, respectively. Transform the plasmids into the L21(DE3)pLysS strain by standard heat-shock transformation. After recovery of the transformant cells at 37 °C with shaking at 200 rpm for 1 h, spread cells onto LB agar plate and incubate at 37 °C overnight. To select for transformants harboring pGEX 4T-1 and pET28, add 100 μg/mL ampicillin and 50 μg/mL kanamycin, respectively, to the medium.
2. Pick up 5–10 colonies, suspend the cells in 30% glycerol, and store at −80 °C until use in protein expression analyses.
3. To prepare GST-HAB1, add 10 mL overnight cell culture to 790 mL LB medium, and incubate at 30 °C with shaking at

200 rpm until OD_{600} = 0.5. Place flask on ice for 5 min, and then add 3.2 mL 1 M $MnCl_2$ (final 4 mM) and 240 μL 1 M IPTG (final 0.3 mM) to the culture. Incubate at 15 °C with shaking at 200 rpm for 16 h.

4. Centrifuge culture at 5000 *g* for 10 min, and then suspend the pellet in 5 mL TBS containing 10 mM $MnCl_2$ (*see* **Note 13**).
5. To prepare cleared lysates, thaw cells, sonicate on ice, and centrifuge lysates at 20,000 *g* for 10 min at 4 °C (*see* **Note 14**).
6. Apply cleared lysates to 1 mL immobilized glutathione column, wash with 30 mL TBS, and elute with 50 mM Tris–HCl, pH 8.0, containing 20 mM reduced glutathione (*see* **Note 15**).
7. Add eluted protein into dialysis membrane tubing, and dialyze against 1000 mL TBS containing 10 mM $MnCl_2$ at 4 °C overnight.
8. Measure protein levels by Bradford protein assay, and store in 30% glycerol with TBS 10 mM $MnCl_2$ at −80 °C until use in the PP2C assay.
9. To prepare HisX6-PYR1/PYLs, add 2 mL overnight cell culture in LB medium to 200 mL TB medium, and incubate at 30 °C with shaking at 200 rpm until OD_{600} = 0.5. Immediately place on ice for 5 min, then add 200 μL 1 M IPTG (final 1 mM) to the culture, and incubate at 15 °C with shaking at 200 rpm for 16 h.
10. Centrifuge the culture at 5000 *g* for 10 min to collect cells. Suspend the pellet in 5 mL Buffer A containing 10 mM imidazole (pH 8.0) (*see* **Note 14**).
11. To prepare cleared lysates, thaw cells, sonicate on ice, and centrifuge lysates at 20,000 *g* for 10 min at 4 °C (*see* **Note 16**).
12. Apply cleared lysates to 400 μL immobilized glutathione column, wash with 15 mL Buffer A containing 30 mM imidazole, and elute with Buffer A containing 250 mM reduced glutathione.
13. Add eluted protein to dialysis membrane tubing and dialyze against 1000 mL TBS at 4 °C overnight.
14. Measure protein levels by Bradford protein assay, and conduct the PP2C assay.
15. To assay inhibition of PP2C enzyme activities by ABA receptor in response to ABA, prepare 80 μL volume as shown in Table 1, and incubate for 20 min at RT.
16. After adding 20 μL 5× PP2C reaction buffer containing 5 mM 4-MP (4-methylumbelliferyl), measure fluorescence intensity twice at 90-s intervals (excitation, 355 nm; emission, 460 nm) on a plate reader (*see* **Note 17**).
17. Determine the chemical-dependent inhibition of PP2C activity by various ABA receptors as shown in Fig. 3 (*see* **Note 18**).

