Supporting Information

Unveiling biosynthesis of the phytohormone abscisic acid in fungi: Unprecedented mechanism of core scaffold formation catalyzed by an unusual sesquiterpene synthase

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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- and ^{2}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) and $CHCl_3$ (Kanto). ^{1}H chemical shifts were reported in δ value based on residual chloroform (7.26 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.

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. *Botrytic cinerea* MAFF 306914 from NARO genebank, Japan was used for extraction of genomic DNA. Sequences of *bcABA1, bcABA2, bcABA3* and *bcABA4* from MAFF 306914 are nearly identical to those of strain B05.10. A fungal host strain used in this study was *A. oryzae* NSAR1, a quadruple auxotrophic mutant (*niaD*$^{-}$, *sC*$^{-}$, *ΔargB*, *adeA*$^{-}$), for fungal expression.

Genomic DNA preparation.

Genomic DNA of *B. cinerea* MAFF 306914 was extracted according to the following method; the mycelia of fungus was collected and dried using paper towel. The dried mycelia was frozen in liquid nitrogen and crushed by SK-mill (Tokken). To the frozen powder was added extraction buffer (400 mM of Tris-HCl (pH 8.0), 500 mM of NaCl, 20 mM of ethylenediaminetetraacetic acid (EDTA) and 1% of sodium dodecyl sulfate) and the suspension was kept at room temperature for 5 min. To the suspension was added phenol:chloroform solution and the mixture was vortexed for 2 sec. After incubation at 65 °C for 60 min, the reaction mixture was centrifuged at 12000 rpm for 5 min. The supernatant was then treated with RNase at 37 °C for 90 min. To the reaction mixture was then added phenol:chloroform solution. After being vortexed for 2 sec, the mixture was centrifuged at 12000 rpm for 5 min. The supernatant was transferred to a new centrifuge tube and re-extracted twice with phenol:chloroform solution followed by chloroform. To the final supernatant was added cold-isopropanol and CH_3COONa solution and genomic DNA was recovered by centrifugation at 12000 rpm for 10 min. The pellet was then washed with 70% ethanol solution and dried for 15 min. Finally, the isolated DNA was resuspended in TE buffer (10 mM of Tris-HCl (pH 8.0) and 1mM of EDTA) and stored at -20 °C for further use.

Construction of *E. coli* expression plasmids.

Previous mRNA analysis showed that the *bcABA3-L* has no intron. Therefore, it was amplified from genomic DNA of *B. cinerea* MAFF 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 *Nde*I site of pColdI using In-Fusion Advantage PCR cloning kit (Clontech Laboratories) to construct expression plasmid pColdI-*bcABA3-L*. The sequence *bcABA3* from MAFF 306914 was identical to that of B05.10. The expression plasmid pColdI-*bcABA3-S* was constructed by using PrimeSTAR Mutagenesis Basal Kit (Takara) with primer sets, *bcABA3m-F* and *bcABA3m-R*, according to the manufacturer's protocol. These plasmids were separately 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 (25 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM imidazole, 5 mM $MgCl_2$) and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni-NTA column to purify the BcABA3-L.

BcABA3 Assays (in vitro).

Typical conditions are as follows; a reaction mixture (100 μL of Tris-HCl buffer (pH 7.4)) containing 130 μM of FPP, 0.5 mM of EDTA, 5 □M of $MgCl_2$, 2 mM of DTT, 2 μg of BcABA3 was incubated at 30 °C for 1 hr. The reaction was quenched by the addition of Hexane (50 μL) and the resultant mixture was vortexed and centrifuged at 12,000 x g. The supernatant was directly analyzed by a GC-MS QP2010 apparatus (Shimadzu, Kyoto, Japan) with a DB-1 MS capillary column (0.32 mm × 30 m, 0.25 □m film thickness; J&W Scientific, Folsom, CA). Each sample was injected onto the column at 100 °C in the splitless mode. After isothermal hold at 100°C for 3 min, the column temperature was increased by 14°C min^{-1} to 268°C. The flow rate of the helium carrier gas was 0.66 $mLmin^{-1}$.

