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A 9-*cis*-epoxycarotenoid dioxygenase inhibitor for use in the elucidation of abscisic acid action mechanisms

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Abstract—The plant hormone abscisic acid (ABA) accumulates in response to drought stress and confers stress tolerance to plants. 9-*cis*-Epoxycarotenoid dioxygenase (NCED), the key regulatory enzyme in the ABA biosynthesis pathway, plays an important role in ABA accumulation. Treatment of plants with abamine, the first NCED inhibitor identified, inhibits ABA accumulation. On the basis of structure–activity relationship studies of abamine, we identified an inhibitor of ABA accumulation more potent than abamine and named it abamineSG. An important structural feature of abamineSG is a three-carbon linker between the methyl ester and the nitrogen atom. Treatment of osmotically stressed plants with 100 μM abamineSG inhibited ABA accumulation by 77% as compared to the control, whereas abamine inhibited the accumulation by 35%. The expression of AB A-responsive genes and ABA catabolic genes was strongly inhibited in abamineSG-treated plants under osmotic stress. AbamineSG is a competitive inhibitor of the enzyme NCED, with a K_i of 18.5 μM. Although the growth of *Arabidopsis* seedlings was inhibited by abamine at high concentrations (>50 μM), an effect that was unrelated to the inhibition of ABA biosynthesis, seedling growth was not affected by 100 μM abamineSG. These results suggest that abamineSG is a more potent and specific inhibitor of ABA biosynthesis than abamine.

1. Introduction

Carotenoid cleavage dioxygenases (CCDs) produce various apocarotenoids that have important biological functions in animals and plants.[1] CCDs catalyze the oxidative cleavage of double bonds at various positions in a variety of carotenoids. Several CCDs have been identified and characterized. An enzyme that cleaves β-carotene at the 15–15′ double bond produces vitamin A, which is essential for development and vision in animals.[2] 9-*cis*-Epoxycarotenoid dioxygenase (NCED) is the best-characterized CCD in plants. NCED from maize, the first carotenoid cleavage enzyme identified, catalyzes the cleavage of 9-*cis*-epoxycarotenoid at the 11–12 double bond to produce a precursor of the plant hormone abscisic acid (ABA).[3,4] CCD1 cleaves several carotenoids symmetrically at the 9–10 and 9′–10′ double bonds to yield C13-norisoprenoid compounds such as β-ionone,[5] which plays a role in flower fragrance. Recently, it has been reported that CCD1 regulates the β-ionone content in petunia, tomato, and grape.[6–8] CCD7 and CCD8 catalyze the sequential cleavage of β-carotene.[9] As the *max3*/*ccd7* and *max4*/*ccd8* mutants of *Arabidopsis* show increased lateral branching, CCD7 and CCD8 appear to be involved in the biosynthesis of an unknown branch-inhibiting factor.[10–12]

ABA is involved in the regulation of many developmental processes in plants, accelerating abscission, inducing dormancy, and stimulating stomatal closure.[13] ABA is

Abbreviations: ABA, abscisic acid; NCED, 9-*cis*-epoxycarotenoid dioxygenase; CCD, carotenoid cleavage dioxygenase.
Keywords: Abscisic acid (ABA); Biosynthesis; Inhibitor; 9-*cis*-Epoxycarotenoid dioxygenase (NCED); Carotenoid cleavage dioxygenase (CCD).
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also involved in responses to environmental stresses such as drought and high salinity.[14] The levels of ABA rapidly increase more than 10-fold within a few hours of osmotic stress, conferring plants with stress tolerance. The accumulation of ABA in response to osmotic stress is thought to be regulated by NCED, the key regulatory enzyme in ABA biosynthesis (Fig. 1). *NCED* genes have been isolated from bean, cowpea, tomato, *Arabidopsis*, and avocado.[15–19] These genes are upregulated by osmotic stress,[15] but are not regulated by ABA.[19,20]

In view of the importance of ABA in plants, it is worthwhile to synthesize and evaluate specific ABA biosynthesis inhibitors that would be useful tools for functional studies of ABA biosynthesis and the effects of ABA in higher plants. In such studies, one advantage of ABA biosynthesis inhibitors over ABA-deficient mutants is that an inhibitor can be applied to any type of plant. Moreover, ABA biosynthesis inhibitors provide a useful method to isolate mutants in which the genes involved in ABA signal transduction have been altered.

