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Elucidation of biosynthetic pathway of a plant hormone abscisic acid in phytopathogenic fungi

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Late stage oxidative modifications of a plant hormone abscisic acid in fungi

Abscisic acid (ABA) is one of the plant hormones that regulates physiological functions in various organisms, including plants, sponges, and humans. The biosynthetic machinery in plants is firmly established, while that in fungi is still unclear. Here, we elucidated the functions of the four biosynthetic genes, *bcABA1-bcABA4*, found in *Botrytis cinerea* by performing biotransformation experiments and in vitro enzymatic reactions with putative biosynthetic intermediates. The first committed step is the cyclization of farnesyl diphosphate to give α-ionylideneethane catalyzed by a novel sesquiterpene synthase, BcABA3, which exhibits low amino acid sequence identities with sesquiterpene synthases. Subsequently, two cytochrome P450s, BcABA1 and BcABA2, mediate oxidative modifications of the cyclized product to afford 1',4'-*trans*-dihydroxy-α-ionylideneacetic acid, which undergoes alcohol oxidation to furnish ABA. Our results demonstrated that production of ABA does not depend on the nucleotide sequence of *bcABA* genes. The present study set the stage to investigate the role of ABA in infections.

Abscisic acid; plant hormone; oxidative modifications; *Aspergillus oryzae*; fungi

Abscisic acid (ABA, **1**) is a sesquiterpene phytohormone that regulates physiological functions in plants and a wide range of organisms from bacteria to humans. For example, **1** mediates adaptive responses towards abiotic stresses such as drought stress and salt stress in plants [1], while it also regulates temperature-induced oxygen consumption and water filtration in sponges [2]. Recent studies have revealed that **1** serves as an activator of human monocytes and vascular smooth muscle cells [3], and that it is a prosurvival factor for megakaryocytes in a Tpo-independent manner [4]. In addition, phytopathogenic fungi, such as *Botrytis cinerea* [5] and *Cercospora* species [6], which cause mold diseases, also produce **1** as a virulence factor [7].

The importance of **1** in several organisms has prompted researchers to elucidate its biosynthetic pathway. In plants, ABA biosynthetic pathway has been firmly established through biochemical and molecular genetic studies (Scheme 1A) [8]. The most notable feature of this pathway is the construction of C15 carbon skeleton by oxidative cleavage of C40 carotenoids derived from β-carotene, imposing complicated biotransformation involving 12 enzyme genes. On the other hand, biosynthesis of **1** in fungi, defined as a direct pathway, is distinct from that in plants [8]. In case of *B. cinerea*, farnesyl diphosphate (FPP) is directly converted into α-ionylideneethane (**2**), which harbors a C15 skeleton [9]. Subsequently, **2** undergoes oxidations at C1, C1', and C4' positions to give **1** (Scheme 1B). Previous labeling experiments using $^{18}O_2$ revealed that molecular oxygen is installed at these positions [9]. The reduction product of **1**, 1',4'-*trans*-dihydroxy-α-ionylideneacetic acid (**5**), was also isolated from *B. cinerea*, while the diastereomeric 1',4'-*cis*-dihydroxy-α-ionylideneacetic acid (**7**) was not [9]. Intermediates **2** and **5** were demonstrated by conversion of [2-^{13}C]-**2** and [$^{2}H_7$]-**5** into **1** in *B. cinerea* [10]. In 2006, a putative biosynthetic gene cluster of **1** was identified in *B. cinerea* [11]. This gene cluster consisted of four genes; two cytochrome P450s, *bcABA1* and *bcABA2*; one short chain dehydrogenase/reductase, *bcABA4*; and one functionally unknown enzyme gene, *bcABA3*. Subsequent gene inactivation experiments of *bcABA1-4* revealed that all mutants lacked the ability to produce **1**, suggesting that these genes might be responsible for the biosynthesis of **1**. However, detailed function of each enzyme remained unclear because of the following reasons; 1) other putative biosynthetic intermediates except **2** and **5** were isolated, and 2) no feeding experiments using putative intermediates were conducted. Especially, little is known about the cyclization enzyme, although circumstantial evidence has suggested that BcABA3 is a potential candidate to catalyze the cyclization.

Previously, we conducted functional analysis of BcABA3 and characterized the cyclization activity from FPP to **2** [12]. This successful functional analysis enabled us to examine the biosynthetic pathway from **2** to **1** through biotransformation experiments using putative biosynthetic intermediates and *in vitro* enzymatic reactions. Here, we described sequence and functional analysis of *bcABA* genes and revealed step-by-step oxidative modification processes via α-ionylideneacetic acid (**3**) and **5**.

