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Abscinazole-E1, a novel chemical tool for exploring the role of ABA 8′-hydroxylase CYP707A

Mariko Okazaki [a,†], Hataitip Nimitkeatkai [b,†], Taku Muramatsu [a,†], Hikaru Aoyama [a], Kotomi Ueno [c], Masaharu Mizutani [c], Nobuhiro Hirai [d], Satoru Kondo [b], Toshiyuki Ohnishi [e], Yasushi Todoroki [a,*]

[a] Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529, Japan
[b] Division of Bioresource Science, Graduate School of Horticulture, Chiba University, Matsudo 271-8510, Japan
[c] Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan
[d] Division of Environmental Science and Technology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8501, Japan
[e] Division of Global Research Leaders, Shizuoka University, Shizuoka 422-8529, Japan

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ABSTRACT

We developed abscinazole-E1 (Abz-E1), a specific inhibitor of abscisic acid (ABA) 8′-hydroxylase (CYP707A). This inhibitor was designed and synthesized as an enlarged analogue of uniconazole (UNI), a well-known plant growth retardant, which inhibits a gibberellin biosynthetic enzyme (*ent*-kaurene oxidase, CYP701A) as well as CYP707A. Our results showed that Abz-E1 functions as a potent inhibitor of CYP707A and a poor inhibitor of CYP701A both in vitro and in vivo. Abz-E1 application to plants resulted in improved desiccation tolerance and an increase in endogenous ABA.

1. Introduction

Abscisic acid (ABA) is a plant hormone involved in stress tolerance, stomatal closure, seed dormancy, and other physiological events.[1–4] The endogenous levels of ABA in plants are cooperatively controlled by biosynthesis, transport, and catabolic inactivation in response to environmental changes.[1–4] A natural or artificial chemical that perturbs this highly controlled system is promising not only as a chemical probe for the mechanism of ABA action,[5] but also because of its potential use in agriculture and horticulture. Although ABA is registered asa farm chemical (plant growth regulator), its practical use has been limited, mainly due to its weak effect in field trials,[6] considered to be due to its rapid inactivation through biodegradation. Catabolic inactivation of ABA is mainly controlled by ABA 8′-hydroxylase, which is the cytochrome P450 catalyzing the C8′-hydroxylation of ABA to 8′-hydroxy-ABA and its more stable tautomer, phaseic acid (PA), which has much lower hormonal activity than ABA (Fig. 1).[4] ABA 8′-hydroxylase was identified as CYP707A1-4 in the model plant *Arabidopsis thaliana* in 2004,[7,8] and since then many CYP707A isozymes have been found in plants.[9–12] Gene knockdown and overexpression studies suggest that ABA 8′-hydroxylase is a key enzyme for controlling ABA concentration during water deficit stress or dormancy maintenance and breaking.[13,14]

S-Uniconazole (UNI), a plant growth retardant developed in the 1980s,[15,16] functions as an inhibitor of CYP707A[17] in addition to *ent*-kaurene oxidase (CYP701A), which catalyzes the three-step oxidation of *ent*-kaurene to *ent*-kaurenoic acid (KA),[18] a biosynthetic precursor of the plant hormone gibberellin (GA). *S*-Diniconazole, the *R* enantiomer of which is a fungicide with a structure very similar to UNI, also inhibits CYP707A,[19] although it arrests plant growth.[20] The broad inhibition spectrum of some azole compounds against P450 enzymes has apparently resulted from the heme coordination of an azole nitrogen, which is a common mechanism of azole P450 inhibitors. In other words, their specificity for individual P450 enzymes depends on structural properties other than the azole group.

In early work,[21] we focused on the small and flexible structure of UNI to develop a more specific inhibitor against CYP707A by three approaches: (1) molecular enlargement;[22] (2) conformational restriction;[23] and (3) modification of the azole ring.[24] All approaches resulted in an increase in specificity for CYP707A. Representative inhibitors are summarized in Table 1. Although these inhibitors are more specific than UNI, they have drawbacks: a low synthetic yield for Abz-F1 and DSI-505M, and a low aqueous solubility for UT4. In the present study, we focused on the molecular enlargement approach to develop a novel UT derivative with higher water solubility than UT4 and with high extensibility to be transformed into diverse functional probes. This paper describes

* Corresponding author. Tel./fax: +81 542384871.
E-mail addresses: aytodor@ipc.shizuoka.ac.jp, aytodor@agr.shizuoka.ac.jp (Y. Todoroki).
† These authors contributed equally to this work.