BcABA3 Assays (in vivo).

The transformant EC-*bcABA3-L* was grown at 37 °C at an OD_{600} of ~0.6 in 500 mL flask in the presence of fosmidomycin (5.5 μM). 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 100 mM of phosphate buffer (pH 7.4). To the cell suspension was added β-farnesene or allofarnesene[1] (1 μg) at 16 °C for 17 h. During those periods, the volatile organic compounds were extracted by a SPME fiber (50/30um DVB/CAR/PDMS. Stableflex, 24Ga, Manual Holder), which was conditioned by inserting it into the GC injector to prevent contamination. After extraction, the fiber was pulled into the needle sheath and the SPME device was removed from the vial and then inserted into the injection port to analyze the sample.

Construction of *A. oryzae* expression plasmids.

The *bcABA1-bcABA4* were amplified from genomic DNA of *B. cinerea* MAFF 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 appropriate restriction site (site 1 and/or site 2) of pTAex3[2], pUSA2[3], or pAdeA2[4] using In-Fusion Advantage PCR cloning kit

(Clontech Laboratories) or NEBuilder HiFi DNA Assembly cloning kit (NEW ENGLAND BioLabs) to construct expression plasmids pTAex3-*bcABA1*, pUSA2-*bcABA24*, and pAdeA2-*bcABA3*.

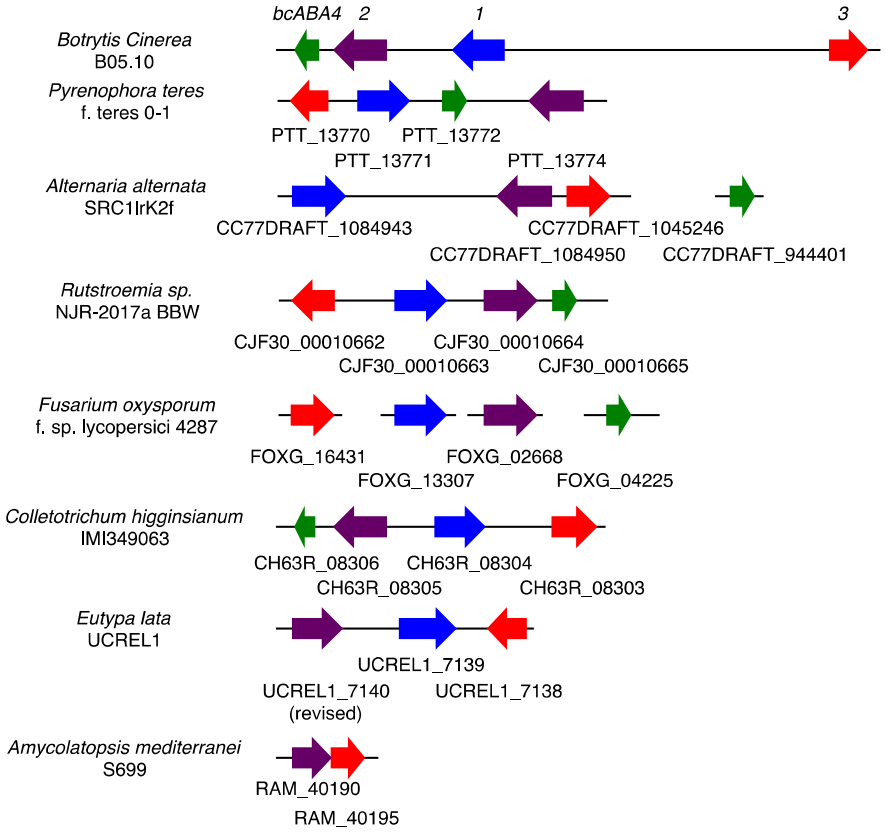
Transformation of *Aspergillus oryzae*.

