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Chemical regulation of abscisic acid catabolism in plants by cytochrome P450 inhibitors

Nobutaka Kitahata,[a,b] Shigeki Saito,[c] Yutaka Miyazawa,[a] Taishi Umezawa,[d] Yukihisa Shimada,[e] Yong Ki Min,[a] Masaharu Mizutani,[c] Nobuhiro Hirai,[f] Kazuo Shinozaki,[d] Shigeo Yoshida[a,b] and Tadao Asami[a,*]

[a]*Plant Functions Laboratory, RIKEN, Saitama 351-0198, Japan*
[b]*Department of Biological and Environmental Sciences, Saitama University, Saitama 338-8570, Japan*
[c]*Institute for Chemical Research, Kyoto University, Kyoto 611-0011, Japan*
[d]*Plant Molecular Biology Laboratory, RIKEN, Tsukuba 305-0074, Japan*
[e]*Plant Science Center, RIKEN, Yokohama 230-0045, Japan*
[f]*The International Innovation Center, Kyoto University, Kyoto 606-8501, Japan*

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Abstract—Plant hormone abscisic acid (ABA) is an important factor for conferring drought stress resistance on plants. Therefore, small molecules that regulate ABA levels in plants can be useful both for investigating functions of ABA and for developing new plant growth regulators. Abscisic acid (ABA) catabolism in plants is primarily regulated by ABA 8′-hydroxylase, which is a cytochrome P450 (P450). We tested known P450 inhibitors containing a triazole group and found that uniconazole-P inhibited ABA catabolism in cultured tobacco Bright Yellow-2 cells. In a structure–activity study of uniconazole, we found a more effective ABA catabolic inhibitor (diniconazole) than uniconazole-P. Diniconazole, a fungicide, acted as a potent competitive inhibitor of recombinant *Arabidopsis* ABA 8′-hydroxylase, CYP707A3, in an in vitro assay. Diniconazole-treated plants retained a higher ABA content and higher transcription levels of ABA response genes during rehydration than did untreated plants and were more drought stress tolerant than untreated plants. These results strongly suggest that ABA catabolic inhibitors that target ABA 8′-hydroxylase can regulate the ABA content of plants and conferred drought stress resistance on plants. The optical resolution of diniconazole revealed that the *S*-form isomer, which is a weak fungicidal isomer, was more active as an ABA catabolic inhibitor than was the *R*-form isomer.
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1. Introduction

The plant hormone abscisic acid (ABA) is involved in the regulation of many developmental processes in plants, including the induction of seed dormancy and the stimulation of stomatal closure.[1] ABA is also involved in plant responses to environmental stresses such as drought and high salinity.[2] The balance between ABA biosynthesis and catabolism controls ABA content, which is an important factor in regulating plant growth and response to environmental stress. One of the practical methods to regulate its balance is a chemical regulation of the ABA biosynthesis and catabolism by inhibitors. In general, inhibitors of plant hormone biosynthesis and catabolism are effective tools for regulating plant growth and response to environmental stresses in a rapid, conditional, reversible, and dose-dependent manner. In the case of ABA, fluridone and norflurazon, which inhibit carotenoid synthesis, reduce ABA content[3,4] and have been useful in studying the physiological function of ABA. Recently, abamine, an inhibitor that targets 9-*cis*-epoxycarotenoid dioxygenase, the enzyme that converts 9-*cis*-neoxanthin to xanthoxin in the ABA biosynthesis pathway, has been developed[5,6] and was used for studying ABA function.[7]

Abbreviations: ABA, abscisic acid; GA, gibberellin; BR, brassinosteroid; RT-PCR, reverse transcriptase polymerase chain reaction; P450, cytochrome P450.

Keywords: Abscisic acid; Catabolic inhibitor; Uniconazole; Diniconazole; Catabolism; ABA hydroxylase; Cytochrome P450; Inhibitor; Triazole.

* Corresponding author. Tel.: +81 48 467 9526; fax: +81 48 462 4674; e-mail: tasami@postman.riken.go.jp

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Several structural analogs of ABA also inhibit ABA catabolism by acting as suicide substrates or competitive inhibitors of ABA 8′-hydroxylase.[8]

Recently, the term 'chemical genetics' is also used in plant science.[9] Auxin mimic or brassinosteroid biosynthesis inhibitor is used for screening new mutants,[10,11] but there has been no example of success in plant chemical genetics dealing with ABA because of the lack of appropriate chemical compounds for its purpose. If we could get small molecule modulator for ABA level, then it can be used in chemical genetics. In this context, we started to develop ABA level modulators. For that purpose, cytochrome P450s (P450s) could be good targets because plant cytochrome P450s participate in many biochemical pathways, including gibberellin (GA), brassinosteroid (BR) biosynthesis[12,13] and ABA catabolism and many types of P450s are inhibited by triazole derivatives. Uniconazole, a triazole-type P450 inhibitor, inhibits both GA biosynthesis[14] and BR biosynthesis[15] and also possesses fungicidal activity.[16] These findings suggest that uniconazole inhibits several types of P450s. Broadly effective P450 inhibitors are useful in developing new specific inhibitors. For example, brassinazole, a specific BR biosynthesis inhibitor, was designed based on its structural analogy to uniconazole and paclobutrazol.[17,18]

