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Bioorganic & Medicinal Chemistry
Bioorganic & Medicinal Chemistry 13 (2005) 3351–3358

Biotin-labeled abscisic acid as a probe for investigating abscisic acid binding sites on plasma membranes of barley aleurone protoplasts☆

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Received 10 December 2004; revised 9 March 2005; accepted 9 March 2005
Available online 6 April 2005

Abstract—Plant hormone abscisic acid (ABA) plays important roles in dormancy and stress responses, but its binding sites have not yet been fully elucidated. In this report, we suggest the utility of biotin-labeled abscisic acid (bioABA) as a probe to investigate ABA-binding sites on the plasma membrane of barley aleurone protoplasts. BioABA was approximately 100 times less effective than ABA in inhibiting expression of gibberellin-inducible α-amylase and in inducing expression of a reporter gene fused to the dehydrin promoter. To ascertain that bioABA could bind to ABA-binding sites on the plasma membrane, we used fluorescence flow cytometry to measure the fluorescence intensity of aleurone protoplasts treated with a combination of bioABA and fluorescence-labeled streptavidin. Addition of bioABA increased the fluorescence of aleurone protoplasts in a concentration-dependent manner, but addition of non-active bioABA derivatives did not. Furthermore, the increase in fluorescence intensity observed upon addition of bioABA was eliminated by co-treatment with excess ABA, but it was not eliminated by co-treatment with other plant hormones. These results suggest that bioABA binds to ABA-binding sites, and that bioABA should be a valuable probe for investigating ABA-binding sites on the plasma membrane.

1. Introduction

The plant hormone abscisic acid (ABA) is involved in regulation of many developmental processes in plants, affecting acceleration of abscission, induction of dormancy, and stimulation of stomatal closure.[1] ABA is also involved in the response to environmental stresses such as drought and high salinity.[2] Thus, ABA plays an important role in the survival of individual plants. Although studies on ABA signal transduction have been carried out in several model systems, and several ABA signal transduction components[3] or ABA-binding proteins[4–6] have been identified, its receptors have not yet been fully elucidated.

The aleurone cells of cereal seeds constitute a well-known model system for studying ABA signal transduction.[7] In aleurone cells, gibberellin (GA) induces α-amylase gene expression, and ABA inhibits this induction. ABA also induces expression of dehydrin genes, which encode proteins that are likely to protect cells from drought stress.[8] Barley aleurone protoplasts are often used for studies of ABA signal transduction because they are responsive to lower concentrations of ABA than are aleurone cells,[9] and a convenient transient assay for dehydrin promoter activity has been established.[10]

Aleurone protoplasts are also used in studies of ABA receptors. These studies have been carried out using various approaches.[11] An approach using ABA microinjection has suggested the existence of both extracellular and intracellular ABA-binding sites in aleurone protoplasts.[12,13] Another approach using ABA derivatives has suggested the existence of multiple ABA receptors with different structural requirements for activity in different response pathways.[14–16] A radio-iodinated

Keywords: Plant hormone; Abscisic acid; Aleurone; Flow cytometer; Binding site.
☆This research was supported in part by the Bioarchitect Research Program at RIKEN from the Science and Technology Agency of Japan.
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doi:10.1016/j.bmc.2005.03.017

ABA photoaffinity probe has been synthesized[17] and is likely to be useful in studies of ABA-binding sites. A radio-iodinated GA photoaffinity probe synthesized for the study of GA binding protein[18] has played an important role in identifying GA-binding polypeptides.[19] Since an approach using ABA derivatives would be relevant to a wide variety of plant species, these derivatives would be important tools for ABA receptor studies.

Fluorescence-labeled derivatives are widely used to identify and analyze functional receptors, and these compounds make it possible to observe the binding between ligands and cognate receptors in real time.[20] This type of observation can provide information on the mode, as well as the site, of binding. In animals, fluorescence-labeled compounds have been used to quantify receptors by flow cytometry or to visualize by fluorescence microscopy.[21] In plants, fluorescein-labeled ABA has been used as a probe for investigating ABA receptors.[22] However, this compound proved unsuitable for the study of binding sites on plasma membranes of barley aleurone protoplasts because it was incorporated into and/or bound non-specifically to the protoplasts to the extent that specific ABA-binding sites could not be identified. To overcome this difficulty, we have applied a different strategy that takes advantage of the particularly high affinity of biotin for bacterial streptavidin. This strategy has previously been important in qualitative and quantitative studies of other plasma membrane receptors,[23] such as ABA receptor on pea guard cells.[24] In this study, we confirm that biotin-labeled ABA (bioABA) has ABA-like activity, and that it is a useful probe for studying ABA-binding sites.

