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Synthesis and biological activity of amino acid conjugates of abscisic acid

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ABSTRACT

We prepared 19 amino acid conjugates of the plant hormone abscisic acid (ABA) and investigated their biological activity, enzymatic hydrolysis by a recombinant *Arabidopsis* amidohydrolases GST-ILR1 and GST-IAR3, and metabolic fate in rice seedlings. Different sets of ABA–amino acids induced ABA-like responses in different plants. Some ABA–amino acids, including some that were active in bioassays, were hydrolyzed by recombinant *Arabidopsis* GST-IAR3, although GST-ILR1 did not show hydrolysis activity for any of the ABA–amino acids. ABA–L-Ala, which was active in all the bioassays, an *Arabidopsis* seed germination, spinach seed germination, and rice seedling elongation assays, except in a lettuce seed germination assay and was hydrolyzed by GST-IAR3, was hydrolyzed to free ABA in rice seedlings. These findings suggest that some plant amidohydrolases hydrolyze some ABA–amino acid conjugates. Because our study indicates the possibility that different plants have hydrolyzing activity toward different ABA–amino acids, an ABA–amino acid may function as a species-selective pro-hormone of ABA.

1. Introduction

Abscisic acid (ABA) is a plant hormone involved in stress tolerance, stomatal closure, seed dormancy, and other physiological events.[1–3] Endogenous levels of ABA in plants are cooperatively controlled by biosynthesis, transport, and catabolic inactivation in response to environmental changes. ABA catabolism includes two major pathways: hydroxylation and glucosyl conjugation (Fig. 1). The principal pathway is mediated by ABA 8′-hydroxylase (CYP707A)[4,5] to produce 8′-hydroxy-ABA, which is thermodynamically unstable and is spontaneously converted into the more stable tautomer phaseic acid. The C1 glucose conjugate ABA-GE is a common metabolite in plants and the major ABA metabolite in some plants.[6]

Some acidic plant hormones, indoleacetic acid (IAA), jasmonic acid (JA), and salicylic acid (SA), are sometimes found as amino acid conjugates.[7–9] Various amino acid conjugates are considered to be a storage form, a deactivated form, or an endogenous bioactive molecule, although no definite role has been established except for (+)-7-*iso*-JA-L-isoleucine.[10] For other classical acidic plant hormones, gibberellin (GA) and ABA, there are no reports of amino acid conjugates being the endogenous metabolites, and based on research on ABA metabolism, we have never found any evidence that ABA–amino acid conjugates are endogenous metabolites. Moreover, ABA–amino acids have never been chemically synthesized, although 2*E*-ABA–L-serine was recently reported as a potential neuroprotective drug.[11]

Plants have amidohydrolase genes, some of which have been identified as IAA–amino acid conjugate hydrolases.[12–17] LeClere et al. suggested the GST fusion of an IAA–amino acid hydrolase, GST-IAR3, may hydrolyze JA–amino acids, as well as IAA–amino acids, on the basis of their preliminary experiments,[14] although amidohydrolases with specificity for JA–amino acids have not yet been identified. Because ABA is not so different in size and polarity from IAA and JA, these amidohydrolases might also hydrolyze ABA–amino acids. If different plant species have a different set of amidohydrolases with distinct substrate specificity, an ABA–amino acid may function as a species-selective ABA pro-hormone. Here we describe the synthesis and biological activity of 19 ABA–L-amino acid conjugates. We also describe the in vitro hydrolase activity of IAA–amino acid hydrolases for ABA–L-amino acids and the in vivo hydrolysis of ABA–L-Ala to release the free ABA.

Abbreviations: ABA, abscisic acid; ABA-GE, abscisic acid glucosyl ester; GA, gibberellin; IAA, indoleacetic acid; JA, jasmonic acid; SA, salicylic acid; Me ABAmide, methyl abscisic amide.

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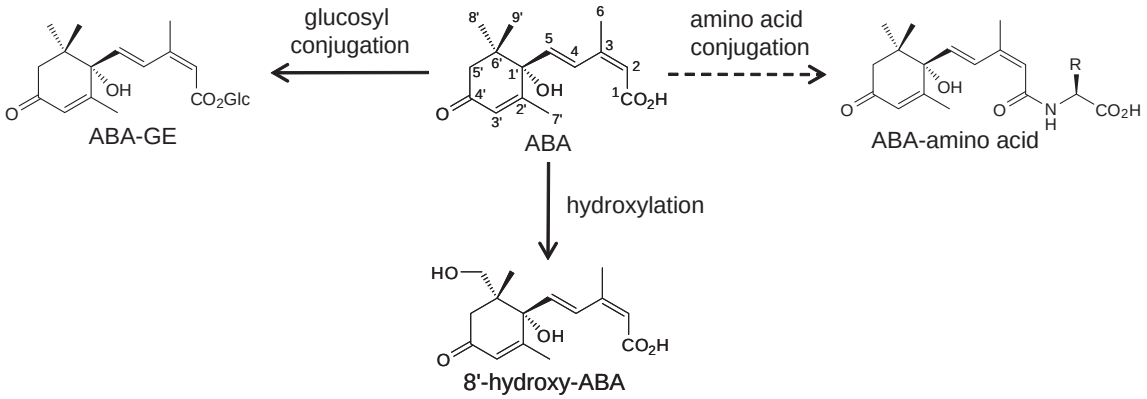

Figure 1. The major catabolic pathway of ABA in plants. Endogenous ABA–amino acid conjugates have not been identified.

2. Results and discussion

2.1. Synthesis

The acid chloride **1** was prepared by reacting *S*-(+)-ABA with phosphorus trichloride (Fig. 2). The coupling reaction of **1** and esters of standard L-amino acids except for L-arginine gave ABA–L-amino acid esters, which were hydrolyzed with sodium hydroxide or formic acid to afford ABA–L-amino acids. In the case of ABA–Ala only, the D-amino acid conjugate was also synthesized. The preparation of ABA–L-Arg was attempted but failed using a method similar to that for the other conjugates. Because ABA–L-Lys was difficult to isolate from the hydrolyzed residue of the methyl ester, it was used for enzyme and biological assays as the methyl ester. An ABA derivative with a simple amide moiety, methyl abscisic amide (Me ABAmide), was also prepared by reacting the acid chloride **1** with methylamine. Hexadeuterated labeled ABA–L-Ala and ABA–D-Ala for an in vivo hydrolysis assay were prepared by treatment of unlabeled compounds with 1 M NaOD solution. The ABA–amino acids prepared were characterized by ESI-TOF-MS (Table 1) and ^{1}H NMR. ABA contamination of the conjugate samples was checked by reverse-phase HPLC analysis (Table 1). Even in the most contaminated sample, the ABA content was less than 0.1%.

