Available online at www.sciencedirect.com

SCIENCE DIRECT®

Phytochemistry 65 (2004) 2667–2678

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

The biosynthetic pathway to abscisic acid via ionylideneethane in the fungus *Botrytis cinerea*

Masahiro Inomata [a], Nobuhiro Hirai [b,*], Ryuji Yoshida [c], Hajime Ohigashi [a]

[a] *Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan*
[b] *International Innovation Center, Kyoto University, Kyoto 606-8501, Japan*
[c] *Department of Agriculture Technology, Toyama Prefectural University, Toyama 939-0311, Japan*

Received 10 March 2004; received in revised form 20 July 2004
Available online 23 September 2004

Abstract

The biosynthetic pathway to abscisic acid (ABA) from isopentenyl diphosphate in the fungus, *Botrytis cinerea*, was investigated. Labeling experiments with $^{18}O_2$ and $H_2{}^{18}O$ indicated that all oxygen atoms at C-1, -1, -1′ and -4′ of ABA were derived from molecular oxygen, and not from water. This finding was inconsistent not only with the known carotenoid pathway via oxidative cleavage of carotenoids, but also with the classical direct pathway via cyclization of farnesyl diphosphate. The fungus produced new C_{15}-compounds, 2*E*,4*E*-α-ionylideneethane and 2*Z*,4*E*-α-ionylideneethane, along with 2*E*,4*E*,6*E*-allofarnesene and 2*Z*,4*E*,6*E*-allofarnesene, but did not apparently produce carotenoids except for a trace of phytoene. The C_{15}-compounds labeled with ^{13}C were converted to ABA by the fungus, and the incorporation ratio of 2*Z*,4*E*-α-ionylideneethane was higher than that of 2*E*,4*E*-α-ionylideneethane. From these results, it was concluded that farnesyl diphosphate was reduced at C-1, desaturated at C-4, and isomerized at C-2 to form 2*Z*,4*E*,6*E*-allofarnesene before being cyclized to 2*Z*,4*E*-α-ionylideneethane; the ionylideneethane was then oxidized to ABA with molecular oxygen. This direct pathway via ionylideneethane means that the biosynthetic pathway to fungal ABA, not only before but also after isopentenyl diphosphate, differs from that to ABA in plants, since plant ABA is biosynthesized using the non-mevalonate and carotenoid pathways.

Keywords: *Botrytis cinerea*; Abscisic acid; Biosynthesis; Allofarnesene; α-Ionylideneethane

1. Introduction

The plant hormone, abscisic acid (ABA, **1**), regulates various physiological reactions including induction of adaptive responses to water deficiency and low temperature in plants (Leung and Giraudat, 1998). It is produced by some phytopathogenic fungi in addition to higher plants, lichens, bryophytes and algae (Hartung and Gimmler, 1994). ABA may control the susceptibility of plant tissues to fungal infection (Kettner and Dörffling, 1995), although its physiological role in fungi is not understood.

Studies on ABA biosynthesis have suggested that its biosynthetic pathway in fungi is different from that in higher plants (Fig. 1). Higher plants use isopentenyl diphosphate (IDP, **2**) derived from the non-mevalonate pathway via 2-*C*-methyl-D-erythritol 4-phosphate (**3**) for its biosynthesis (Hirai et al., 2000a); in turn, the IDP is converted to 9*Z*-epoxycarotenoid, which is cleaved by dioxygenase to form ABA (Schwartz et al., 2003). In contrast to higher plants, IDP for ABA of the fungi, *Botrytis cinerea*, *Cercospora pini-densiflorae* and *C. cruenta*, is biosynthesized by the mevalonate pathway via mevalonic acid (**4**) (Hirai et al., 2000a;

---

[*] Corresponding author. Tel.: +81-75-753-6282; fax: +81-75-753-6284.
E-mail address: hirai@kais.kyoto-u.ac.jp (N. Hirai).

0031-9422/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2004.08.025

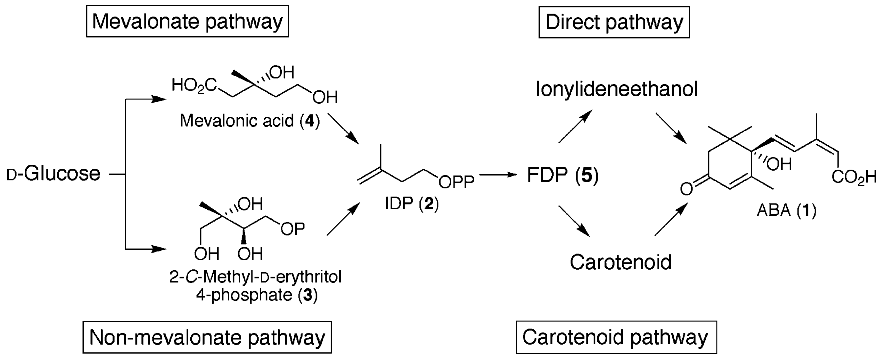


Fig. 1. Outline of ABA biosynthetic pathway.

Yamamoto et al., 2000). Two pathways, the direct and carotenoid pathways, have been proposed for the pathway after IDP in fungi.

In the direct pathway, farnesyl diphosphate (FDP, **5**) is hydrolyzed, isomerized, cyclized and desaturated to form ionylideneethanol, and oxidation of ionylideneethanol gives ABA, the classical direct pathway in Fig. 2. This pathway has been suggested based on administration experiments with potential C_{15} precursors in two fungi. *C. rosicola* converted FDP to ABA (Bennett et al., 1984), and *C. rosicola* and *C. cruenta* metabolized 2*Z*,4*E*-α-ionylideneethanol (**6**) and 2*Z*,4*E*-γ-ionylideneethanol (**7**), respectively, to ABA (Neill et al., 1987; Oritani et al., 1985; Kato et al., 1987). However, putative intermediates between FDP and ionylideneethanol, 4,5-didehydrofarnesol and monocyclofarnesol, were not converted to ABA, and their production by these fungi has not been confirmed (Bennett et al., 1990; Yamamoto and Oritani, 1997; Yamamoto et al., 2001). This is a shortcoming in support of the direct pathway.