2. Results

2.1. Chemical structure of biotin-labeled abscisic acid

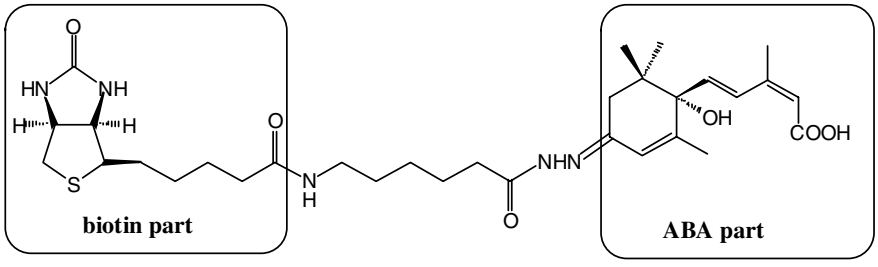
It has been reported previously that ABA retains its activity in barley aleurone protoplasts after substitution of other functional groups for the carbonyl group at its 4′-position.[25] Thus, we synthesized bioABA, which possesses a biotin group at the 4′-position of ABA. The structure of bioABA is shown in Figure 1.

2.2. Inhibition of GA-induced α-amylase expression by bioABA

The inhibition of GA-induced α-amylase induction is a well-established effect of ABA and has been used to test ABA-like activity of various ABA analogues.[15] To test whether bioABA possesses ABA-like activity, we carried out a GA-induced α-amylase synthesis test in barley aleurone protoplasts. Concentrations at which α-amylase activity was inhibited by 50% (IC_{50}) were estimated from a plot of α-amylase induction activity versus bioABA concentration (Fig. 2). As shown by Figure 2A, IC_{50} was about 0.02 μM for ABA and about 2 μM for bioABA. Thus, bioABA possessed approximately 1/100th of the ability of ABA to inhibit induction of α-amylase activity by GA. Inhibition increased with bioABA concentration up to 100 μM. Since it was possible that the observed inhibitory effect of bioABA was actually due to free ABA in the medium arising from degradation of bioABA, we performed HPLC analysis of the incubation medium. This analysis showed that free ABA was not detected in incubation medium (data not shown).

2.3. Dehydrin induction by bioABA

Although bioABA inhibited GA-inducible α-amylase induction, its effect could have been due to a mechanism other than that of ABA on the GA/α-amylase induction pathway. Thus, we also used a different ABA-responsive pathway to examine bioABA activity. In response to drought stress, ABA mediates a rapid physiological response and also slower responses such as dehydrin synthesis. Exogenous ABA induces the accumulation of dehydrin in unstressed plants. To examine whether bioABA mimics ABA in this respect, the effect of bioABA on the induction of dehydrin genes was tested in barley aleurone protoplasts by measuring dehydrin promoter activity in transient assays (Fig. 2B). ABA (10 μM) increased β-glucuronidase (GUS) activity by about four-fold over control levels, whereas 10, 100, and 1000 μM bioABA increased GUS activity about 1.5-, 2.5-, and 3.3-fold over control levels, respectively. Thus, bioABA possessed about 1/100th the activity of ABA in inducing expression from the dehydrin promoter. This relative activity is similar to that described above for induction of GA-inducible α-amylase activity.



biotin labeled abscisic acid (bioABA)

Figure 1. Structure of biotin-labeled ABA (bioABA).

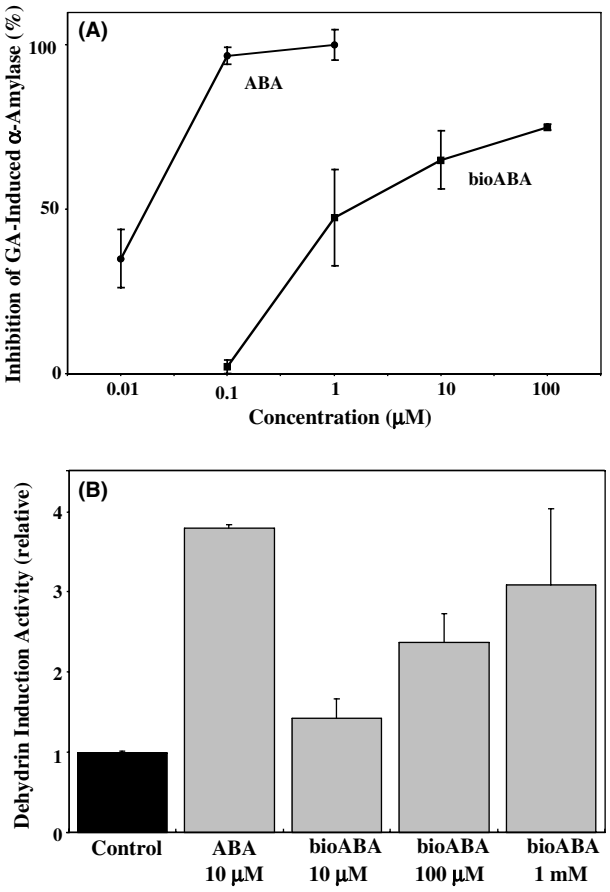


Figure 2. (A) Inhibitory activity of ABA and bioABA on GA-inducible α-amylase expression in barley aleurone protoplasts. Protoplasts were incubated with ABA or bioABA. The activity of each sample is expressed as % inhibition, where the activity of aleurone protoplasts treated with GA alone is defined as 0% inhibition, and that of the control solution (no GA) is defined as 100% inhibition. (B) Activation of the dehydrin promoter in barley aleurone protoplasts by ABA and bioABA. Promoter activity was measured as GUS reporter gene activity in transient transfection assays. The mean and SE of triplicate samples are shown.