Table 1

Composition and preincubation for PP2C assay

	4-MP		*p*NPP	
	Volume μL	Final	Volume μL	Final
GST-HAB1	X	50 nM	X	600 nM
6xHis-PYLs	X	100 nM	X	1200 nM
1 M $MnCl_2$	1	12.5 mM	1	12.5 mM
10% BME	1	0.125%	1	0.125%
BSA (3 mg/mL)	1	3 μg	–	–
Compound solution	1	–	1	–
Water	X	–	X	–
Total	80 μL	–	80 μL	–

After mixing reagents, incubate at RT for 20 min

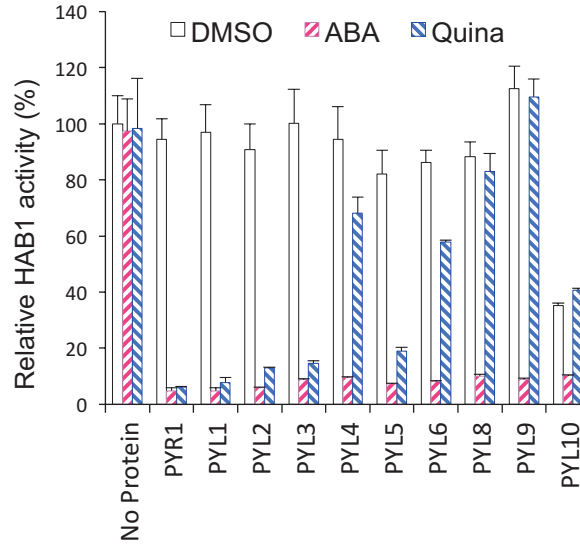

Fig. 3 Agonist assays for various receptors. Inhibition of HAB1 activities by various ABA receptors in response to ABA and quinabactin (Quina). HAB1 enzyme activities in the absence of receptor protein and chemical are shown as 100%. Reaction solutions contain 50 nM PP2Cs and 100 nM various receptors. Assay is conducted in the presence or absence of each test compound at 10 μM (Ref. [5])

3.5 In Vivo Evaluation of ABA Agonists Using Arabidopsis ABA-Responsive GUS Reporter System

1. Sow seeds of ABA-responsive reporter transgenic plants on a square plate (½ MS, 0.5% w/v sucrose, and 0.5% w/v Gelzan™) and then keep at 4 °C for 4 days.
2. Place the plate vertically, and grow the seedlings at 22 °C under a 16-h-light/8-h-dark photoperiod (*see* **Note 19**).
3. Transfer 6-day-old seedlings to a multi-well plate containing a solution consisting of ½ MS salts and 0.5% w/v sucrose, and incubate the seedlings at 22 °C under light conditions. After overnight incubation, add various types and concentrations of chemicals to the wells, and incubate seedlings at 22 °C under light conditions for 6 h.
4. Remove incubation solution, add GUS reaction buffer to the wells. Place the multi-well plate in a vacuum chamber, and gently deaerate seedlings for 10 min. Then, incubate seedlings at 37 °C in the dark overnight.
5. After checking GUS staining, remove the reaction buffer, add 70% EtOH to stop the reaction, and incubate at 60 °C to bleach chlorophyll pigments. Exchange 70% EtOH two or three times.
6. Check the GUS staining under stereoscopic and optical microscopes (*see* Fig. 4 and *see* **Note 20**).