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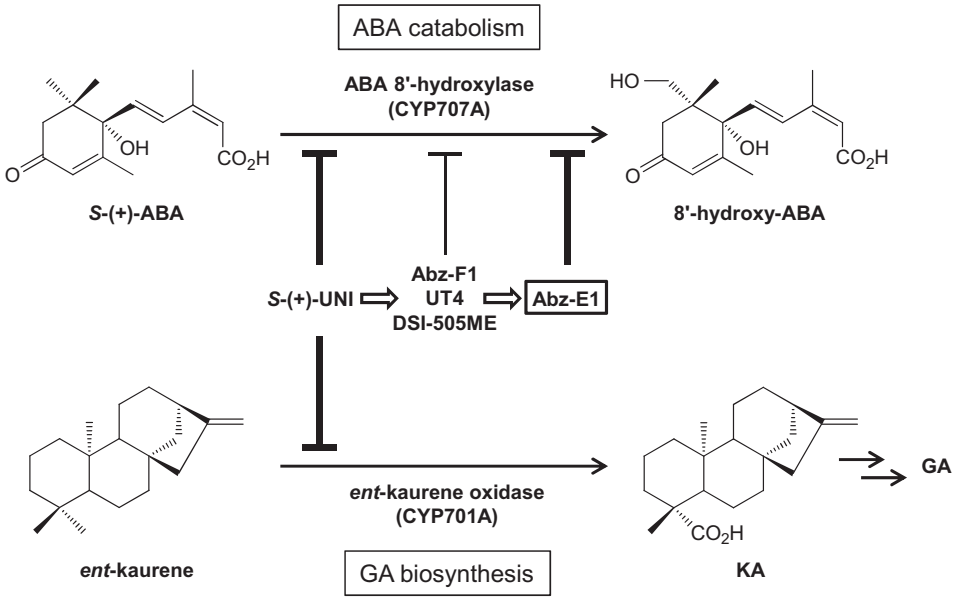
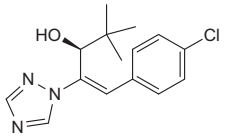
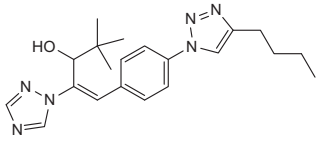
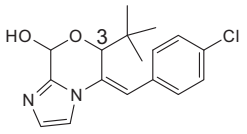
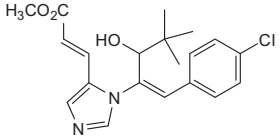


Figure 1. Uniconazole (UNI) is a potent inhibitor of ABA 8′-hydroxylase and *ent*-kaurene oxidase.

Table 1
Azole inhibitors of CYP707A

Name	Structure	CYP707A inhibition[d] K_I (nM)	Growth inhibition[e] IC_{50} (μM)
UNI[a]		10	0.18
UT4[a]		195	42
Abz-F1[b]		420 (3*R*) 970 (3*S*)	>100
DSI-505ME[c]		120	>100

[a] Ref. 22.
[b] Ref. 23.
[c] Ref. 24.
[d] *Arabidopsis* recombinant CYP707A3 coexpressed with *Arabidopsis* P450 reductase (ATR2) in *E. coli*.
[e] Rice seedlings.

the design and preparation of UT1-E2Ts, which we named abscinazole-E1 (Abz-E1), and its in vitro and in vivo inhibition of CYP707A and CYP701A.

2. Results and discussion

2.1. Design, preparation, and water solubility

In a previous study, we found that the CYP707A inhibitory potency of UT with protic groups such as hydroxy and carboxy groups at the C4′ side chain is poor compared with that of UT with an alkyl side chain, although the 1,2,3-triazolyl moiety had no effect on the inhibitory potency.[22] This finding suggests that the CYP707A active site is relatively, but nor entirely, hydrophobic, like P450 active sites generally. Therefore, we selected ethylene glycol ether, which is a hydrophilic moiety with no protic group. Considering also extensibility to be transformed into diverse functional probes, we designed UT1-E2Ts with the tosylate of diethylene glycol as a novel UT derivative. UT1-E2Ts was easily prepared from the 4′-azide derivative of UNI, compound **2**, using click chemistry,[25] like other

UT derivatives[22] (Scheme 1). Water solubility of UT1-E2Ts was compared with UNI and UT4. Table 2 shows that UT1-E2Ts had higher water solubility than UT4, but slightly lower solubility than UNI.

2.2. Inhibition of *Arabidopsis* CYP707A3 and rice CYP701A6 in an in vitro enzyme assay

Inhibitory activity of UT1-E2Ts against ABA 8′-hydroxylase was examined using recombinant *Arabidopsis* CYP707A3 co-expressed with *Arabidopsis* P450 reductase (ATR2) in *Escherichia coli*.[22] The activity was evaluated based on the decrease in the amount of the enzymatic product, PA. The mode of inhibition was competitive (Fig. 2) and the inhibition constant (K_I) was 27 ± 8 nM, which was almost equal to that of UNI (10 nM) (Table 3). This means that UT1-E2Ts is the most potent CYP707A inhibitor, at least in vitro, among all the UT derivatives as well as Abz-F1 and DSI-505M.

To construct an assay system for evaluating inhibitory activity against *ent*-kaurene oxidase, CYP701A, we expressed recombinant rice (*Oryza sativa* L. cv. Nipponbare) CYP701A6 in insect cells using a baculovirus vector. This enzyme catalyzes all three steps to KA via the corresponding alcohol and aldehyde,[18] meaning that kinetic analysis is very complicated. Therefore, instead of the K_I value, we evaluated the IC_{50} value for formation of the end product, KA (Fig. 2b and Table 3). The IC_{50} value of UNI was 45 nM when 1 μM *ent*-kaurene was used as the substrate, whereas that of UT1-E2Ts was 1 μM under the same assay conditions. This means that the inhibitory activity of UT1-E2Ts against CYP701A6 was substantially reduced compared with UNI. We renamed UT1-E2Ts as abscinazole-E1 (Abz-E1) before testing it in bioassays.

