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BIOSYNTHESIS OF ABSCISIC ACID IN *CERCOSPORA PINI-DENSIFLORAE*

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Key Word Index—*Cercospora pini-densiflorae*; Deuteromycetes; abscisic acid; biosynthesis; 1′,4′-*trans*-diol of abscisic acid, 1′,4′-dihydroxy-α-ionylideneacetic acid; 1′-deoxyabscisic acid; 4′-oxo-α-ionylideneacetic acid; 4′-hydroxy-α-ionylideneacetic acid.

Abstract—Out of 12 families of phytopathogenic fungi examined, four strains produced abscisic acid, the 1′,4′-*trans*-diol of abscisic acid, or both. *Cercospora pini-densiflorae* produced large amounts of the 1′,4′-*t*-diol of abscisic acid and small amounts of 4′-hydroxy-α-ionylideneacetic acids, 1′-deoxyabscisic acid, and ABA. It converted 1′,4′-*t*-diolABA into ABA more efficiently than 1′-deoxyABA into ABA. This result establishes that the main biosynthetic route for the formation of ABA in *C. pini-densiflorae* is via 1′,4′-*t*-diolABA.

INTRODUCTION

Prompted by the discovery that the phytopathogenic fungus *Cercospora rosicola* synthesizes (+)-abscisic acid (ABA, **1**) in culture medium [1], several groups have started to study the pathway of ABA biosynthesis in fungi. A general biosynthetic precursor of ABA common to all fungi has not yet been found. Thus 1′-deoxyabscisic acid (1′-deoxyABA, **8**) is regarded as an immediate precursor of ABA in *C. rosicola* [2] whereas 1′,4′-dihydroxy-γ-ionylideneacetic and the (+)-1′,4′-*trans*-diol of abscisic acid (1′,4′-*t*-diol-ABA, **4**) fulfill the same function in *C. cruenta* [3] and *Botrytis cinerea* [4] respectively. 1′4′-*t*-DiolABA is one of the precursors of ABA in higher plants [5]. We have screened phytopathogenic fungi for other fungi that produce ABA, to find if 1′,4′-*t*-diolABA occurs widely as a precursor in such fungi. Here, we describe the results of screening and identification of six ABA-related metabolites produced by *C. pini-densiflorae*. Feeding experiments showed that 1′,4′-*t*-diolABA was the major immediate precursor of ABA in this fungus rather than 1′-deoxyABA.

RESULTS AND DISCUSSION

Eighty strains of phytopathogenic fungi beloging to 12 families, *Botryosphaeria*, *Cercospora*, *Cladosporium*, *Diaporthe*, *Fusarium*, *Leucostoma*, *Monilinia*, *Pestatoria*, *Phomopsis*, *Physalospora*, *Sphaerotheca*, and *Verticillium*, were cultured on potato-dextrose-agar media for one month at 25°. Extracts of the culture media were analysed by HPLC, GC-ECD, or both. Unlike the supposition by Dörffling *et al.* that the distribution of ABA is widespread among phytopathogenic fungi [6], we could find only four strains of phytopathogenic fungi that produced any ABA or 1′,4′-*t*-diolABA. Several other groups have also reported that attempts to detect ABA in a number of fungi responsible for leaf spot or leaf rotting disease were unsuccessful [4, 7–10]. These results strongly indicate that the distribution of ABA is limited to certain species of phytopathogenic fungi. The fungal strains that produced ABA and its related metabolite are listed in Table 1. These fungi have not been reported to produce ABA or 1′,4′-*t*-diolABA before now. *Cercospora pini-densiflorae* No. 26 released a large amount of 1′4′-*t*-

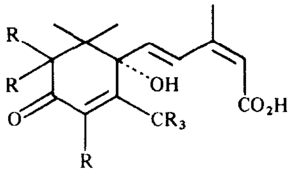
Table 1. Concentration of ABA and 1′,4′-*t*-diolABA in culture media of different phytopathogenic fungi

				Concentration (ng/ml)	
Species	Strain	Host	Medium*	ABA	1′,4′-*t*-diolABA
Cercospora pini-densiflorae	No. 26	Pine	PDA	110	5.5×10^3
			CD	380	2.0×10^4
			ME	13	700
C. theae	Breda de Haan [2]	Pine	PDA	10	5
C. fici	Heald et Wolf [1]	Pine	PDA	—	8
Verticillium dahliae	VBr-Na	Chinese cabbage	PDA	67	—