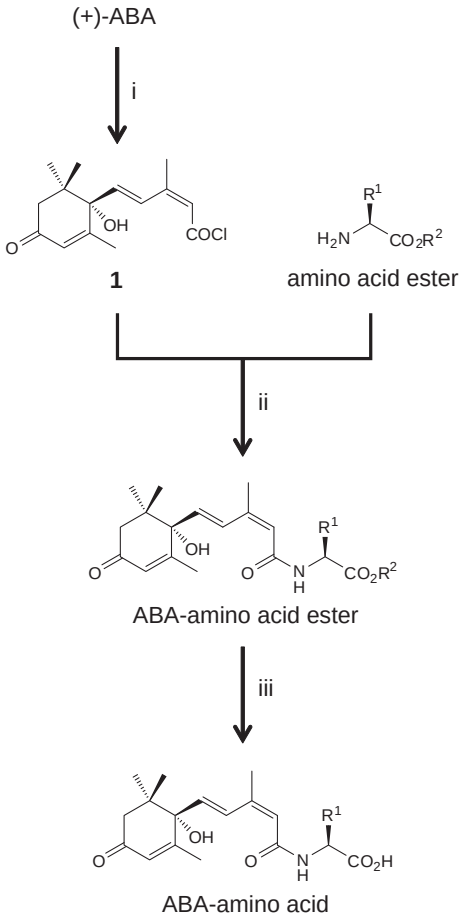

Figure 2. Preparation of ABA–amino acids. Reagents and conditions: (i) PCl_3 in CH_2Cl_2, rt, 1 h; (ii) rt, 1 h; (iii) 0.5–1 M NaOH in MeOH or formic acid, rt, 1–3 h.

2.2. Biological activity

The biological activity of ABA–L-amino acids and ABA–D-Ala was examined by four bioassays: germination assays using *Arabidopsis thaliana*, lettuce and spinach seeds, and a rice seedling growth assay (Fig. 3). All the compounds were tested at the same concentration, 10 μM in the *Arabidopsis* assay and 100 μM in other assays, at which the inhibitory ratio of ABA was 100%. Some L-amino acid conjugates showed significant activity, especially in the rice growth assay; seven conjugates, ABA–Gly, ABA–L-Ala, ABA–L-Met, ABA–L-Ser, ABA–L-Asn, ABA–L-Gln, and ABA–L-Asp, exhibited more than 80% inhibition of rice seedling elongation. The dose response effect of these conjugates was examined and IC_{50} values were determined to be 6–16 μM (Table 2), indicating that their activity is almost equivalent to that of ABA. On the other hand, in contrast to ABA–L-Ala, ABA–D-Ala showed no significant activity. In germination assays, the effect of ABA–amino acids was generally weaker than in the rice growth assay. In the *Arabidopsis* assay, ABA–L-Ala and ABA–L-Lys inhibited germination, whereas in the spinach assay, ABA–L-Ala, ABA–L-Glu, and ABA–L-Gln functioned as relatively effective inhibitors. These observations allowed us to conclude that the activity of ABA–amino acid conjugates is different in different species.

There are three possibilities for specific conjugates exhibiting ABA-like activity: (1) the conjugates themselves act as ABA mimics; (2) free ABA was released by nonenzymatic hydrolysis of the conjugates owing to their chemical instability; and (3) free ABA was released by enzymatic hydrolysis of the conjugates. The first possibility is not plausible, because the free carboxylate group of ABA is significantly involved in ABA activity,[18] and the crystal structure of the ABA receptor bound with ABA shows that the carboxylate of ABA forms an ion pair with the ε-amino group of the lysine residue at the bottom of the binding cavity.[19–23]To investigate the second possibility, we examined the stability of the active

Table 1
Exact mass data (ESI-TOF, positive/negative) and reverse-phase HPLC-based purity of ABA–amino acids

Compound	$[M+Na]^+/[M+H]^+/[M-H]^-$		Δ (mmu)	Elemental composition	HPLC-based ABA contaminant (%)
	Theoretical	Experimental			
ABA–Gly	344.1474	344.1474	0	$C_{17}H_{23}NNaO_5^+$	nd[b]
–L-Ala	358.1630	358.1626	−0.4	$C_{18}H_{25}NNaO_6^+$	nd
–D-Ala	358.1630	358.1637	0.7	$C_{18}H_{25}NNaO_6^+$	nd
–L-Val	386.1943	386.1937	−0.6	$C_{20}H_{29}NNaO_5^+$	0.10
–L-Leu	400.2100	400.2099	−0.1	$C_{21}H_{31}NNaO_5^+$	nd
–L-Ile	400.2100	400.2100	0	$C_{21}H_{31}NNaO_5^+$	nd
–L-Met	394.1688	394.1689	0.1	$C_{20}H_{28}NO_5S^+$	nd
–L-Pro	384.1787	384.1784	−0.3	$C_{20}H_{27}NNaO_5^+$	nd
–L-Phe	434.1943	434.1947	0.4	$C_{24}H_{29}NNaO_5^+$	nd
–L-Trp	473.2052	473.2051	−0.1	$C_{26}H_{30}N_2NaO_5^+$	nd
–L-Ser	374.1580	374.1577	−0.3	$C_{18}H_{25}NNaO_6^+$	nd
–L-Thr	364.1760	364.1759	−0.1	$C_{19}H_{26}NO_6^-$	nd
–L-Asn	401.1689	401.1687	−0.2	$C_{19}H_{26}N_2NaO_6^+$	nd
–L-Gln	415.1845	415.1848	0.3	$C_{20}H_{28}NNaO_6^+$	nd
–L-Tyr	450.1893	450.1892	−0.1	$C_{24}H_{29}NNaO_6^+$	nd
–L-Cys	366.1375	366.1371	−0.4	$C_{24}H_{29}NNaO_6^-$	nd
–L-Lys[a]	407.2546	407.2547	0.1	$C_{22}H_{34}N_2O_5^+$	nd
–L-His	424.1848	424.1846	−0.2	$C_{21}H_{27}N_3NaO_5^+$	nd
–L-Asp	378.1553	378.1552	−0.1	$C_{19}H_{24}NO_7^-$	nd
–L-Glu	416.1685	416.1684	−0.1	$C_{20}H_{27}NNaO_7^+$	nd

[a] The methyl ester.
[b] Not detected.