Materials and methods

General

All reagents commercially supplied were used as received. Column chromatography was carried out on 60N silica gel (Kanto Chemicals). Optical rotations were recorded on JASCO P-2200 digital polarimeter. ^{1}H-NMR spectra were recorded on Bruker DRX-500 or Bruker AMX-500 spectrometer (500 MHz for ^{1}H-NMR). NMR spectra were recorded in $CDCl_3$ (99.8 atom% enriched, Kanto) or CD_3OD (99.8 atom% enriched, TCI). ^{1}H chemical shifts were reported in δ value based on residual chloroform (7.26 ppm) and methanol (3.34 ppm) as a reference. GC-MS and UPLC analyses were conducted with a MS-2010 (Shimadzu) and a Waters ACQUITY UPLC, respectively.

Oligonucleotides for polymerase chain reactions (PCRs) were purchased from Hokkaido System Science Co., Ltd. PCRs were performed with a BioRad S1000 thermal cycler. Analysis of the samples during protein purification was performed using SDS-polyacrylamide gel electrophoresis, and the proteins were visualized by using Coomassie Brilliant Blue staining. Protein concentration was determined by the Bradford method with bovine serum albumin as a standard.

Compounds **2**, **3**, **5**, and **7** were synthesized according to the literature procedures [9, 13, 14].

Strain and culture conditions

Escherichia coli HST08 was used for cloning and following standard recombinant DNA techniques. *E. coli* BL21-Gold(DE3) was used for protein expression. *Botrytis cinerea* 306914 was used for extraction of genomic DNA. A fungal host strain used in this study was *A. oryzae* NSAR1 [15], a quadruple auxotrophic mutant (*niaD*$^{-}$, *sC*$^{-}$, *ΔargB*, *adeA*$^{-}$), for fungal expression.

Genomic DNA preparation

Genomic DNA of *B. cinerea* 306914 was extracted according to the literature procedure [12].

Construction of A. oryzae expression plasmids

The *bcABA2* was amplified from genomic DNA of *B. cinerea* 306914 with primer set as shown in Supplemental Table S1. PCR reactions were performed with the KOD-Plus-Neo (TOYOBO). Each PCR product was inserted into *Kpn*I site of pTAex3 using In-Fusion Advantage PCR cloning kit (Clontech Laboratories) to construct expression plasmid pTAex3-*bcABA2*. Other plasmids, pTAex3-*bcABA1* and pUSA2-*bcABA24* were constructed in our previous study [12].

Transformation of Aspergillus oryzae

Transformation of *A. oryzae* NSAR1 (1.0×10^8 cells) was performed by the protoplast-polyethylene glycol method reported previously to construct following transformants [12]; AO-*bcABA1* (plasmids used in the transformation; pTAex3-*bcABA1*), AO-*bcABA2* (pTAex3-*bcABA2*), and AO-*bcABA124* (pTAex3-*bcABA1* and pUSA2-*bcABA24*). AO

is an abbreviation of *Aspergillus oryzae* and AO-*bcABAx* means a transformant harboring *bcABAx* gene.

Analysis of the metabolites

Mycelia of AO-*bcABA1234* was inoculated into a MPY medium (5 mL) in 20 mL Erlenmeyer flask. The culture was incubated at 30 °C for 2 d. The fermentation broth was soaked in acetone (10 mL) for 6 h. After filtration, the filtrate was concentrated *in vacuo*. The residual water was adjusted to pH 1.0 and then extracted with EtOAc, and the organic layers were concentrated *in vacuo*. This crude extracts were directly analyzed by a UPLC-MS equipped with a ACQUITY UPLC BEH C18 (ϕ2,1 x 50 mm) at the following conditions (**method A**): flow rate; 0.7 mL/min, solvent system; acetonitrile in H_2O containing 0.1 % of formic acid, 0-1 min: 10 %; 1-3 min: a linear gradient 10 %-95 %; 3-5 min: 95 %).

cDNA preparation

AO-*bcABA124* was grown on MPY medium containing 0.925% $(NH_4)_2SO_4$, 0.01 % adenine for 3-5 d at 30 °C. Total RNA was extracted from each dried mycelia using TRIzol® Reagent (Invitrogen) according to the manufacturer's instructions and then treated with DNase I (Life Technologies) for reverse transcription. Complementary DNA (cDNA) was synthesized with PrimeScriptTM II 1st strand cDNA synthesis kit (Takara) using the oligo dT primer according to the manufacturer's instructions. The cDNA was used as a template of the PCR reactions for direct sequencing and subcloning of each STS gene into the pColdI vector.