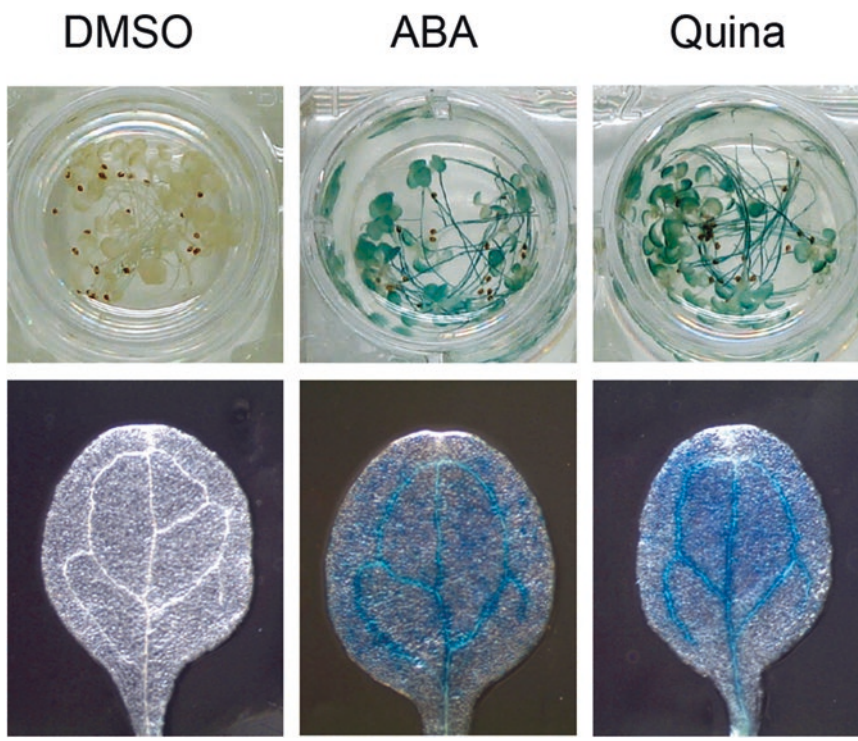

Fig. 4 Induction of *MAPKKK18* expression after treatment with 10 μM ABA and quinabactin. Strong GUS staining in *Arabidopsis* seedling after treatment with ABA and quinabactin (Quina). No GUS staining in *Arabidopsis* seedling treated with DMSO (control) (Ref. [5])

3.6 In Vivo Evaluation of Agonists Using Arabidopsis Seed Germination and Seedling Growth

1. Drop 1 μL chemical solution at optimal concentration (1–25 μM) into 96-well plate.
2. Keep medium (½ MS containing 0.5% w/v sucrose and 0.75% w/v agar) at 60 °C until use, and then add 90 μL medium into each well using a multichannel electronic pipette (*see* **Note 21**).
3. Sterilize *Arabidopsis* seeds in a solution containing 5% (v/v) NaClO and 0.05% (v/v) Tween-20 for 10 min, and rinse with water four times. Suspend the sterilized seeds in 0.1% (w/v) agar solution, and then drop 10 μL agar solution containing approx. 25 seeds into the center of each well using a multichannel electronic pipette (*see* **Note 22**).
4. Keep plate in the dark for 4 days at 4 °C, and then transfer the plate to 22 °C in the dark.
5. After incubation for some days, check inhibition of germination and hypocotyl growth (*see* **Note 23**).

3.7 Expression Analysis of ABA-Responsive Genes by Real-Time RT-PCR

1. Sow the seeds of an ABA-responsive reporter transgenic plant on a square plate (½ MS, 0.5% w/v sucrose, and 0.5% w/v Gelzan™), and then keep plate at 4 °C for 4 days.
2. Place the plate vertically, and grow the seedlings at 22 °C under a 16-h-light/8-h-dark photoperiod.
3. Transfer 10-day-old seedlings to 24-well plate containing a solution consisting of ½ MS salts and 0.5% w/v sucrose, and incubate the seedlings at 22 °C under light conditions overnight. Then, add various types and concentrations of chemicals to the wells, and incubate seedlings at 22 °C under light conditions for 6 h.
4. Harvest the seedling into 2 mL tubes containing 5 and 2 mm stainless beads, freeze with liquid nitrogen, and then crush by vortexing.
5. Isolate total RNA using an RNA isolation kit, and synthesize cDNA using a reverse transcription kit.
6. Analyze the transcript levels of ABA-responsive genes by real-time PCR detection system with PCR master mix containing SYBR® Green. The primer sequences used in our laboratory are shown in Table 2.