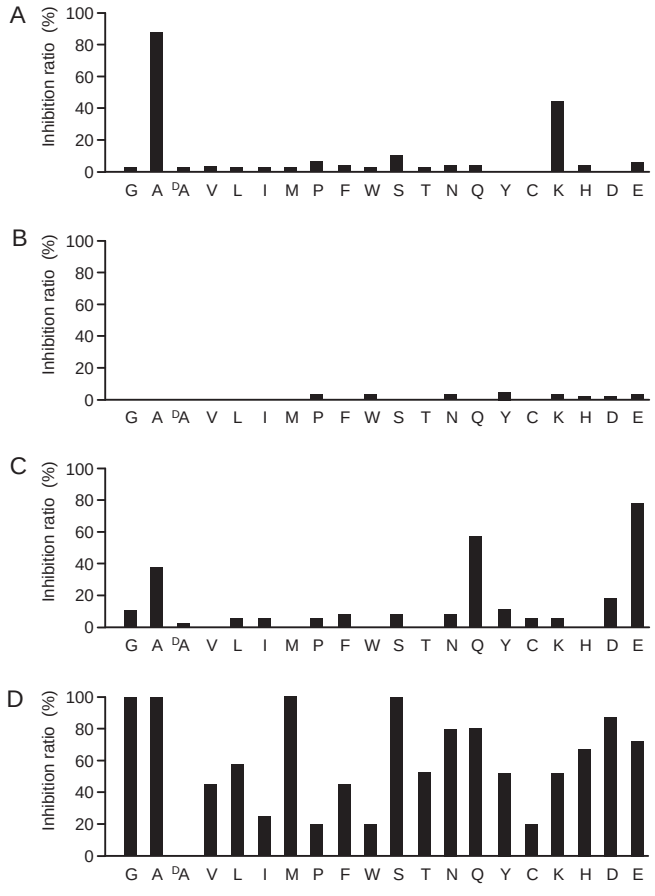

Figure 3. Inhibitory activity of ABA–amino acid conjugates in four bioassays. The conjugate is showed by the one-letter amino acid symbol. ABA–L-Lys was the methyl ester. (A) *Arabidopsis* seed germination. (B) Lettuce seed germination. (C) Spinach seed germination. (D) Rice seedling elongation. ABA exhibited 100% inhibition in all the assays, whereas ABA–methylamide and free amino acids showed no inhibition (data not shown).

Table 2
The IC_{50} values of ABA and ABA–amino acids in the rice elongation assay

Compound	IC_{50} (μM)
ABA–Gly	5.7
–L-Ala	7.0
–L-Met	16
–L-Ser	13
–L-Asn	9.3
–L-Gln	8.3
–L-Asp	6.4
ABA	2.1

conjugates under the rice assay conditions. ABA–L-Ala was not decomposed to release free ABA by incubation in an aqueous solution at 25 °C for 7 days under continuous light conditions, although it may have partially isomerized into 2*E*-ABA–L-Ala (not identified) (Fig. 4). The other active conjugates also were stable in the aqueous solution. Therefore, the second possibility is also not plausible. The active ABA–amino acids may have been enzymatically hydrolyzed to release free ABA after incorporation in plants.

2.3. Enzymatic amidohydrolysis

We expressed two GST fusions of *Arabidopsis* IAA–amino acid hydrolases, GST-IAR3 and GST-ILR1, according to a reported method[14] to examine whether the ABA–amino acids are enzymatically hydrolyzed by plant amidohydrolases. After checking that these GST-hydrolase fusions hydrolyzed IAA–L-Ala as reported, ABA–amino acid hydrolysis activity was examined. GST-ILR1 did not show hydrolysis activity for any of the ABA–amino acids, whereas GST-IAR3 hydrolyzed four ABA–L-amino acids, ABA–L-Gly, ABA–L-Ala, ABA–L-Met, and ABA–L-Asn, although activity was lower than that for IAA–L-Ala (Table 3). ABA–D-Ala was not hydrolyzed by GST-IAR3. The ABA–L-amino acids hydrolyzed by GST-IAR3 coincide with the corresponding IAA–amino acids hydrolyzed by the same enzyme,[14] meaning the activity is probably dependent on

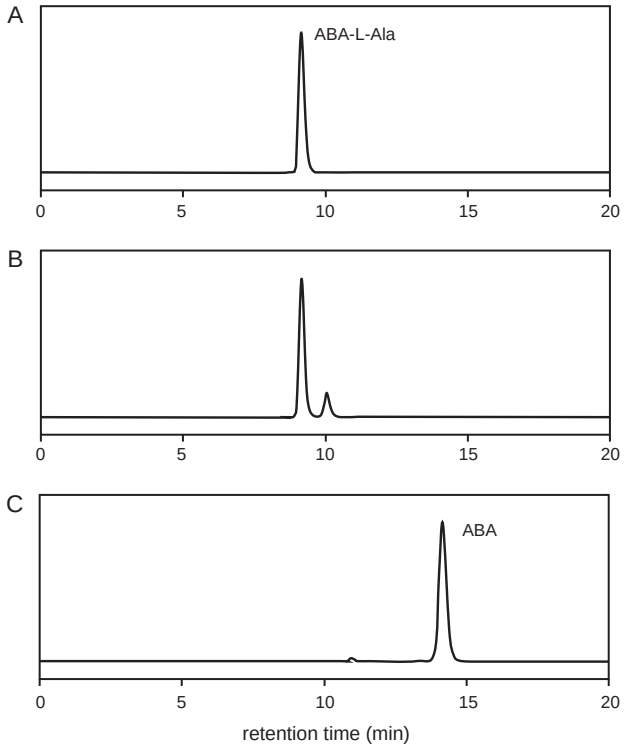

Figure 4. Stability of ABA–L-Ala in aqueous solution under the same conditions as those in the rice seedling elongation assay (25 °C, continuous light, 7-day incubation period). HPLC chromatograms: (A) ABA–L-Ala solution at day 0; (B) ABA–L-Ala solution at day 7; and (C) ABA standard solution.