The carotenoid pathway has been proposed for *C. cruenta*, which produces β-carotene and 2*Z*,4*E*-γ-ionylideneacetaldehyde (**8**) along with **7** (Oritani et al., 1985; Yamamoto et al., 2000). This fungus converts **8** to ABA and incorporated ^{18}O from $^{18}O_2$ into C-1 of **8** and into C-1, -1′ and -4′ of ABA (Yamamoto et al., 2000). This result apparently coincides with the biosynthesis of ABA via **8** formed by oxidative cleavage of carotenoids. However, 9*Z*-carotenoids possessing a γ-ring that should be precursors for **8** have not been found in this fungus. *C. rosicola* also produces β-carotene (Norman, 1991), but production of carotenoids by other ABA-producing fungi has not been reported. Thus, the carotenoid and direct pathways in fungi remain unclear.

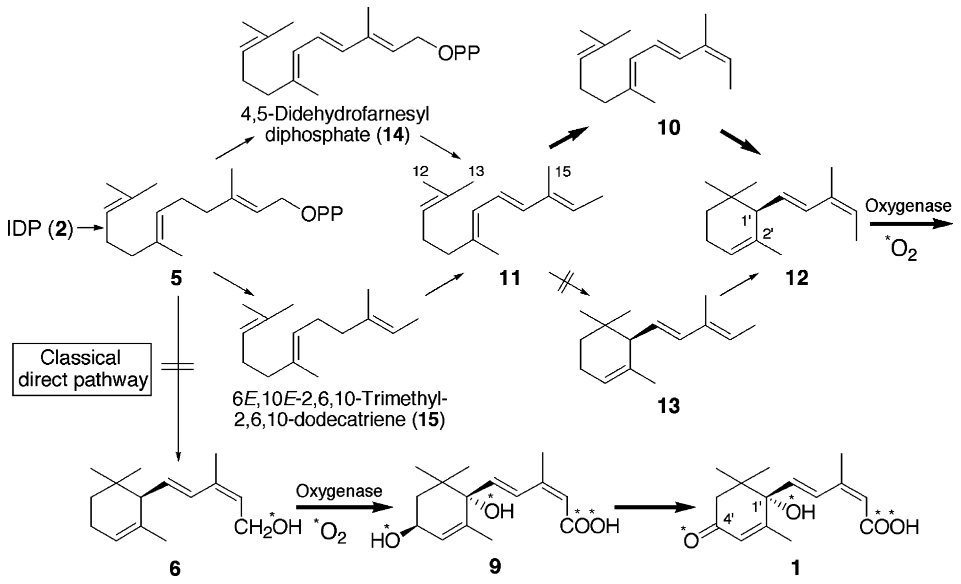
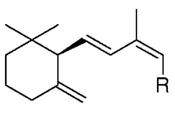


Fig. 2. The direct pathway via ionylideneethane for ABA biosynthesis in *B. cinerea*. Bold arrows show the pathway confirmed by the feeding experiments. The oxygen atoms with an asterisk are derived from molecular oxygen.



7: R = CH_2OH
8: R = CHO

The different origin of IDP between higher plants and fungi suggests that in fungi the genes for ABA biosynthesis are quite distinct from that in higher plants. However, IDP is the common precursor of all terpenoids, so elucidation of the biosynthetic pathway after IDP in fungal ABA and comparison of those between fungi and higher plants are important to discuss the origin of genes for ABA biosynthesis. We investigated the biosynthetic pathway between IDP and ABA in *B. cinerea* that produces 1′,4′-*t*-diol-ABA (**9**) as the precursor of ABA (Hirai et al., 1986; Kettner and Dörffling, 1995). This paper proposes a direct pathway via ionylideneethane based on results from labeling experiments with $^{18}O_2$ and $H_2{}^{18}O$, precise analyses of ABA-related metabolites, and administration experiments with labeled metabolites.

2. Results and discussion

2.1. Labeling experiment with $^{18}O_2$ and $H_2{}^{18}O$

A labeling experiment of ABA with $^{18}O_2$ may distinguish between the carotenoid and direct pathways. In the direct pathway, oxidation at C-1 of ionylideneethanol by an oxidase with oxygen from H_2O will form the carboxyl group of ABA. This means that no ^{18}O from $^{18}O_2$ is introduced into C-1 of ABA in the direct pathway. If ABA in the fungus is biosynthesized by the carotenoid pathway as in plants, a precursor carotenoid would be oxidatively cleaved by a dioxygenase with molecular oxygen, and the resulting C_{15}-aldehyde could be oxidized at C-1 by an oxidase with oxygen from H_2O to form the carboxyl group of ABA (Seo et al., 2000a,b). In this case, one ^{18}O from $^{18}O_2$ is introduced into C-1 (Creelman and Zeevaart, 1984; Zeevaart et al., 1989). Thus, the number of ^{18}O labels at C-1 would be different between the two pathways. ^{18}O-Labeling at C-1′ and -4′ may also provide information about the pathway.