2.4. Effect of bioABA on fluorescence intensity in barley aleurone protoplasts

Fluorescence flow cytometry is a technique that allows measurement of the fluorescence intensity of a single cell, which has been used to analyze the surface components of animal cells. However, flow cytometry is rarely used in plant cell-surface analyses because detection of fluorescence changes is impeded by the direct interaction of fluorescent chemical tags with components on the plant cell wall. However, removal of the cell wall to generate protoplasts makes it possible to analyze the direct interaction of bioABA with bioABA-binding components on the plasma membrane. Thus, we applied fluorescence flow cytometry in conjunction with fluorescence-labeled streptavidin. First, bioABA was incubated with protoplasts to allow it to bind to ABA-binding sites on the plasma membrane. Then, fluorescence-labeled streptavidin was added and allowed to bind to the resultant bioABA-binding complexes. Although streptavidin has less non-specific binding affinity than does avidin, fluorescence increased upon the addition of labeled streptavidin even in the absence of bioABA (data not shown). However, this fluorescence did not prevent detection of changes in fluorescence caused by the addition of bioABA (Fig. 3A).

We examined the influence of bioABA concentration on fluorescence intensity (Fig. 3B). BioABA (1, 10, or 100 μM) caused fluorescence intensity to increase by about 1.7-, 2.0-, or 2.3-fold over control levels, respectively. Since sufficient fluorescence intensity was observed with 10 μM bioABA, we used 10 μM as the optimum bioABA concentration in this study.

2.5. The bioABA-induced increase in fluorescence intensity is eliminated by co-treatment with ABA

Although we demonstrated an increase in fluorescence intensity of aleurone protoplasts upon the addition of bioABA, the possibility remained that the increase was due to non-specific binding of bioABA to the plasma membrane. To address this possibility, we performed additional assays in the presence of an excess of ABA. We reasoned that ABA should inhibit the bioABA-induced fluorescence if and only if bioABA is coupled specifically to ABA-binding sites. When aleurone protoplasts were co-treated with bioABA (10 μM) and a 100-fold molar excess of ABA (1 mM), the increase in fluorescence seen upon addition of bioABA was eliminated and the fluorescence level was reduced to that of bioABA non-treated samples (fluorescence intensity = 1.0) (Fig. 3B). We performed a more detailed examination of the effect of ABA on bioABA binding. Fluorescence intensity decreased with ABA concentration up to 1 mM, although co-treatment with equimolar concentrations of bioABA and ABA (10 μM) did not cause elimination of fluorescence intensity (Fig. 3C). This may be due to non-specific binding of bioABA to aleurone cells. That is, binding of bioABA to protoplasts may consist of specific binding and non-specific binding.

2.6. Effect of bioABA derivatives and plant hormones other than ABA on protoplast fluorescence

To examine whether the increase of fluorescence intensity observed upon addition of bioABA was specific for bioABA, we measured the fluorescence of aleurone protoplasts after treatment with biotin or bioABA derivatives containing modified ABA moieties (Fig. 4). The structures of these bioABA derivatives are shown in Figure 4A. We found that treatment with biotin or bioABA derivatives did not alter the fluorescence intensity of aleurone protoplasts in the presence of fluorescence-labeled streptavidin (Fig. 4B). Furthermore, to examine whether the inhibition of bioABA-induced fluorescence upon co-treatment with ABA was specific for ABA, we performed additional experiments with other plant hormones. Unlike ABA, co-treatment with GA or jasmonate (JA) did not affect the fluorescence intensity of labeled aleurone protoplasts (Fig. 5). Proteinase K is a stable and highly reactive serine protease. Pretreatment of aleurone protoplasts with proteinase K blocked the binding of bioABA. This inhibition of