Table 3
Amidohydrolase activity of *Arabidopsis* GST-IAR3 to release ABA from ABA–amino acids

Substrate	nmol ABA released/h/mg[b]
ABA–Gly	230
–L-Ala	310
–D-Ala	nd
–L-Val	nd
–L-Leu	nd
–L-Ile	nd
–L-Met	22
–L-Pro	nd
–L-Phe	nd
–L-Trp	nd
–L-Ser	nd
–L-Thr	nd
–L-Asn	19
–L-Gln	nd
–L-Tyr	nd
–L-Cys	nd
–L-Lys[a]	nd
–L-His	nd
–L-Asp	nd
–L-Glu	nd
IAA–L-Ala	2100 (nmol IAA released/h/mg)

[a] The methyl ester.
[b] Mean values of two independent experiments are given.

the amino acid preference of GST-IAR3. TaIAR3, an ortholog for the *Arabidopsis* IAR3, hydrolyzes the amino acid conjugates of indole-3-butyric acid.[15] These findings indicate that some IAA amidohydrolases may primarily recognize the amino acid part of conjugates rather than IAA. Rice (*Oryza sativa*) has a homolog (Os01g0560000) of *Arabidopsis* IAR3, and other related genes. Lettuce (*Lactuca sativa*) also has genes homologous to *ILR1* and *IAR3*.[24]

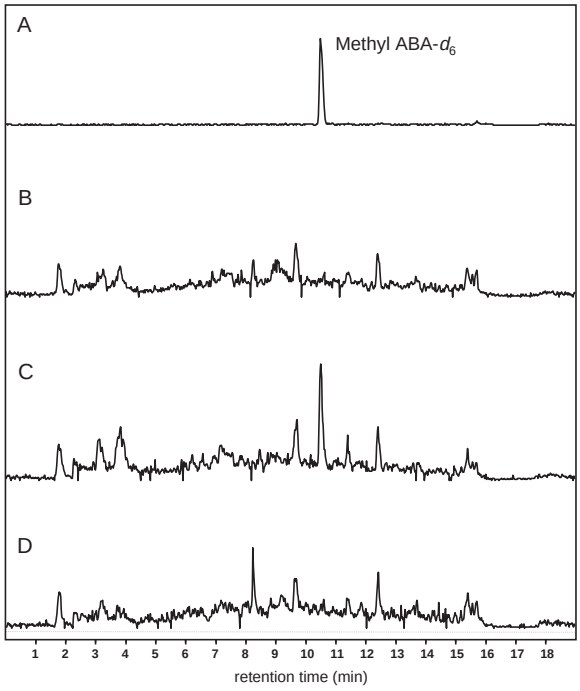

Figure 5. Mass chromatograms at m/z 267 $[M-H_2O+H]^+$ in an LC–MS analysis of methylated 80% acetone extract of rice seedlings fed ABA–L-Ala or ABA–D-Ala. (A) Methyl ABA-d_6 standard sample. (B) Unfed control sample. (C) Sample fed ABA–L-Ala. (D) Sample fed ABA–D-Ala.

This result indicates that ABA–amino acids exhibit the biological activity of ABA after enzymatic hydrolyzation. However, the bioactive ABA–amino acids in the *Arabidopsis* assay do not completely coincide with ABA–amino acids hydrolyzed by *Arabidopsis* GST-IAR3. This inconsistency may suggest that other amidohydrolases are responsible for ABA–amino acid hydrolysis in *Arabidopsis* seeds. In addition, uptake efficiency may be involved in the efficiency of in vivo hydrolysis of ABA–amino acids. The difference in the activity of ABA–amino acids in different bioassays may depend on differential expression or distinct amidohydrolase combinations.

2.4. Rice seedlings hydrolyze ABA–L-Ala, but not ABA–D-Ala, to release free ABA

To test whether the active ABA–amino acids are hydrolyzed in plants, ABA–d_6-L-Ala and ABA-d_6–D-Ala were fed to 3-day-old rice seedlings for 4 days. The 80% acetone extracts of the seedlings were methylated with TMS-diazomethane for purification and analyzed by LC-MS in neutral conditions, in which ABA-d_6 must be stable enough to not revert to the nondeuterated form. ABA-d_6 was detected only from seedlings administered ABA-d_6–L-Ala (Fig. 5). This means that rice seedlings hydrolyzed ABA–L-Ala, but not ABA–D-Ala, to release free ABA. This finding suggests that amidohydrolases in rice seedlings, just like *Arabidopsis* GST-IAR3, recognize an amide moiety originating from L-amino acids, not D-amino acids.

3. Conclusions

We prepared 19 ABA–amino acid conjugates and investigated their biological activity, enzymatic hydrolysis by recombinant *Arabidopsis* amidohydrolases, and metabolic fate in rice seedlings. Different sets of ABA–amino acids induced ABA-like responses in different plants. Some ABA–amino acids, partially including active

ones in bioassays, were hydrolyzed by recombinant *Arabidopsis* GST-IAR3. ABA–L-Ala, which was active except in a lettuce bioassay and was hydrolyzed by GST-IAR3, was hydrolyzed to free ABA in rice seedlings. These findings suggest that some plant amidohydrolases, including those reported as IAA amidohydrolases, hydrolyze some ABA–amino acid conjugates. No ABA–amino acids have been identified from plant materials. We also have not yet investigated whether ABA–amino acids are endogenous metabolites in plants. Thus, it is not clear whether plants have specific amidohydrolases to ABA–amino acids. In our preliminary experiment, GA_3-L-Ala, -Gly, -Met, and -Ile induced GA-like activity in rice seedlings (data not shown). Taken together, the substrate specificity of plant amidohydrolases may depend mainly on the amino acid part of the conjugate, not on the phytohormone part. Because our study indicates the possibility that different plants have hydrolyzing activity toward different ABA–amino acids, ABA–amino acids may function as a species-selective pro-hormone of ABA.

4. Experimental

4.1. General

^{1}H NMR spectra were recorded with tetramethylsilane as the internal standard using a JEOL JNM-EX270 (270 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). The purity of synthesized compounds was confirmed by HPLC using a Shimadzu LC-10. LC–MS analysis was performed by a Shimadzu LCMS-2010 system.