Botrytis cinerea was cultured under $^{18}O_2$ for 20 days, and the methyl esters of ABA and compound **9** produced by the fungus were analyzed by EI-MS. The mass spectrum of the ABA methyl ester gave $[M + 6]^+$, $[M + 4]^+$, $[M + 2]^+$ and $[M]^+$ ions at *m/z* 284, 282, 280 and 278, respectively, indicating incorporation of three ^{18}O atoms into ABA (Table 1). The fragment ion derived from the side-chain that appears at *m/z* 125 in the non-labeled ABA methyl ester was detected at *m/z* 129, 127 and 125 (Gray et al., 1974); this showed that two oxygen atoms at C-1 at maximum were labeled with ^{18}O. A fragment ion containing 1′,4′-oxygen atoms was detected at *m/z* 164 in addition to *m/z* 162 corresponding to the ion without ^{18}O (Gray et al., 1974), indicating that either C-1′ or -4′ was labeled with ^{18}O. The labeled position was determined by analysis of the dehydrated ion. Dehydrated ions $[M + 6\text{-}H_2O]^+$, $[M + 6\text{-}H_2{}^{18}O]^+$/$[M + 4\text{-}H_2O]^+$, $[M + 4\text{-}H_2{}^{18}O]^+$/$[M + 2\text{-}H_2O]^+$ and $[M + 2\text{-}H_2{}^{18}O]^+$/$[M - H_2O]^+$ were observed at *m/z* 266, 264, 262 and 260, respectively. More than 60% of the dehydrated ion of the ABA methyl ester is formed by elimination of the 1′-hydroxyl group, and either the 4- or 5-hydrogen (Hirai et al., 2000b). We found by analyzing a mass spectrum of authentic [4′-^{18}O]ABA methyl ester that a residual 32% of the dehydrated ion was formed by elimination of the 4′-oxygen and the 4- or 5-hydrogen after migration of the 1′-*O*-hydrogen to the 4′-oxygen (Table 1).[1] This finding means that [1′-^{18}O]ABA and [4′-^{18}O]ABA methyl esters can be distinguished by their ratios of relative intensity of the dehydrated ions $[M + 2\text{-}H_2O]^+$ and $[M + 2\text{-}H_2{}^{18}O]^+$; the former ester should give a ratio of about 32:68, and the latter a ratio of about 68:32. The ratio of the relative intensity of the dehydrated ions at *m/z* 266 and 264 was 29:71 after subtraction of the relative intensity derived from an $[M + 4\text{-}H_2O]^+$ ion from the relative intensity of the ion at *m/z* 264. This ratio was close to that of the dehydrated ions $[M + 2\text{-}H_2O]^+$ and $[M + 2\text{-}H_2{}^{18}O]^+$ of the [1′-^{18}O]ABA methyl ester, indicating that the 1′-oxygen was labeled with ^{18}O, but the 4′-oxygen was not. These results revealed that C-1, -1 and -1′ of ABA were labeled with ^{18}O from $^{18}O_2$.

The mass spectrum of the methyl ester of **9** showed molecular ions corresponding to $[M + 8]^+$, $[M + 6]^+$, $[M + 4]^+$, $[M + 2]^+$ and $[M]^+$, indicating that all four oxygen atoms of **9** at maximum were derived from ^{18}O (Table 2). This result did not coincide with that of ABA. It is possible that the 4′-^{18}O label of ABA was lost by exchange with ^{16}O from water during culture after [1,1,1′,4′-$^{18}O_4$]ABA had been biosynthesized from [1,1,1′,4′-$^{18}O_4$]-**9**. To examine this possibility, authentic [4′-^{18}O]ABA was incubated in buffer solutions of pH 3.0, 5.0 and 7.0 for 6 days. The result showed that the 4′-^{18}O content decreased from 84% to 3% and 78% after incubation at pH 3.0 and 5.0, respectively, and changed very little after incubation at pH 7.0. Measurement of the pH of the medium during culturing showed that the pH decreased from 5.8 to 2.7 during the first 5 days, and then gradually increased to 4.4 until day 20 after inoculation. This fungus began to produce ABA from day 5 after inoculation, and ABA accumulated in the

---

[1] The dehydration mechanism involving 4′-oxygen will be reported elsewhere.

Table 1
Relative intensities[a] and compositions[b] of molecular and major fragment ions of methyl esters of ABA isolated from *B. cinerea* cultured under $^{18}O_2$, or in $H_2{}^{18}O$

Methyl ester of non-labeled ABA				Methyl ester of [4′-^{18}O]ABA				Methyl ester of ABA isolated from *B. cinerea* cultured under $^{18}O_2$				Methyl ester of ABA isolated from *B. cinerea* cultured in $H_2{}^{18}O$			
m/z	Ion species	Rel. int. (%)	Comp. (%)	*m/z*	Ion species	Rel. int. (%)	Comp. (%)	*m/z*	Ion species	Rel. int. (%)	Comp. (%)	*m/z*	Ion species	Rel. int (%)	Comp. (%)
278	[M]$^+$	2.2	100	280	[M + 2]$^+$	2.1	84	284	[M + 6]$^+$	0.9	17	280	[M + 2]$^+$	1.4	82
				278	[M]$^+$	0.4	16	282	[M + 4]$^+$	1.4	26	278	[M]$^+$	0.3	18
								280	[M + 2]$^+$	1.8	34				
								278	[M]$^+$	1.2	23				
260	[M − H_2O]$^+$	4.8	100	262	[M + 2-H_2O]$^+$	3.2	57 (68)[e]	266	[M + 6-H_2O]$^+$	0.6	5 (29)[e]	262	[M + 2-H_2O]$^+$	2.6	55 (67)[e]
				260	[M + 2-$H_2{}^{18}O$]$^+$	2.4	27 (32)[e]	264	[M + 6-$H_2{}^{18}O$]$^+$	2.6	12 (71)[e]	260	[M + 2-$H_2{}^{18}O$]$^+$	2.1	27 (33)[e]
					[M − H_2O]$^+$		16		[M + 4-H_2O]$^+$		10		[M − H_2O]$^+$		18
								262	[M + 4-$H_2{}^{18}O$]$^+$	4.9	16				
									[M + 2-H_2O]$^+$		25				
								260	[M + 2-$H_2{}^{18}O$]$^+$	3.8	9				
									[M − H_2O]$^+$		23				
162	[M − 116]$^+$[c]	27.3	100	164	[M + 2-116]$^+$	25.4	78	164	[M + 6-120]$^+$	30.7	17	164	[M + 2-116]$^+$	27.5	73
				162	[M − 116]$^+$	7.1	22		[M + 4-118]		29[f]	162	[M − 116]$^+$	10.0	27
									[M + 2-116]$^+$						
								162	[M + 4-120]$^+$	36.4	31[f]				
									[M + 2-118]$^+$						
									[M − 116]$^+$		23				
125	[M − 153]$^+$[d]	26.3	100	125	[M + 2-155]$^+$	30.4	84	129	[M + 6-155]$^+$	15.3	17	125	[M + 2-155]$^+$	34.5	82
					[M − 153]$^+$		16		[M + 4-153]$^+$		3		[M − 153]$^+$		18
								127	[M + 4-155]$^+$	30.3	23				
									[M + 2-153]$^+$		16				
								125	[M + 2-155]$^+$	31.9	18				
									[M − 153]$^+$		23				

[a] Relative intensities were corrected by natural ^{13}C-, ^{2}H- and ^{17}O-isotopic abundance.