BcABA4 cloning and expression in Escherichia coli

Intronless *bcABA4* was amplified from cDNA with primer set as shown in Supplemental Table S1. PCR reactions were performed with the KOD-Plus-Neo (TOYOBO). Each PCR product was inserted into *Nde*I site of pColdI using In-Fusion Advantage PCR cloning kit (Clontech Laboratories) to construct expression plasmid pColdI-*bcABA4*. This plasmid was introduced into *E. coli* BL21-Gold(DE3) for overexpression. The transformant was grown at 37 °C at an OD_{600} of ~0.6 in 500 mL flask. After cooling at 4 °C, isopropyl β-D-thiogalactopyranoside (0.1 mM) was added to the culture. After incubation at 16 °C for 17 h, the cells were harvested by centrifugation at 4000 rpm. Harvested cells were resuspended in disruption buffer (100 mM Tris-HCl (pH 8.0), 20 % glycerol, 1 mM DTT) and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni-NTA column to purify the expressed protein.

Biotransformations

Mycelia of the transformant (AO-*bcABA1*, AO-*bcABA2*, or AO-*bcABA124*) were inoculated into 5 mL of MPY (maltose-peptone-yeast extract: 3 % maltose, 1 % polypeptone, 0.5 % yeast extract) medium containing appropriate nutrients (0.925 % $(NH_4)_2SO_4$, 0,15 % methionine, 0.01 % adenine, 0.06 % arginine) in 20 mL Erlenmeyer flask. After 2 d incubation at 30 °C, substrate (100 μg, methanol solution) was then administered to the culture broth. After an additional 6 h incubation at 30 °C, the mycelia were collected by filtration and soaked in acetone (10 mL). The organic layer was then concentrated *in vacuo*. The residual water was adjusted to pH 1.0 and then extracted with EtOAc, and the organic layers were concentrated *in vacuo*. The crude extracts were directly analyzed by a UPLC-MS apparatus according to **method A**.

Isolation of biosynthetic intermediates

α-ionylideneethane (2); Mycelia of AO-*bcABA3* were inoculated into a solid medium containing polished rice (20 g) in 500 mL Erlenmeyer flasks. Each culture was incubated at 30 °C for 7 d. After extraction with hexane, the organic layers were washed with brine, dried over anhydrous Na_2SO_4, and concentrated *in vacuo*. Purification of the residue by silica gel column chromatography (hexane) gave **2** (4.0 mg from 0.2 kg of rice medium). $[\alpha]_D^{20}$ +254.6° (*c* 0.50, $CHCl_3$); FI-HR-MS: calcd. for $C_{15}H_{24}$ $[M]^+$: 204.1878, found: 204.1884. The ^{1}H-NMR data are in good agreement with the reported data [9].

α-ionylideneacetic acid (3); The crude extracts of the biotransformation experiment of **2** (4.6 mg) using AO-*bcABA1* were purified with silica gel column chromatography (hexane/ethyl acetate = 1/5) followed by further purification utilizing HPLC equipped with Wakosil-II 5C18 (ϕ4.6 mm x 250 mm) at the following conditions (λ = 210 nm, flow rate; 1.0 mL/min, solvent system; acetonitrile in H_2O containing 0.1 % of trifluoroacetic acid, 0-1 min: a linear gradient 90 %-100 %; 10-20 min: 100 %) to afford **3** (1.9 mg). $[\alpha]_D^{22}$ +179.7° (*c* 0.16, EtOH). [lit. $[\alpha]_D^{21}$ +450.3° (*c* 0.6, EtOH)] [16]. ESI-HR-MS: calcd. for $C_{15}H_{21}O_2$ $[M-H]^-$: 233.1547, found: 233.1549. The ^{1}H-NMR data are in good agreement with the reported data [14].

1'-deoxyABA (6); In this case, fermentation period was changed to 2 d after administration of **3**. The crude extracts were purified with silica gel column chromatography (hexane/ethyl acetate = 1/1) followed by further purification utilizing HPLC to give **6** (0.4 mg from 5.0 mg of **3**). ESI-HR-MS: calcd. for $C_{15}H_{19}O_3$ $[M-H]^-$: 247.1340, found: 247.1343. The ^{1}H-NMR data are in good agreement with the reported data [17].

Results and discussion

Sequence and functional analyses of bcABA3

In the previous report [12], we initially described functional analysis of BcABA3 to simplify the discussion although we actually elucidated the function of BcABA124 first. It may be worthwhile to describe how we conducted the experiments for elucidating the function of totally new terpene synthase BcABA3. In the report describing identification of the ABA gene cluster [11], authors used DNA sequences from three strains of *B. cinerea*; ABA overproducer ATCC58025 (for gene inactivation), SAS56 (for making a genomic DNA library), and B05.10 (for checking the gene organization). Among these strains, the phytopathogenic strains SAS56 and B05.10 do not produce ABA in axenic culture [18]. This paper did not provide any sequence data of *bcABA3* from the three strains. We speculated that all three sequences may be nearly identical because they did not mention their differences.