Although carotenoid biosynthesis inhibitors such as fluridone and norflurazon have been used as ABA biosynthesis inhibitors,[21,22] these compounds cause lethal damage during plant growth because carotenoids play an important role in protecting photosynthetic organisms against damage by photooxidation.[23] Therefore, the use of these inhibitors in the investigation of ABA functions is limited to narrow physiological aspects. Abamine is a novel inhibitor of ABA biosynthesis that targets NCED and does not cause lethal damage.[24,25] Thus, abamine could be used to examine a broad range of physiological aspects involved in the functions of ABA. Abamine has already helped reveal that ABA plays a role in the control of the number of nodules on roots of leguminous plants.[26]

However, abamine has some points to be improved. Treatment with abamine suppresses the levels of ABA accumulation in *Arabidopsis* plants exposed to osmotic stress by 40% at the maximum. Moreover, the growth of *Arabidopsis* seedlings is inhibited by abamine at high concentrations, an effect that is unrelated to the inhibition of ABA biosynthesis. To overcome these problems, we designed and synthesized derivatives of abamine and carried out structure–activity relationship studies on these molecules. This approach led to the identification of an ABA biosynthesis inhibitor that is more potent and specific than abamine–abamineSG.

2. Results

2.1. A screen for potent ABA biosynthesis inhibitors

Table 1 shows the structures of abamine and the abamine derivatives that were tested in this study. The abamine derivatives have a modified phenyl ring of the *N*-benzyl group (compounds **1**–**4**), a modified linker between the ester and the nitrogen atom (compounds **5**–**8**), or a modified alkyl group at the ester moiety (compounds **9**–**14**).

To screen the above chemicals for ABA biosynthesis inhibitory activity more potent than that of abamine, we determined the ABA content of *Arabidopsis* plants that were incubated for 4 h in 0.4 M mannitol containing individual abamine derivatives. Following the mannitol treatment, the ABA content in mannitol-treated

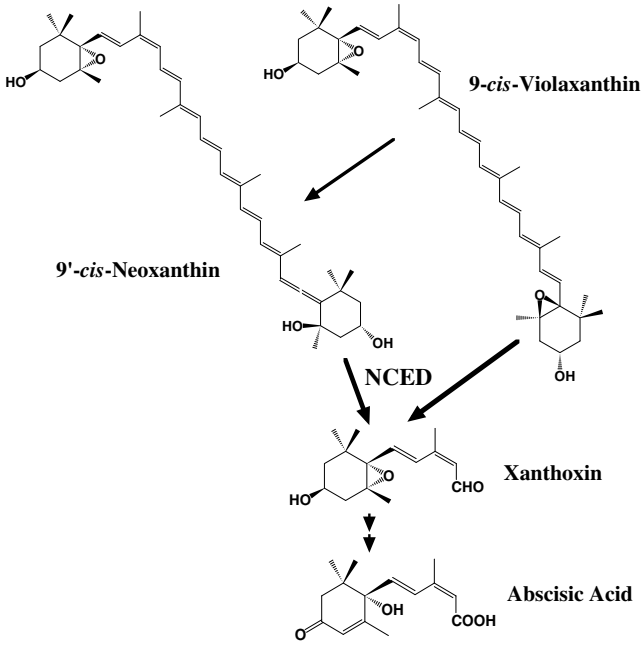
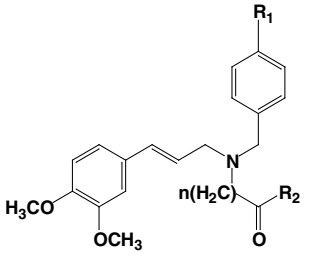


Figure 1. The ABA biosynthesis pathway in higher plants. ABA is synthesized from C_{40}-carotenoids, such as 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin, via the oxidative cleavage catalyzed by NCED. This step is the key regulatory step in the ABA biosynthesis pathway.