Transformation of *A. oryzae* NSAR1 (1.0×10^8 cells) was performed by the protoplast-polyethylene glycol method reported previously[5] to construct AO-*bcABA1234*.

Analysis of the metabolites.

Mycelia of AO- *bcABA1234* was inoculated into a MPY medium (2 mL) in 10 mL test tube. Each culture was incubated at 30 °C for 4 days. The fermentation broth was soaked in acetone (2.5 mL) for 12 hr. 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 C10 (ϕ2,1 x 50 mm) at the following conditions (conditions 1): 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 %).

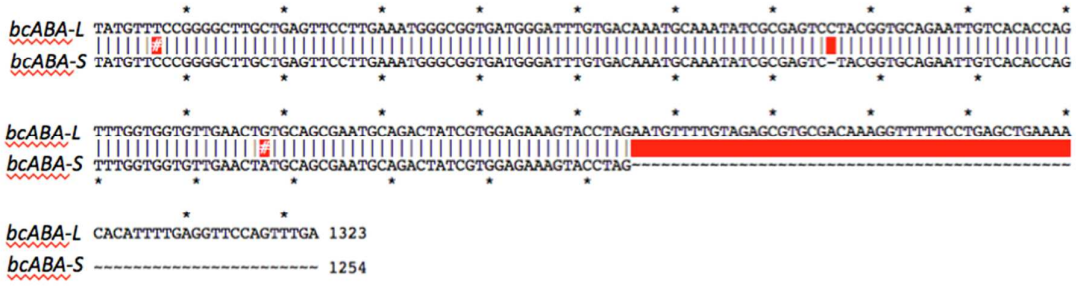
Figure S1. Schematic view of the abscisic acid biosynthetic gene cluster in several fungi. The accession number and the identity of each BcABA3 homolog are summarized in the table.



Strain	Accession number	identity (%)
Rutstroemia sp. NJR-2017a BBW	PQE10665.1	86
Colletotrichum higginsianum IMI 349063	XP_018158055.1	70
Pseudogymnoascus sp. WSF 3629	OBT40576.1	67
Eutypa lata UCREL1	EMR65886.1	69
Leptosphaeria maculans JN3	XP_003843016.1	67
Amycolatopsis mediterranei S699	AEK46506.1	65
Aspergillus tubingensis CBS 134.48]	OJI80076.1	63
Pyrenophora tritici-repentis Pt-1C-BFP	XP_001939959.1	63
Pyrenophora teres f. teres 0-1	EFQ89822.1	61
Exophiala xenobiotica	XP_013319909.1	60
Elsinoe australis	PSK60221.1	58
Alternaria alternata	XP_018379073.1	55
Stemphylium lycopersici	KNG44597.1	51
Fusarium oxysporum f. sp. lycopersici 4287	XP_018257345.1	52
Streptomyces sp. NRRL F-6131	WP_030303341.1	51

Figure S2. Comparison of BcABA-S and BcABA-L sequences

(A) Alignment of genomic DNA sequences of *bcABA-L* (B05.10 strain, XM_024694602.1) and *bcABA-S* (SAS56 strain, AM237449.1)