At the beginning, we considered that slight differences of the *bcABA* gene sequences from three strains might affect the expression analysis. In addition, we knew many researchers involved in the study of fungal ABA biosynthesis focused on the terpene synthase [19] and mostly failed. Therefore, before going to the detailed analysis of previously uncharacterized BcABA3, we decided to confirm the functions of BcABA124 because their biosynthetic intermediates were predicted experimentally [9, 10] and bioconversions of these intermediates were easier to be employed. For functional analysis of *bcABA* genes, we used *B. cinerea* MAFF306914 strain, which is genetically relevant to strain B05.10 (99% nucleotide sequence identities with *bcABA1-4*) [20]. As described later sections, all *bcABA124* sequences from an ABA non-producer MAFF306914 (B05.10) are functional. With these experimental data, we are now confident to employ functional analysis of BcABA3.

At first, we focused on the initially deposited sequence *bcABA3-S* (accession number CAJ87067, 1254 bp) from SAS56 because that from ATCC58025 was not available. The nucleotide sequence of *bcABA3-S* was prepared by PCR-based removal of an extra base from the longer sequence *bcABA3-L* (BCIN_08g03880, 1323 bp). A recombinant His_6-tagged BcABA3-S expressed in *E. coli* was obtained as an insoluble form. Solubility was improved when it was expressed as a fusion protein with maltose binding protein. However, no enzymatic activity was detected. Being different from BcABA3-S, His_6-tagged BcABA3-L was expressed as a soluble form. In vitro enzymatic reaction revealed that BcABA3-L mediated a cyclization of FPP to yield **2** in the presence of Mg^{2+} [12]. The nucleotide sequence of *bcABA3-L* is highly conserved in strains that did not produce **1**, B05.10 and T4, suggesting that production of **1** does not depend on the nucleotide sequence of *bcABA3*, despite SAS56 strain harboring *bcABA3-S*. It should be emphasized that BcABA3 is a novel terpene synthase, which exhibits low amino acid sequence similarities with those of well-known sesquiterpene synthases, thus limiting the assessment whether the amino acid sequences predicted from the corresponding nucleotide sequences are functional or not.

Heterologous expression of bcABA genes in A. oryzae

Production of **2** by BcABA3 allowed us to speculate that remaining three modification enzymes, BcABA1, BcABA2, and BcABA4, participated in the transformation of **2** to **1**. To determine whether the proposed genes are sufficient for production of **1**, we prepared three plasmids, pTAex3-*bcABA1*, pAdeA2-*bcABA3*, and pUSA2-*bcABA24*, which were used for transformations to yield AO-*bcABA1234*. Each DNA clone, *bcABA1*, *bcABA2*, and *bcABA4*, exhibited more than 99% nucleotide sequence identities with those of the corresponding gene from strains B05.10 and T4

(Figure S1). Semi-synthetic intermediates, such as **2**, **3**, **5**, and **7** [9, 13, 14], and natural product **1** were used as chromatographic standards. Careful UPLC analysis of the metabolites from AO-*bcABA1234* revealed production of **1** (estimated yield based on the peak area of **1**; 8 mg/L in MPY medium, 69 mg/kg of rice medium) as well as small amount of 1'-deoxyABA (**6**). The structure was determined by NMR analysis described in the following section. Since **6** was not isolated from *B. cinerea*, it appeared to be a byproduct possibly produced by unexpected oxidation of 1'-hydroxyionylideneacetic acid (**4**) (Scheme 2). On the other hand, putative biosynthetic intermediates such as **2**, **3**, **5**, and **7** were not detected in the metabolites, suggesting that they might efficiently be converted into **1** in this transformant (Scheme 2).