[b] Composition of relative intensities of each ion group. Composition of fragment ions [M + 2-$H_2{}^{18}O$]$^+$ and [M − H_2O]$^+$ at *m/z* 260 for [4′-^{18}O]ABA-Me were calculated as follows: 43% (total percent dehydrated ion at *m/z* 260) − 16% (percent [M]$^+$ ion at *m/z* 278) = 27% (percent [M + 2-$H_2{}^{18}O$]$^+$ ion); percent [M − H_2O]$^+$ was identical to that [M]$^+$ ion. Composition of other fragment ions was calculated by the same method as that described above.

[c] The fragment ion contains oxygen atoms at C-1′- and 4′.

[d] The fragment ion contains two oxygen atoms at C-1.

[e] The values in parentheses show the ratios of compositions of dehydrated ions [M + 2-H_2O]$^+$ and [M + 2-$H_2{}^{18}O$]$^+$, or [M + 6-H_2O]$^+$ and [M + 6-$H_2{}^{18}O$]$^+$.

[f] The sum of compositions of fragment ions [M + 4-118]$^+$ and [M + 2-116]$^+$, or [M + 4-120]$^+$ and [M + 2-118]$^+$.

Table 2
Relative intensities[a] and compositions[b] of molecular and major fragment ions of methyl esters of compound **9** isolated from *B. cinerea* cultured under $^{18}O_2$, or in $H_2{}^{18}O$

	Methyl ester of non-labeled **9**			Methyl ester of **9** isolated from *B. cinerea* cultured under $^{18}O_2$			Methyl ester of **9** isolated from *B. cinerea* cultured in $H_2{}^{18}O$		
	m/z	Rel. int. (%)	Comp. (%)	*m/z*	Rel. int. (%)	Comp. (%)	*m/z*	Rel. int. (%)	Comp. (%)
Molecular ion	280	1.4	100	288	0.3	8	280	1.3	100
				286	0.7	19			
				284	0.9	24			
				282	1.0	27			
				280	0.8	22			
First dehydrated ion	262	27.4	100	268	8.1	15	262	19.9	100
				266	15.7	28			
				264	19.6	35			
				262	12.1	22			
Second dehydrated ion	244	21.7	100	248	14.7	29	244	17.4	100
				246	19.8	39			
				244	15.8	31			
$[M-155]^+$ ion[c]	125	100.0	100	129	69.9	26	125	100.0	100
				127	99.7	37			
				125	98.5	37			

[a] Relative intensities were corrected by natural ^{13}C-, ^{2}H- and ^{17}O-isotopic abundance.

[b] Composition of relative intensities of each ion group.

[c] The fragment ion contains two oxygen atoms at C-1.

medium. The acidity of the medium strongly supported the above possibility. Therefore, all oxygen atoms at C-1, -1, -1′ and -4′ of ABA may be derived from molecular oxygen.

The ^{18}O labels of ABA and compound **9** may be derived from $H_2{}^{18}O$ that could be produced from $^{18}O_2$ by respiration of the fungus during culture. To avoid this possibility, the fungus was cultured in a medium made from $H_2{}^{18}O$. The mass spectrum of the methyl ester of ABA produced showed an $[M+2]^+$ ion at *m/z* 280, dehydrated ions $[M+2\text{-}H_2O]^+$ and $[M+2\text{-}H_2{}^{18}O]^+$ at *m/z* 262 and 260, respectively, with a ratio of 67:33, and a fragment ion containing 1′,4′-oxygen at *m/z* 164, indicating that the ABA was labeled at C-4′ with ^{18}O (Table 1). The ^{18}O label at C-4′ would be incorporated by non-enzymatic exchange of ^{16}O with ^{18}O from $H_2{}^{18}O$ of a medium as described above. Compound **9** was not labeled with ^{18}O (Table 2). This result further confirmed that all oxygen atoms at C-1, -1, -1′ and -4′ of ABA were derived from molecular oxygen, and not from water. The difference of our result from the $^{18}O_2$ labeling result of *C. cruenta* (Yamamoto et al., 2000) may be explained by the exchange of 1-^{18}O of **8** with ^{16}O from $H_2{}^{16}O$ via a hydrate of the aldehyde group due to a slow conversion of **8** to ABA in *C. cruenta*.

The above finding was inconsistent not only with the direct pathway but also with the carotenoid pathway. Labeling of both oxygen atoms at C-1 of ABA with ^{18}O from $^{18}O_2$ suggested that oxidation at C-1 of a putative C_{15}-aldehyde intermediate was catalyzed not by oxidase with oxygen from H_2O, but by a monooxygenase with molecular oxygen. This oxidation mechanism may be applied to the carotenoid pathway. However, we could not exclude a pathway different from the direct and carotenoid pathways. In bile acid biosynthesis, oxidation of the 27-methyl group of 5β-cholestan-3α,7α,12α-triol to the carboxyl group proceeds by multiple monooxygenations with molecular oxygen (Holmberg-Betsholtz et al., 1993). Carotenoids and C_{15} compounds produced by the fungus were carefully analyzed for biosynthetic intermediates for ABA that suggest the biosynthetic pathway of ABA.

2.2. Isolation and identification of ABA-related compounds

If the carotenoid pathway is operative in *B. cinerea*, the precursor carotenoid could be a 9*Z*-isomer possessing an ε-ring. Carotenoids in the extract from the mycelia were analyzed by HPLC with a photodiode array detector at a detection limit of 0.3 ng for all-*E*-β-carotene. Three compounds having absorption spectra similar to that of phytoene were detected at R_ts 3.8, 4.0, and 9.0 min, whereas no other carotenoids were detected. Compounds **10** and **11** detected at R_ts 3.8 and 4.0 min, respectively, were isolated, and their ^1H NMR and mass spectra suggested that **10** and **11** were 2*Z*,4*E*,6*E*- and 2*E*,4*E*,6*E*-allofarnesenes, respectively (Miyaura et al., 1982; Sakai and Hirose, 1969). The structures of **10** and **11** were confirmed by comparison of their spectral data with those of chemically synthesized 2*Z*,4*E*,6*E*- and 2*E*,4*E*,6*E*-allofarnesenes. Compounds **10** and **11** are known to occur in the essential oil of *Perilla frutescens f. viridis* Makino (Sakai and Hirose, 1969) and *Melissa officinalis* L. (Hose et al., 1997), and in virgin olive oil (Lanzon et al., 1994). The compound eluted at R_t 9.0 min was identified as 15,15′*Z*-phytoene. The contents of **10**, **11** and 15,15′*Z*-phytoene were 990, 1300 and 5 ng/g fresh weight of mycelia, respectively.