(B) Alignment of BcABA-S (SAS56 strain, CAJ87067.1) and BcABA-L (B05.10 strain, XP_024550392.1)

```
CAJ87067.1          MQQVITQTLVDDRFIQISDSKKSEGLATDSTKRQSQEQPIHDKDPIKAATAAMATTPLVK
XP_024550392.1      MQQVITQTLVDDRFIQISDSKKSEGLATDSTKRQSQEQPIHDKDPIKAATAAMAATPLVK
                    ***********************************************************:*****

CAJ87067.1          EHQDTWYYPPDIANDLQSINLPAELKGEIFACAWEYTRCVIPNYTNWNRYVAFMRIIIMG
XP_024550392.1      EHQDTWYYPPDIANDLQSINLPAELKGEIFACAWEYTRCVIPNYTNWNRYVAFMRIIIMG
                    ************************************************************

CAJ87067.1          IIAEFRGEMVDVTASNNLLGYDLDATLAALFEGTPGHKEMAREYKTFLLITADKASERRD
XP_024550392.1      IIAEFRGEMVDVTASNNLLGYDLDATLAALFEGTPGHKEMAREYKTFLLITADKASERRD
                    ************************************************************

CAJ87067.1          GELFRRYVNALAQSPRHWFRMRDCDALARFTIASALACNDLDDIWFTEDQFEILTEIGDT
XP_024550392.1      GELFRRYVNALAQSPRHWFRMRDCDALARFTIASALACNDLDDIWFTEDQFEILTEIGDT