Table 1. Structures of abamine derivatives used in this study



	R_1	R_2	$n(CH_2)$
Abamine	F	OCH_3	1
1	Cl	OCH_3	1
2	OCH_3	OCH_3	1
3	CH_3	OCH_3	1
4	H	OCH_3	1
5	F	OCH_3	2
6	F	OCH_3	3
7	Cl	OCH_3	2
8	Cl	OCH_3	3
9	F	OH	1
10	F	OCH_2CH_3	1
11	F	$OCH_2CH_2CH_3$	1
12	F	$OCH_2CH{=}CH_2$	1
13	F	$OCH(CH_3)_2$	1
14	F	CH_3	1

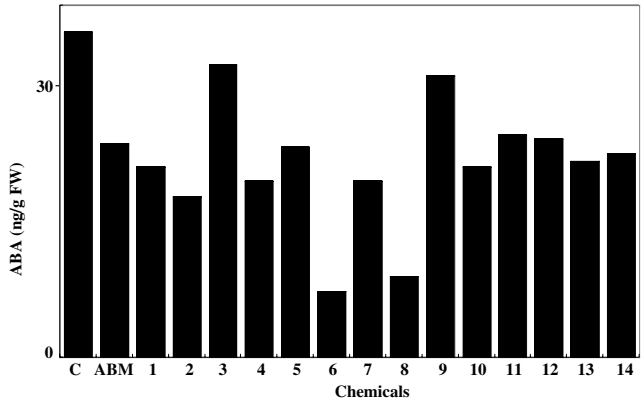


Figure 2. The inhibition of ABA accumulation under osmotic stress by abamine derivatives. Ten-day-old plants were incubated for 4 h in 0.4 M mannitol containing 100 μM of abamine or abamine derivatives. Three independent experiments were performed, with similar results.

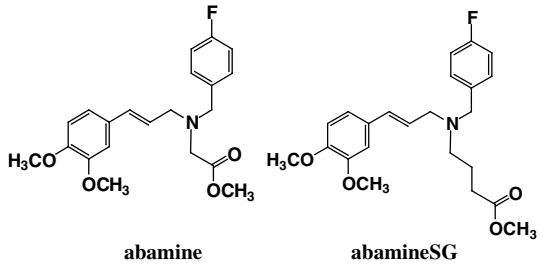


Figure 3. Structures of abamine and abamineSG. The length of the linker between the methyl ester and the nitrogen atom was crucial for the inhibition of ABA biosynthesis.

plants was about 17 times higher than in unstressed plants. Addition of abamine at 100 μM inhibited ABA accumulation by 35%. Two of the abamine derivatives tested, compounds **6** and **8**, had stronger inhibitory effects than abamine (Fig. 2). The structures of these compounds differed from those of the other abamine derivatives in the number of carbons between the methyl ester and the nitrogen atom. The most potent ABA biosynthesis inhibitors had the structural feature of a three-carbon linker between the methyl ester and the nitrogen atom. Since the inhibitory effect of compound **6** on ABA accumulation was slightly stronger than that of compound **8**, we selected compound **6** as a candidate for a novel potent ABA biosynthesis inhibitor, naming it abamineSG. AbamineSG was tested in all subsequent experiments (Fig. 3). The inhibitory activities of the 10 other abamine derivatives were similar to that of abamine, except for those of two, which were almost negligible (Fig. 2).

2.2. Effect of abamineSG on ABA accumulation under osmotic stress

To assess the inhibition of ABA accumulation by abamineSG, we compared its inhibitory activity with that of abamine. AbamineSG inhibited ABA accumulation in a dose-dependent manner within the concentration range of 10–100 μM (Fig. 4A) and retained strong inhibitory activity after incubation for 10 h in 0.4 M mannitol