Functional analysis of BcABA4

Previous gene inactivation experiment revealed that *ΔbcABA4* accumulated one compound, the molecular weight of which is identical to that of 1',4'-*trans*-dihydroxy-α-ionylideneacetic acid (**5**) [10], indicating that BcABA4 mediates oxidation of **5** into **1** in the last step of ABA biosynthesis (Scheme 2). This proposed oxidation was confirmed by *in vitro* enzymatic reactions. The cDNA sequence of BcABA4, which was prepared from AO-*bcABA1234*, was cloned into the expression vector pColdI for protein expression in *Escherichia coli* BL21-Gold(DE3). The *N*-terminal His_6-tagged protein was successfully expressed in a soluble form. The purified enzymes were subjected to SDS-PAGE analysis to confirm their molecular size and purity (Figure 1). When the recombinant BcABA4 was incubated with racemic **5** (rac-**5**) in the presence of NAD^+, **1** was produced as a sole product (Figure 1). In contrast, no product was detected when using $NADP^+$ instead of NAD^+, suggesting that BcABA4 oxidizes rac-**5** using NAD^+. As **7**, which is a diastereomeric compound of **5**, was also converted into **1**

in *B. cinerea*, rac-**7** was used as a substrate instead of rac-**5**. UPLC analysis demonstrated that **1** was also produced by the action of BcABA4. Time course experiments showed preference of BcABA4 toward natural diastereomer **5** (4.1 mol/mg*min), although BcABA4 also accommodates **7** (0.87 mol/mg*min) in the active site cavity. The reverse reaction of **1** was not detected under the same reaction conditions. These results provided conclusive evidence that **5** is unambiguously a biosynthetic intermediate in *B. cinerea*.

Functional analysis of modification enzyme genes

The function of remaining P450s, BcABA1 and BcABA2, was then examined by biotransformation experiments using three AO-transformants, AO-*bcABA1*, AO-*bcABA2*, and AO-*bcABA124*. These transformants were prepared by introducing expression plasmids, pTAex3-*bcABA1*, pTAex3-*bcABA2*, and pUSA2-*bcABA24*, into the wild type strain, *A. oryzae* NSAR1.

Initially, racemic **2** (rac-**2**) was incubated with AO-*bcABA124*. UPLC analysis of the metabolites showed production of **1** as well as **6**, confirming that biotransformation is effective to analyze the function of BcABA1 and BcABA2 (Figure S2). However, during the functional analysis of BcABA1, we found that AO-*bcABA1* produced optically active **6** when incubated with rac-**2**. The CD spectrum was in good agreement with the reported data of (1'*R*)-(+)-**6** [21] (Figure S3), demonstrating that a stereochemistry of the C1' side chain of **6** is the same as that of **1**. This optical resolution of rac-**2** occurs during the oxidative modifications catalyzed by BcABA1 (Figure S3). However, to avoid complicated discussions caused by kinetic optical resolution, we just focused on determining the biosynthetic pathway. For this purpose, optically active **2** was used as a substrate for biotransformation experiments. It was

isolated from AO-*bcABA3*, which produces **2** in solid medium (isolation yield: 20 mg/kg of rice). UPLC analysis of the metabolites revealed that AO-*bcABA124* converted **2** into **1** and a small amount of **6** and that AO-*bcABA1* converted **2** into two products, **3** and **6** (Figure 2). On the other hand, both AO-*WT* and AO-*bcABA2* yielded no product from **2** (Figure 2). HR-MS analysis revealed the molecular formula of **6** as $C_{15}H_{20}O_3$. The ^{1}H-NMR spectrum was identical to that of 1'-deoxyABA reported in the previous paper [17]. Typical signals are as follows; four vinyl protons (δ_H 7.70, 6.10, 5.93, and 5.72), one methine proton (δ_H 2.83), two allylic methyl protons (δ_H 2.03 and 1.95), one methylene proton (δ_H 2.48 and 2.06), and two methyl protons (δ_H 1.06 and 1.00). Multiplicities at H5 (δ_H 6.10, dd, J = 15.8, 9.35 Hz) showed the presence of methine proton (δ_H 2.83, d, J = 9.35 Hz) at C1' position. The degree of oxidation suggested that **6** is an oxidative product of **3**, indicating that carboxylic acid formation on the terminal methyl group of **2** precedes the ketone formation leading to **6**.

To further elucidate the late-stage oxidations, similar biotransformation experiments were conducted with optically active **3**, which was prepared from large-scale biotransformation of **2** with AO-*bcABA1*. We identified **1** as a major product when **3** was incubated with AO-*bcABA124*, confirming that **3** is a biosynthetic intermediate (Figure 2). Diol **5** was observed as a major product among the metabolites obtained from AO-*bcABA2* whereas the diastereomeric compound **7** was not. This metabolite profile is consistent with that of *B. cinerea*. Accumulation of **6** was observed even during the biotransformation experiment of **3** using AO-*WT*, which indicated that allylic oxidation of **3** is catalyzed by inherent enzymes in *A. oryzae* and that **6** is a shunt product. This was further validated by the fact that no conversion of **6** was observed using AO-*bcABA124* (Figure S4).