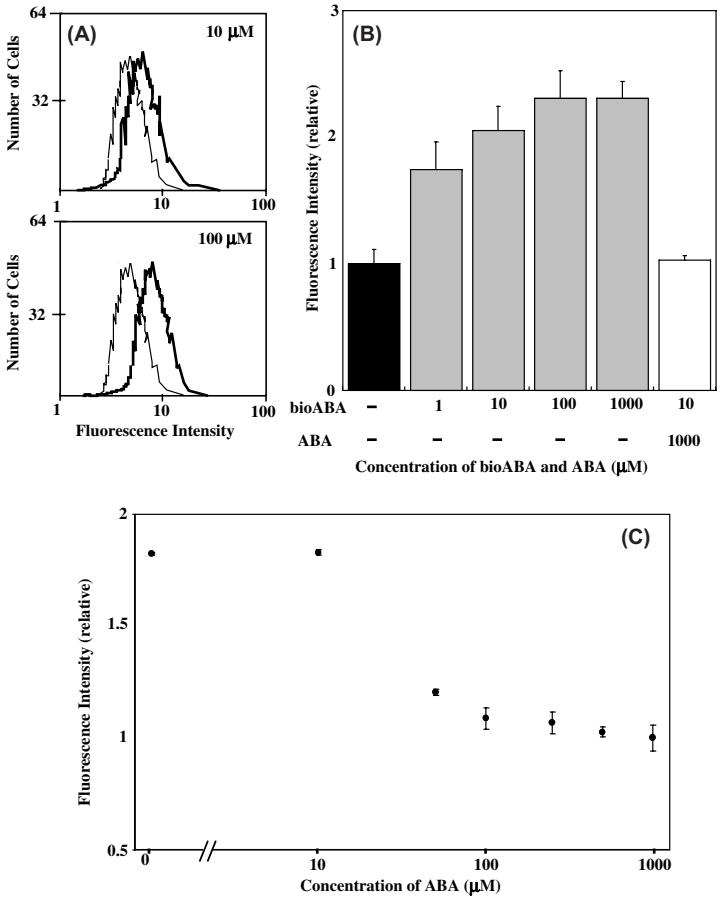


Figure 3. Effects of treatment with bioABA, ABA, or both, on fluorescence of labeled barley aleurone protoplasts. (A) Fluorescence histogram. In each sample, 5000 protoplasts were analyzed. Solid line histogram shows control (fluorescence-labeled streptavidin only). Bold line histogram shows 10 or 100 μM bioABA. (B) Protoplasts were incubated with ABA or bioABA and then incubated with fluorescence-labeled streptavidin. (C) Protoplasts were incubated with bioABA (10 μM) in the presence and absence of ABA and then incubated with fluorescence-labeled streptavidin. In the control samples, only fluorescence-labeled streptavidin was added. The mean and SE of triplicate samples are shown.

bioABA binding by proteinase K was suppressed in the case when denatured enzyme was treated (Fig. 6). These results suggest that ABA perception is dependent on extracellular proteinaceous domains.

3. Discussion

We use the avidin–biotin complex system to investigate plant hormone binding sites on protoplast plasma membranes by flow cytometry. In animals, the avidin–biotin complex system has been used to investigate plasma membrane receptors, but it has rarely been used for this purpose in plants. In this study, we used bioABA as a probe for investigating ABA-binding sites (Fig. 1). BioABA showed ABA-like activity although it was approximately 100-fold less active than ABA in barley aleurone protoplasts (Fig. 2). This implies that bioABA is recognized as ABA may be at ABA perception sites. Increase of fluorescence intensity was observed upon addition of both bioABA and fluorescence-labeled streptavidin, and this increase was eliminated by co-treatment with ABA (Fig. 3). The increase of fluorescence intensity was also observed even in the absence of bioABA probably due to non-specific binding. However, since the amount of fluorescence-labeled streptavidin used in each experiment was constant, the amount of non-specific binding should have been constant. In addition, since avidin does not have access to internal membrane components in platelets due to its molecular size,[26] fluorescence-labeled streptavidin also would not have access to internal membrane components in aleurones. Thus, the observed effects of bioABA on protoplast fluorescence should only reflect binding of bioABA to ABA-binding sites on the plasma membrane. Fluorescence intensity was not affected by bioABA derivatives, which did not show any ABA-like activity (Fig. 4). Considering that the total shape and physico-chemical properties of these bioABA derivatives are similar to those of bioABA, ABA part of bioABA plays a key role in exerting the affinity of bioABA to ABA-binding sites. Other plant hormones, jasmonic acid and gibberellic acid, was examined to test their effect on the bioABA-induced increase in fluorescence (Fig. 5). Jasmonic acid shows biological activities similar to ABA and gibberellic acid shows antagonistic biological activity against ABA, and more these hormones have carboxyl moiety in their molecules as ABA does. However, they did not affect the bioABA-induced increase in fluorescence. We examined the effects of several proteases in our fluorescence flow