                    ************************************************************

CAJ87067.1          LYDAVAFYKHRAEGETNSTFAYMPEDLRIKAYSECREILWALDAAWARNPKLANVINFVR
XP_024550392.1      LYDAVAFYKHRAEGETNSTFAYMPEDLRIKAYSECREILWALDAAWARNPKLANVINFVR
                    ************************************************************

CAJ87067.1          FFGGPIHMMMRRYRFVEENLTIGKSETDKVVDQTRKNFKLWNRVDANKRSVLNTQRYKAL
XP_024550392.1      FFGGPIHMMMRRYRFVEENLTIGKSETDKVVDQTRKNFKLWNRVDANKRSVLNTQRYKAL
                    ************************************************************

CAJ87067.1          IARSEELMFPGLAEFLEMGGDGICDKCKYRES----------TVQNCHTS----LVV
XP_024550392.1      IARSEELMFPGLAEFLEMGGDGICDKCKYRESYGAELSHQFGGVELCSECRLSWRKYLEC
                    ********************************              .:*. *    *

CAJ87067.1          LNYAANADYRGEST------
XP_024550392.1      FVERATKVFPELKTHFEVPV
                    :   *.  :   .*
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Figure S3. GC-MS chromatograms of the headspace volatile organic compounds from (A) EC-*WT*, (B) EC-*bcABA3*, and (C) EC-*amABA3*.

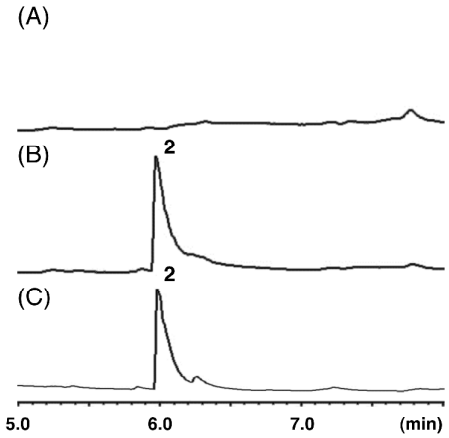


Figure S4. SDS-PAGE analysis of BcABA3. M: marker and lane 1: purified BcABA3.

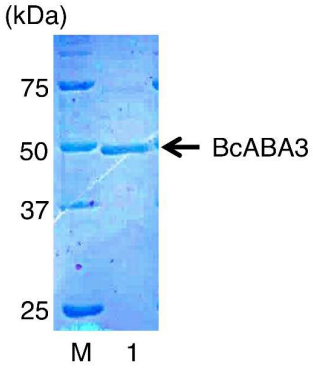


Figure S5. GC-MS chromatograms of the enzymatic reaction products: (A) without BcABA3, (B) in the presence of EDTA (20 mM), and (C) with BcABA3.

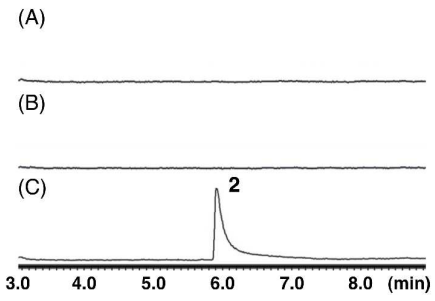


Figure S6. GC-MS chromatograms of the products from in vivo biotransformation (D) without substrate (*m/z* 148), (E) with **4** (*m/z* 135 and 148), and (F) with **5** (*m/z* 148).

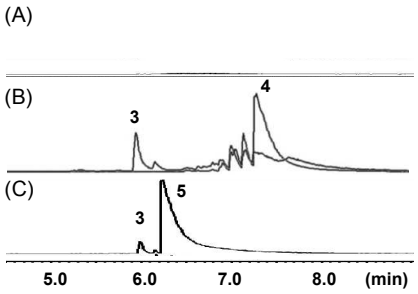


Figure S7. (A) ^{1}H-NMR spectrum (0.5 – 7.5 ppm) of **2**. (B) ^{2}H-NMR spectrum of **2** prepared by enzymatic reaction in the presence of 90% D_2O.

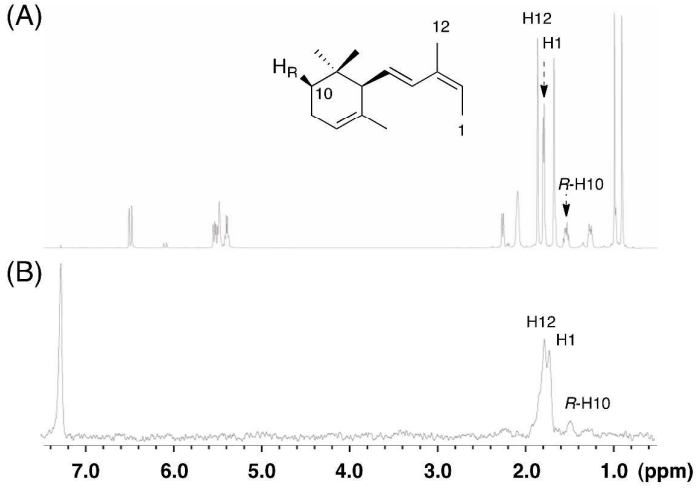


Figure S8. MS spectra of deuterium labeled **1**. The observed mass shifts are shown in the parenthesis.