The predominant ABA catabolic pathway is thought to be the ABA 8′-hydroxylation pathway, which catalyzes the conversion of ABA to phaseic acid (PA). ABA 8′-hydroxylase belongs to the plant P450 family[19] and *Arabidopsis* ABA 8′-hydroxylases (CYP707A family members) have been cloned.[20,21] Non-specific inhibitor of P450 enzyme, such as tetcyclasis, was shown to be a potent inhibitor of 8′-hydroxylase in vitro.[19,20] Thus, ABA 8′-hydroxylase is an attractive target for the regulation of the ABA content of plants. In this report, we screened chemicals that inhibit the conversion of ABA to PA and found that uniconazole and one of the synthetic compounds inhibited ABA catabolism. Biochemical and physiological studies revealed that a compound that showed the highest activity among the tested compounds targets ABA 8′-hydroxylase, affects the ABA content of plants, and confers drought stress resistance on plants.

2. Results

2.1. Screen for ABA catabolic inhibitors

When 20 μM ABA was added to BY-2 cells grown for one day, half of the ABA was catabolized to PA within 40 h under our experimental conditions. We used this catabolic reaction to search for candidate ABA catabolic inhibitors. ABA 8′-hydroxylase, a P450, is a key enzyme in the ABA catabolic pathway. Many types of P450s are inhibited by triazole derivatives. In addition, several triazole compounds are predicted to inhibit ABA catabolism because endogenous ABA levels accumulate after the compounds are applied.[22] In this context, we tested the effects of known triazole-type P450 inhibitors on ABA catabolism to find promising compounds for the development of new and potent ABA catabolic inhibitors (Fig. 2A). Among the nine triazole compounds tested, uniconazole-P, an optical isomer of uniconazole, strongly inhibited ABA catabolism (Fig. 2B). Armed with this finding, we searched for ABA catabolic inhibitors among triazole compounds synthesized on the basis of their structural analogy to uniconazole (Table 1). Of the compounds tested, compound **4** (known as fungicide diniconazole) was the most effective ABA catabolic inhibitor (Fig. 3). To assess the effect of compound **4** on the inhibition of ABA catabolism in BY-2 cells, we compared its activity with that of its *Z*-form isomer (compound **3**) and of uniconazole-P. Compound **4** inhibited ABA degradation in a dose-dependent manner within the concentration range of 0.3–10 μM. It exhibited approximately three times the inhibitory activity of uniconazole-P (Fig. 4). On the other hand, the *Z*-form isomer did not inhibit ABA degradation (Fig. 4). Later from this point, we called compound **4** as diniconazole. In these catabolic reactions using BY-2 cells, more than 80% of ABA treated in these experiments was recovered as unreacted ABA and PA. The recovery rate was almost the similar level in all experiments.

2.2. In vitro CYP707A3 inhibition kinetic analysis of diniconazole

Although diniconazole inhibited ABA catabolism in BY-2 cells, it was possible that its effect could be

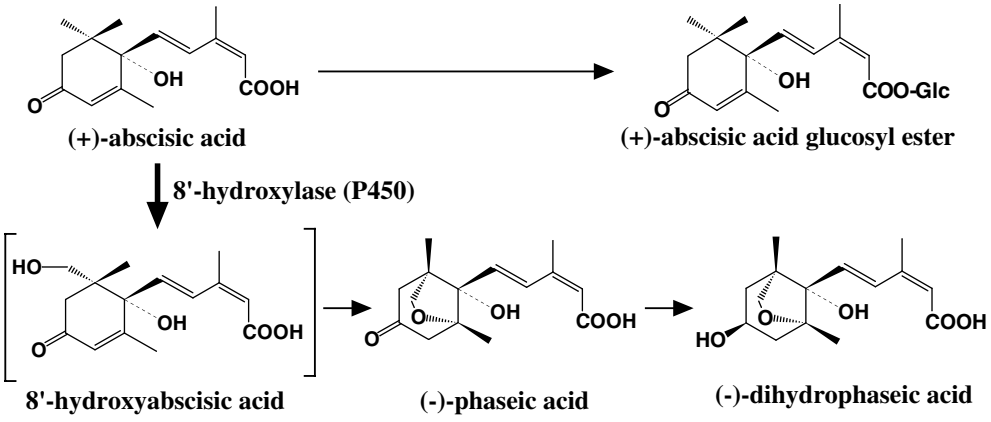


Figure 1. Catabolic pathway of ABA in plants.