4.2. Chemicals

(+)-ABA was a gift from Toray Industries Inc., Tokyo, Japan. IAA-L-alanine was commercially purchased.

4.3. Synthesis of ABA–amino acid conjugates

Conjugates were typically synthesized by adding the methyl, ethyl or *t*-butyl ester of amino acids (3–5 equiv) to (+)-ABA chloride, followed by hydrolysis with NaOH or formic acid. Amino acid esters were prepared by stirring the commercially available ester hydrochloride and triethylamine (3–5 equiv) in dry DMF for 10 min. The mixture was filtered through absorbent cotton to remove the resulting salt before adding it to (+)-ABA chloride. (+)-ABA chloride was prepared by stirring (+)-ABA and PCl_3 (1.2–3 equiv) in dry CH_2Cl_2 for 1 h. After adding the amino acid ester to (+)-ABA chloride, the reaction mixture was stirred for 1 h. After adding water, the mixture was extracted with CH_2Cl_2. 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 a stepwise gradient of hexane–EtOAc. Except for ABA–L-Asn, ABA–L-Gln, and ABA–L-Lys, 0.5–1 M NaOH was added to each ABA–amino acid conjugate ester dissolved in MeOH. The mixture was stirred for 1–2 h. After acidifying with 1 M HCl to pH 2, the mixture was extracted with EtOAc. 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 a stepwise gradient of hexane–EtOAc containing 1% AcOH to afford the ABA amino acid conjugate as a white solid. The esters of ABA–L-Asn and ABA–L-Gln were dissolved in formic acid and stirred for 3 h. After adding satd $NaHCO_3$, the mixture was extracted with CH_2Cl_2. 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 a stepwise gradient of $CHCl_3$–MeOH containing 1% AcOH to afford the corresponding ABA amino acid conjugate as a white solid. The methyl ester of ABA–L-Lys was not hydrolyzed.

4.3.1. ABA–Gly

Yield: 37%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.93 and 2.01 (each 3H, s, H_3-6 and H_3-7′), 2.17 and 2.54 (each 1H, d, J = 16.8 Hz, H_2-5′), 3.92 (2H, s, H-C_α-Gly), 5.85 and 5.91 (each 1H, s, H-2 and H-3′), 6.16 (1H, d, J = 16.2 Hz, H-5), 7.76 (1H, d, J = 16.2 Hz, H-4).

4.3.2. ABA–L-Ala

Yield: 49%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.40 (3H, d, J = 7.3 Hz, H_3-C_β-Ala), 1.93 and 2.00 (each 3H, d, J = 1.2 Hz, H_3-6 and H_3-7′), 2.17 and 2.55 (1H, d, J = 16.8 Hz, H_2-5′), 4.40 (1H, m, H-C_α-Ala), 5.84 and 5.92 (each 1H, s, H-2 and H-3′), 6.14 (1H, d, J = 16.1 Hz, H-5), 7.73 (1H, d, J = 16.1 Hz, H-4).

4.3.3. ABA–D-Ala

Yield: 57%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.39 (3H, d, J = 7.3 Hz, H_3-C_β-Ala), 1.93 and 2.00 (each 3H, d, J = 1.0 Hz, H_3-6 and 7′), 2.16 and 2.54 (each 1H, d, J = 17.0 Hz, H_2-5′), 4.40 (1H, m, H-C_α-Ala), 5.84 and 5.91 (each 1H, s, H-2 and H-3′), 6.15 (1H, d, J = 16.2 Hz, H-5), 7.72 (1H, d, J = 16.2 Hz, H-4).

4.3.4. ABA–L-Val

Yield: 39%. ^{1}H NMR (270 MHz, CD_3OD): δ 0.97 (6H, m, H_3-C_γ-Val ×2), 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.93 and 2.01 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 2.1-2.2 (2H, overlapped, H-5′ and H-C_β-Val), 2.54 (1H, d, J = 16.8 Hz, H-5′), 4.35 (1H, d, J = 5.6 Hz, H-C_α-Val), 5.90 and 5.93 (each 1H, s, H-2 and H-3′), 6.14 (1H, d, J = 16.2 Hz, H-5), 7.74 (1H, d, J = 16.2 Hz, H-4).

4.3.5. ABA–L-Leu

Yield: 22%. ^{1}H NMR (270 MHz, CD_3OD): δ 0.94 and 0.97 (each 3H, d, J = 6.2 Hz, H_3-C_δ-Leu × 2), 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.5–1.8 (3H, overlapped, H-C_γ- and H_2-C_β-Leu), 1.93 and 2.00 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 2.17 and 2.54 (each 1H, d, J = 16.8 Hz, H_2-5′), 4.45 (1H, m, H-C_α-Leu), 5.86 and 5.91 (each 1H, m, H-2 and 3′), 6.14 (1H, d, J = 16.2 Hz, H-5), 7.73 (1H, d, J = 16.2 Hz, H-4).

4.3.6. ABA–L-Ile

Yield: 21%. ^{1}H NMR (270 MHz, acetone-d_6): δ 0.9–1.0 (6H, overlapped, H_3-C_γ- and H_3-C_δ-Ile), 1.04 and 1.07 (each 3H, s, H_3-8′ and H_3-9′), 1.26 and 1.53 (each 1H, m, H_2-C_γ-Ile), 1.91 and 1.98 (each 3H overlapped 1H, s, H_3-6 and H_3-7′, overlapped H-C_β-Ile), 2.14 and 2.54 (each 1H, d, J = 16.8 Hz, H-5′), 4.40 (1H, s, HO-1′), 4.52 (1H, dd, J = 8.6 and 5.6 Hz, H-C_α-Ile), 5.81 and 5.94 (each 1H, s,H-2 and 3′), 6.25 (1H, d, J = 16.2 Hz, H-5), 7.23 (1H, d, J = 8.6 Hz, HN-C_α-Ile), 8.08 (1H, d, J = 16.2 Hz, H-4).

4.3.7. ABA–L-Met

Yield: 39%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.93 and 2.01 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 2.09 (3H, s, H_3-C_ε-Met), 1.9–2.2 (3H, overlapped, H-5′ and H_2-C_β-Met), 2.5–2.6 (3H, overlapped, H-5′ and H_2-C_γ-Met), 4.57 (1H, m, H-C_α-Met), 5.86 and 5.91 (each 1H, s, H-2 and H-3′), 6.15 (1H, d, J = 16.2 Hz, H-5), 7.73 (1H, d, J = 16.2 Hz, H-4).