*PDA Potato-dextrose-agar; CD, Czapek–Dox; ME, Malt extract.

diolABA into the medium. This fungus produced more ABA and 1′,4′-*t*-diolABA on Czapek–Dox medium than on either potato-dextrose-agar or malt extract medium. The onset of ABA and 1′,4′-*t*-diolABA production was 24–30 days after inoculation. This fungus did not produce ABA or its related metabolites in shake culture. When cultured on Czapek–Dox medium in the dark, ABA and 1′4′-*t*-diolABA production decreased to 29 and 37%, respectively, of that in the control culture. These observations provided additional evidence that ABA production by this fungus is much influenced by environmental factors, as reported for *C. rosicola* [11], *C. cruenta* [12] and *B. cinerea* [4, 13].

We cultured *C. pini-densiflorae* No. 26 on a large scale to search for other ABA-related metabolites. The EtOAc-soluble acidic fraction of the culture medium was chromatographed on silica gel. ABA (yield 1 mg) was found in the 40% EtOAc eluate, and 1′,4′-*t*-diolABA (15 mg) was isolated from the same fraction. From the 10%-EtOAc eluate, 1′-deoxyABA (500 μg, **8**) and its 2-*trans*-isomer (500 μg, **10**) were obtained, both metabolites are found in *C. rosicola* and *C. cruenta* [2, 12]. The 20% EtOAc eluate was further purified by preparative TLC and HPLC to give three metabolites, **11**, (730 μg), **12** (60 μg), and **13** (50 μg) as oils. The ORD of **11** showed a positive Cotton effect and an absorption maximum was observed at 265 nm due to the α, β and γ, δ-unsaturated carboxyl group. The GC/MS of the methyl ester (**11**-Me) showed the dehydrated ion at *m*/*z* 246. The base ion peak at *m*/*z* 125 was assignable to an ABA-like side chain. The ^{1}H NMR spectrum of **11** showed the presence of an ABA-like side chain at δ1.99 (3H, *s*, Me-3), 5.68 (1H, *br s*, 2-H), 6.02 (1H, *dd*, *J* = 9 and 16 Hz, 5-H), and 7.53 (1H, *d*, *J* = 16 Hz, 4-H), a 2′-methyl group on the double bond and the 4′-allyl proton attached to a hydroxyl bearing carbon at δ1.65 (3H, *d*, *J* = 1.5 Hz) and 4.23 (1H, *m*), respectively, and a 1′-allyl proton at δ2.34 (1H, *d*, *J* = 9 Hz). These spectral data strongly suggested that **11** was a 4′-dihydro-derivative of (+)-1′*R*-deoxyABA (**8**); either (1′*R*)-4′*S*-or (1′*R*)-4′*R*-hydroxy-α-ionylideneacetic acid (4′-OH-α-ionylideneacetic acid) The UV, ORD, and GC/MS analysis of the methyl esters of **12** and **13** suggested that they were epimeric or geometric isomers of **11**. Authentic methyl esters of 4′-OH-α-ionylideneacetic acid and its 2-*trans*-isomer prepared by reduction of (+)-1′-*R*-deoxyABA-Me (**8**-Me) with sodium borohydride were used to check these structures including the absolute configuration at C-4′. The reduction product was found to be a mixture of four compounds by HPLC analysis. The first and the third compounds eluted were (1′*R*)-4′-OH-α-ionylideneacetic acid methyl ester and the second and the fourth ones were the 2-*trans*-isomer of (1′*R*)-4′-OH-α-ionylideneacetic acid methyl ester, according to the ^{1}H NMR spectrum [14]. In the ^{1}H NMR spectra of the first and the third compound, the C-1′ proton of the latter was observed at a lower field (δ2.53) than that of the former (δ2.31). This downfield shift was assigned to the anisotropic effect of a 4′-hydroxyl group that was *cis* to the C-1′ proton. Thus the absolute configuration at C-4′ of the first and the third compound could be *S* and *R*, respectively. Furthermore, (1′*R*)-4′*S*-OH-α-ionylideneacetic acid was eluted faster than (1′*R*)-4′*R*-OH-α-ionylideneacetic acid when chromatographed on ODS as reported by Ichimura *et al.* [12]. This is also



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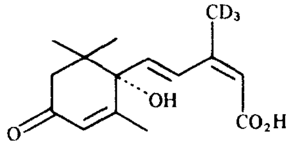
2 R = D