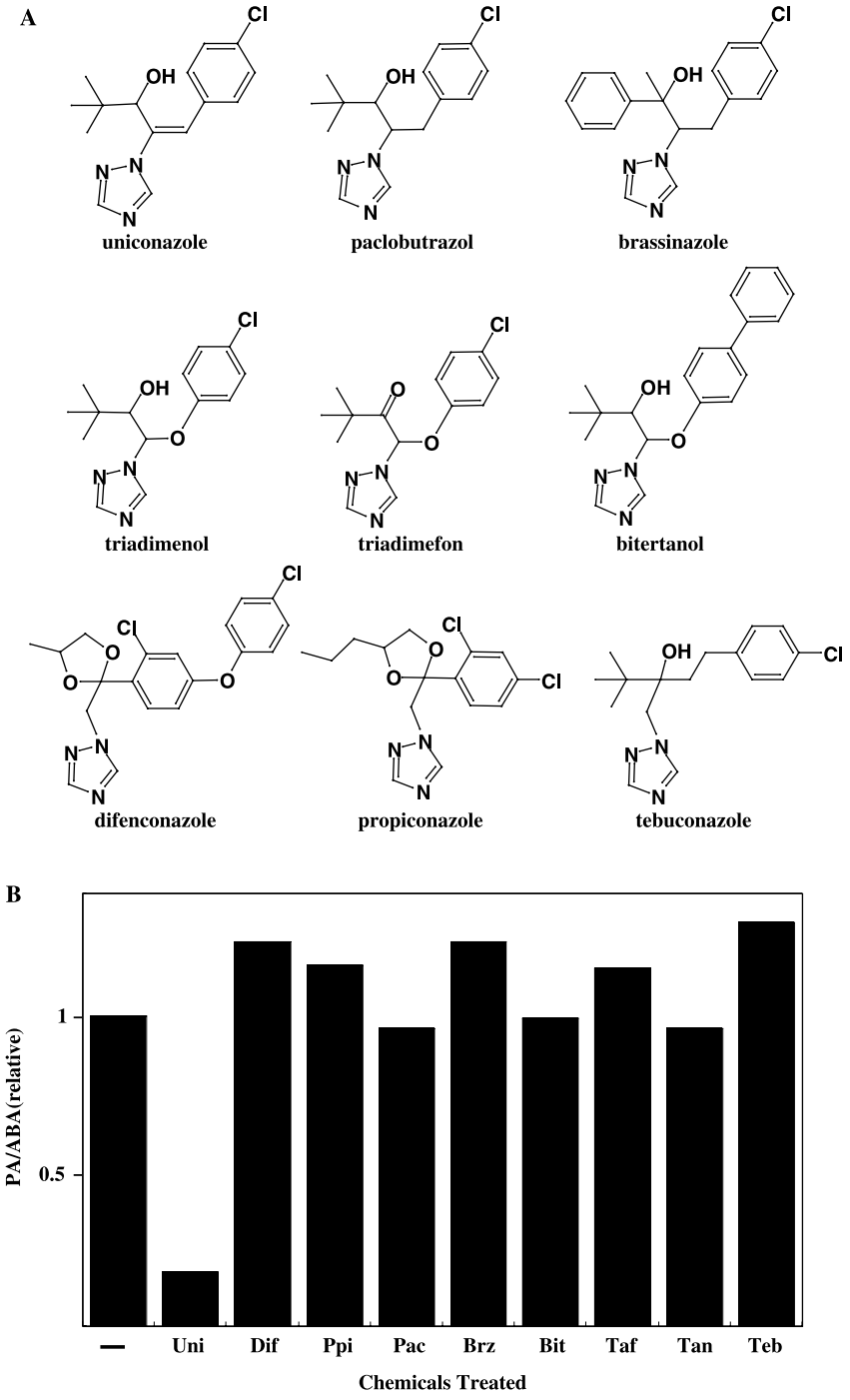


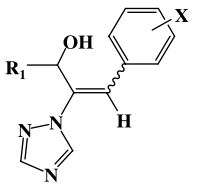
Figure 2. Inhibition of ABA catabolism by nine known triazole-type P450 inhibitors, in BY-2 cells. (A) Structure of known triazole-type P450 inhibitors assayed in this report. (B) Inhibitors were added at concentrations of 3 μM. Two independent experiments were performed with similar results. Uni, uniconazole-P; Dif, difenconazole; Ppi, propiconazole; Pac, paclobutrazol; Brz, brassinazole; Bit, biteratanol; Taf, triadimefon; Tan, triadimenol; and Teb, tebuconazole.