2.3. Effect on seed germination and early growth

We tested the effect of Abz-E1 on germination of *Arabidopsis* and lettuce seeds. Although Abz-E1 exhibited no effect on rooting of *Arabidopsis* and lettuce seeds, it affected leaf emergence of *Arabidopsis* slightly at 100 μM, similarly to UNI at 10 μM (Fig. 3). Abz-E1 enhanced the effect of ABA on early growth of lettuce upon simultaneous application of 100 μM of Abz-E1 (Fig. 4). We also tested the effect of Abz-E1 on early growth of rice seedlings and found it had little effect on seedling growth (Fig. 5). These results suggest that Abz-E1 does not function as a potent inhibitor of P450 enzymes (e.g., CYP701A) involved in seed germination and seedling growth, whereas it may inhibit the catabolism of ABA in vivo.

Table 2
Water solubility of UT1-E2Ts (Abz-E1)

Compound	Solubility (%) in water (2 mL)[a]	
	20 nmol	200 nmol
UNI	79.4	39.5
UT4	40.7	—[b]
UT1-E2Ts (Abz-E1)	68.9	21.3

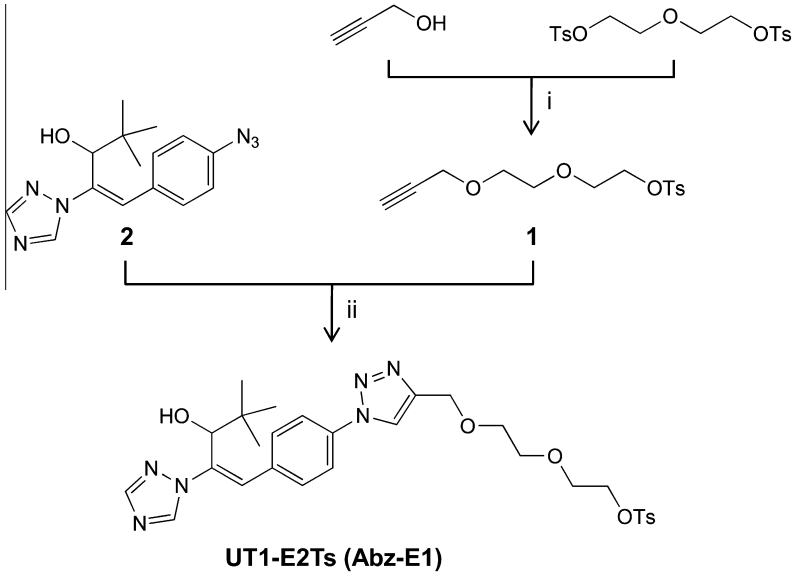
[a] Calculated based on the in water/in MeOH ratio of HPLC peak area.
[b] Not tested.

2.4. Effect on stomatal closure, drought tolerance, and endogenous ABA and PA

To determine whether in vivo inhibition of Abz-E1 against CYP707A is sufficient to intensify ABA-induced physiological responses, we tested the effect of Abz-E1 on stomatal aperture, drought tolerance, and endogenous amount of ABA and its catabolite PA. We tested 90-day-old apple seedlings by spraying them with an aqueous solution containing Abz-E1 at 10 μM. The seedlings were water-stressed for 12 days beginning 24 h after treatment with the inhibitor, and then rehydrated on the 13th day. Application of Abz-E1 before dehydration induced drought tolerance (Fig. 6a) and stomatal closure (Fig. 6b) during dehydration. The endogenous amount of ABA during dehydration increased significantly after treatment with Abz-E1 (Fig. 6c), whereas the amount of PA decreased (Fig. 6d). This indicates that Abz-E1 inhibited the hydroxylation of ABA to PA by CYP707A in apple seedlings.

3. Conclusion

UNI, which was developed as an inhibitor of a GA biosynthetic enzyme (CYP701A), is a low selectivity P450 inhibitor that inhibits multiple P450 enzymes including ABA 8′-hydroxylase (CYP707A). Based on our speculation that the low selectivity of UNI may have resulted from its small size, we earlier developed enlarged UNI



Scheme 1. Synthesis of UT1-E2Ts (Abz-E1). Reagents and conditions: (i) NaH, dry DMF, rt, 78%; (ii) $CuSO_4$, sodium ascorbate, THF, rt, 96%.