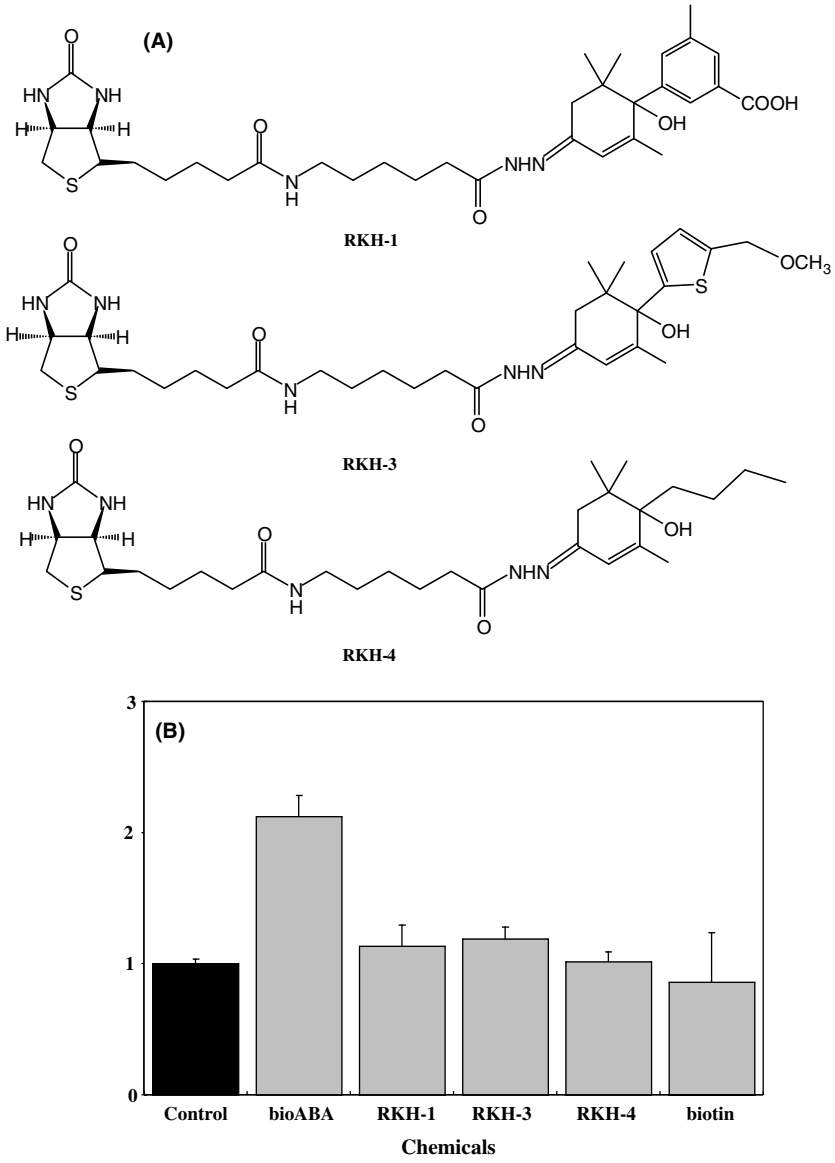


Figure 4. Effect of bioABA derivatives on fluorescence of barley aleurone protoplasts. (A) Structures of bioABA derivatives used in this study. (B) Protoplasts were incubated with 10 μM bioABA derivatives and then incubated with fluorescence-labeled streptavidin. In the control samples, only fluorescence-labeled streptavidin was added. The mean and SE of triplicate samples are shown.

cytometry assay, and found that protoplast fluorescence intensity was decreased by proteinase K treatment (Fig. 6), suggesting that the extracellular domain of ABA receptors is cleaved by proteinase K. These results suggest that the bioABA-binding sites studied here act as perception sites for ABA.

This study would open the new way for studying plant hormone binding sites. In further studies, bioABA might be used to visualize ABA-binding sites by fluorescence microscopy or to purify ABA-binding components by affinity chromatography. In animals, the avidin–biotin complex system has been used for visualization and purification of various receptors, including κ opioid receptor,[21] vasopressin receptor,[27] and parathyroid hormone receptor.[23] In plants, GTPase proteins[28] and fusicoccin-binding proteins[29] were purified using biotin-labeled GTP and fusicoccin, respectively. Likewise, bioABA could be used for histological analysis of ABA-binding sites or purification of ABA-binding components. Furthermore, bioABA may be useful in conjunction with proteolytic approaches to characterize ABA-binding components on membranes.

BioABA is likely a useful probe in other ABA signal transduction studies, such as those concerning stomatal closure or response to drought stress. In broad bean guard cells, bioABA has been shown to have ABA-like activity and to bind specifically to ABA-binding sites in the plasma membrane.[24] Methods for protoplast preparation of *Arabidopsis* mesophyll cells have been established,[30] and they have been used in signal transduction studies[31] including those examining ABA-dependent signal transduction involved in response to drought stress.[32] Studies of ABA-binding sites of broad bean guard cells or *Arabidopsis* mesophyll cells using

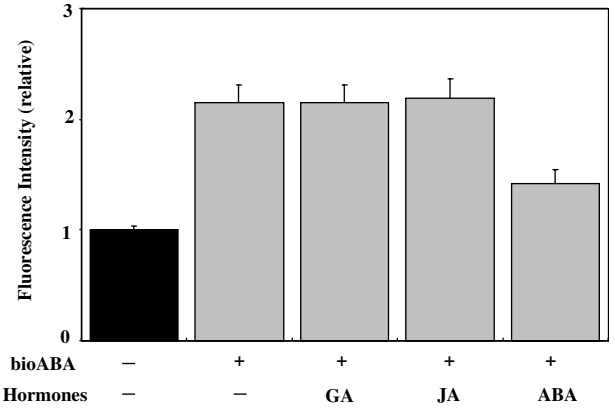


Figure 5. Effect of bioABA on fluorescence intensity of labeled barley aleurone protoplasts in the presence of other plant hormones. Protoplasts were co-incubated with 10 μM bioABA and 100 μM plant hormones (GA, JA, or ABA) and then incubated with fluorescence-labeled streptavidin. In the control samples, only fluorescence-labeled streptavidin was added. The mean and SE of triplicate samples are shown.

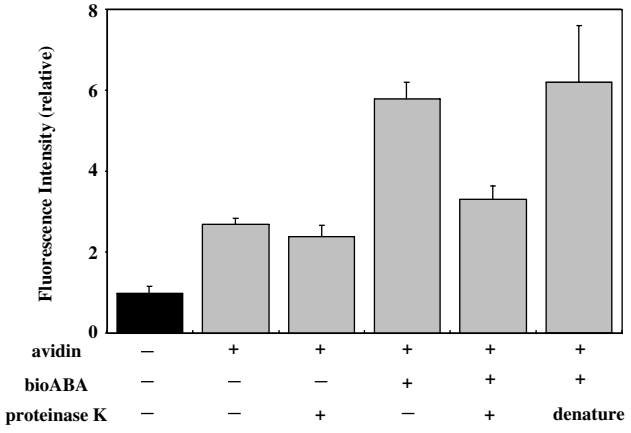


Figure 6. Suppression of bioABA binding to aleurone protoplasts by pretreatment with proteinase K. Protoplasts were preincubated with proteinase K (1 mg/mL) for 1 h at 30 °C and incubated with or without 10 μM bioABA. Denatured proteinase K was prepared by boiling for 10 min, and added in place of proteinase K. The mean and SE of triplicate samples are shown.

bioABA might reveal different profiles for ABA receptors relevant to dormancy, stomatal closure, and response to drought stress.