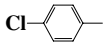
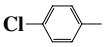
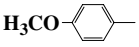
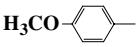
ascribed to the inhibition of a pathway other than that mediated by ABA 8′-hydroxylase. Therefore, to directly demonstrate the effect of diniconazole, we carried out an in vitro ABA 8′-hydroxylase assay using the recombinant *Arabidopsis* ABA 8′-hydroxylase, CYP707A3. In the presence of diniconazole, the activity of ABA 8′-hydroxylase was inhibited strongly, whereas with the *Z*-form (compound **3**), little effect on ABA 8′-hydroxylase activity was seen (data not shown). Subsequently, we performed an inhibition kinetics analysis. The data in Figure 4 shows that diniconazole is a potent competitive inhibitor of CYP707A3, with a K_i of 2 nM, as determined from a Dixon plot (Fig. 5).

2.3. Effect of diniconazole on drought stress

In plants, ABA accumulates during dehydration, and its levels decrease during the recovery process. This reg-

Table 1. Structure of uniconazole derivatives assayed in this report



R_1	X	*Z*	*E*
t-Bu	3-OCH_3	1	2
t-Bu	2,4-DiCl	3	4
t-Bu	4-O-*n*-C_4H_9	5	6
t-Bu	4-F	7	8
t-Bu	4-CH_3	9	10
t-Bu	4-CN	11	12
	4-Cl	13	14
	4-Cl	15	16
	4-Br	17	18
	4-Cl	19	20
	4-Br	21	22

ulation of ABA content is related to the expression levels of not only 9-*cis*-epoxycarotenoid dioxygenase, which is thought to be a rate-limiting step in ABA biosynthesis, but also of ABA 8′-hydroxylase. Therefore, using LC–MS/MS, we examined the effects of diniconazole on the ABA content during dehydration and rehydration. Two-week-old *Arabidopsis* plants possessed about 2–4 ng of ABA per gram fresh weight (data not shown). When the plants were exposed to dehydration conditions for 4 h, ABA accumulated to about 10-fold the level of controls. Diniconazole-treated plants slightly accumulate more ABA than control plants during dehydration (Table 2). After a 4-h recovery, ABA was reduced to approximately one-fifth of the 4-h dehydration level in plants without treatment and to about half the 4-h dehydration level in plants treated with 10 μM diniconazole (Table 2). The experiments were repeated three times with similar results. ABA induces the expression of ABA response genes such as *RD29b* and *RAB18*.[23,24] To examine whether the expression of ABA response genes was influenced by diniconazole treatment during rehydration, RT-PCR analysis was performed on samples collected after 8 h of rehydration (Fig. 6). Diniconazole-treated plants retained high transcription levels of ABA response genes during rehydration (Fig. 6). These results were consistent with the ABA content levels. Next, we examined whether ABA catabolic inhibition affected drought stress tolerance. After 2-h dehydration treatment, diniconazole-treated plants clearly showed stronger drought stress tolerance than plants without treatment (Fig. 7).

2.4. Optical resolution of diniconazole

Diniconazole is a stronger fungicide than is uniconazole, but a far less effective growth retardant as a GA biosynthesis inhibitor,[25] making diniconazole a more useful ABA catabolic inhibitor than uniconazole. Diniconazole has an asymmetric center in the molecule, and thus it consists of two optical isomers (*S*-form and *R*-form). The *S*-form isomer of uniconazole (or uniconazole-P) is much more active as GA biosynthesis inhibitor than are the *R*-forms; the *S*-form is also less active as fungicides than are the *R*-forms.[26] Therefore, we anticipated that the optical resolution of diniconazole might separate its strong fungicidal activity from its inhibitory activity toward ABA catabolism. Upon performing optical resolution and examining the inhibitory activity of the two isomers, we found that the *S*-form of diniconazole, which is the less active fungicidal isomer, was