Biosynthetic pathway of abscisic acid in B. cinerea

The oxidative modifications of **2** leading to **1** in *B. cinerea* are summarized in Scheme 2. They can be divided into three steps: step 1) allylic oxidations of the terminal methyl group of **2** to give **3**, step 2) allylic oxidations at C1' and C4' positions of **3** to yield **5**, and step 3) an alcohol oxidation of **5** to furnish **1**. The former two are catalyzed by the two cytochrome P450s, BcABA1 and BcABA2, respectively. Among them, BcABA2 catalyzing two rounds of allylic oxidations is remarkable in that the face selectivity is different during the first- and second-step oxidation (first step: β-face of C4', second step: α-face of C1'). The intermediate **3** also undergoes allylic oxidations catalyzed by the action of inherent enzymes in *A. oryzae*. However, such unexpected oxidation could not affect the production of **1** when the downstream oxidation enzyme genes, *bcABA2* and *bcABA4*, were co-expressed with *bcABA1*, because rapid conversion of **3** into **5** suppressed the side reaction. Similar phenomena were reported in the reconstitution studies of pyripyropenes (side reaction: hydrolysis) [22], paxilline (hydrolysis) [23], and solanapyrone (oxidation) [24]. Alcohol oxidation of **5** catalyzed by BcABA4 is also interesting in that BcABA4 accommodates diastereomeric compounds, **5** and **7**, although the conversion rate of **5** is much higher than that of **7**.

ABA biosynthesis in fungi

ABA was widely distributed in phytopathogenic fungi including *Cercospora* species [6], *Rhizopus* species [25], and *Fusarium* species [25]. Labeling experiments using *Cercospora* species, such as *C. rosicola* [26-28], *C. cruenta* [28, 29], and *C. pini-densiflorae* [30], revealed that the biosynthetic pathway of **1** depends on the species, although all fungi commonly applied the C15 direct pathway. For example, in *C. rosicola*, **6** is considered as an intermediate instead of **5**, suggesting that the order of

oxidation from **2** to **1** is different from the biosynthetic pathway in *B. cinerea*. On the other hand, in *C. cruenta*, γ-ionylideneethane is likely a biosynthetic intermediate, although subsequent oxidation processes are identical to those in *B. cinerea*. These differences in the biosynthetic pathways can be explained by assuming that functionally relevant ABA proteins can be evolved by a minute change in the active site residues of BcABA proteins. Therefore, once the corresponding enzymes are found in the genomes of *C. rosicola* and *C. cruenta*, we can provide conclusive evidences on this hypothesis through well-established biotransformation experiments.

Author contribution

J.T., T.K., A.M., and H.O. designed research; J.T., and T.K. performed research; A.M., J.T., T.K., T.O., C.L., A.M., and H.O. analyzed data; and A.M. and H.O. wrote the paper.

Footnotes

J.T. and T.K. contributed equally to this work.

Disclosure statement

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Figure legends

Scheme 1. Proposed biosynthetic pathways of **1** in (A) plants and (B) fungi.

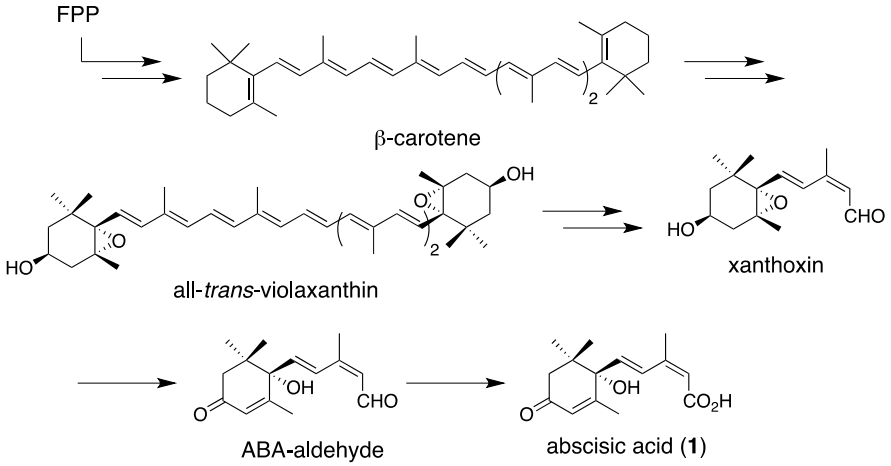
Scheme 2. Biosynthetic pathway of **1** in *B. cinerea*. AO is an abbreviation of *Aspergillus oryzae*.

Figure 1. (A) UPLC profiles of (a) BcABA4 reaction with **5** and (b) BcABA4 reaction with **7**. (B) SDS-PAGE analysis of recombinant BcABA4.