4. Experimental

4.1. Chemicals

S-(+)-ABA was a kind gift from Toray Co., Ltd (Chuo, Tokyo, Japan) and biotinamidocaproylhydrazide was purchased from Sigma–Aldrich (St. Louis, MO, USA).

4.2. Synthesis of bioABA

The procedure used for synthesis of bioABA was essentially the same as described previously,[17] with minor modifications. (+)-*S*-ABA (26.4 mg) and biotinamidocaproylhydrazide (37.1 mg) were stirred in 1 mL acetic acid in 5 mL methanol for 48 h. The solvent was evaporated, H_2O (5 mL) was added, and the aqueous solution was extracted three times with 5 mL of ethyl acetate. The combined organic phase was dried over anhydrous sodium sulfate, and the solvent was evaporated. The resulting residue was purified by silica gel thin-layer chromatography (chloroform–ethyl acetate–acetic acid = 10:10:1). The product was analyzed by ^{1}H NMR (300 MHz, $CDCl_3$): 7.61 (1H, d, J = 16 Hz), 6.15 (1H, s), 5.97 (1H, d, J = 16 Hz), 5.71 (1H, s), 4.57 (1H, m), 4.34 (1H, m), 3.56 (1H, m), 3.18 (2H, t, J = 12 Hz), 2.92 (1H, dd, J = 15, 5 Hz), 2.72 (1H, d, J = 15 Hz), 2.42 (2H, t, J = 12 Hz), 2.4–2.2 (3H, m), 2.20 (2H, t, J = 12 Hz), 1.91 (3H, s), 1.76 (3H, s), 1.18–1.66 (14H, m), 1.03 (3H, s), 0.97 (3H, s). The mass of the monoisotopic ion was determined by fast atom bombardment mass spectrometry to be 618. Three RKH compounds were prepared similarly. The products were analyzed by ^{1}H NMR (300 MHz, $CDCl_3$). RKH1: 7.92 (1H, s), 7.78 (1H, s), 7.45 (1H, s), 6.48 (1H, s), 4.54 (1H, m), 4.31 (1H, m), 3.62 (1H, m), 3.18 (2H, t, J = 12 Hz), 2.92 (1H, dd, J = 15, 5 Hz), 2.71 (1H, d, J = 15 Hz), 2.40 (3H, s), 2.20 (2H, t, J = 12 Hz), 1.76 (3H, s), 1.18–1.66 (14H, m), 1.13 (3H, s), 0.78 (3H, s). RKH3: 6.88 (1H, d, J = 15 Hz), 6.71 (1H, d, J = 15 Hz), 6.21 (1H, s), 4.54 (2H, s), 4.45 (1H, m), 4.31 (1H, m), 3.34 (1H, m), 3.21 (3H, s), 3.17 (2H, t, J = 12 Hz), 2.92 (1H, dd, J = 15, 5 Hz), 2.71 (1H, d, J = 15 Hz), 2.35 (2H, t, J = 11 Hz), 2.19 (2H, t, J = 12 Hz), 1.84 (3H, s), 1.24–1.72 (14H, m), 1.10 (3H, s), 0.95 (3H, s). RKH4: 6.08 (1H, s), 4.51 (1H, m), 4.32 (1H, m), 3.33 (1H, m), 3.19 (2H, t, J = 12 Hz), 2.91 (1H, dd, J = 15, 5 Hz), 2.72 (1H, d, J = 15 Hz), 2.42 (2H, t, J = 11 Hz), 2.33 (2H, t, J = 12 Hz), 1.94 (3H, s), 1.27–1.66 (20H, m), 1.10 (3H, s), 0.96 (3H, s), 0.90 (3H, s).

4.3. Isolation of barley aleurone protoplasts

Barley grains, *Hordeum vulgare* cv. Himalaya, harvested in Canberra, Australia, in 1998 were used. The procedure used for barley aleurone protoplast isolation was essentially the same as described previously,[33] with modifications. Twenty quarter-seeds were sterilized by treatment with 1.5% sodium hypochlorite for 20 min followed by five washes with sterile water. They were then soaked in a sterile 50-mL tube with 20 mL of protoplast soaking solution containing 20 mM methylethanesulfonate (MES) (pH 5.4), 14 mM L-arginine HCl, and 70 mM $CaCl_2$. The tube was left in the dark for 24 h at room temperature. After the starchy endosperm was removed, the aleurone layers were transferred to a sterile 5-mL flask and incubated with 1.5 mL of protoplast isolation medium (PIM) consisting of 10 mM MES (pH 5.4), 10 mM L-arginine HCl, 360 mM KCl, 90 mM $CaCl_2$, 1% polyvinylpyrrolidone, and 4.5% Onozuka R-10 cellulase. The flask was left in the dark for 22 h at room temperature. The PIM was then removed and replaced with 1.5 mL fresh PIM, and the flask was incubated for another 22 h. The PIM was removed and replaced with 1.5 mL of KCl medium consisting of 10 mM MES (pH 5.4), 10 mM L-arginine HCl, 360 mM KCl, and 90 mM $CaCl_2$. Protoplasts were re-