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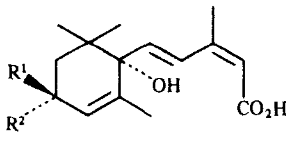
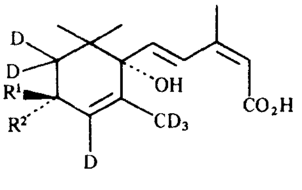


4 R¹ = OH, R² = H
5 R¹ = H, R² = OH



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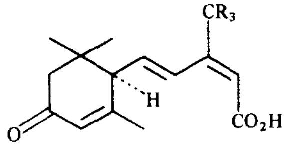
7 R¹ = D, R² = OH



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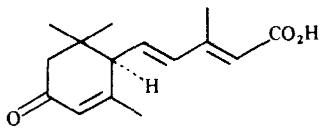

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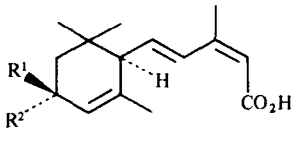
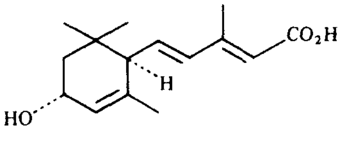


11 R¹ = OH, R² = H
12 R¹ = H, R² = OH



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evidence of the reliability of this assignment. A similar downfield shift was seen in the second and the fourth compounds eluted off the column. This effect was compatible with the presence of a 2-*trans*-isomer because the difference of the chemical shift of C-1′ ($\Delta\delta$) between the 2-*cis*- and 2-*trans*-isomers was less than 0.10 for 1′-deoxyABA-Me and α-and γ-ionylideneacetic acid [15]. From these observations, the absolute configuration at C-4′ of the second and the fourth compounds was assigned as *R* and *S*, respectively. Methyl esters of **11**, **12**, and **13** were co-chromatographed on GC and HPLC with methyl esters of (1′*R*)-4′*S*-OH-α-ionylideneacetic acid, (1′*R*)-4′*R*-OH-α-ionylideneacetic acid and *t*-(1′*R*)-4′*R*-OH-α-ionylideneacetic acid, respectively, and found to be identical by direct comparison of the spectra. Thus, **11**, and **12** were identified as (1′*R*)-4′*S*- and (1*R*)-4′*R*-OH-α-ionylideneacetic acid, respectively, and **13** was the 2-*trans*-isomer of **12**. That there is fungal production of (1′*R*)-4′*S*- and (1′*R*)-4′*R*-OH-α-ionylideneacetic acid (**11**) and **12**) was shown in this experiment, although these compounds have been tentatively identified as metabolites of *C. rosicola* by GC/MS [16].

It is likely that **11** is the direct precursor of 1′,4′-*t*-diolABA, and that both 4′-epimers of 4′-OH-α-ionylideneacetic acid are converted into 1′-deoxyABA.

The amounts of *t*-1′-deoxyABA and *t*-(1′*R*)-4′*R*-OH-α-ionylideneacetic acid produced were about the same as those of the corresponding 2-*cis*-isomers. Ichimura *et al.* reported that the yield of *t*-1′-deoxyABA from *C. cruenta* was 5% that of 1′-deoxyABA [12]. 1′-DeoxyABA and (1′*R*)-4′*R*-OH-α-ionylideneacetic acid produced by *C. pini-densiflorae* might isomerize to the 2-*trans*-isomers when exposed to light during growth of the organism. Another possibility is that the C-4′ oxidizing and hydroxylating enzymes in *C. pini-densiflorae* are insensitive to the geometry of the side chain, because the 2-*trans*-isomers of α-ionylidene ethanol and α-ionylideneacetic acid was converted to 2-*trans*-isomer of 1′-deoxyABA in high yield [17].