4.3.8. ABA–L-Pro

Yield: 38%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.91and 2.02 (each 3H, s, H_3-6 and H_3-7′), 1.9–2.1 and 2.27 (4H, m, H_2-C_β- and H_2-C_γ-Pro), 2.17 and 2.53 (1H, d, J = 16.8 Hz, H_2-5′), 3.58 (2H, m, H_2-C_δ-Pro), 4.45 (1H, m,

H-C_α-Pro), 5.90 and 6.02 (each 1H, s,H-2 and H-3′), 6.14 (1H, d, J = 16.1 Hz, H-5), 7.26 (1H, d, J = 16.1 Hz, H-4).

4.3.9. ABA–L-Phe

Yield: 72%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.01 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.91 and 1.97 (each 3H, s, H_3-6 and H_3-7′), 2.16 and 2.53 (each 1H, d, J = 16.8 Hz, H_2-5′), 2.97 (1H, dd, J = 13.9 and 8.6 Hz, H-C_β-Phe), 3.21 (1H, dd, J = 13.9 and 5.3 Hz, H-C_β-Phe), 4.68 (1H, dd, J = 8.6 and 5.3 Hz, H-C_α-Phe), 5.79 (1H, s, H-2), 5.90 (1H, s, H-3′), 6.13 (1H, d, J = 16.2 Hz, H-5), 7.1–7.3 (5H, m, phenyl-Phe), 7.71 (1H, d, J = 16.2 Hz, H-4).

4.3.10. ABA–L-Trp

Yield: 47%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.00 and 1.04 (each 3H, s, H_3-8′ and H_3-9′), 1.90 and 1.96 (each 3H, d, J = 1.2 Hz, H_3-6 and H_3-7′), 2.16 and 2.53 (each 1H, d, J = 16.8 Hz, H_2-5′), 3.18 (1H, dd, J = 14.5 and 7.6 Hz, H-C_β-Trp), 3.36 (1H, dd, J = 14.5 and 5.3 Hz, H-C_β-Trp), 4.76 (1H, m, H-C_α-Trp), 5.79 and 5.89 (each 1H, s, H-2 and H-3′), 6.13 (1H, d, J = 16.2 Hz, H-5), 6.9–7.1, 7.31 and 7.55 (5H, overlapped, indolyl-Trp), 7.73 (1H, d, J = 16.2 Hz, H-4), 10.25 (1H, m, HN-indolyl-Trp).

4.3.11. ABA–L-Ser

Yield: 38%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.92 and 2.00 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 2.17 and 2.54 (1H, d, J = 16.8 Hz, H_2-5′), 3.83 and 3.91 (each 1H, dd, J = 11.2 and 4.2 Hz, H_2-C_β-Ser), 4.52 (1H, dd, J = 4.9 and 4.2 Hz, H-C_α-Trp), 5.91 (2H, s, H-2 and H-3′), 6.16 (1H, d, J = 16.2 Hz, H-5), 7.76 (1H, d, J = 16.2 Hz, H-4).

4.3.12. ABA–L-Thr

Yield: 43%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.19 (3H, d, J = 6.6 Hz, H_3-C_γ-Thr), 1.93 and 2.02 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 2.17 and 2.54 (each 1H, d, J = 16.8 Hz, H_2-5′), 4.32 (1H, m, H-C_β-Thr), 4.45 (1H, d, J = 3.3 Hz, H-C_α-Thr), 5.90 and 5.95 (each 1H, s, H-2 and H-3′), 6.16 (1H, d, J = 16.2 Hz, H-5), 7.76 (1H, d, J = 16.2 Hz, H-4).

4.3.13. ABA–L-Asn

Yield: 18%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.06 (each 3H, s, H_3-8′ and H_3-9′), 1.93 and 2.00 (each 3H, s, H_3-6 and H_3-7′), 2.17 and 2.54 (1H, d, J = 16.8 Hz, H-5′), 2.77 (2H, m, H_2-C_β-Asn), 4.72 (1H, m, H-C_α-Asn), 5.84 and 5.91 (each 1H, s, H-2 and H-3′), 6.15 (1H, d, J = 16.2 Hz, H-5), 7.74 (1H, d, J = 16.2 Hz, H-4).

4.3.14. ABA–L-Gln

Yield: 35%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.92 and 2.00 (each 3H, d, J = 1.0 Hz, H_3-6 and H_3-7′), 2.17 (1H, d, J = 16.2 Hz, H-5′), 1.9–2.3 (4H, m, H_2-C_β- and H_2-C_γ-Gln), 2.55 (1H, d, J = 16.8 Hz, H-5′), 4.40 (1H, m, H-C_α-Gln), 5.86 and 5.90 (each 1H, s, H-2 and H-3′), 6.15 (1H, d, J = 16.2 Hz, H-5), 7.73 (1H, d, J = 16.2 Hz, H-4).

4.3.15. ABA–L-Tyr

Yield: 43%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.01 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.91 and 1.98 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 2.16 and 2.53 (each 1H, d, J = 16.8 Hz, H_2-5′), 2.88 (1H, dd, J = 13.9 and 8.6 Hz, H-C_β-Tyr), 3.10 (1H, dd, J = 13.9 and 5.6 Hz, H-C_β-Tyr), 4.61 (1H, dd, J = 8.6 and 5.6 Hz, H-C_α-Tyr), 5.81 and 5.90 (each 1H, s, H-2 and H-3′), 6.13 (1H, d, J = 16.5 Hz, H-5), 6.69 and 7.03 (each 2H, m, phenyl-Tyr), 7.70 (1H, d, J = 16.5 Hz, H-4).

4.3.16. ABA–L-Cys

Yield: 30%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.03 and 1.06 (each 3H, s, H_3-8′ and H_3-9′), 1.93 and 2.01 (each 3H, s, H_3-6 and H_3-7′), 2.17 and 2.55 (each 1H, d, J = 17.1 Hz, H_2-5′), 3.11 (1H, dd, J = 15.2 and 7.3 Hz, H-C_β-Cys), 2.8–3.1 (1H, m, H-C_β-Cys), 4.5–4.6 (1H, m, H-C_α-Cys), 5.91 (2H, s, H-2 and H-3′), 6.16 (1H, d, J = 16.2 Hz, H-5), 7.74 (1H, d, J = 16.2 Hz, H-4).