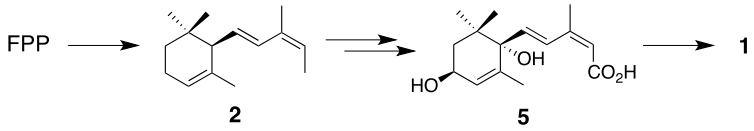
Figure 2. UPLC profiles. (a) authentic samples, and metabolites produced by (b) AO-*WT* with **2**, (c) AO-*bcABA1* with **2**, (d) AO-*bcABA2* with **2**, (e) AO-*bcABA124* with **2**, (f) AO-*WT* with **3**, (g) AO-*bcABA1* with **3**, (h) AO-*bcABA2* with **3**, and (i) AO-*bcABA124* with **3**.

Table 1. *bcABA1-4* genes identified from *B. cinerea*.

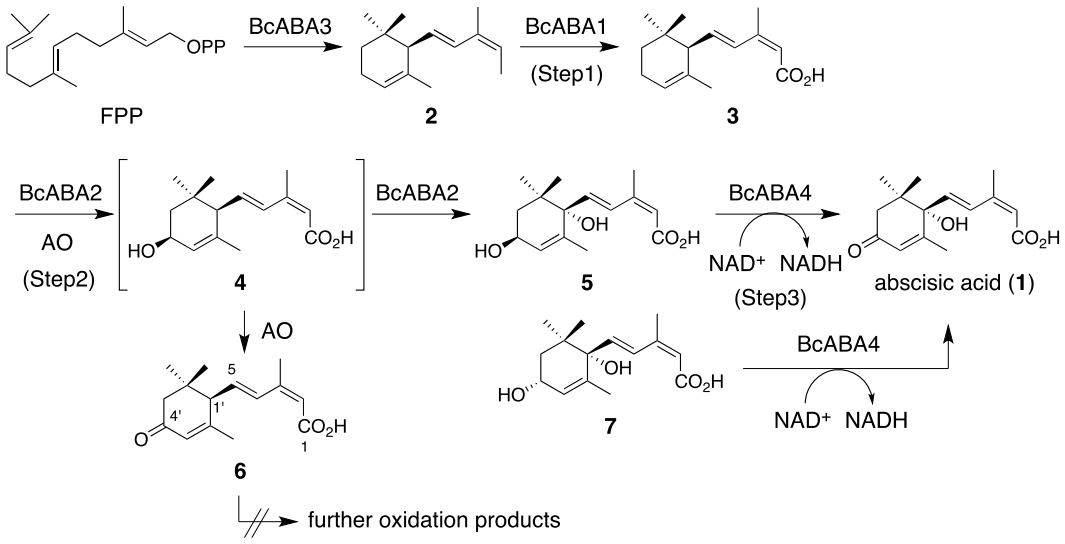
(A) ABA biosynthesis in plants



(B) ABA biosynthesis in fungi



Scheme 1.


OPP
BcABA3
BcABA1
(Step1)
CO2H
FPP
2
3
BcABA2
AO
(Step2)
HO
CO2H
4
BcABA2
OH
HO
CO2H
5
BcABA4
NAD+ NADH
(Step3)
O
OH
CO2H
abscisic acid (1)
AO
5
1'
4'
O
CO2H
1
6
OH
HO
CO2H
7
BcABA4
NAD+ NADH
further oxidation products

Scheme 2.