1′-DeoxyABA has been proposed as a precursor of ABA, as both *C. rosicola* and *C. cruenta* convert this compound into ABA [2, 3]. However, there is the possibility that 1′,4′-*t*-diolABA is a metabolite formed from ABA [4, 16, 18–21]. (+)-[$^2H_6$]ABA (**2**) was fed to *C. pini-densiflorae* to find if 1′,4′-*t*-diolABA is a precursor or a metabolite of ABA. The synthesized 1′,4′-*t*-diolABA was assayed and purified by HPLC and then its methyl ester was analysed by GC/MS to calculate the deuterium of the labelled ABA. The relative intensity of the monodehydrated ion of 1′,4′-*t*-diolABA-Me was made use of in this quantitative assessment. The 1′,4′-*t*-diolABA-Me recovered from the medium with added [$^2H_6$]ABA had only one monodehydrated ion at *m/z* 262 assignable to non-labelled 1,′,4′-*t*-diolABA-Me. This result showed that ABA was not converted into 1′,4′-*t*-diolABA under these experimental conditions. Furthermore, this fungus could not convert (±)-[$^2H_3$]ABA (**3**) into 1′,4′-*t*-diolABA under the same experimental conditions. Thus, the possibility that (+)-[$^2H_6$]ABA could not be reduced because of the steric hindrance and isotopic effect of deuterium atoms in the ring system was ruled out.

Because of previous results [2–4], we assumed that ABA is derived from two precursors, 1′,4′-*t*-diolABA and 1′-deoxyABA, in this fungus. To identify which metabolite is the main immediate precursor, (+)-[$^2H_3$]-1′-deoxyABA (**9**), (+)-[$^2H_7$]-1′,4′-*t*-diolABA (**6**), and its *cis*-diol isomer (**7**) were fed individually to the fungus. The ABA produced was assayed by HPLC and its methyl ester were analysed by GC/MS. The GC/MS of ABA-Me obtained from the medium after administration of [$^2H_7$]-1′,4′-*t*-diolABA showed six monodehydrated ions assignable to [^1H]-and [$^2H_{1,2,3,5,6}$]ABA-Me. The percentage of biosynthesized ABA from the [$^2H_7$]-1′,4′-*t*-diolABA added was 17%. The incorporation ratio was calculated from the expression DT/B x 100, where D was the percentage of ^2H content determined by GC/MS, T was the total amount of ABA assayed by HPLC, and B was the amount of ^2H-labelled precursor added. The (+)-[$^2H_7$]-1′,4′-*t*-diolABA was converted into ABA in a 3.3% yield. When [$^2H_3$]-1′-deoxyABA (**9**) was added to refloated cultures of *C. pini-densiflorae* the deuterium contents of the synthesized ABA was 44% and the incorporation ratio was 1.5%. The difference between the observed amount of deuterium of ABA may reflect differences in the endogenous level of each metabolite. (+)-[$^2H_7$]-1′,4′-*cis*-DiolABA (**7**) and (+)-[$^2H_7$]-1′,4′-*t*-diolABA were both converted into ABA by this fungus with the incorporation ratio of 4.5%. These results indicated that both 1′,4′-*t*-diolABA and 1′-deoxyABA were converted into ABA by *C. pini-densiflorae*. The oxidation of these compounds to ABA was not automatic, because they are stable in aqueous solution [2, 4]. 1′,4′-*t*-DiolABA was converted to ABA with a higher incorporation ratio than that of 1′-deoxyABA, so the biosynthetic pathway of ABA via 1,′,4′-*t*-diolABA could be the main route in *C. pini-densiflorae* rather than via 1′-deoxyABA. 1′,4′-*cis*-DiolABA (**5**) also is a possible precursor, but it was not detected in the culture medium of *C. pini-densiflorae* by GC-SIM. Whether the 4′-hydroxyl group is oxidized by one slightly stereospecific enzyme or two stereospecific enzymes is not known.

Earlier reports show that a number of higher plants can not convert 1′-deoxyABA into ABA [22–24], and 1′-deoxyABA has not been found to occur in higher plants in nature. On the other hand, 1′,4′-*t*-diolABA was found in plants and also the three fungi reported here in addition to *B. cinerea* [4, 5], and was converted easily into ABA [4, 5, 16, 20, 21]. These facts strongly suggest that 1′,4′-*t*-diolABA is a more common precursor of ABA than 1′-deoxyABA.

EXPERIMENTAL

HPLC. HPLC systems: A: YMC Pack A311 ODS column (6 × 100 mm), eluted with MeOH–H_2O–HOAc (400:600:1), 2.0 ml/min, detection UV_{254}; B:Chemcosorb 5-ODS-H ODS column (4.6 × 150 mm), eluted with MeOH–H_2O–HOAc (60:40:0.1), 1.0 ml/min, detection UV_{254}; C: Cosmosil 5SL silica gel column (4.6 × 150 mm), eluted with *n*-hexane–*iso*-PrOH (400:1), 2.0 ml/min, detection UV_{254}; D: Cosmosil 5SL silica gel column (4.6 × 150 mm), eluted with *n*-hexane–EtOH (200:1), 2.0 ml/min, detection UV_{266}; E: YMC Pack A311 ODS column (6 × 100 mm), eluted with MeOH–H_2O–HOAc (60:40:0.1), 1.5 ml/min, detection UV_{254}; F: YMC Pack A311 ODS column (6 × 100 mm), eluted with MeOH–H_2O–HOAc (550:450:1) 1.5 ml/min, detection UV_{254}.