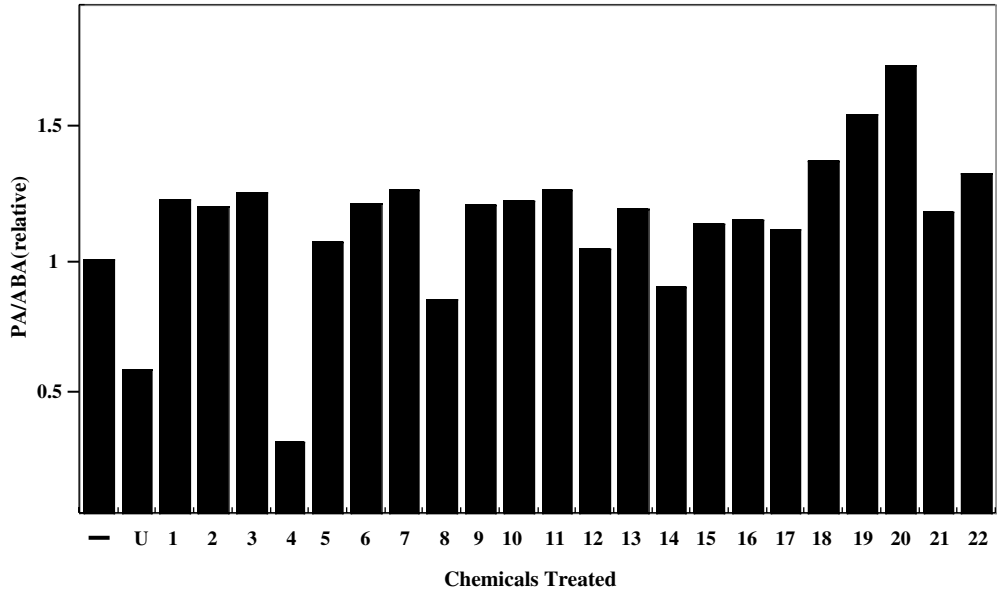


Figure 3. Inhibition of ABA catabolism by uniconazole derivatives with triazole, in BY-2 cells. Inhibitors were added at concentrations of 3 μM. Two independent experiments were performed with similar results. U indicates uniconazole-P.

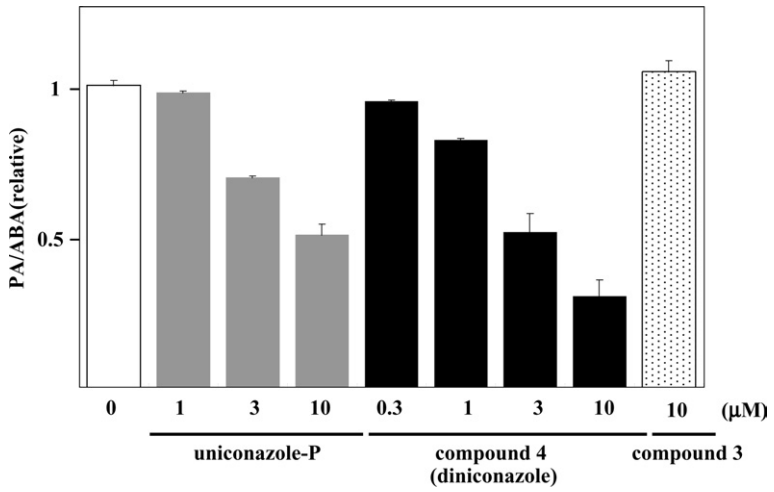


Figure 4. Inhibition of ABA catabolism by diniconazole, uniconazole-P, and compound **3** at various concentrations, in BY-2 cells. Data are means ± SD obtained from triplicate samples. Two independent experiments were performed with similar results.

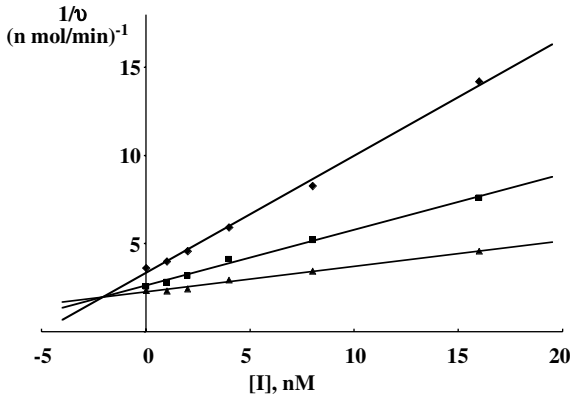


Figure 5. Kinetic analysis of the inhibition of CYP707A3 by diniconazole. CYP707A3 activity was measured in the presence of 0.5 μM (diamond), 1 μM ABA (square), and 2 μM ABA and 25 μg/mL microsomal protein with the indicated concentrations of diniconazole at pH 7.25 at 30 °C. Diniconazole is a potent competitive inhibitor of CYP707A3, with a K_i of 2 nM as determined from a Dixon plot.

Table 2. Endogenous levels of ABA after dehydration and rehydration treatment of *Arabidopsis*

Dose of diniconazole (μM)	Experiment		
	1 ABA (ng/g FW)	2 ABA (ng/g FW)	3 ABA (ng/g FW)
Dehydration			
Control	18.3	39.5	30.2
3	20.0	40.1	32.7
10	20.9	41.8	32.2
Rehydration after dehydration			
Control	3.6	5.8	6.5
3	10.8	15.9	18.1
10	14.5	22.1	18.7

All experiments were performed under the same experimental conditions. Ten-day-old *Arabidopsis* plants were treated in 0.4 M mannitol solution with or without diniconazole for 4 h (dehydration). After dehydration, the plants were transferred into solutions without mannitol but with the same concentration of diniconazole for 4 h (rehydration). FW indicates fresh weight.

more active as an ABA catabolic inhibitor than was the *R*-form (Fig. 8).