The occurrence of **10** and **11** suggested that *B. cinerea* produced ionylideneethane, a cyclized derivative of allofarnesene. Further analysis of the mycelia extract by GC–MS resulted in detection of two compounds **12** and **13** that gave molecular ions at *m*/*z* 204, coinciding with the molecular weight of ionylideneethane. Compounds **12** and **13** had $[M - 56]^+$ ions at *m*/*z* 148, suggesting that they possessed a partial 1,5,5-trimethyl-1-cyclohexene structure (Biemann, 1962). Compounds **12** and **13** were obtained as a mixture, but whose amounts were too small to obtain a ^{1}H NMR spectrum. We chemically synthesized 2*Z*,4*E*- and 2*E*,4*E*-α-ionylideneethanes, and confirmed by comparison of their R_ts in the GC and mass spectra with those of **12** and **13** that **12** and **13** were 2*Z*,4*E*- and 2*E*,4*E*-α-ionylideneethanes, respectively. Compounds **12** and **13** are new compounds, and were present in amounts of 7.7 and 1.1 ng/g fresh weight of mycelia, respectively. β-Ionylidenethane was not detected in the mycelium extract.

The absence of precursor carotenoids and the occurrence of C_{15} compounds **10**–**13**, suggested that another pathway via allofarnesene and α-ionylideneethane was operative in *B. cinerea*. This direct pathway can be envisaged to contain the following reaction steps: reduction at C-1 and desaturation at C-4 of FDP, isomerization at C-2 of **11** or **13**, cyclization of **10** or **11**, and oxidation at C-1, -1, -1′ and -4′ of **12** with molecular oxygen (Fig. 2). We thus performed an administration experiment with [2-^{13}C]-**10**, **11**, **12** and **13** to examine the direct pathway via ionylideneethane and to distinguish the two routes for isomerization and cyclization.

*2.3. Administration of [2-^{13}C]-**10**, **11**, **12** and **13** to B. cinerea*

Compounds [2-^{13}C]-**10**, **11**, **12** and **13** were synthesized, and administered to *B. cinerea*. The incorporation ratios of these compounds into **9**, **10**, **11**, **12**, **13** and ABA were examined using ^{13}C NMR or EI-MS analyses (see Table 3). All of the [2-^{13}C]-**10**, **11**, **12** and **13** were converted to ABA and **9**, indicating that these compounds were biosynthetic precursors of ABA. The low incorporation of the labeled compounds into **9** was due to its small amount compared with amounts of ABA. The incorporation ratio (0.7%) of **11** to ABA was higher than that (0.2%) of **10**. This result demonstrates the apparent preference of cyclization of **11** to **13** before isomerization of **11** to **10**. However, **10** and **11** are extremely unstable in an acidic aqueous solution in addition to being volatile, and the medium of the fungus was acidic as described above. Conversion of [2-^{13}C]-**10** and **11** to their respective isomers may affect the incorporation ratios also. Therefore, the small difference between the low incorporation ratios of **10** and **11** seemed insignificant. The incorporation ratios of **12** and **13** were more significant than those of **10** and **11**, and isomerization of **12** and **13** to **13** and **12**, respectively, was negligible. The higher incorporation ratio (17.1%) of **12** to ABA than that (2.6%) of **13** to ABA suggested that isomerization of **11** to **10** preceded cyclization of **11** to **13**. The content ratio (4:3) of **11** and **10**, and that (9:1) of **12** and **13** in the mycelium extract also preferred isomerization before cyclization. At present, it is unknown which pathway via 4,5-didehydrofarnesyl diphosphate (**14**) or 6*E*,10*E*-2,6,10-trimethyl-2,6,10-dodecatriene (**15**) is operative between FDP and **11**.

Stereospecificity of oxygenase converting **12** to ABA was examined by an HPLC analysis of ABA that incorporated 17.1% of (±)-[2-^{13}C]-**12**. If both enantiomers of [2-^{13}C]-**12** are converted to ABA, the enantiomeric excess of the ABA should be 96.52%, and if (*S*)-[2-^{13}C]-**12** is selectively converted to ABA, the enantio-

Table 3
Incorporation of 2-^{13}C labeled **10**, **11**, **12** and **13**

Products	Substrates			
	[2-^{13}C]-**11** I.R.[a] (I.A.)[b]	[2-^{13}C]-**10** I.R. (I.A.)	[2-^{13}C]-**13** I.R. (I.A.)	[2-^{13}C]-**12** I.R. (I.A.)
11	–[c]	0.5 (82.1)	–	–
10	0.7 (85.3)	–	–	–
13	ND[d]	ND	–	0.6 (100)
12	Trace (1.4)	ND	0.5 (65.1)	–
9	Trace (1.1)	Trace (1.1)	0.1 (1.3)	0.8 (4.4)
ABA	0.7 (1.2)	0.2 (1.2)	2.6 (1.3)	17.1 (4.2)

[a] Incorporation ratio (%).

[b] ^{13}C-isotopic abundance (%).

[c] Not tested.

[d] Not detected.

meric excess of ABA should be 93.04%. The result showed that the ABA was a natural (*S*) isomer with 99.77 ± 0.03% *ee*. This enantiomeric excess indicated that (*R*)-[2-^{13}C]-**12** was selectively converted to ABA.