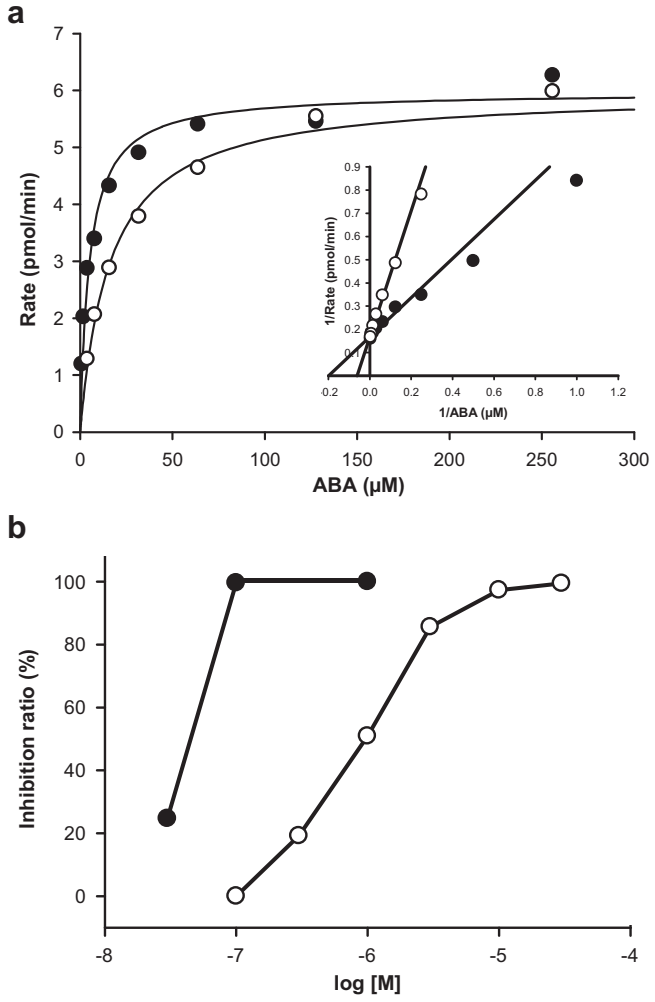


Figure 2. (a) Competitive inhibition of CYP707A3 by UT1-E2Ts (Abz-E1). Assays contained *S*-ABA (●) or *S*-ABA and 40 nM Abz-E1 (○). The inset is a double-reciprocal plot of the same data. (b) Dose response in inhibition of CYP701A6 by UNI (●) and UT1-E2Ts (Abz-E1) (○).

Table 3
UNI and UT1-E2Ts (Abz-E1) K_I values for *Arabidopsis* CYP707A3 and IC_{50} values for rice CYP701A6

Compound	K_I for CYP707A3 (nM)	IC_{50} for CYP701A6 (nM)
UNI	10[a]	45
UT1-E2Ts (Abz-E1)	27 ± 8	970

[a] Ref. 22 and 23.

analogues, UT compounds, that have a 1,2,3-triazolyl alkyl chain. Although UT compounds showed strong CYP707A inhibition, the biological activity of most of UT compounds was not tested because of their water insolubility. Because the introduction of protic functional groups in the alkyl chain diminished the inhibitory activity, in the present study we developed Abz-E1, which has a diethylene glycol chain with a terminal tosylate. Abz-E1 showed good water solubility and strong inhibitory activity against CYP707A in vitro and in vivo, equivalent to that of UNI. On the other hand, against CYP701A, Abz-E1 was a poorer inhibitor than UNI. Abz-E1 is expected to be a useful tool in chemical biology and chemical genetics as well as a novel plant growth regulator. Moreover, because Abz-E1 has a terminal tosylate, it is expected to be a useful precursor for synthesis of multifunctional chemical probes.

4. Experimental

4.1. General

(+)-ABA was a gift from Toray Industries Inc., Tokyo, Japan. *ent*-Kaurene and *ent*-kaurenoic acid were a gift from Professor Tomonobu Toyomasu (Yamagata University) and Dr. Shinjiro Yamaguchi (RIKEN Plant Science Center), respectively. *ent*-Kaurenoic acid was also purchased from Olchemlm Ltd, Czech Republic. ^{1}H NMR spectra were recorded with tetramethylsilane as the internal standard using JEOL JNM-EX270 (270 MHz) and JNM-LA500 (500 MHz) NMR spectrometers. ^{13}C NMR and 2D-correlation NMR experiments were recorded using a JNM-LA500 (500 MHz) NMR spectrometer. High resolution mass spectra were obtained with a JEOL JMS-T100LC 'AccuTOF'. Column chromatography was performed on silica gel (Wakogel C-200).

4.2. Synthesis of UT1-E2Ts (Abz-E1)

4.2.1. 2-(2-(Prop-2-ynyloxy)ethoxy)ethyl 4-methylbenzenesulfonate (1)

To a stirred solution of propargyl alcohol (100 mg, 1.8 mmol) in dry DMF (30 mL) was added NaH (60% in oil, 900 mg, 22 mmol) at 0 °C under Ar. After stirring for 30 min at room temperature, diethylene glycol bis(*p*-toluenesulfonate) (2.8 g, 6.7 mmol) was added to the mixture at 0 °C. The mixture was stirred for 1 h at room temperature. After quenching with sat. NH_4Cl at 0 °C, the resulting mixture was extracted with EtOAc (250 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residual oil was purified by silica gel column chromatography with 20% EtOAc in hexane to obtain **1** (410 mg, 78%) as a colorless oil. ^{1}H NMR (270 MHz, $CDCl_3$): δ 2.43 (1H, t, J = 2.6 Hz, H-1), 2.45 (3H, s, C*H*$_3$ in tosylate), 3.59–3.66 (4H, m, –OC*H*$_2$-C*H*$_2$O–), 3.70 (2H, m, –OC*H*$_2$CH$_2$OSO$_2$–), 4.16 (2H, m, HCCC*H*$_2$O–), 4.17 (2H, m, –OCH$_2$C*H*$_2$OSO$_2$–), 7.34 and 7.80 (each 2H, m, tosylate); HRMS (ESI-TOF, positive mode): calcd for $C_{14}H_{18}O_5SNa$ $[M+Na]^+$ 321.0773, found 321.0771.