4.3.17. ABA–L-Lys-Me

Yield: 40%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.06 (each 3H, s, H_3-8′ and H_3-9′), 1.2–1.8 (6H, m, H_2-C_β-, H_2-C_γ-, H_2-C_δ-Lys), 2.92 (2H, m, H_2-C_ε-Lys), 1.93 and 2.01 (each 3H, s, H_3-6 and H_3-7′), 2.17 and 2.54 (each 1H, d, J = 16.8 Hz, H_2-5′), 3.72 (3H, s, H_3COC(O)-Lys), 4.41 (1H, m, H-C_α-Lys), 5.89 and 5.91 (each 1H, s, H-2 and H-3′), 6.16 (1H, d, J = 16.2 Hz, H-5), 7.72 (1H, d, J = 16.2 Hz, H-4).

4.3.18. ABA–L-His

Yield: 38%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.01 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.92 and 1.99 (each 3H, s, H_3-6 and H_3-7′), 2.17 and 2.53 (each 1H, d, J = 16.8 Hz, H_2-5′), 3.11 (1H, dd, J = 15.2 and 7.3 Hz, H-C_β-His), 3.25 (1H, m, H-C_β-His), 4.63 (1H, m, H-C_α-His), 5.84 and 5.90 (each 1H, s, H-2 and H-3′), 6.14 (1H, d, J = 16.2 Hz, H-5), 7.26 and 8.63 (each 1H, s, H-C_δ- and H-C_ε-His), 7.71 (1H, d, J = 16.2 Hz, H-4).

4.3.19. ABA–L-Asp

Yield: 57%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.06 (each 3H, s, H_3-8′ and H_3-9′), 1.93 and 2.00 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 2.17 and 2.54 (each 1H, d, J = 16.8 Hz, H_2-5′), 2.83 (2H, m, H_2-C_β-Asp), 4.76 (1H, m, H-C_α-Asp), 5.85 and 5.91 (each 1H, s, H-2 and H-3′), 6.16 (1H, d, J = 16.2 Hz, H-5), 7.76 (1H, d, J = 16.2 Hz, H-4).

4.3.20. ABA–L-Glu

Yield: 15%. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and H_3-9′), 1.93 and 2.00 (each 3H, d, J = 1.3 Hz, H_3-6 and H_3-7′), 1.9–2.2 (3H, overlapped, H-5′ and H_2-C_β-Glu), 2.40 (2H, m, H_2-C_γ-Glu), 2.55 (1H, d, J = 16.8 Hz, H-5′), 4.44 (1H, m, H-C_α-Glu), 5.86 and 5.91 (each 1H, s, H-2 and H-3′), 6.15 (1H, d, J = 16.2 Hz, H-5), 7.73 (1H, d, J = 16.2 Hz, H-4).

4.3.21. ABA-d_6–L-Ala and –D-Ala

A solution of ABA–L-Ala (7.0 mg, 21 μmol) in D_2O (0.5 ml) was stirred, and 1 M NaOD (1.5 ml) was added; the mixture was stirred for 70 h at rt in the dark. After quenching with 1 M DCl (2 ml), the mixture was extracted with EtOAc. The organic layer was washed with D_2O, dried over Na_2SO_4, and concentrated in vacuo. The residual oil was purified by silica gel column chromatography with a stepwise gradient of hexane–EtOAc to give ABA-d_6–L-Ala (3.0 mg, 8.8 μmol, 42% yield) as a pale yellow oil. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and 9′), 1.39 (3H, d, J = 7.3 Hz, H_3-3″), 2.00 (3H, d, J = 1.0 Hz, H_3-6), 4.39 (1H, q, J = 7.3 Hz, H-2″), 5.84 (1H, s, H-2), 6.14 (1H, d, J = 16.2 Hz, H-5), 7.73 (1H, d, J = 16.2 Hz, H-4). ESI-TOF-MS (positive): $[M+Na]^+$ at m/z 364.2007 ($C_{18}H_{19}D_6NO_5Na$ requires 364.2007).

A similar approach for ABA–D-Ala (19.0 mg, 57 μmol) gave ABA-d_6–D-Ala (17.0 mg, 50 μmol, 88% yield) as a pale yellow oil. ^{1}H NMR (270 MHz, CD_3OD): δ 1.02 and 1.05 (each 3H, s, H_3-8′ and 9′), 1.39 (3H, d, J = 7.3 Hz, H_3-3″), 2.00 (3H, s, H_3-6), 4.40 (1H, q, J = 7.3 Hz, H-2″), 5.84 (1H, s, H-2), 6.15 (1H, d, J = 16.0 Hz, H-5), 7.72 (1H, d, J = 16.0 Hz, H-4); ESI-TOF-MS (positive): $[M+Na]^+$ at m/z 364.2004 ($C_{18}H_{19}D_6NO_5Na$ requires 364.2007).

4.3.22. ABA–methyl amide

ABA–methyl-amide was prepared by the similar manner as the preparation of ABA–amino acid conjugates. After adding methyl amine (2.0 M THF solution, 250 μl, 500 μmol) in THF (1.5 ml) to (+)-ABA chloride prepared from (+)-ABA (30 mg, 114 μmol) in CH_2Cl_2 (1.5 ml) at rt, the reaction mixture was stirred for 0.8 h.

After adding water, the mixture was extracted with EtOAc. 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 a stepwise gradient of hexane–EtOAc to afford ABA–methyl amide (20.3 mg, 73 μmol, 64%). ^{1}H NMR (270 MHz, $CDCl_3$): δ 1.01 and 1.10 (each 3H, s, H_3-8′ and 9′), 1.93 (3H, d, J = 1.3 Hz, H_3-6 or H_3-7′), 1.96 (3Hd, J = 1.3 Hz, H_3-6 or H_3-7′), 2.28 and 2.47 (each 1H, d, J = 17.1 Hz, H_2-5′), 2.84 (3H, d, J = 4.9 Hz, H_3C-NH-), 5.64 (1H, s, H-2 or H-3′), 5.79 (1H, br, HN-), 5.91 (1H, s, H-2 or H-3′), 6.08 (1H, d, J = 16.2 Hz, H-5), 7.93 (1H, d, J = 16.2 Hz, H-4); ESI-TOF-MS (positive): $[M+Na]^+$ at m/z 300.1572 ($C_{16}H_{23}NO_3Na$ requires 300.1576).