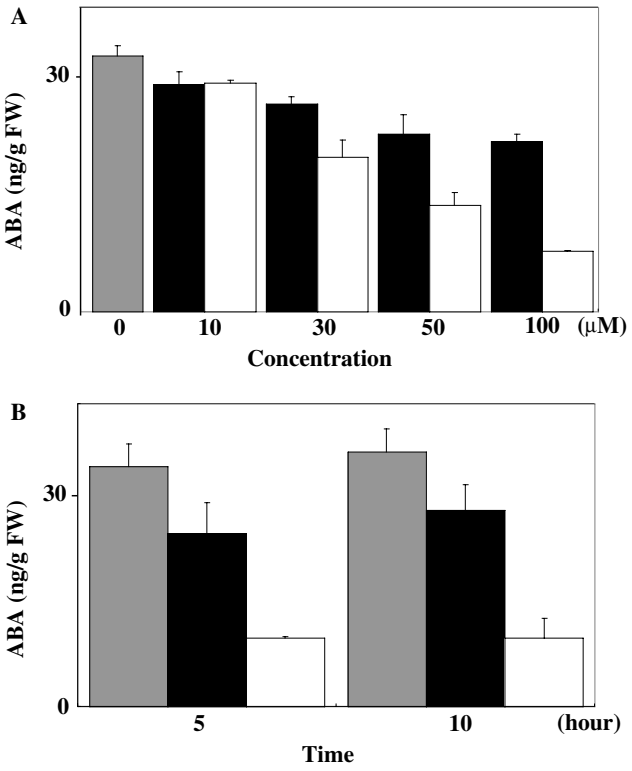


Figure 4. The effects of abamine and abamineSG on the inhibition of ABA accumulation under osmotic stress. (A and B) Ten-day-old plants were incubated in 0.4 M mannitol with the indicated concentration of abamine or abamineSG for 4 h (A), or with 100 μM abamine or abamineSG for the indicated times (B). Black bars indicate abamine treatment, white bars indicate abamineSG treatment, and gray bars indicate control treatment. The data presented are means ± SD of three samples. Two independent experiments were performed, with similar results.

(Fig. 4B). At 100 μM, abamineSG inhibited the ABA accumulation in plants exposed to osmotic stress by 77%, whereas abamine inhibited ABA accumulation by 35% (Fig. 4A).

To examine whether this difference in ABA accumulation influenced the transcription levels of ABA-inducible genes, RT-PCR analysis was performed (Fig. 5). The ABA-inducible genes *RD29B* and *RAB18* are not expressed under normal conditions.[27,28] In plants immersed in 0.4 M mannitol, expression of these genes was induced. In mannitol-immersed plants treated with abamine, the induction of these genes was slightly repressed as compared to the levels in the above plants, but in mannitol-immersed plants treated with abamineSG, these genes were strongly repressed (Fig. 5A).

We also examined the expression levels of ABA biosynthetic and catabolic genes. The expression of *NCED3*, a drought-stress-inducible ABA biosynthetic gene, was induced by 0.4 M mannitol. Additional treatment with either abamine or abamineSG did not influence the transcription of *NCED3* (Fig. 5B). The expression of the *CYP707A* genes, which are ABA catabolic genes, increased moderately in response to the increased ABA content during osmotic stress. Although abamine

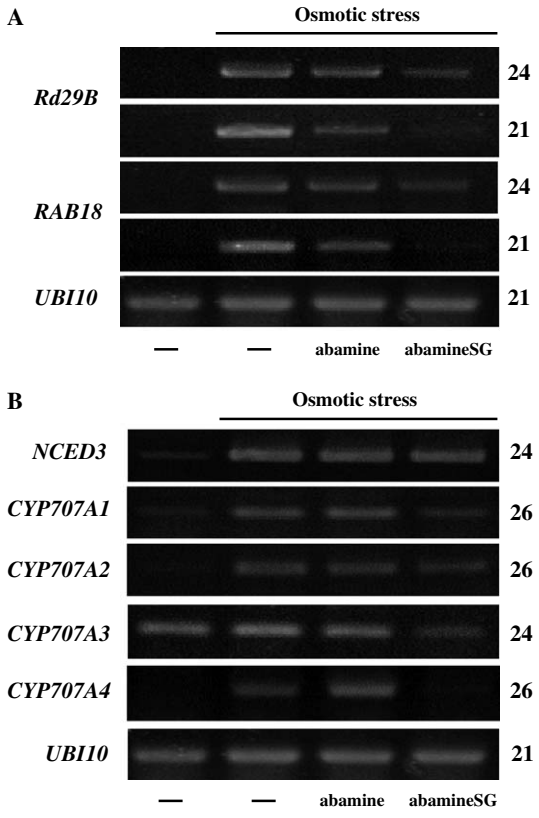


Figure 5. Transcription levels of ABA-inducible, ABA biosynthetic, and ABA catabolic genes under osmotic stress. (A) Transcription levels of the ABA-inducible genes *RD29B* and *RAB18* were analyzed by RT-PCR. The transcription levels of *UBI10* were similar in each treatment. Numbers to the right indicate the number of PCR cycles. (B) The transcription levels of the ABA biosynthetic gene *NCED3* and the ABA catabolic gene *CYP707As* were analyzed by RT-PCR.