3. Discussion

In this report, we demonstrate that some of the triazole derivatives target ABA 8′-hydroxylase and inhibit ABA catabolism. From among the tested compounds, diniconazole showed the highest activity (Fig. 3). Compounds with substituents on the phenyl ring of (*E*)-4,4-dimethyl-1-phenyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (*E*-form analogs), including uniconazole and diniconazole, have higher fungicidal activity than do the corresponding *Z*-form analogs or ketone analogs.[16] The *Z*-form and ketone analogs of uniconazole and diniconazole were found to have no inhibitory activity toward ABA catabolism (Fig. 3, data not shown). In addition, the inhibitory activities of uniconazole and diniconazole toward ABA catabolism, which are likely

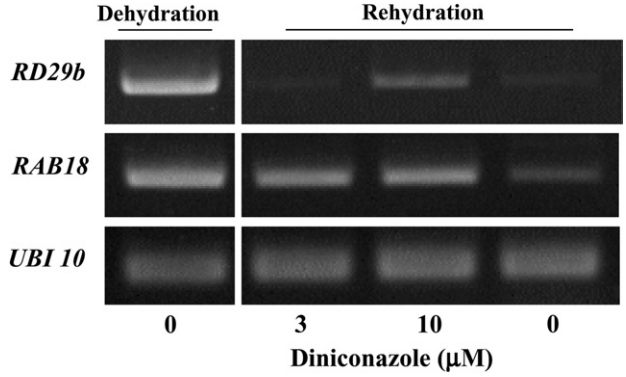


Figure 6. The expression of ABA response genes after rehydration. The expression of *UBI10* showed no difference in transcript levels for each treatment. Ten-day-old *Arabidopsis* plants were treated in 0.4 M mannitol solution with or without diniconazole for 4 h (D). After dehydration, the plants were transferred into solutions without mannitol but with the same concentration of diniconazole for 4 h (R).

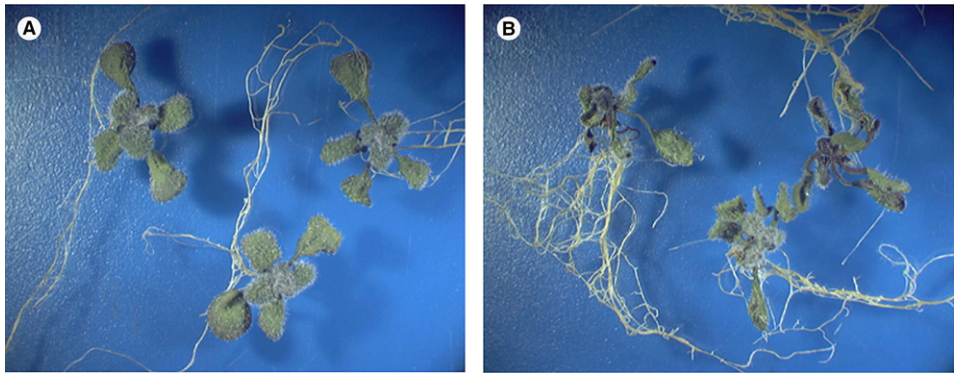


Figure 7. Drought tolerance of plants with or without diniconazole. Ten-day-plants grown on MS agar plate were transferred to plate with or without diniconazole and then grown for one day. Drought stress was by 2-h desiccation. The photographs showed dehydrated plant treated with (A) or without (B) 30 μM diniconazole.

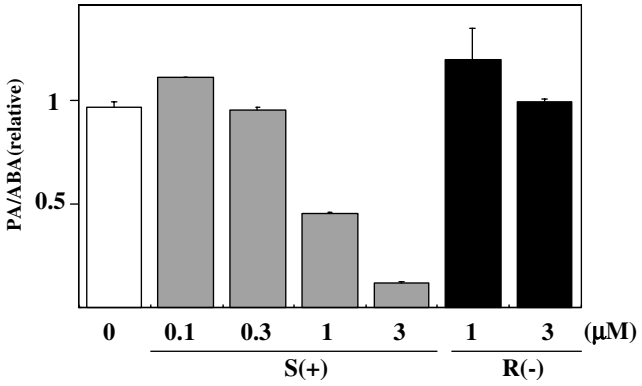


Figure 8. Inhibition of ABA catabolism by two optical isomers of diniconazole. Effect in BY-2 cells was examined. Data are means ± SD obtained from triplicate samples. Two independent experiments were performed with similar results.

fungicidal activities, were higher than those of other *E*-form analogs (Fig. 3, Ref. 16). That is, diniconazole is the most potent ABA catabolic inhibitor among the compounds tested in this report.