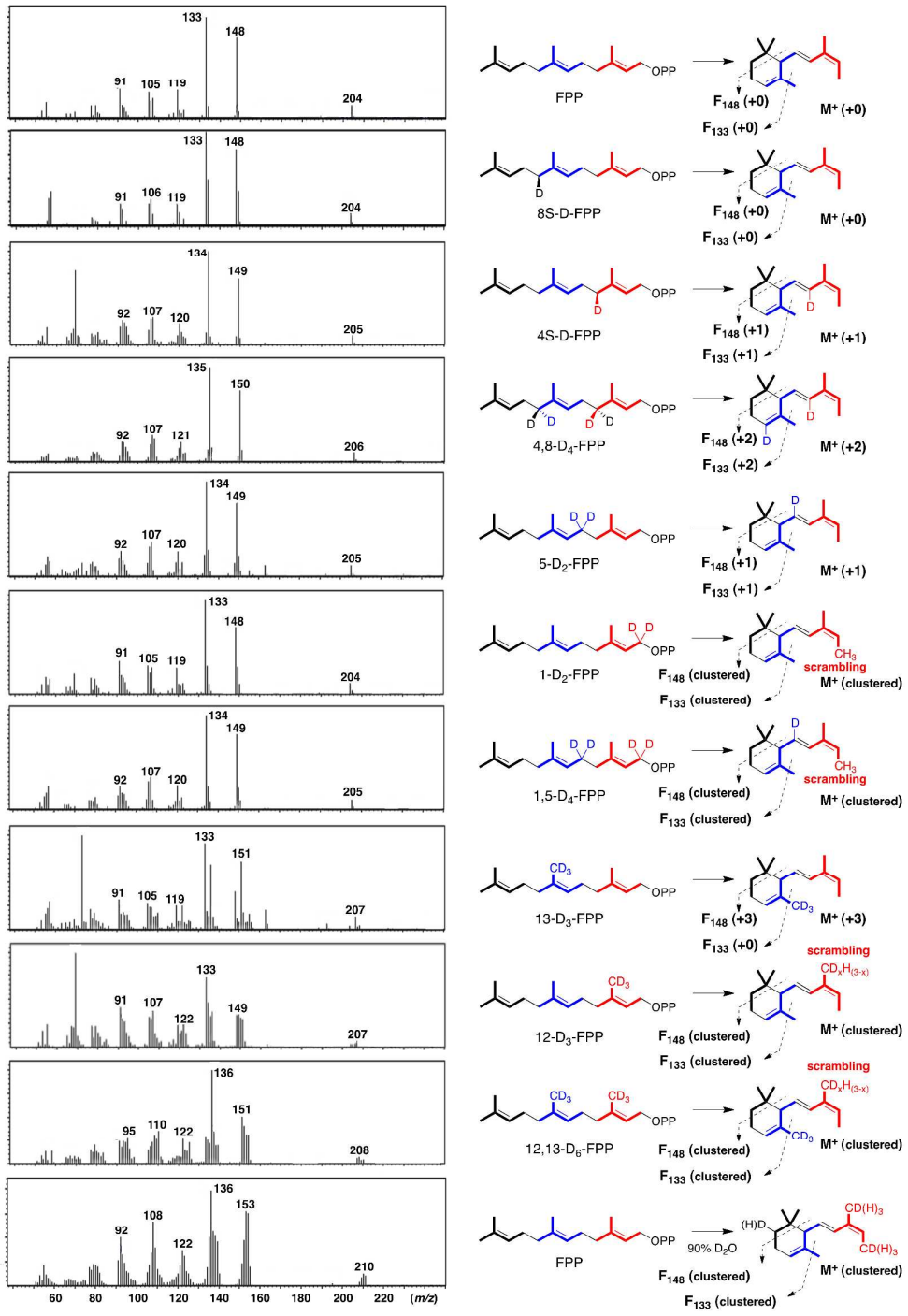


Figure S8. Continued.

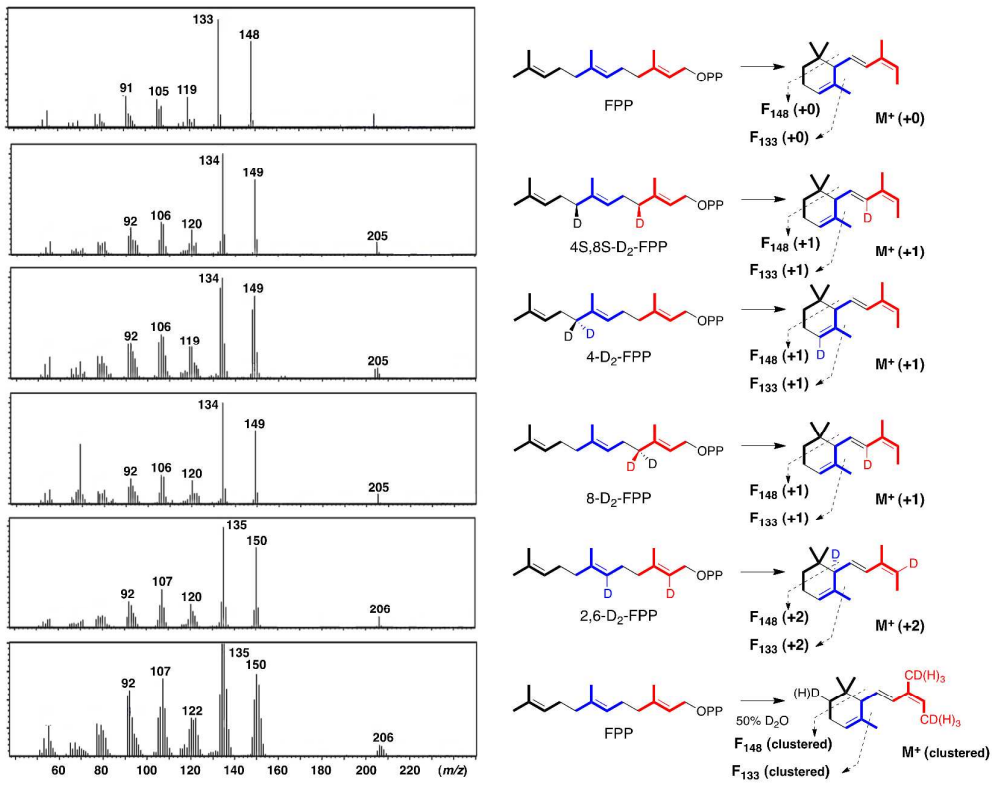


Figure S9. Proposed deprotonation-protonation sequence of β-farnesene via **A2**$^{+}$ and **B**$^{+}$.

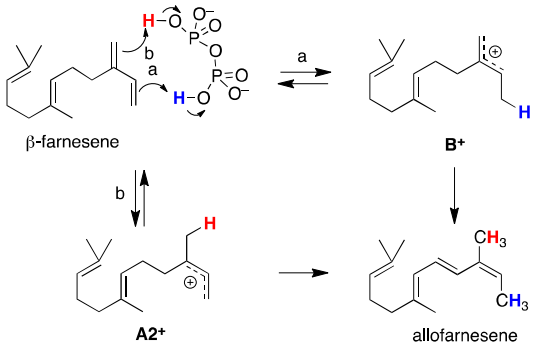


Figure S10. Proposed stereochemical course of the BcABA3 catalyzing cyclization via (A) a chair conformation and (B) a boat conformation.

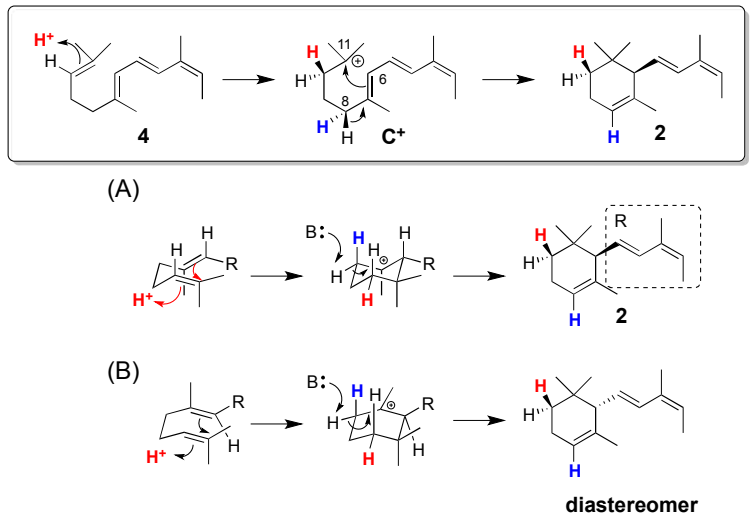
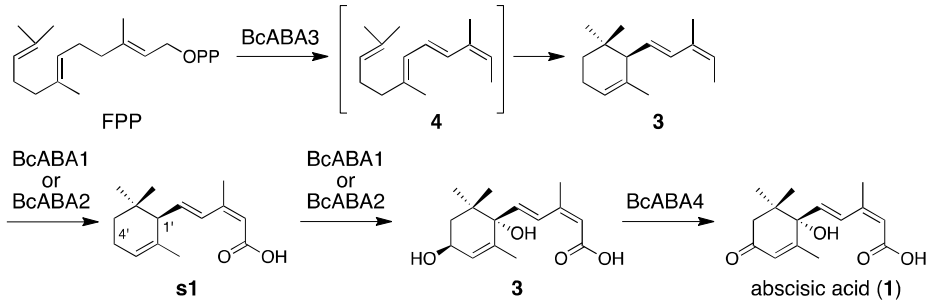
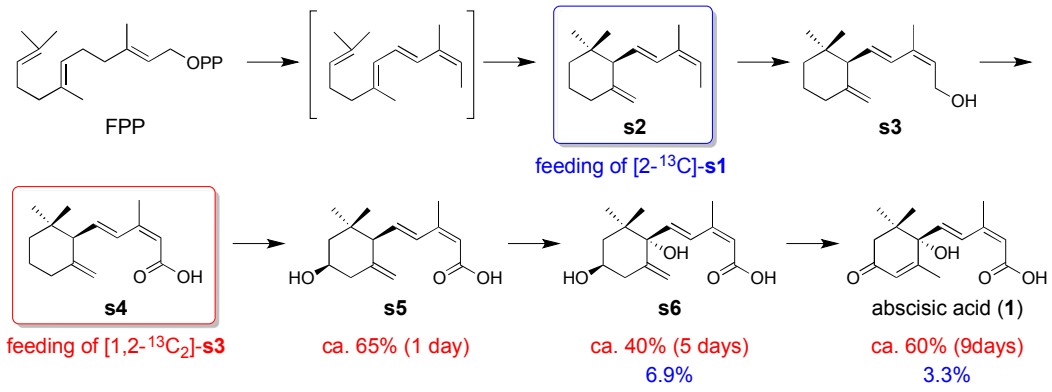


Figure S11. Alignment of amino acid sequences of homologous enzymes with BcABA3. Highly conserved regions are shown in region 1 to region 3.

Scheme S1. Proposed biosynthetic pathway of abscisic acid in *B. cinerea*.



Scheme S2. Proposed biosynthetic pathway of abscisic acid in *C. cruenta*. Incorporation ratios of each compound are shown below the structure.



Scheme S3. Alternative mechanism catalyzed by BcABA3.

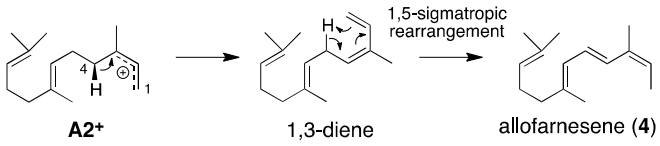