Screening for fungi that produce ABA. The fungus was inoculated on to potato-dextrose-agar medium (Nissui Corp. Ltd) and incubated for one month at 25°. Each culture medium (16 ml) was extracted with 40 ml of Me_2CO containing 20 μg of BHT. The Me_2CO extract was dried and dissolved in 1 ml MeOH. A portion of the MeOH soln was analysed by HPLC (system A) or GC-ECD [1% OV-17 glass column (3 mm × 2 m), N_2 flow rate 60 ml/min, oven temp 190°] after methylation. The amount of ABA in the medium (16 ml) was below the detection limit of GC-ECD. Detection limits of ABA were 2.5 ng/injection for HPLC, and 400 pg/injection for GC-ECD, respectively.

Isolation of ABA-related metabolites. C. pini-densiflorae No. 26 was cultured on 4 1 of Czapek–Dox medium for 33 days in the laboratory. The EtOAC-soluble acidic fraction of the culture medium was chromatographed on silica gel (20 g) and eluted with a mixture of toluene–EtOAc containing 1% HOAc. The fraction eluted with 10% EtOAc was subjected to silica gel TLC (toluene–EtOAc, 7 : 3) and the metabolities that absorbed UV at R_f 0.23 were methylated and purified by prep. HPLC (system C). The UV-absorbing peaks at R_t 18.0 and 19.5 min were collected and concd, yielding 500 μg 1′-deoxyABA-Me (**8**-Me) and 500 μg of its 2-*trans*-isomer (**10**-Me), respectively. 1′-DeoxyABA-Me (**8**-Me); $[\alpha]_D^{25}$ + 278° (EtOH, *c* 0.0072); UV λ_{max}^{EtOH} nm (ε): 266 (14 500); ^{1}H NMR (400 MHz, $CDCl_3$): δ0.98 (3H, *s*), 1.06 (3H, *s*) 1.98 (3H, *d*, *J* = 1.2 Hz), 2.00 (3H, *d*, *J* = 1.3 Hz), 2.14 (1H, *d*, *J* = 16.8 Hz), 2.37 (1H, *d*, *J* = 16.8 Hz), 2.72 (1H, *d*, *J* = 9.5 Hz), 3.71 (3H, *s*), 5.71 (1H, *s*), 5.93 (1H, *dd*, *J* = 9.5 and 15.6 Hz), 5.94 (1H, *s*), 7.72 (1H, *d*, *J* = 15.6 Hz); EIMS (GC) 70 eV *m/z* (rel. int.) 262 $[M]^+$ (0.8), 231 (2), 174 (13), 146 (41), 125 (100). *t*-1′-DeoxyABA-Me (**10**-Me); $[\alpha]_D^{25}$ + 198° (EtOH, *c* 0.0101); UV λ_{max}^{EtOH} nm (ε): 266 (17 000); ^{1}H NMR (400 MHz, $CDCl_3$): δ0.97 (3H, *s*), 1.06 (3H, *s*), 1.89 (3H, *d*, *J* = 1.2 Hz), 2.12 (1H, *d*, *J* = 16.8 Hz), 2.28 (3H, *d*, *J* = 1 Hz), 2.35 (1H, *d*, *J* = 16.8 Hz), 2.65 (1H, *d*, *J* = 9.5 Hz), 3.72 (3H, *s*), 5.78 (1H, *s*), 5.95 (1H, *s*), 5.97 (1H, *dd*, *J* = 9.5 and 15.6 Hz), 6.21 (1H, *d*, *J* = 15.6 Hz); EIMS (GC) 70 eV *m/z* (rel. int.): 262 $[M]^+$ (4), 231 (6), 206 (14), 189 (5), 174 (62), 146 (100), 125 (70). The 20% EtOAc eluate was separated by prep. TLC (silica gel, toluene–EtOAc–HOAc, 14:6:1 development x 6) to give the crude acids (R_f 0.73). They were purified by prep. HPLC (system B). The peaks at R_t 9.6, 11.0, and 