4.2.2. (*E*)-2-(2-((1-(4-(3-Hydroxy-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pent-1-en-1-yl)phenyl)-1*H*-1,2,3-triazol-4-yl)methoxy)ethoxy)ethyl 4-methylbenzenesulfonate (Abz-E1)

To a stirred solution of **1** (2.5 g, 8.5 mmol) and **2**[22] (1.4 g, 4.6 mmol) in THF (280 mL) was added aqueous $CuSO_4$ (10 mg mL^{-1}, 380 mL) and aqueous sodium ascorbate (10 mg mL^{-1}, 340 mL) at room temperature. The mixture was stirred for 1 h before extraction with CH_2Cl_2 (600 mL × 3). The organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residual oil was purified by silica gel column chromatography with 20% EtOAc in hexane to obtain Abz-E1 (2.6 g, 96%) as a pale yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.68 (9H, s, *t*-butyl), 2.44 (3H, s, C*H*$_3$ in tosylate), 3.64–3.67 (2H, m, –OCH$_2$C*H*$_2$O–), 3.69–3.72 (4H, m, –OC*H*$_2$CH$_2$OC*H*$_2$CH$_2$OSO$_2$–), 4.18 (2H, m, –OCH$_2$-C*H*$_2$OSO$_2$–), 4.42 (1H, d, J = 8.5 Hz, *H*O-3), 4.62 (1H, d, J = 8.5 Hz, H-3), 4.77 (2H, s, 1,2,3-triazole-C*H*$_2$O–), 7.01 (1H, s, H-1), 7.33 (2H, m, tosylate), 7.55 (2H, m, 1-phenyl), 7.78 (2H, m, tosylate), 7.83 (2H, m, 1-phenyl), 8.07 (1H, s, 1,2,3-triazole), 8.12 and 8.60 (each 1H, s, 1,2,4-triazole); ^{13}C NMR (125 MHz, $CDCl_3$): δ 21.6 (methyl in tosylate), 26.1 (methyl in *t*-butyl), 36.2 (tertiary carbon in *t*-butyl), 64.7, 68.7, 69.2, 69.8, and 70.7 (diethylene glycol linker), 75.6 (C3), 120.6 (C3′ and C5′), 120.7 (C3″ and 1,2,3-triazole), 127.6 (C1), 127.9 (tosylate), 129.8 (tosylate), 130.3 (phenyl), 132.9 (tosylate), 134.4 (phenyl), 136.6 (phenyl), 137.9 (C2), 144.9 (tosylate), 146.1 (phenyl and 1,2,3-triazole); UV λ_{max} (MeOH) nm (ε): 271.2 (20,000); HRMS (ESI-TOF, positive mode): calcd for $C_{29}H_{36}N_6O_6SNa$ $[M+Na]^+$ 619.2315, found 619.2312.

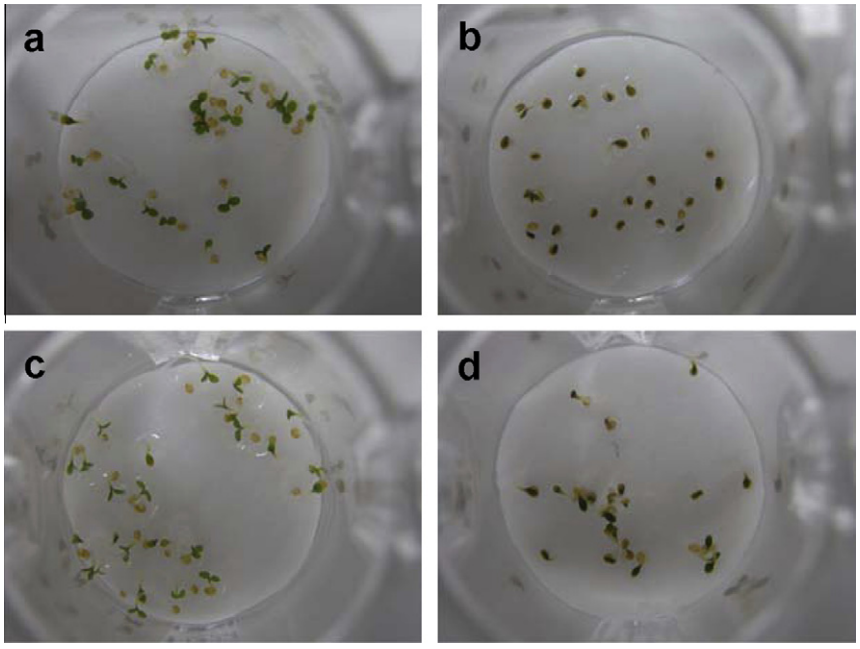


Figure 3. Effect of Abz-E1 on leaf emergence in *Arabidopsis* 54 h after imbibition: (a) control, (b) 10 μM UNI, (c) 30 μM Abz-E1, and (d) 100 μM Abz-E1.

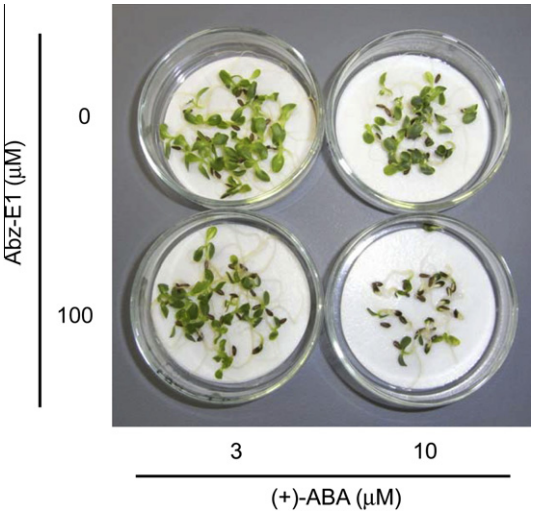


Figure 4. Enhancement of Abz-E1 on ABA activity in early growth (14 days after germination) of lettuce.