Table S1. Oligonucleotides used for construction of expression plasmids.

Insert	Sequence 5'-3'	Size Vector
bcABA1	F: **CAAGCTCCGGAATTC**ATGTCTAATTCTATATTG	1769 bp
	R: **TACCGAGCTCGAATTC**CTATTTGTATTCTGTTC	pTAex3
bcABA2	F: **AATTCGAGCTCGGTAC**ATGCTGCTTAGCATTAAAG	1810 bp
	R: **CTACTACAGATCCCCGG**CTATCTAGGAACCTCTTTTAAC	pUSA2
linker	F: AGAGGTTCCTAGATAGCCGGGGATCTGTAGTAGCTC	860 bp
	R: ATGGTTGAGAGGACATTGCGGCCGCTAGCTCAAATC	pUSA2
bcABA4	F: **TGAGCTAGCGGCCGCA**ATGTCCTCTCAACCATTC	840 bp
	R: **CTACCCGGGTCACTAG**CTAACATCTCCATCCGCC	pUSA2
bcABA3-Ao	F: **ATCGATTTGAGCTAGC**ATGCAGCAAGTTATTACTCAA	1323 bp
	R: **TAGTGCGGCCGCTAGC**TCAAACTGGAACCTCAAAATG	pAdeA2
bcABA3-Ec	F: **ATATCGAAGGTAGGCAT**ATGCAGCAAGTTATTAC	1323 bp
	R: **GGGTACCGAGCTCCATATG**TCAAACTGGAACCTCAAAATG	pColdI
bcABA3m	F: TCGCGAGTCTACGGTGCAGAATTGTCACACCAG	1254 bp
	R: ACCGTAGACTCGCGATATTTGCATTTGTCACAA	

[References]

1. Inomata, M.; Hirai, N.; Yoshida, R.; Ohigashi, H. *Phytochemistry* **2004**, *65*, 2667-2678.
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