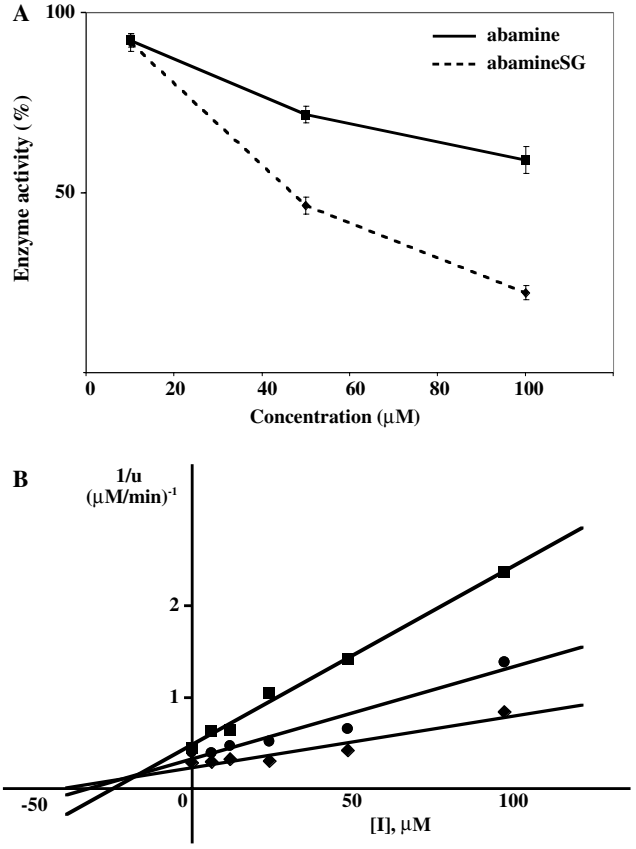


Figure 6. The effect of abamineSG on the inhibition of NCED activity. (A) The inhibitory activities of abamineSG and abamine were compared. The data shown are means ± SD of three independent experiments. (B) Inhibition kinetic analysis was performed for abamineSG. NCED activity was measured in the presence of 30 μM (■), 45 μM (●) or 60 μM (♦) of 9′-*cis*-neoxanthin and enzyme with the indicated concentrations of abamineSG at pH 6.7, and 25 °C. AbamineSG is a competitive inhibitor of NCED, with a K_i of 18.5 μM as determined using a Dixon plot.

treatment inhibited this induction only slightly, abamineSG strongly inhibited it (Fig. 5B). Interestingly, expression of *CYP707A3* was lower in abamineSG-treated plants than in unstressed plants (Fig. 5B).

2.3. In vitro NCED inhibition kinetic analysis of abamineSG

To assess the inhibition of NCED activity by abamineSG, the inhibitory activities of abamine and abamineSG were tested in an in vitro assay using NCED expressed in *E. coli*. At 100 μM, abamineSG inhibited the NCED activity by about 80%, whereas abamine inhibited the activity by about 40% (Fig. 6A). Next, an inhibition kinetic analysis of abamineSG was performed. Figure 6B shows that abamineSG is a competitive inhibitor of NCED, with a K_i of 18.5 μM determined using a Dixon plot. This K_i value is approximately half that of abamine.

The *NCED* gene is a member of the carotenoid cleavage dioxygenase (CCD) gene family. It has recently been revealed that CCD enzymes play important roles in plant development.[1] CCD1 regulates the synthesis of aroma compounds,[6–8] and CCD7 is involved in the synthesis of an unknown branch-inhibiting factor.[10–12] To examine whether abamineSG has an inhibitory effect on the activities of other CCD enzymes, an in vitro assay was performed with CCD1 and CCD7 expressed in *E. coli*. At 100 μM, abamineSG inhibited the NCED activity by 78%, whereas the inhibitory effects of abamineSG on the CCD1 or CCD7 activities were 20% or less (data not shown). The inhibitory effects of abamine on the CCD1 or CCD7 were similar to those of abamineSG (data not shown).

2.4. Effect of abamineSG on seedling growth

The growth of *Arabidopsis* seedlings is strongly inhibited by high concentrations of abamine (Fig. 7B). This effect is unrelated to the inhibition of ABA biosynthesis, because ABA biosynthesis mutants do not show this phenotype. In contrast, seedling growth was unaffected by 100 μM abamineSG (Fig. 7C). The fresh weight of abamineSG-treated plants was almost equal to that of untreated plants, but that of abamine-treated plants was only 30% that of untreated plants (Fig. 7D).