3.8 Physiological Analyses of Water Stress (See Fig. 5)

1. For the thermo-imaging analysis and water-loss assay using detached rosette leaves, grow *Arabidopsis* plants in soil at 22 °C under a 16-h-light/8-h-dark photoperiod. Spray a solution of the chemical and 0.05% v/v Tween-20 onto rosette leaves. After overnight incubation, measure leaf surface temperature using a thermal imaging camera under light conditions. Subsequently, cut the aerial shoot from the root, place

Table 2

Sequences of primers used for expression analysis of ABA-responsive genes

Gene name	Forward primer	Reverse primer
MAPKKK18	AAGCGGCGCGTGGAGAGAGA	GCTGTCCATCTCTCCGTCGC
RD29B	TATGAATCCTCTGCCGTGAGAGGTG	ACACCACTGAGATAATCCGATCCT
RD29A	TGAAGTGATCGATGCACCAGG	GACACGACAGGAAACACCTTTG
ACT2 (internal control)	CTCATGAAGATCCTTACAG	CTTTCAGGTGGTGCAACGAC

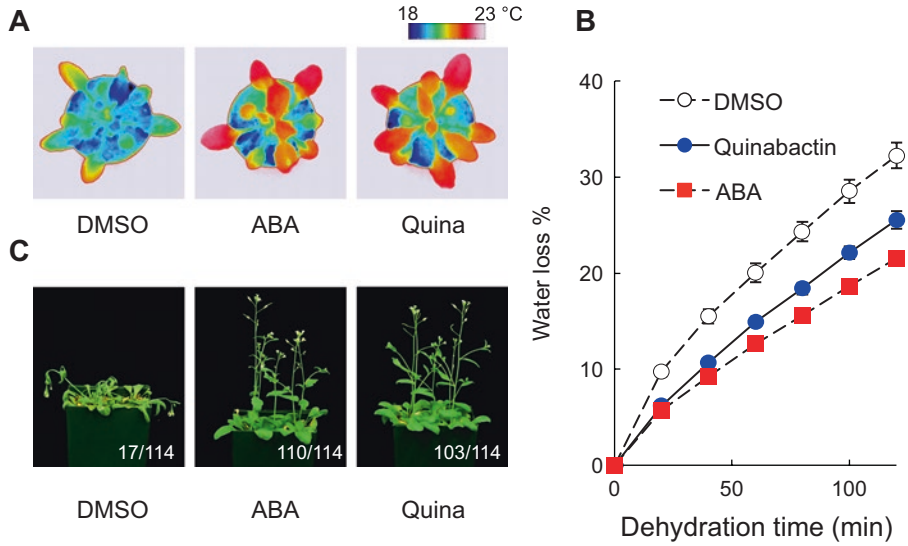

Fig. 5 Physiological analyses of water stress. Thermal imaging of intact leaves (**a**) and water loss from detached leaves (**b**) of *Arabidopsis* plants after applying 25 μM compound. (**c**). Effect of quinabactin (Quina) on *Arabidopsis* drought tolerance. Two-week-old plants were subjected to drought stress by withholding water and were photographed after 12 days. During the drought period, chemical solution containing 0.05% Tween-20 was applied by aerosol spraying onto plants at daily intervals over a 3-day period. Number of surviving plants (out of total number tested) for each treatment is shown at the bottom right of each image (Ref. [5])

the shoot on a dish, and weigh at 20-min intervals for 2 h under light conditions to determine water loss (*see* **Note 24**).

2. For the drought stress assay, weigh an equal amount of soil for each seedling so that the results are comparable. Transplant *Arabidopsis* seedlings into soil and grow for 2 weeks at 22 °C under a 16-h-light/8-h-dark photoperiod. Impose drought stress by withholding water (*see* **Note 25**). During the drought period, apply a solution of the chemical and 0.05% v/v Tween-20 by aerosol spraying onto plants at daily intervals

over a 3-day period. Observe the degree of wilting in the plants during the drought stress treatment. Recovery after rewatering can also be monitored (*see* **Note 26**).