14.2 min were collected and concd, yielding 730 μg **11**, 60 μg **12**, and 50 μg **13**, respectively. (1′*R*)-4′*S*-OH-α-Ionylideneacetic acid (**11**); $[\alpha]_D^{22}$ + 356° (EtOH, *c* 0.015); UV λ_{max}^{EtOH} nm (ε): 265 (19 700); ^{1}H NMR (400 MHz, $CDCl_3$): δ0.85 (3H, *s*), 0.96 (3H, *s*), 1.43 (1H, *m*), 1.65 (4H, Me-2′ and H-5′ overlapping, *d* and *m*, *J* = 2 Hz), 1.99 (3H, *s*), 2.34 (1H, *d*, *J* = 9 Hz), 4.23 (1H, *br s*), 5.56 (1H, *br s*) 5.68 (1H, *br s*), 6.02 (1H, *dd*, *J* = 9 and 16 Hz), 7.53 (1H, *d*, *J* = 16 Hz); **11**-Me; EIMS (GC) 70 eV *m/z* (rel. int.): 246 $[M-H_2O]^+$ (12), 231 (3), 214 (11), 199 (19), 187 (23), 185 (31), 171 (20), 170 (35), 159 (12), 157 (15), 145 (24), 143 (20), 133 (11), 125 (100), 119 (23). (1′*R*)-4-*R*-OH-α-Ionylideneacetic acid methyl ester (**12**-Me); ORD $[\alpha]_{285}^{26}$ +8000 (peak), $[\alpha]_{265}^{26} \pm 0$, $[\alpha]_{248}^{26} - 7100$ (trough) (EtOH, *c* 0.00113); UV λ_{max}^{EtOH} nm (ε): 265 (20 000); EIMS (GC) 70 eV *m/z* (rel. int.): 246 $[M-H_2O]^+$ (8), 231 (6), 214 (10), 199 (28), 187 (14), 185 (27), 171 (42), 170 (37), 159 (5), 157 (14), 155 (30), 145 (24), 143 (21), 133 (11), 125 (100), 119 (16). *t*-(1′*R*)-4′R-OH-α-Ionylideneacetic acid methyl ester (**13**-Me); ORD $[\alpha]_{285}^{27}$ + 9500 (peak), $[\alpha]_{264}^{27} \pm 0$, $[\alpha]_{240}^{27} - 14\ 500$ (trough) (EtOH, *c* 0.0017); UV λ_{max}^{EtOH} nm (ε): 262 (20 000); EIMS (GC) 70 eV *m/z* (rel. int.): 246 $[M-H_2O]^+$ (33), 213 (16), 207 (21), 186 (45), 185 (43), 183 (24), 180 (15), 183 (24), 180 (15), 171 (61), 169 (34), 159 (19), 157 (46), 133 (100), 125 (73). The 40% EtOAc eluate was further purified on a silica gel column eluted with a mixture of toluene–EtOAc–HOAc (160:40:1) to afford ABA (1 mg, **1**) and 1′,4′-*t*-diolABA (15 mg, **4**). ABA was identified by GC/MS after methylation. ABA-Me (**1**-Me); EIMS (GC) 70 eV *m/z* (rel int.): 278 $[M]^+$ (1), 260 (20), 245 (11), 233 (6), 213 (8), 190 (100), 162 (34), 125 (29), 107 (10). 1′,4′-*t*-DiolABA (**4**), colourless needles (EtOAc), mp 183–184°, $[\alpha]_D^{28}$ + 298° (EtOH, *c* 0.034); UV λ_{max}^{EtOH} nm (ε): 262 (19 600). 1′,4′-*t*-DiolABA-Me; ^{1}H NMR (400 MHz, $CDCl_3$): δ0.91 (3H, *s*), 1.05 (3H, *s*), 1.59 (1H, *dd*, *J* = 13.4 and 9.8 Hz), 1.67 (3H, *s*), 1.83 (1H, *dd*, *J* = 13.4 and 6.7 Hz), 2.02 (3H, *br s*), 3.69 (3H, *s*), 4.22 (1H, *m*), 5.70 (2H, *br s*), 6.10 (1H, *d*, *J* = 15.9 Hz), 7.66 (1H, *d*, *J* = 15.9 Hz); EIMS (probe) 30 eV *m/z* (rel. int.): 280 $[M]^+$ (1), 262 (20), 244 (19), 230 (15), 206 (19), 174 (46), 159 (18), 146 (57), 125 (100), 111 (41).