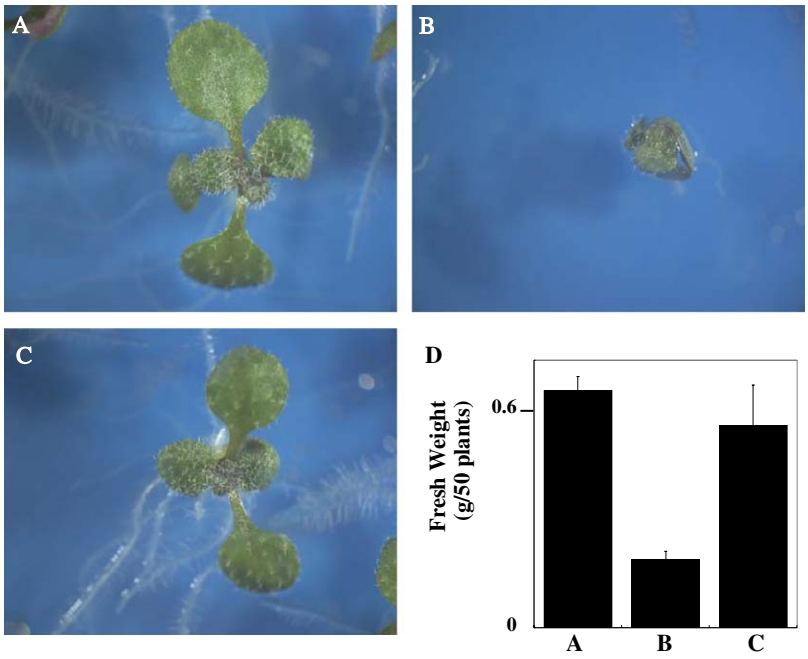


Figure 7. Effects of high concentrations of abamine and abamineSG on plant growth. (A–C) Ten-day-old *Arabidopsis* seedlings on a one-half-strength MS agar plate lacking inhibitor (A), with 100 μM abamine (B), or with 100 μM abamineSG (C). (D) The growth conditions in (A–C) correspond to those in Fig. 6A–C. The data shown are means ± SD of three independent experiments. *Arabidopsis* seedlings were grown in a growth chamber at 22 °C under continuous light.

3. Discussion

To develop more potent ABA biosynthesis inhibitors, abamine derivatives were synthesized (Table 1) and evaluated for their effects on osmotic-stress-induced ABA accumulation (Fig. 2). On the basis of a study of structure–activity relationships, we developed abamineSG, an ABA biosynthesis inhibitor that is more potent and specific than abamine (Fig. 3). The ABA content in mannitol-stressed plants treated with 100 μM abamine was 10 times that in unstressed plants, but the ABA content in mannitol-stressed plants treated with 100 μM abamineSG was only three times greater than in unstressed plants (Fig. 4). Therefore, abamineSG suppresses ABA responses in plants more potently than abamine.

Neither substitutions of the phenyl ring of the *N*-benzyl group nor modification of the methyl group in the ester moiety enhanced the inhibitory activity of abamine, suggesting that these groups are unrelated to the inhibitory activity of abamine. However, abamine derivatives with a three-carbon linker between the methyl ester and the nitrogen atom showed inhibited ABA biosynthesis more strongly than abamine. These results suggest that the length of the linker is important in the inhibition of ABA biosynthesis. It would be interesting to evaluate the inhibition of ABA biosynthesis by compounds with linkers consisting of four or more carbons.

Induction of the *CYP707A* genes in response to osmotic stress was strongly inhibited in abamineSG-treated plants, but not in abamine-treated plants (Fig. 5). These results show that abamineSG inhibits ABA biosynthesis more strongly than abamine. Interestingly, expression of *CYP707A3* was lower in abamineSG-treated plants under osmotic stress than in unstressed plants. An unknown mechanism that represses the *CYP707A3* gene might operate in plants when the increase of ABA content is suppressed by abamineSG, even if the plants are exposed to mannitol stress. Further studies using abamineSG might reveal whether this ABA catabolism repressive mechanism exists. These results show that abamineSG could be a valuable tool for elucidating the mechanisms that regulate ABA content. Under osmotic stress, the levels of *NCED3* transcripts in abamineSG-treated plants were similar to those in untreated plants (Fig. 5). It has been reported that ABA does not regulate expression of the *NCED* genes,[19,20] and our results are consistent with the results of those reports.

The growth of seedling is inhibited not only by high concentrations of abamine (Fig. 7B), but also by lipoxygenase inhibitor nordihydroguaiaretic acid, which is the lead compound of abamine (data not shown). Thus, the growth inhibition by abamine might be derived from inhibition of lipoxygenase. As abamineSG does not have the side effect, abamineSG should be a more specific NCED inhibitor than abamine.