leased by swirling the flask gently and were pipetted carefully into a 50-mL culture tube. The pooled protoplasts were allowed to settle under gravity. The medium was discarded and 20 mL of KCl medium was added to the tube. Protoplast suspensions were then purified by centrifugation through a percoll/sucrose gradient. The layers on top of the sucrose containing live protoplasts were collected, diluted with 20 mL of KCl medium, mixed gently and centrifuged for 5 min at 50*g*.

4.4. α-Amylase activity assays

Assays for α-amylase activity were performed in triplicate by essentially the same method as described previously.[34] Protoplasts were resuspended in an appropriate volume of low-KCl medium consisting of 10 mM MES (pH 5.4), 10 mM L-arginine HCl, 180 mM KCl, 90 mM $CaCl_2$. Resuspended protoplasts were treated with ABA or bioABA at various concentrations and incubated in the dark at 25 °C for 24 h in the presence of 1 μM GA. After incubation, the protoplasts were lysed completely by vortexing for 10 min at 4 °C. An appropriate volume of enzyme extract was mixed with 0.1% starch (soluble) (Merck Japan Ltd, Meguro, Tokyo, Japan) in 1 mM acetate buffer (pH 5.4) containing 2 mM $CaCl_2$ and incubated at 37 °C for 5 min. The reaction was stopped by adding 1% iodine in 50 mM HCl, and A_{620} was measured in a spectrometer.

4.5. Transient expression assays

Protoplasts were resuspended in 1 mL of incubation medium (IM) consisting of 10 mM MES (pH 5.5), 500 mM mannitol, 100 mM glucose, 55 mM sucrose, 14 mM L-arginine, and 0.32% B5-Gamborg salt. They were then mixed gently with 100 μg sheared salmon sperm DNA and 100 μg Hv41(-935)-IGN plasmid DNA,[35] and the suspension was left undisturbed for 1 min. Three volumes of filter-sterilized protoplast transfection medium consisting of 10 mM Tris–HCl (pH 9.0), 17.3% (w/v) polyethylene glycol 6000, 670 mM mannitol, and 133 mM $Ca(NO_3)_2$ were added to the protoplasts, which were then mixed and left at room temperature for 20 min with occasional swirling. Next, 40 mL of IM was added in 10-mL aliquots with 2 min between additions. The protoplasts were collected by centrifugation for 2 min at 50*g* and washed twice in 30 mL of IM. The pelleted protoplasts were resuspended in an appropriate volume (generally 15 mL for protoplasts isolated from 150 grains) of IM containing 50 unit/mL nystatin, 150 μg/mL cefotaxime, 20 mM $CaCl_2$, 1.5 μg/mL aprotinin, and 1.5 μg/mL leupeptin. Aliquots of the protoplasts (1 mL) were transferred into flasks and incubated in the dark at 25 °C for 24 h in the presence of ABA or bioABA. Fluorometric assays of GUS activity were then performed in triplicate for each sample as described previously.[36]

4.6. Analysis of bioABA binding to aleurone protoplasts with flow cytometer

Protoplasts were resuspended in an appropriate volume of low-KCl medium. For each sample, a 1-mL aliquot of resuspended protoplasts was incubated with or without bioABA or various other compounds in the dark at 25 °C for 5 min. After washing with low-KCl medium, samples were treated with 16 μg/mL BODIPY FL-streptavidin (Molecular Probes, Eugene, OR, USA), incubated in the dark at 25 °C for 5 min, and washed with low-KCl medium. Flow cytometry was carried out as described previously[37] except that the method of quantitation differed. In brief, samples were analyzed with an Epics Elite (Coulter, Fullerton, CA, USA) instrument equipped with a 15-mW argon-ion laser for excitation at 488 nm. In each sample, 5000 protoplasts were analyzed. Fluorescence was measured on a logarithmic scale with a 530/30-nm filter. We compared the fluorescence intensity involving the largest number of cells. The fluorescence intensity of the sample treated only with fluorescence-labeled streptavidin was used as a control, and the fluorescence intensity of each sample was reported relative to the intensity of the control. Each assay was performed in triplicate.

Acknowledgements

We thank Mr. D. Yamazaki for helpful advice concerning this work and Ms. Yoko Miura for the synthesis of chemicals. This work was supported partly by Bioarchitech Research Programme at RIKEN.