Preparation of 4′-OH-α-ionylideneacetic acid methyl ester and their 2-trans-*isomers.* (+)-(1′*R*)-DeoxyABA-Me (1 mg) was dissolved in 0.8 ml MeOH, and *ca* 2 mg $NaBH_4$ added. The reaction mixture was left for 23 hr at room temp. After dilution with 30 ml H_2O, the soln was extracted with EtOAc (10 ml × 4). The EtOAc extract was purified by prep. HPLC (system D). The peaks at R_t 9.6 ((1′*R*)-4′*S*-OH-α-ionylideneacetic acid methyl ester, 180 μg), 13.0 (*t*-(1′*R*)-4′*R*-OH-α-ionylideneacetic acid methyl ester, 310 μg), 14.8 (1′*R*)-4′*R*-OH-α-ionylideneacetic acid methyl ester, 230 μg), and 18.8 min (*t*-(′*R*)-4′*S*-OH-α-ionylideneacetic acid methyl ester, 200 μg) were collected and concd. (1′*R*)-4′*S*-OH-α-Ionylideneacetic acid methyl ester; ^{1}H NMR (400 MHz, $CDCl_3$): δ0.86 (3H, *s*), 0.96 (3H, *s*), 1.40 (1H, *dd*, *J* = 13 and 10 Hz), 1.65 (4H, Me-2′ and H-5′ overlapping, *d* and *dd*, *J* = 1.5 Hz and 13.7 Hz), 1.99 (3H, *d*, *J* = 1.5 Hz), 2.31 (1H, *d*, *J* = 9 Hz), 3.70 (3H, *s*), 4.22 (1H, *m*), 5.55 (1H, *s*), 5.64 (1H, *s*), 5.98 (1H, *dd*, *J* = 9 and 16 Hz), 7.59 (1H, *d*, *J* = 16 Hz). (1′*R*)-4′*R*-OH-α-Ionylideneacetic acid methyl ester; ^{1}H NMR (400 MHz, $CDCl_3$): δ0.85 (3H, *s*), 1.02 (3H, *s*), 1.39 (1H, *dd*, *J* = 7 and 13 Hz), 1.63 (3H, *d*, *J* = 1 Hz), 1.84 (1H, *dd*, *J* = 6 and 13 Hz), 1.97 (3H, *d*, *J* = 2 Hz), 2.53 (1H, *d*, *J* = 10 Hz), 3.70 (3H, *s*), 4.26 (1H, *m*), 5.57 (1H, *s*), 5.65 (1H, *s*), 5.84 (1H, *dd*, *J* = 10 and 16 Hz), 7.59 (1H, *d*, *J* = 16 Hz). *t*-(1′*R*)-4′*R*-OH-α-Ionylideneacetic acid methyl ester; ^{1}H NMR (400 MHz, $CDCl_3$): δ0.85 (3H, *s*), 1.00 (3H, *s*), 1.38 (1H, *dd*, *J* = 7 and 13 Hz), 1.61 (3H, *d*, *J* = 2 Hz), 1.84 (1H, *dd*, *J* = 6 and 13 Hz), 2.27 (3H, *d*, *J* = 1 Hz), 2.45 (1H, *d*, *J* = 10 Hz), 3.71 (3H, *s*), 4.26 (1H, *br s*), 5.58 (1H, *s*), 5.74 (1H, *s*), 5.85 (1H, *dd*, *J* = 10 and 15 Hz), 6.12 (1H, *d*, *J* = 15 Hz). *t*-(1′*R*)-4′*S*-OH-α-Ionylideneacetic acid methyl ester; ^{1}H NMR (400 MHz, $CDCl_3$): δ0.85 (3H, *s*), 0.95 (3H, *s*), 1.37 (1H, *dd*, *J* = 10 and 13 Hz), 1.63 (3H, *d*, *J* = 2 Hz), 1.67 (1H, *dd*, *J* = 6 and 13 Hz). 2.20 (1H, *d*, *J* = 10 Hz), 2.27 (3H, *d*, *J* = 1 Hz), 3.71 (3H, *s*), 4.24 (1H, *br s*), 5.53 (1H, *s*), 5.75 (1H, *s*), 5.94 (1H, *dd*, *J* = 10 and 15 Hz), 6.11 (1H, *d*, *J* = 15 Hz). EIMS (GC) 70 eV *m/z* (rel. int.) 246 $[M-H_2O]^+$ (53), 213 (25), 199 (49), 185 (35), 183 (20), 171 (81), 125 (75), 91 (100). When $MeOH-H_2O$ (2:1) mixture was used as solvent, reduction of (+)-1′-deoxyABA-Me with $NaBH_4$ did not give 2-*trans*-isomers of 4′-OH-α-ionylideneacetic acid methyl esters.