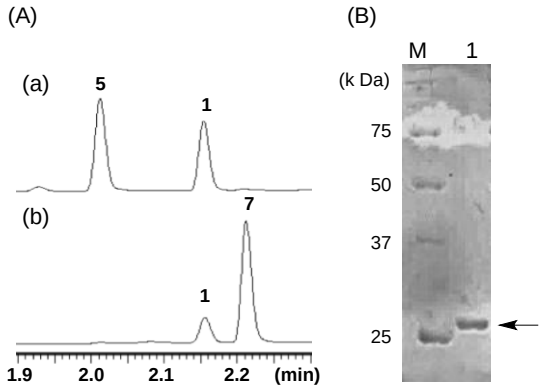
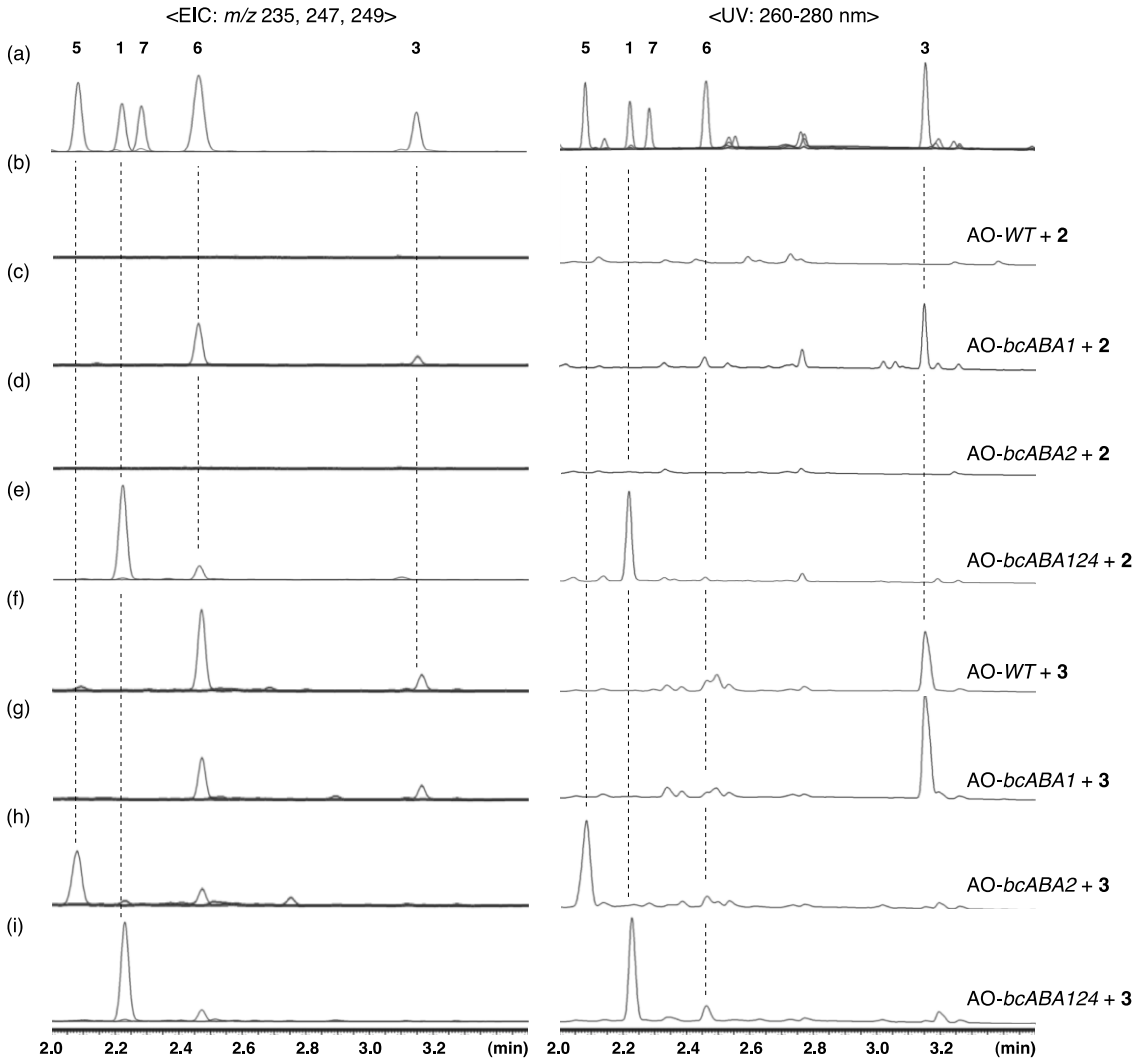


Figure 1.


<EIC: m/z 235, 247, 249>
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(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
5
1
7
6
3
AO-WT + 2
AO-bcABA1 + 2
AO-bcABA2 + 2
AO-bcABA124 + 2
AO-WT + 3
AO-bcABA1 + 3
AO-bcABA2 + 3
AO-bcABA124 + 3
2.0
2.2
2.4
2.6
2.8
3.0
3.2
(min)

Figure 2.

Table 1.

Gene	Strain	ABA production	DNA length (bp)	Protein size (aa)	Number of introns
bcABA1	SAS56	No production	1769	509	4
	B05.10	No production	1925	493	4
	T4	No production	1769	509	4
	ATCC58025	6.0 g/L	*	*	*
bcABA2	SAS56	No production	1810	527	4
	B05.10	No production	1507	443	3
	T4	No production	1810	528	4
	ATCC58025	6.0 g/L	*	*	*
bcABA3	SAS56	No production	1254	417	0
	B05.10	No production	1323	422	1
	T4	No production	1323	440	0
	ATCC58025	6.0 g/L	*	*	*
bcABA4	SAS56	No production	842	258	1
	B05.10	No production	842	258	1
	T4	No production	842	258	1
	ATCC58025	6.0 g/L	*	*	*

* Only raw sequence reads (accession number; PRJNA421408) are available.