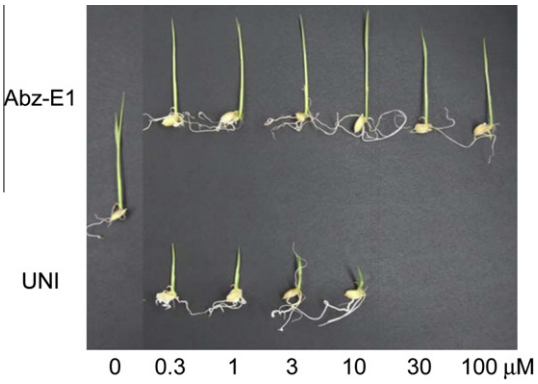


Figure 5. Comparison of effect of Abz-E1 and UNI on rice seedling growth on day 7 after treatment.

4.3. Water-solubility test

MeOH solutions of test samples were put in a glass vial and concentrated in vacuo. Distilled water (2 mL) or MeOH (2 mL) was added to the vial. After shaking several times, 10 μL of the solution was subjected to HPLC. HPLC conditions were: ODS column, Hydrosphere C18 (150 × 6.0 mm, YMC); solvent, 65% MeOH in H_2O; flow rate, 1.0 mL min^{-1}; detection, 254 nm. Solubility (%) in water was calculated based on the in water/in MeOH ratio of the HPLC peak area.

4.4. Preparation of recombinant enzymes

4.4.1. Coexpression of recombinant *Arabidopsis* CYP707A3 and *Arabidopsis* P450 reductase (ATR2) in *E. coli*

A truncated *Arabidopsis* CYP707A3 (707A3d28), which lacked the putative membrane-spanning segment of the N-terminus, residues 3–28, was constructed. Cells of *E. coli* strain BL21 were transformed with the constructs pCW-CYP707A3d28 and pACYC-AR2. Cultures (3 mL) were grown overnight in Luria-Bertani medium supplemented with ampicillin (50 μg mL^{-1}) and chloramphenicol (100 μg mL^{-1}). Then, 50 mL of Terrific Broth medium supplemented with ampicillin (50 μg mL^{-1}), chloramphenicol (100 μg mL^{-1}), and aminolevulic acid (0.5 mM) was inoculated with an aliquot of the overnight culture (0.5 mL). The culture was incubated with gentle shaking (225 rpm) at 37 °C until A_{600} reached 0.6 (at 2.5–3 h), and expression of the P450 enzyme was induced by the addition of isopropyl β-D-thiogalactopyranoside (0.1 mM). The culture was shaken continuously (150 rpm) at 25 °C and cells were harvested 48 h later by centrifugation at 2330*g* for 20 min at 4 °C. Pelleted cells were suspended in 2.5 mL of buffer A (50 mM potassium phosphate buffer, pH 7.25, 20% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol). The suspension was sonicated for 30 s, and the enzyme solution in the supernatant was collected by centrifugation at 23,470*g* for 30 min at 4 °C. The P450 content was determined by spectrophotometric analysis, using the extinction coefficient of a reduced CO difference spectrum (91.1 mM^{-1} cm^{-1}).