In conclusion, the direct pathway via ionylideneethane contains the following steps; FDP is dehydrogenated at C-4, 5 and reduced at C-1 to give **11**, cyclization of **10** following isomerization at C-2 of **11** gives (*R*)-**12**, and then (*R*)-**12** is converted to (*S*)-ABA via **6** and **9** by oxidation at C-1, -1, -1′ and -4′ by monooxygenase with molecular oxygen (Fig. 2). This direct pathway may occur in other ABA-producing fungi. Some algae which do not produce carotenoid in spite of containing a considerable amount of ABA (Hartung and Gimmler, 1994) might biosynthesize ABA by the direct pathway via ionylideneethane. This study revealed that fungal ABA biosynthesis not only before, but also after IDP formation, differs from that in higher plants. Fungi probably obtained the genes for ABA biosynthesis independently of higher plants.

3. Experimental

3.1. General

^{1}H and ^{13}C NMR, NOESY, HMQC and HMBC spectra were measured using a Bruker ARX500 instrument (500 MHz for ^{1}H and 125 MHz for ^{13}C), with TMS as the internal standard. Direct EI-MS was carried out with a JEOL JMS-600H mass spectrometer, the temperature of the direct probe being increased from 50 to 450 °C at a rate of 128 °C/min. The instrument was operated at a chamber temperature of 250 °C, an accelerating voltage of 3 kV, an ionization voltage of 70 eV, and an ionization current of 300 μA in the positive ion mode, the resolution being 1,000 during measurements. GC–EI mass spectra were recorded on the above mass spectrometer equipped with a Hewlett–Packard HP6890 instrument, using a HP-5 column (30 m × 0.32 mm, 5% diphenyl–95% dimethylpolysiloxane, film thickness 0.25 μm, Hewlett–Packard Co., Wilmington, DE, USA), and 1.0 ml/min of He flow. The parameters of the mass spectrometer were as for direct EI-MS analyses. The oven temperature for GC was programmed from 120 to 270 °C at a rate of 5 °C/min for methyl ester of ABA and **9**, and from 100 to 190 °C at a rate of 3 °C/min for **10**, **11**, **12** and **13**. The ^{18}O contents were calculated after corrections for natural ^{13}C-, ^{2}H- and ^{17}O-isotopic abundance. IR spectra were obtained by a Shimadzu FT-IR-8100AI spectrometer. UV–Vis spectra and optical rotations were measured with a Shimadzu UV 2200AI and a JASCO DIP-1000 polarimeter, respectively. HPLC was performed with a Hitachi L-7100 pump, a Hitachi L-7400 UV detector and a Hitachi D-7500 chromato-integrator. For carotenoid analysis, HPLC was performed with a Waters 600E multisolvent delivery system using a photodiode array detector Waters 991J with a detection range of 200–600 nm. HPLC columns used were a YMC-Pack ODS-AQ 311 column (100 × 6 mm, YMC Co. Ltd., Kyoto, Japan), a YMC-Pack SIL A-003 column (silica gel, 250 × 4.6 mm, YMC Co. Ltd., Kyoto, Japan), a YMC Carotenoid column (250 × 4.6 mm, YMC Inc., Wilmington, NC, USA) and a Chiralcel OD column (250 × 4.6 mm, Daicel Chemical Industries Ltd., Niigata, Japan). CC was carried out on Wakogel C-200 (silica gel; particle size, 0.075–0.15 mm; Wako Pure Chemical Industries Ltd., Osaka, Japan) or Al_2O_3 90 (Merck, Darmstadt, Germany).

3.2. Material

The phytopathogenic fungus, *B. cinerea* No. 403, was separated from lesions on a leaf of geranium, and cultured on a PSA-agar medium. The single conidium was isolated from the colony of the fungus under a microscope, and germinated on a PSA-agar medium (Yoshida and Takano, 1992). (±)-ABA was purchased from Wako Pure Chemical Industries Ltd., whereas $^{18}O_2$ (99 atom% ^{18}O) and $H_2{}^{18}O$ (>95 atom% ^{18}O) were from Isotec Inc., Miamisburg, OH, USA. [1-^{13}C]Bromoethane (99 atom% ^{13}C) as obtained from Aldrich Chemical Co. Inc., Milwaukee, WI, USA.

3.3. Labeling experiment with $^{18}O_2$

Culturing under an $^{18}O_2$ atmosphere was performed according to the method of Yamamoto et al. (2000), who reported that more than a 6 M equivalent of $^{18}O_2$ to glucose was required for normal fungal growth and ABA production. Potato dextrose medium [15 ml, containing 0.3 g (1.7 mmol) of D-glucose] was added to an Erlenmeyer flask (300 ml) sealed with a stopcock. After inoculation of *B. cinerea*, the flask was immediately evacuated and then replaced with N_2 to purge the air from the flask; this procedure was then repeated twice. The air bag (5 l) containing 0.25 l (10 mmol, 23 °C) of $^{18}O_2$ and 1.0 l of N_2 was fitted to the flask, and *B. cinerea* was statically cultured at 23 °C in the dark for 20 days. The atmosphere in the air bag was not replenished with $^{18}O_2$ during the culture.

After filtration of the culture, the filtrate was acidified with 25% H_3PO_4 to pH 3.0, and partitioned with EtOAc (3 × 20 ml). The organic layers were combined, dried (Na_2SO_4), filtered and concentrated in vacuo to give a yellow oil (6.8 mg), which was subjected to silica gel (3 g) CC using mixtures of toluene and EtOAc containing 1% HOAc as eluants. The material (2.9 mg), eluted with 20–30% EtOAc in toluene, was subjected to preparative HPLC using a YMC-Pack ODS-AQ 311 column (solvent, 0.1% HOAc–45% MeOH–55%

H_2O; flow rate, 1.0 ml/min; detection, 254 nm). Materials eluted at R_ts 7.7 and 10.9 min were collected and concentrated in vacuo to give **9** (4.8 μg) and ABA (0.48 mg), respectively. These compounds were separately methylated by ethereal CH_2N_2 to give methyl esters of **9** (4.8 μg) and ABA (0.48 mg). Methyl ester of ABA: EI-MS (probe) 70 eV: *m/z* (rel. int.): 284 $[M + 6]^+$ (0.9), 282 $[M + 4]^+$ (1), 280 $[M + 2]^+$ (2), 278 $[M]^+$ (1), 266 (0.7), 264 (3), 262 (5), 260 (4), 194 (60), 192 (100), 190 (82), 164 (32), 162 (36) 129 (16), 127 (30), 125 (32); the total ^{18}O content was calculated from the relative intensities of the molecular ions to be 46%. Methyl ester of **9**: EI-MS (probe) 70 eV: *m/z* (rel. int.): 288 $[M + 8]^+$ (0.4), 286 $[M + 6]^+$ (0.8), 284 $[M + 4]^+$ (1), 282 $[M + 2]^+$ (1), 280 $[M]^+$ (0.8), 268 (8), 266 (16), 264 (20), 262 (12), 248 (15), 246 (20), 244 (16), 129 (71), 127 (100), 125 (99); the total ^{18}O content was calculated from the relative intensities of the molecular ions to be 42%.