Preparation of labelled compounds. (+)-$[^2H_7]$-1′,4′-*cis*- and *t*-diolABA (**7** and **6**) and (+)-$[^2H_6]$ABA (**2**) were synthesized as described previously [7]. (±)-$[^2H_3]$ABA (**3**) was provided by Dr S. Yoshida, University of Tokyo. The proportion of labelled (±)-$[^2H_3]$ABA was 42% as estimated by GC/MS analysis after methylation. $[^2H_3]$-1′-DeoxyABA (**9**) was prepared by biotransformation from (±)-α-ionylideneacetic acid with ^{2}H content of 70% estimated by GC/MS analysis in *C. pini-densiflorae* No. 26 as follows. The fungus was sub-cultured on 600 ml (200 ml × 3) of medium for 30 days. Mycelia were removed from the medium, carefully washed with sterile H_2O, and refloated on a new medium. (±)-$[^2H_3]$-α-Ionylideneacetic acid (4.5 mg) was fed to the mycelia of this fungus as a methanolic soln (150 μl) and incubated for another 14 days. The medium was filtered and extracted with EtOAc (150 ml × 4) at pH 3.0. The EtOAc extract (45 mg) was chromatographed on silica gel (11 g) eluted with a mixture of toluene and EtOAc containing 0.1% HOAc. The fraction eluted with 10% EtOAc was purified by prep. TLC (silica gel, toluene–EtOAc–HOAc, 18:2:1, development × 6) to give the fraction (R_f 0.57) containing $[^2H_3]$-1′-deoxyABA (**9**). This fraction was further purified by prep. HPLC (system E). The peak at R_t 4.8 min was collected and concd. to afford $[^2H_3]$-1′-deoxyABA (400 μg). The proportion of the labelled 1′-deoxyABA was 57% as estimated by GC/MS after methylation. No *t*-1'-deoxyABA was detected by GC-FID (1% OV-17). The enantiomeric ratio of $[^2H_3]$-1'-deoxyABA (**9**) was 1'*R*: 1'*S* = 16:9 as calculated by ORD analysis.

Addition of ^{2}*H-labelled compounds to the culture.* The ^{2}H-labelled compounds were added to the culture (200 ml) as for the preparation of ^{2}H-labelled 1'-deoxyABA. The ^{2}H-labelled 1'-deoxyABA (250 μg), 1',4'-*t*-diolABA (210 μg) and 1',4'-*cis*-diolABA (800 μg) were used for the feeding experiments. The EtOAc extract (3–7 mg) of the culture medium was purified by prep. TLC (silica gel, toluene–EtOAc–HOAc, 12:8:1) to afford ABA. At times, the fraction containing ABA was further purified by prep. HPLC (system A). The synthesized ABA was methylated and analysed by EIMS (GC) 70 eV. ABA levels were assayed by HPLC (system A) in portions of the EtOAc extract after the labelled compds were added. The levels of ABA were corrected for extraction losses.

Analysis of endogenous 1',4'-cis-*diolABA in* C. pini-densiflorae. The EtOAc extract (460 mg) obtained from 6 l of culture medium was chromatographed on silica gel (63 g) eluted with a mixture of toluene and EtOAc containing 1% HOAc. The 60% EtOAc eluate (64 mg) was purified by prep. HPLC (system F). The eluent containing the compound of interest was collected (R_t 4 to 8 min fraction) and concd. When GC-SIM (EI) analysis was done, the ions at *m/z* 262 $[M-H_2O]^+$, 244 $[M-2H_2O]^+$, and 230 $[M-H_2O-MeOH]^+$ were monitored. The fracttion corresponding to 1.1 mg of the 60% EtOAc eluate was purified by prep. HPLC and analysed by GC-SIM [2% OV-1, glass column (3 mm × 1 m), He flow rate 30 ml/min, oven temp. 220°]. The detection limit for 1',4'-*cis*-diolABA was 25 ng/injection.

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