Biosynthesis inhibitors have advantages over mutants because they allow rapid, conditional, reversible, selective, and dose-dependent control of biological functions. They can be tested on every type of plant to investigate the function of the target enzymes. However, abamine is difficult to use in such studies, because it is not potent enough for this purpose and produces the side effect of

growth inhibition. AbamineSG has more potent inhibitory activity than abamine both in vivo and in vitro (Figs. 4 and 6), and does not cause growth inhibition (Fig. 7). Thus, abamineSG should aid in the elucidation of the functions of ABA in cells or plants.

'Chemical genetics' approaches are increasingly being used in plant science studies.[29] For example, an auxin mimic and a brassinosteroid biosynthesis inhibitor have been used to screen for new mutants,[30,31] and novel mutants that affect the ABA sensitivity of *Arabidopsis* germination and seedling growth have recently been identified using an ABA analog.[32–34] Until the development of abamineSG, there were no chemicals that could be used to markedly decrease ABA content without fatal side effects. AbamineSG, which is currently used to decrease the ABA content in plants, could also be used in chemical genetics approaches to identify novel ABA mutants that show increased sensitivity to ABA.

The *NCED* gene is part of the *CCD* gene family. It has recently been shown that some *CCD* genes have important physiological functions. *CCD1* regulates the β-ionone content of plants,[6–8] and *CCD7* is related to the biosynthesis of unknown branch-inhibiting factors.[10–12] AbamineSG has weak inhibitory activities on these CCD enzymes. Thus, further chemical modifications and corresponding structure–activity relationship studies could make it possible to develop specific inhibitors for these CCD enzymes.

4. Experimental

4.1. Chemicals

9′-*cis*-Neoxanthin and all-*trans*-violaxanthin for the NCED assay were purified from spinach (*Spinacea oleracea*) leaves. β-Carotene for the CCD assay was purchased from Sigma (USA). Carotenoid standards were purchased from Wako Pure Chemical (Japan).

4.2. Synthesis of abamine derivatives

Abamine derivatives were synthesized essentially as reported by Han et al.[24] The chemical data for compound **6** (abamineSG) and compound **8** are as follows:

4-[[3-(3,4-Dimethoxyphenyl)allyl](4-fluorobenzyl)amino]-butyric acid methyl ester (**6**): (69%), pale yellow oil; ^{1}H NMR ($CDC1_3$) δ: 7.31 (2H, m), 7.04–6.90 (4H, m), 6.83 (1H, d, $J = 8.2$ Hz), 6.45 (1H, d, $J = 15.8$ Hz), 6.10 (1H, dt, $J = 15.8$, 6.5 Hz), 3.93 (3H, s), 3.89 (3H, s), 3.64 (3H, s), 3.59 (2H, s), 3.20 (2H, d, $J = 6.5$ Hz), 2.51 (2H, t, $J = 7$ Hz), 2.37 (2H, t, $J = 1$ Hz), 1.85 (2H, quint, $J = 1$ Hz). Anal. Calcd for $C_{23}H_{28}FNO_4$: C, 68.81; H, 7.03; N, 3.49. Found: C, 68.92; H, 6.98; N, 3.48.

4-{(4-Chlorobenzyl)[3-(3,4-dimethoxyphenyl)allyl]amino}-butyric acid methyl ester (**8**): (63%), pale yellow oil; ^{1}H NMR ($CDC1_3$) δ: 7.29 (4H, m), 6.94–6.89 (2H, m), 6.83 (1H, d, $J = 8.2$ Hz), 6.45 (1H, d, $J = 15.8$ Hz), 6.09 (1H, dt, $J = 15.8$, 6.5 Hz), 3.93 (3H, s), 3.90 (3H, s), 3.64 (3H, s), 3.58 (2H, s), 3.20 (2H, *d*, $J = 6.5$ Hz), 2.50 (2H, t, $J = 7$ Hz), 2.37 (2H, t, $J = 7$ Hz), 1.84 (2H, quint, $J = 1$ Hz). Anal. Calcd for $C_{23}H_{28}C1NO_4$ 1/3 H_2O: C, 65.15; H, 6.83; N, 3.30. Found: C, 65.19; H, 6.70; N, 3.25.