For the development of inhibitors targeting a specific enzyme, we must consider the specificity of inhibitors. Uniconazole-P is known as a GA biosynthesis inhibitor and diniconazole is known as a fungicide. A fungicidal effect on plant function can be negligible for use as a ABA catabolic inhibitor, but the effect as GA biosynthesis inhibitor should be in mind. In our preliminary test, diniconazole slightly retarded rice stem elongation and such retardation was rescued by treatment with GA (Ref. 18; data not shown). Although this result suggests that diniconazole may inhibit GA biosynthesis, this effect of diniconazole on the retardation of rice stem elongation is far less than that of uniconazole as reported.[25] Moreover, as ABA also retarded rice stem elongation and this retardation was recovered by the co-application of GA with ABA, more detailed studies on the effect of diniconazole on GA biosynthesis inhibition should be essential for clarifying the side effect of diniconazole. Finally, we found diniconazole as a ABA catabolism inhibitor in a small chemical library prepared in our laboratory, but the substitution on the phenyl ring may not be optimal for specifying the inhibitory activity of these chemicals for ABA catabolism. The design of brassinazole, a specific BR biosynthesis inhibitor, was based on its analogy to uniconazole and paclobutrazol.[18] Although uniconazole and paclobutrazol inhibit GA and BR biosynthesis, the substitution of a phenyl group with a planar structure for the *tert*-butyl group with a tetrahedral structure succeeded in enhancing the inhibitory effect on BR biosynthesis while eliminating the inhibitory effect on GA biosynthesis. The substitutions of the functional group of uniconazole examined in this report separated the inhibitory effect on ABA metabolism from that on GA biosynthesis. However, the substitution of a different group for the *tert*-butyl group may lead to the development of more specific and potent ABA catabolic inhibitors. Tetcyclacis, a cytochrome P450 inhibitor, also targets ABA 8′-hydroxylase and causes ABA to accumulate in plants[19,20,27] but it was less potent than diniconazole in our in vitro test (data not shown).

In our experiment, diniconazole-treated *Arabidopsis* plants accumulate slightly more ABA during dehydration than control (Table 2). That is, diniconazole is not efficient in dehydration stage. This result can be ascribed to the fact that the expression level of CYP707As genes was suppressed under dehydration condition. That is, catabolic reaction does not efficiently work in this condition. Contradictory results have been reported with respect to uniconazole-induced changes in ABA levels.[28,29] Inconsistencies have also been reported for other triazole compounds and have been thought to reflect differences of plant species or of the times of measurement after triazole treatment.[30] Thus, the accumulation of ABA in diniconazole-treated *Arabidopsis* plants may differ with the diniconazole concentration used as well as with other experimental conditions, such as the time of measurement after treatment.

ABA-treated plants display increased resistance to several types of environmental stress, including drought, salinity, ozone, heat, chilling, and freezing.[1] Therefore, an increase of ABA is important for conferring environmental stress tolerance on plants. It has been reported that drought-stress tolerance was increased in uniconazole-treated turfgrass.[31] We achieved similar results with

Arabidopsis, which acquired drought-stress resistance when treated with a high concentration of diniconazole (Fig. 7). Although we did not examine the ABA content of these plants, the drought-stress tolerance may be attributable to the inhibition of ABA catabolism. These findings suggest that diniconazole may effectively confer stress tolerance on plants by inhibition of ABA catabolism. This result that diniconazole treated plants show a different phenotype from that of untreated plants will open the way for using diniconazole in mutant screening. The mutants insensitive to diniconazole can be ABA signal transduction mutants as was seen in the brassinosteroid research[11] and the identification of the genes of the mutants will provide a new way of practical application of such genes for agriculture.

4. Experimental

4.1. Chemicals

S-(+)-ABA was a kind gift from Toray Co., Ltd (Chuo, Tokyo, Japan). [1,2-$^{13}C_2$]-(±)-ABA was prepared as described by Asami et al.[32] The cytochrome P450 inhibitors listed in Figure 1 were purchased from Wako Pure Chemicals (Osaka, Japan). The *E*,*Z*-mixtures of the tested compounds were synthesized and the geometric isomers were separated by column chromatography or high-performance liquid chromatography as previously reported.[16,18] The ^{1}H NMR ($CDCl_3$, 300 MHz) data for diniconazole (compound **4**) are as follows: 0.63 (s, 9H), 4.36 (d, 1H), 4.40 (d, 1H), 6.86 (s, 1H), 7.35 (d, 1H), 7.45 (d, 1H), 7.49 (s, 1H), 8.07 (s, 1H), 8.51 (s, 1H). The diniconazole enantiomers (*S*-form and *R*-form) were obtained by separating diniconazole on a chiral stationary phase column (Daicel Chem. Ltd, Chiralpak AS, 4.6 × 250 mm), using *n*-hexane/2-propanol (95:5) as the eluant at a flow rate of 1.4 mL/min, with detection at a wavelength of 254 nm. Chemical data for the enantiomers are consistent with those of the previous work.[33]