4 Notes

1. Freeze-thawing of chemical DMSO solutions should be minimized. Generally, chemical libraries are commercially available at a concentration of approx. 10 mM in a 96-well plate format. If chemical libraries are purchased as a powder, prepare a stock chemical solution in DMSO with a Biomeck liquid-handling workstation. Chemical library management software such as Instant JChem (https://www.chemaxon.com/) is convenient to create a chemical database, which provides a means to store, search, and view chemical structures.
2. pAD GAL4 can be used as an alternative to pACT. However, AtPYL5–13 cannot be used for chemical screening in this system, because these constitutively bind to HAB1 in yeast in an ABA-independent manner.
3. Other uracil auxotrophic strains can be used instead of MAV99 in this system.
4. Storage of yeast for more than 10 days will reduce its activity, so that the ABA sensitivity of yeast is weak in the screening step. Ideally, keep yeast cells for 10 days or fewer.
5. Generally, lanes 1 and 12 are empty wells in chemical plates. In our assay, ABA is added to one side as the positive control. Replicate preparations can be performed faster using a multichannel electronic pipette.
6. The percentage of agar in the SD medium is 0.75% (w/v), but the optimal concentration of agar in medium might vary depending on the product.
7. To exclude manual mistakes and false positives, plate experiments should be performed in duplicate.
8. The MAV99 system will detect chemicals mimicking uracil as well as ABA agonists. To overcome this disadvantage of MAV99 system, the X-gal reporter system is used for the second screening.
9. The use of multi-well plates reduces the amount of chemicals.
10. This operation can be skipped if chloroform-resistant plates are used.
11. If the agar temperature is too low or the agar volume is too small, the medium will solidify before it covers the plate completely.

12. This step is not required if the main purpose of screening is to find ABA agonists. The ABA-responsive GUS reporter assay can quickly reveal the effect of the chemical in vivo.
13. Cells can be stored at −80 °C until use for protein purification.
14. Sonication can be difficult if there is a high concentration of cells; therefore, dilute cells appropriately with TBS containing 10 mM $MnCl_2$.
15. One milliliter of immobilized glutathione resin fills a 5 mL polypropylene column.
16. Sonication can be difficult if there is a high concentration of cells; therefore, dilute cells appropriately with Buffer A.
17. Instead of 4-MP, *p*NPP (*p*-nitrophenyl phosphate) can be used as an alternative substrate in the PP2C enzyme assay. Dissolve *p*NPP to 125 mM in reaction buffer (156 mM Tris–OAc (pH 7.9), 330 mM potassium acetate), and add 20 μL reaction buffer to the preincubation buffer as shown in Table 1. Monitor reaction products of *p*NPP at 405 nm on a plate reader.
18. To determine the IC_{50} for each chemical in the receptor-mediated inhibition of PP2C activity, the chemical should be tested at various concentrations.
19. In our laboratory, *MAPKKK18* promoter::GUS reporter transgenic plants are used for in vivo evaluation of ABA agonists. *RD29B* promoter::GUS reporter transgenic plants can also be used.
20. The eMolecules® web tool (https://www.emolecules.com/) can be used to search for analogs of the hit chemicals. It is possible that some analogs will show strong or different selectivity.
21. The agar concentration used in our laboratory is 0.75% w/v. However, the optimum agar concentration may vary depending on the product.
22. The use of both ABA-insensitive and -deficient mutants can reveal whether the chemical is related to ABA signaling.
23. As well as inhibiting seed germination, most bioactive chemicals will inhibit the growth of etiolated hypocotyls and roots in the dark.
24. The optical dose of chemicals varies among plant species. Ensure that plants are not under a strong airflow when leaf surface temperature and water loss measurements are conducted. Soil is a 1:1 mixture of vermiculite:Promix.
25. *Arabidopsis* seedlings are grown in square 6 × 6 × 5 cm pots containing 100 g soil (four seedlings per pot). Soil is a 1:1 mixture of vermiculite:Promix.
26. The timing of rehydration is determined by trial and error, and depends on the growth conditions and plant species.

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