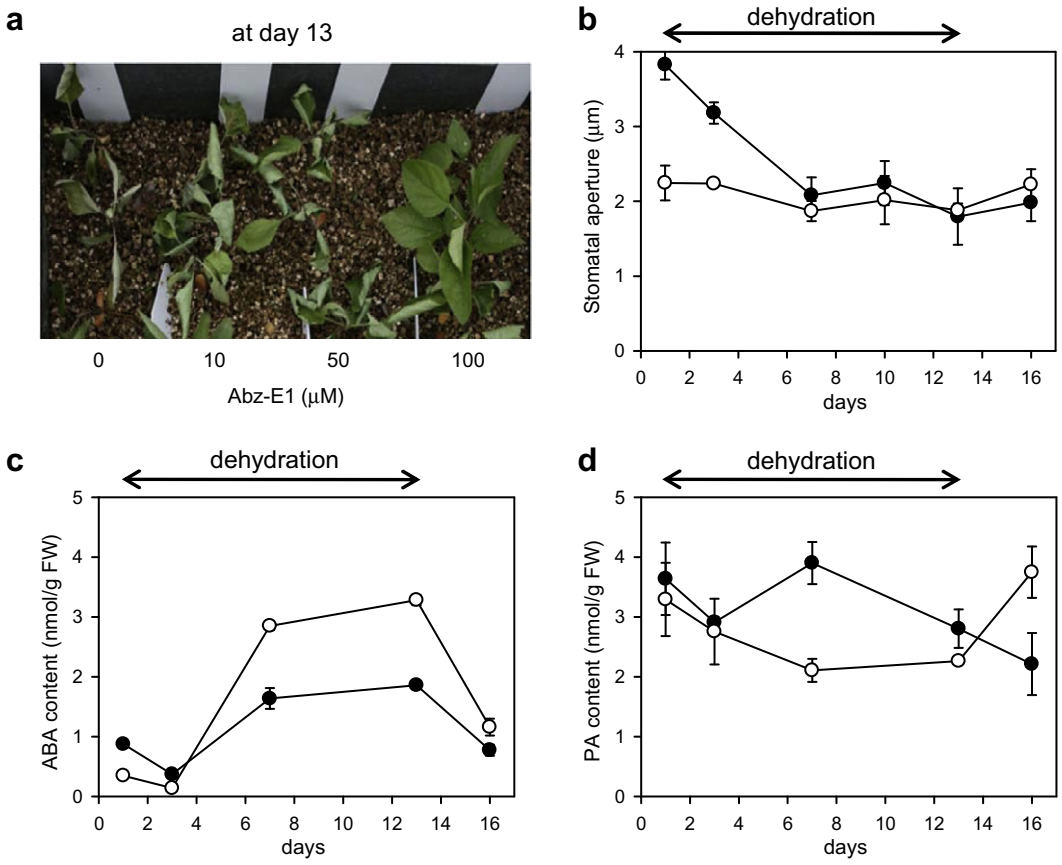


Figure 6. Effect of Abz-E1 on drought tolerance (a), stomatal aperture (b), ABA content (c), and PA content (d) during dehydration: control (●) and 10 μM Abz-E1 (○). Arrows show dehydration period (day 1 to day 13).

4.4.2. Cloning and heterologous expression of OsCYP701A6 with a baculovirus-insect cell system

cDNA containing the entire open-reading frame of OsCYP701A6 was amplified by RT-PCR. The full-length OsCYP701A6 (AK066285) was obtained from the Rice Genome Resource Center (the National Institute of Agrobiological Sciences, Japan), and the OsCYP701A6 ORF was amplified by PCR with the full-length cDNA as template. Nucleotide sequences of gene-specific primers were as follows: TO-35: 5′-AAA*ACTAGT*ATGGAGGCGTTCGTGC-3′ (*Spe*I site in italics and start codon underlined) and TO-36: 5′-AAAA*TTCGAA*TCA-CATCCTTCCTCTGCG-3′ (*Hind*III site in italics and stop codon underlined). A 1-ng aliquot of the full-length cDNA was used as a template for the PCR reaction in a 20-μL reaction mixture containing 5 × PrimeSTAR Buffer (Mg^{2+} plus) (4 μL), PrimeSTAR HS DNA Polymerase (0.5 U), 200 μM dNTP mixture, and 0.2 μM of the gene-specific primers described above. The PCR product was gel-purified and cloned into pJET1.2/bluntusing a CloneJET PCR Cloning Kit (Fermentas, Canada). The cloned inserts were sequenced with pJET1.2 forward and reverse sequencing primers to confirm the absence of PCR errors in the inserts. Full-length cDNA of OsCYP701A6 in pJET1.2/blunt plasmid vector was excised with the restriction enzymes *Spe*I and *Hind*III (TaKaRa Bio) and purified by 1% (w/v) agarose gel electrophoresis. This cDNA was ligated into pFastBac1 vector (Invitrogen, USA) digested with the same set of restriction enzymes. The pFastBac1-OsCYP701A6 constructs were used for the preparation of recombinant bacmid DNA by transformation of *E. coli* strain DH10Bac (Invitrogen). Preparation of recombinant baculovirus DNA containing OsCYP701A6 cDNA and transfection of *Sf9* (*Spodoptera frugiperda 9*) cells were performed according to the manufacturer's protocol (Invitrogen). For expression of OsCYP701A6, *Sf9* cells infected by baculovirus containing OsCYP701A6 cDNA were incubated in Grace's insect cell medium, 0.1% (w/v) Pluronic F-68 (Invitrogen), 10% (v/v) fetal bovine serum, 100 μM 5-aminolevulinic acid and 100 μM ferrous citrate on a rotary shaker at 27 °C and 150 rpm for 96 h. Insect cells were then harvested by centrifugation at 3000*g* for 10 min and washed with PBS buffer three times. After centrifuging again, insect cells were sonicated in buffer A containing 50 mM potassium phosphate (pH 7.3), 20% (v/v) glycerol, 1 mM EDTA, 0.1 mM dithiothreitol and centrifuged at 8000*g* for 10 min. The supernatant was further centrifuged at 100,000*g* for 1 h and the resulting pellet (microsomal fraction) was homogenized with buffer A. The concentration of active P450 was estimated from the carbon monoxide difference spectrum.[26]

4.5. Enzyme assay

4.5.1. CYP707A3

A reaction mixture containing 25 μg mL^{-1} of CYP707A3 microsomes coexpressed AR2 in *E. coli*, (+)-ABA (final conc. 0.5–128 μM), inhibitors (0 for control, 2–1000 nM in 5 μL DMF) and 50 μM NADPH in 50 mM potassium phosphate buffer (pH 7.25) were incubated for 10 min at 30 °C. Reactions were initiated by adding NADPH, and stopped by addition of 50 μL of 1 M NaOH. Each reaction mixture was acidified with 100 μL of 1 M HCl. To extract the reaction products, the mixture was loaded onto an Oasis HLB cartridge (1 mL, 30 mg; Waters) and washed with 1 mL of 10% MeOH in H_2O containing 0.5% AcOH. The enzyme products were then eluted with 1 mL of MeOH containing 0.5% AcOH, and the eluate was concentrated in vacuo. The dried sample was dissolved in

50 μL of MeOH, and 10 μL was subjected to HPLC. HPLC conditions were: ODS column, Hydrosphere C18 (150 × 6.0 mm, YMC); solvent, 35% MeOH or 20% MeCN in H_2O containing 0.1% AcOH; flow rate, 1.0 mL min^{-1}; detection, 254 nm. Enzyme activity was confirmed by determining the amount of PA in control experiments before each set of measurements. Inhibition constants were determined using the Enzyme Kinetics module of SigmaPlot 10 software[27] after determining the mode of inhibition by plotting the reaction velocity in the presence and absence of inhibitor on a double-reciprocal plot. For the uninhibited enzymatic reaction, the K_M for (+)-ABA was calculated to be 3.4 ± 0.6 μM, based on five separate experiments. All assays were conducted at least three times.