4.2. Cell culture

Suspension-cultured tobacco (*Nicotiana tabacum*) Bright Yellow-2 cells (BY-2 cells) were maintained in conventional medium as described by Miyazawa et al.[34]

4.3. Screen for ABA catabolic inhibitors

To assess the effects of the tested compounds on ABA metabolism, ABA (20 μM) and a test compound (3 μM) dissolved in DMSO at 1000× concentration were added to a suspension of log-phase cells grown for 24 h in conventional medium. After 40 h, the cell cultures were collected in tubes and centrifuged at 13,000 rpm. The supernatant was filtered and 100 μL of the filtrate was loaded onto an HPLC equipped with a Capcell Pac C18 column (250 × 4.6 mm; Shiseido, Tokyo). The column was eluted at a flow rate of 0.5 mL/min with methanol/water (55:45, v/v) containing 0.1% acetic acid. The amounts of ABA and PA present were determined from the chromatogram.

4.4. In vitro 8′-hydroxylase assay

Heterologous expression of CYP707A3 using a baculovirus-insect cell system and preparation of recombinant CYP707A3 microsomes was described by Saito et al.[21] Typical incubations contained 50 mM potassium phosphate (pH 7.25), 25 μg/mL recombinant CYP707A3 microsomes, and (+)-ABA in a final reaction volume of 2 mL. For determination of K_i value, 0.5, 1.0 or 2.0 μM (+)-ABA was incubated with diniconazole (ranging from 1 to 16 nM). Reactions were initiated by addition of NADPH to a final concentration of 100 μM and carried out at 30 °C for 10 min and was stopped by addition of 100 μL of 1 N HCl. The reaction products were extracted four times with an equal volume of ethyl acetate. The organic layers were combined, dried over Na_2SO_4, filtered, and concentrated to give ethyl acetate-soluble materials. The materials were dissolved in 50 μL of methanol, and 10 μL of the sample was subjected to HPLC: column, YMC AQ-311 (Kyoto; ODS, 100-mm length × 6 mm i.d.); solvent, 45% (v/v) methanol in water containing 0.1% (v/v) acetic acid; flow rate, 1.0 mL/min; detection at 254 nm. Retention times of authentic samples were 5.8 min for PA, 9.6 min for 2*E*-ABA, and 12.1 min for ABA. The kinetic constants were calculated from triplicated data sets. The K_i value was determined by a Dixon plot of the initial velocity (v_0) versus inhibitor concentration.

4.5. Measurement of ABA level

Measurement of ABA level was performed essentially as reported by Zhou et al.[35] Samples were homogenized and extracted in 5 mL of methanol/water/acetic acid (90:9:1, v/v/v) with 2,6-di-*tert*-butyl-4-methylphenol (200 mg/L). $^{13}C_2$-ABA was added as an internal standard at the beginning of each extraction. After extraction, 17.5 mL water was added, and the samples were clarified by centrifugation at 13,000 rpm for 10 min. The samples were loaded onto Oasis HLB cartridges (Waters, Mississauga, Canada) that had been conditioned with methanol and equilibrated with methanol/water/acetic acid (9:90:1, v/v/v); ABA and PA were eluted with 1 mL methanol/water/acetic acid (90:9:1, v/v/v) and collected into a fresh tube. An aliquot (5 μL) of each sample was loaded onto a Capcell Pac C18 column (150 × 2 mm; Shiseido, Tokyo, Japan), and HPLC was performed using a binary solvent system of methanol and water with 0.1% formic acid (1:1) at a flow rate of 0.2 mL/min. The compounds were analyzed by tandem mass spectrometry with MRM in negative-ion mode. The precursor (m/z) > product (m/z) for each compound was as follows: 263 > 153 for the ABA standard and 265 > 153 for the $^{13}C_2$-ABA internal standard.

4.6. RT-PCR analysis

Total RNA was isolated from *Arabidopsis* plants by using RNeasy (QIAGEN). RT-PCR was performed using SuperscriptII (Invitrogen, California, USA). Gene-specific primer pairs were as follows: *RD29b* (At5g52300): 5′-AATTATCAGTCCAAAGTTACTGAT-3′ and 5′-TTTCTGCCCGTAAGCAGTAACAGA-3′;

Rab18 (At5g66400): 5′-AGCAGCAGTATGACGAGTAC-3′ and 5′-CTGGCAACTTCTCCTTGATC-3′; and *UBQ10* (At4g05320): 5′-TAAAAACTTTCTCTCAATTCTCTCT-3′ and 5′-TTGTCGATGGTGTCGGAGCTT-3′.

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