4.4. Hydrolysis of ABA–amino acid conjugates by GST-IAR3 and GST-ILR1

4.4.1. Generation and purification of GST-IAR3 and GST-ILR1

The amidohydrolases IAR3 and ILR1 were expressed in *Escherichia coli* as a fusion to the C terminus of GST. pGEX-*IAR3* and pGEX-*ILR1* were made as reported by Davies et al.[13] For protein expression, single colonies from freshly transformed BL21 (DE3) *E. coli* cells were inoculated into 100 ml of Luria broth containing 100 μg ml^{-1} ampicillin and grown for 6 h at 37 °C. Cultures were allowed to cool to rt, induced by adding isopropyl-1-thio-β-D-galactopyranoside to 50 μM and grown overnight with shaking at 18 °C. Cells were harvested by centrifugation and lysed by sonication, and the soluble lysate was incubated with glutathione-agarose overnight at 4 °C. Fusion proteins were eluted with 5 mM reduced glutathione in 50 mM Tris (pH 8.0) for 3 h at 4 °C.

4.4.2. Hydrolysis assays

A hydrolysis buffer containing 50 mM Tris–HCl (pH 8.0), 1 mM dithiothreitol, 1 mM $MnCl_2$, and 1 mM ABA–amino acids was incubated for 5 min at 30 °C. Hydrolysis was started by adding 15 ng μl^{-1} GST-IAR3 (or GST-ILR1) and incubating at the same temp for 5 h. At each time point, 15 μl of the reaction was stopped by dilution in 135 μl of MeOH containing 1% AcOH. Time points were taken at 2, 3, and 5 h for IAA–L-Ala, ABA–Gly, –L-Ala, –D-Ala, –L-Ser, –L-Thr, and –L-Asp; at 2, 4, and 7 h for ABA–L-Asn, –L-Gln, –L-Lys-Me, –L-His, and –L-Glu; at 2, 5, and 7 h for ABA–L-Val, –L-Met, –L-Pro, –L-Tyr, and –L-Cys; and at 4, 6, and 24 h for ABA–L-Leu, –L-Ile, –L-Phe, and –L-Trp. The reaction mixture was loaded onto an Oasis HLB cartridge (30 mg) and the enzyme products were eluted with MeOH containing 0.1% AcOH. After concentrating in vacuo, the dried sample was dissolved in MeOH (final concn 200 ng μl^{-1}) and 10 μl of each sample was used in HPLC analysis: reverse phase column, YMC Hydrosphere C18 (150 × 6 mm) or YMC-Pack ODS-AQ (150 × 6 mm); mobile phase, 45% or 40% MeOH in H_2O (0.1% AcOH); flow rate, 1 ml min^{-1}; detection, 254 nm.

4.5. Bioassays

4.5.1. *A. thaliana* seed germination

Twenty-five seeds of *A. thaliana* 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 in which two sheets of filter paper had been placed and allowed to germinate under continuous light for 24 h at 22 °C. The percentage of seeds with an emerged radicle was calculated. All tests were conducted twice. The IC_{50} value of ABA was 0.20 μM.

4.5.2. Lettuce seed germination

Twenty-five seeds of lettuce (*Lactuca sativa* L. cv. Grand Rapids) were placed on two sheets of filter paper soaked in 2 ml of a test solution in a petri dish and allowed to germinate under continuous light for 24 h at 22 °C. The percentage of seeds with an emerged radicle was calculated. All tests were conducted twice. The IC_{50} value of ABA was 5.7 μM.

4.5.3. Spinach seed germination

Seeds of spinach (*Spinacia oleracea* L. cv. Jiromaru) were sterilized with EtOH for 5 min and washed with running tap water for 2 h. The sterilized seeds were placed on two sheets of filter paper soaked in 2 ml of a test solution in a petri dish and allowed to germinate under continuous light for 24 h at 22 °C after incubation at 5 °C for 2 days. The percentage of seeds with an emerged radicle was calculated. All tests were conducted twice. The IC_{50} value of ABA was 25 μM.

4.5.4. Rice seedling growth

Seeds of rice (*Oryza sativa* L. cv. Nipponbare) were sterilized with EtOH for 5 min and washed with running tap water. The sterilized seeds were soaked in water to germinate for 3 days at 25 °C. 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 tests were conducted twice. The IC_{50} value of ABA was 2.1 μM.

4.6. Test of ABA–L-Ala stability

An aqueous solution (2 ml) of ABA–L-Ala (100 μM) was prepared in a glass tube as for the rice growth assay. The tube was sealed with a plastic cap and incubated under continuous light at 25 °C for 7 days. The solution was subjected to HPLC analysis: reverse phase column, YMC-Pack ODS-AQ (150 × 6 mm); mobile phase, 45% MeOH in H_2O (0.1% AcOH); flow rate, 1 ml min^{-1}; detection, 254 nm.

4.7. Metabolic assay

Forty-five germinated rice seeds were placed in a glass tube containing 2 ml of water before incubating for 3 days. The water in the tube was removed, and 2 ml of an aqueous solution of ABA–L-Ala-d_6 or ABA–D-Ala-d_6 was added. The seedlings were incubated for 4 days under continuous light at 25 °C, and then frozen in liquid nitrogen. The frozen material was pulverized and extracted with 80% (v/v) acetone for 24 h at 5 °C. 1′,4′-*trans*-Diol-ABA was added as an internal standard for quantification of ABA after concentrating in vacuo. The residual solution was washed with hexane (15 ml × 3) and extracted with EtOAc (15 ml × 3). The combined EtOAc layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residual oil was dissolved in MeOH (2 ml) before treating with TMS-diazomethane (2.0 M solution in hexane, 1 ml). The mixture was concentrated in vacuo. The residue was loaded onto Sep-Pak cartridge Plus C18 (Waters) and eluted with MeOH (10 ml). The eluate was concentrated in vacuo and dissolved in MeOH (0.5 ml) for LC–MS (column, Capcell Pak C18 UG120, 150 × 2.0 mm I.D., Shiseido; solvent, 20–68% MeCN in H_2O; flow rate, 0.2 ml min^{-1}; oven, 40 °C; detection, 190–366 nm; MS condition, ESI (positive), SIM mode).

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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.2011.01.019.

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