4.3. Dehydration treatment

Arabidopsis seeds were surface sterilized in 1.25% NaOCl (w/v) for 10 min, washed five times in sterile distilled water, and sown on 0.8% (w/v) agar-solidified medium containing one-half-strength Murashige and Skoog salts and 1% (w/v) sucrose. The plates were incubated for 3 days at 4 °C and then transferred to 22 °C under continuous light. Ten-day-old seedlings were pretreated with or without abamine at various concentrations for 2 h, and then each sample was immersed in 0.4 M mannitol (10 mM HEPES, pH 6.5) containing or lacking abamine. After 4 h of incubation, the seedlings were homogenized and soaked in methanol.

4.4. Measurement of ABA levels

ABA levels were measured essentially as reported by Kitahata et al.[35] Samples were extracted with 5 ml of methanol–water–acetic acid (90:9:1, v/v). Internal standards of $^{13}C_2$-ABA were added at the beginning of the extraction. Following the extraction, 17.5 ml of water was added. The samples were clarified by centrifugation at 15,000 rpm for 10 min. Oasis HLB cartridges (Waters, Mississauga, Canada) were conditioned with methanol and equilibrated with methanol–water–acetic acid (9:90:1, v/v/v). Then, the samples were loaded onto the cartridges and washed 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 in clean tubes; 5 μl of each sample was loaded onto an HPLC equipped with a C18 column (150 mm × 2 mm, 5 μM; Shiseido, Tokyo, Japan) using a flow rate of 0.2 ml/min and a binary solvent system comprising methanol (A) and water with 0.1% formic acid (B). The compounds were analyzed by tandem mass spectrometry with MRM in negative-ion mode. The precursor (*m*/*z*) > product (*m*/*z*) of each compound was: 263 > 53 for ABA standard, 265 > 153 for the $^{13}C_2$-ABA internal standard.

4.5. In vitro NCED assay

The procedure used to assay NCED activity was described previously.[28] NCED from cowpea was expressed in *E. coli* and purified.[19] Recombinant protein was incubated with 9-*cis*-neoxanthin in a solution of 100 mM bis-Tris, 0.05% Triton X-100, 10 mM ascorbate, and 0.5 mM $FeSO_4$ at room temperature for 10 min. The reaction mixture was extracted twice with ethyl acetate. The extractions were combined, concentrated, dissolved in methanol–water–chloroform (80:15:5, v/v/v), and subjected to HPLC analysis. The putative C25 product was eluted with a linear gradient between solvent A (85:5 v/v, methanol–water) and sol-

vent B (1:1 v/v, methanol–chloroform) at a flow rate of 1.5 ml/min and monitored with a UV/visible detector at 440 nm. The enzyme-inhibitor inhibition constant K_i and the mechanism of inhibition were determined using Dixon plots.

4.6. RT-PCR analysis

Total RNA was isolated using the RNeasy kit (Qiagen) from *Arabidopsis* plants that were exposed to osmotic stress for 4 h. The RT reaction was performed using Superscript II (Invitrogen, California, USA). The gene-specific primer pairs used were as follows: for *RD29B* (At5g52300), 5′-AATTATCAGTCCAAAGTTACTG AT-3′ and 5′-TTTCTGCCCGTAAGCAGTAACAG A-3′; for *RAB18* (At5g66400), 5′-AGCAGCAGTATG ACGAGTAC-3′ and 5′-CTGGCAACTTCTCCTTGA TC-3′; for *UBQ10* (At4g05320), 5′-TAAAAACTTTCT CTCAATTCTCTCT-3′ and 5′-TTGTCGATGGTGT CGGAGCTT-3′; for *NCED3* (At3gl4440), 5′-ATGG CTTCTTTCACGGCAAC-3′ and 5′-GCGATCTGAA CACTAGGATC-3′; for *CYP707A1* (At4gl9230), 5′-G AATCCATCGCTCAAGACTC-3′ and 5′-GGATCTG TGAGAGTTCCTTC-3′; for *CYP707A2* (At2g29090), 5′-TTATGCTGATGAACCGGCAC-3′ and 5′-GCTCA CTAACACCATTCGAG-3′; for *CYP707A3* (At5g45340), 5′-CTTGGGATG GAACTCAACTC-3′ and 5′-GAAT GATCCGAGGA GATCTG-3′; and for *CYP707A4* (At3gl9270), 5′-AGTAGAGGTAAGCTTCCTCC-3′ and 5′-GTCCTATCA GCTTCTCTTTG-3′.

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