3.4. Preparation of [4′-^{18}O]ABA and its incubation in buffer

[4′-^{18}O]ABA was prepared according to the method of Gray et al. (1974). NaOH (1 mg, 0.025 mmol) was dissolved in $H_2{}^{18}O$ (100 μl). ABA (6.0 mg, 0.023 mmol) was dissolved in the $Na^{18}OH$ solution and heated at 85–90 °C for 1 h. After cooling, distilled H_2O (20 ml) was added to the solution, the latter being acidified with 25% H_3PO_4 to pH 3.0 and partitioned with EtOAc (3 × 10 ml). The organic layers were combined, dried (Na_2SO_4), filtered and concentrated in vacuo to give a yellow oil (5.4 mg). This oil was subjected to silica gel (2 g) CC using mixtures of toluene and EtOAc containing 1% HOAc as eluants. The material eluted with 30–40% of EtOAc in toluene was collected and concentrated in vacuo to give [4′-^{18}O]ABA (5.0 mg). [4′-^{18}O]ABA (0.1 mg) was dissolved in MeOH (0.1 ml), and ethereal CH_2N_2 was added to the solution to give the methyl ester of [4′-^{18}O]ABA. Methyl ester of [4′-^{18}O]ABA: EI-MS (probe) 70 eV: *m/z* (rel. int.): 280 $[M + 2]^+$ (2), 278 $[M]^+$ (0.4), 262 (3), 260 (2), 192 (100), 190 (13), 164 (26), 162 (7), 136 (16), 134 (15), 125 (30), 91 (11); the total ^{18}O content was calculated from the relative intensities of the $[M + 2]^+$ and $[M]^+$ ions to be 84%. This content was adopted as the 4′-^{18}O content, since the ^{18}O content 78% calculated from the relative intensity of fragment ions containing 1′,4′-oxygen at *m/z* 164 and 162 was close to the above content.

[4′-^{18}O]ABA (0.1 mg) dissolved in MeOH (0.1 ml) was added to 0.3 ml each of a 50 mM buffer solution, NaOAc–HCl at pH 3.0 and 5.0, and Na_2HPO_4–NaH_2PO_4 at pH 7.0, before being kept at 23 °C in the dark for 6 days. After addition of distilled H_2O (5 ml), the solutions were acidified to pH 3.0 with 25% of H_3PO_4, and partitioned with EtOAc (3 × 2 ml). The organic layers were combined, dried (Na_2SO_4), filtered, with the filtrates concentrated in vacuo and individually dissolved in MeOH (0.1 ml) and ethereal CH_2N_2 to give the corresponding methyl esters. The methyl ester of ABA incubated at pH 3.0, EI-MS (probe) 70 eV: *m/z* (rel. int.): 278 $[M]^+$ (2), 260 (5), 190 (100), 164 (1), 162 (26), 134 (23), 125 (27), 91 (8); the total ^{18}O content was 3%. The methyl ester of ABA incubated at pH 5.0: EI-MS (probe) 70 eV: *m/z* (rel. int.): 280 $[M + 2]^+$ (2), 278 $[M]^+$ (0.5), 262 (3), 260 (3), 192 (100), 190 (29), 164 (23), 162 (10), 136 (14), 134 (16), 125 (35), 91 (10); the total ^{18}O content was 78%. The methyl ester of ABA incubated at pH 7.0: EI-MS (probe) 70 eV: *m/z* (rel. int.): 280 $[M + 2]^+$ (2), 278 $[M]^+$ (0.3), 262 (3), 260 (2), 192 (100), 190 (18), 164 (24), 162 (8), 136 (15), 134 (14), 125 (30), 91 (10); the total ^{18}O content was 83%.

3.5. Labeling experiment with $H_2{}^{18}O$

Difco's potato dextrose broth (0.24 g, Becton Dickinson, Sparks, MD, USA) was dissolved in $H_2{}^{18}O$ (10 ml), with the resulting medium sterilized by filtration using a 0.1 μm-pore sized syringe filter (Asahi Technoglass Corp., Tokyo, Japan), and added to a sterilized Erlenmeyer flask (50 ml). After inoculation of *B. cinerea* in the medium, the fungus was statically cultured at 23 °C in the dark for 20 days. The culture was filtered, and the mycelium was washed with distilled H_2O (30 ml). The filtrate was then acidified with 25% H_3PO_4 to pH 3.0, and immediately partitioned with EtOAc (3 × 20 ml). The organic layers were combined, dried (Na_2SO_4), filtered and concentrated in vacuo to give a yellow oil (8.0 mg), which was subjected to silica gel (3 g) CC using mixtures of toluene and EtOAc containing 1% HOAc as the eluant. The colorless oil (3.8 mg) eluted with 30–40% EtOAc in toluene was dissolved in MeOH (2 ml), and then ethereal CH_2N_2 was added to the solution. After leaving the solution to stand at room temperature for 1 h, the solution was concentrated in vacuo to give a colorless oil (3.8 mg), which was subjected to preparative HPLC using a YMC-Pack SIL A-003 column (solvent, 95% *n*-hexane–5% 2-PrOH; flow rate, 1.0 ml/min; detection, 254 nm). Materials eluted at R_ts 9.6 and 14.0 min were collected and concentrated in vacuo to give the methyl esters of ABA (1.4 mg) and **9** (39 μg), respectively. Methyl ester of ABA: GC–EI-MS (R_t 15.1 min) 70 eV: *m/z* (rel. int.): 280 $[M + 2]^+$ (1), 278 $[M]^+$ (0.3), 262 (3), 260 (2), 192 (100), 190 (22), 164 (29), 162 (10), 136 (19), 134 (19), 125 (35), 91 (15); the total ^{18}O content was calculated from the relative intensities of the molecular ions to be 82%. Methyl ester of **9**: GC–EI-MS (R_t 13.8 min) 70 eV: *m/z* (rel. int.): 280 $[M]^+$ (1), 262 (20), 244 (17), 230 (20), 206 (26), 174 (64), 146 (81), 125 (100), 111 (46), 95 (49).