4.5.2. CYP701A6

The microsomal fractions of OsCYP701A6 were combined with purified NADPH-P450 reductase.[28] Assays of 400 μL contained 50 mM potassium phosphate (pH 7.25), 25 pmol mL of OsCYP701A6 microsomal fraction, purified NADPH-P450 reductase (0.1 U), *ent*-kaurene (10 μM) as substrate, and inhibitor (0.01–10 μM). Reactions were initiated by addition of NADPH, and were carried out at 30 °C for 30 min. After termination by adding 1 M HCl (100 μL) and EtOAc (200 μL), 5 μL of 1 mM abietic acid was added as an internal standard. The reaction products were extracted three times with an equal volume of ethyl acetate and the organic phase was collected. Anhydrous Na_2SO_4 was added to remove residual water. To derivatize the reaction products, methanol (100 μL) and TMS-CHN_2 (100 μL) (2.0 M in Et_2O) were added and the reaction mixture was incubated at room temperature for 15 min. Organic solvent was removed under Ar and samples were adjusted to 200 μL with Et_2O before GC–MS analysis (QP2010-plus, Shimadzu Corp., Japan). GC conditions were: column, DB-5 ms (0.25 mm id × 15 m; 0.25 μm film thickness; J&W Scientific); carrier gas, He; flow rate, 1.84 mL min^{-1}; injection port temperature, 280 °C; splitless injection; column oven temperature, 80 °C (1 min), 80–200 °C (18 °C min^{-1}), 200–210 °C (2 °C min^{-1}), 210–280 °C (30 °C min^{-1}), 280 °C (3 min). The content of KA was calculated from the area ratio of molecular and fragment ions of methyl KA (*m*/*z* 316, 273, and 257) to those of methyl abietate (*m*/*z* 316 and 256).

4.6. Bioassays

4.6.1. *Arabidopsis* seed germination assay

Twenty-five seeds (Col-0) were sterilized successively with 70% (v/v) EtOH for 30 min and reagent grade EtOH for 1 min. The sterilized seeds were soaked in 250 μL of a test solution and incubated in the dark for 3 days at 5 °C. The vernalized seeds in the test solution were transferred to 24-well plates placed on two sheets of filter paper, and allowed to germinate under continuous light for 24 h at 22 °C. All assays were conducted at least twice.

4.6.2. Lettuce seed germination and growth assays

Five seeds (*Lactuca sativa* L. cv. Grand Rapids) were placed in 24-well plates on two sheets of filter paper soaked in 0.2 mL of a test solution and allowed to germinate and grow under continuous light for 23 days at 22 °C. All assays were conducted at least twice.

4.6.3. Rice seedling elongation assay

Seeds of rice (*O. sativa* L. cv. Nipponbare) were sterilized with EtOH for 5 min and washed with running tap water. The sterilized seeds were soaked in water for 3 days at 25 °C to germinate. The seeds were then placed in a glass tube containing 2 mL of a test solution and grown with the tube sealed with a plastic cap under continuous light at 25 °C. When the seedlings were 7 days old, the length of the second leaf sheath was measured. All assays were conducted at least twice.

4.6.4. Drought tolerance assay

Apple [*Malus sylvestris* (L.) Mill. var. *domestica* (Borkh.) Mansf.] cultivar Fuji seeds were soaked overnight in water, then sown in moist vermiculite and grown in a greenhouse. Seedlings (90 days old) were sprayed uniformly with 2 mL of a test solution (10, 50, or 100 μM) containing Abz-E1 or distilled water (control) 24 h before water-stressing them for 12 days. Stomatal aperture was measured as follows: the extent of opening of 10 stomatal apertures per leaf was observed using a TM-1000 microscope (Hitachi High-Technologies Co., Tokyo, Japan); three leaves from three randomly selected seedlings (one leaf per seedling) were used for a total of 30 stomata per treatment.

4.6.5. ABA and PA analysis and quantification in apple seedlings

3′,5′,5′,7′,7′,7′-Hexadeuterated ABA (ABA-d_6) was purchased from Shoko Co. (Tokyo, Japan). PA was received as described by Hirai et al.[29] An internal standard of PA, 7′,7′,7′-trideuterated PA(PA-d_3), was prepared according to a previously reported method.[30] The extraction and quantification of ABA and PA in apple seedlings were performed according to a previously reported method[31] using HPLC and gas chromatography–mass spectrometry–selective ion monitoring (GC–MS–SIM) (Shimadzu QP5000). ABA was calculated by the ratio of peak areas for *m*/*z* 190(d_0)/194(d_6). PA was determined from *m*/*z* 276(d_0)/279(d_3).

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Supplementary data

Supplementary data (^{1}H NMR spectra of new compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.11.011.

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