References and notes

1. Zeevaart, J. A. D.; Creelman, R. A. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1988**, *39*, 439.
2. Shinozaki, K.; Yamaguchi-Shinozaki, K. *Plant Physiol.* **1997**, *115*, 327.
3. Leung, J.; Giraudat, J. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 199.
4. Zhang, D. P.; Wu, Z. Y.; Li, X. Y.; Zhao, Z. X. *Plant Physiol.* **2002**, *28*, 714.
5. Pedron, J.; Brault, M.; Nake, C.; Miginiac, E. *Eur. J. Biochem.* **1998**, *252*, 385.
6. Razem, F. A.; Luo, M.; Liu, J. H.; Abrams, S. R.; Hill, R. D. *J. Biol. Chem.* **2004**, *279*, 9922.
7. Lovegrove, A.; Hooley, R. *Trends Plant Sci.* **2000**, *5*, 102.
8. Close, T. J.; Kort, A. A.; Chandler, P. M. *Plant Mol. Biol.* **1989**, *13*, 95.
9. Taiz, L.; Jones, R. L. *Planta* **1971**, *101*, 95.
10. Jacobsen, J. V.; Close, T. J. *Plant Mol. Biol.* **1991**, *16*, 713.
11. Finkelstein, R. R.; Gampala, S. S.; Rock, C. D. *Plant Cell* **2000**, *14*, S15.
12. Gilroy, S.; Jones, R. L. *Plant Physiol.* **1994**, *104*, 1185.
13. Gilroy, S. *Plant Cell* **1996**, *8*, 2193.
14. Asami, T.; Min, Y. K.; Han, S. Y.; Kitahata, N.; Oh, K.; Murofhushi, N.; Yoshida, S. *Plant Growth Regul.* **2002**, *38*, 237.
15. Hill, R. D.; Liu, J. H.; Durnin, D.; Lamb, N.; Shaw, A.; Abrams, S. R. *Plant Physiol.* **1995**, *108*, 573.
16. Kim, B. T.; Min, Y. K.; Asami, T.; Park, N. K.; Kwon, O. Y.; Cho, K. Y.; Yoshida, S. *J. Agric. Food Chem.* **1999**, *47*, 313.
17. Kohler, A. D.; Beale, M. H.; Rollason, R.; Barratt, D. H. P.; Lewis, M. J.; van der Muelen, R. M.; Wang, M. *J. Chem. Soc., Perkin Trans. 1* **1997**, 1543.

18. Beale, M. H.; Hooley, R.; Lewis, M. J.; Smith, S. J.; Ward, J. L. *J. Chem. Soc., Perkin Trans. 1* **1995**, 657.
19. Lovegrove, A.; Barratt, D. H. P.; Beale, M. H.; Hooley, R. *Plant J.* **1998**, *15*, 311.
20. Hiratsuka, T.; Kato, T. *J. Biol. Chem.* **1987**, *262*, 6318.
21. Lawrence, D. M. P.; el-Hamouly, W.; Archer, S.; Leary, J. F.; Bidlack, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1062.
22. Asami, T.; Tao, L.; Yamamoto, S.; Robertson, M.; Min, Y. K.; Murofushi, N.; Yoshida, S. *Biosci. Biotech. Biochem.* **1997**, *61*, 1198.
23. Newman, W.; Beall, L. D.; Levine, M. A.; Cone, J. L.; Randhawa, Z. I.; Bertolini, R. B. *J. Biol. Chem.* **1989**, *264*, 16359.
24. Yamazaki, D.; Yoshida, S.; Asami, T.; Kuchitsu, K. *Plant J.* **2003**, *35*, 129.
25. Asami, T.; Min, Y. K.; Nakano, T.; Matsuyama, T.; Murofushi, N.; Yamaguchi, I.; Yoshida, S. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 1571.
26. Komiotis, D.; Wencel-Drake, J. D.; Dieter, J. P.; Lim, C. T.; le Breton, G. C. *Biochem. Pharmacol.* **1996**, *52*, 763.
27. Jans, D. A.; Bergmann, L.; Peters, R.; Fahrenholz, F. *J. Biol. Chem.* **1990**, *265*, 14599.
28. de Boar, A. H.; van Hunnik, E.; Korthout, H. A. A. J.; Sedee, N. J. A.; Wang, M. *FEBS Lett.* **1994**, *337*, 281.
29. Korthout, H. A. A. J.; van der Hoeven, P. C. J.; Wagner, M. J.; van Hummik, E.; de Boer, A. H. *Plant Physiol.* **1994**, *105*, 1281.
30. Damm, B.; Willlmitzer, L. *Mol. Gen. Genet.* **1988**, *213*, 15.
31. Sheen, J. *Plant Physiol.* **2001**, *127*, 1466.
32. Uno, Y.; Furihata, T.; Abe, H.; Yoshida, R.; Shinozaki, K.; Yamaguchi-Shinozaki, K. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 11632.
33. Lin, W.; Gopalakrishnan, B.; Muthukrishnan, S. *Protoplasma* **1996**, *192*, 93.
34. Asami, T.; Robertson, M.; Yamamoto, S.; Yoneyama, K.; Takeuchi, Y.; Yoshida, S. *Plant Cell Physiol.* **1998**, *38*, 342.
35. Robertson, M.; Cuming, A. C.; Chandler, P. M. *Physiol. Plant* **1995**, *94*, 470.
36. Jefferson, R. A. *Plant Mol. Biol. Rep.* **1998**, *5*, 387.
37. Lawrence, D. M. P.; Hutchinson, I.; Seyed-Mozaffari, A.; Archer, S.; Bidlack, J. M. *J. Immunol. Method* **1997**, *201*, 173.