*3.6. Detection of **10**, **11** and 15,15′Z-phytoene by HPLC*

Botrytis cinerea was grown on a surface culture with potato-dextrose medium (2 l) at 23 °C in the dark for 8 days. The mycelia (200 g) were harvested by filtration, washed with distilled H_2O, homogenized in MeOH (50 ml) with sea sand (particle size, 850–1400 μm), and extracted with EtOAc–MeOH (3 × 1 l, 1:1). The extracts were then combined, filtered, with the filtrate concentrated in vacuo to give a brown aqueous solution (150 ml). The solution was partitioned with EtOAc (5 × 50 ml), with the combined organic layers washed with distilled H_2O, dried (Na_2SO_4), filtered and concentrated in vacuo to give a black oil (1.2 g). The resulting oil was analyzed by HPLC using a photodiode array detector (200–600 nm): column, YMC Carotenoid column; solvent, 15–85% *t*-butyl methyl ether in MeOH (0–30 min, linear); flow rate, 1.0 ml/min. The contents of **10**, **11** and 15,15′*Z*-phytoene were calculated from the calibration curves between the peak areas and amounts of synthetic **10** and **11**, and 15,15′*Z*-phytoene isolated from *C. cruenta*, respectively.

3.7. Isolation of 15,15′Z-phytoene

Botrytis cinerea was grown on a surface culture with potato-dextrose medium (4 l) at 23 °C in the dark for 8 days. After filtration, the mycelia (460 g) was washed with distilled H_2O, homogenized in MeOH (100 ml) with sea sand and extracted with EtOAc–MeOH (3 × 2 l, 1:1). The extracts were combined, filtered, with the filtrate concentrated to give a brown aqueous solution (200 ml). The residue was partitioned with EtOAc (5 × 100 ml), with the organic layers combined, washed with 1% $NaHCO_3$ (100 ml) and then distilled H_2O (100 ml), dried (Na_2SO_4), filtered and concentrated in vacuo to give a black oil (2.3 g). The latter was subjected to silica gel (50 g) CC using mixtures of *n*-hexane and toluene as eluants. The material (3.5 mg) eluted with 10% toluene in *n*-hexane was subjected to Al_2O_3 (15 g) CC with mixtures of *n*-hexane and toluene as eluants. The material (1.9 mg) eluted with 5% toluene in *n*-hexane was purified by preparative HPLC using a YMC Carotenoid column (solvent, 75% MeOH–25% *t*-butyl methyl ether; flow rate, 1.0 ml/min; detection, 254 nm). The material eluted at R_t 9.6 min was collected and concentrated in vacuo to give 15,15′*Z*-phytoene (2.2 μg). For UV, EI-MS and ^1H NMR spectral data of 15,15′*Z*-phytoene, see the literature (Ebenezer and Pattenden, 1993; Britton et al., 1977).

*3.8. Detection of **12** and **13** by GC–MS, and isolation of **10**, **11** and a mixture of **12** and **13***

The culture condition of *B. cinerea* was the same as that described for the isolation of 15,15′*Z*-phytoene. The culture (4 l in total) was filtered after 8 days, with the mycelia (490 g) washed with distilled H_2O, homogenized in acetone (100 ml) with sea sand and extracted with acetone (3 × 1 l). Following filtration of the extract, the filtrate was concentrated in vacuo to give a yellow aqueous solution (400 ml), with the later being partitioned with *n*-hexane (5 × 100 ml); the resulting organic layers were combined, washed with 1% $NaHCO_3$ (100 ml) and distilled H_2O (100 ml), dried (Na_2SO_4), filtered and concentrated in vacuo to give an orange oil (570 mg). The latter was subjected to silica gel (15 g) CC with mixtures of *n*-hexane and toluene as eluants. The colorless oil (3.4 mg) eluted with 5% toluene in *n*-hexane was analyzed by GC–EI-MS under the conditions described in Section 3.1, with compound **12** and **13** being detected at R_ts 6.7 and 7.3 min, respectively. Compound **12**. GC–EI-MS 70 eV *m*/*z* (rel. int.): 204 $[M]^+$ (29), 148 $[M - 56]^+$ (100), 133 (86), 119 (21), 105 (18), 91 (23), 77 (10), 55 (12). Compound **13**. GC–EI-MS 70 eV *m*/*z* (rel. int.): 204 $[M]^+$ (31), 148 $[M - 56]^+$ (100), 133 (86), 120 (15), 105 (14), 91 (13), 77 (8). These compounds were not detected in the material eluted with 10% toluene in *n*-hexane.

The colorless oil was subjected to Al_2O_3 (15 g) CC using mixtures of *n*-hexane and toluene as eluants. Materials eluted with *n*-hexane and 5% toluene in *n*-hexane (3.3 mg) were purified by preparative HPLC using a YMC Carotenoid column (solvent, 90% MeOH–10% H_2O; flow rate, 1.0 ml/min; detection, 254 nm). Materials eluted at R_ts 10.6, 12.1 and 14.7 min were separately collected, partitioned with *n*-hexane (3 × 10 ml), with the combined *n*-hexane layers concentrated in vacuo to give a mixture (1.4 μg) of **12** and **13**, **10** (0.3 mg) and **11** (0.4 mg), respectively. 2*E*,4*E*,6*E*-allofarnesene (**11**). Colorless oil; UV: λ_{max} (*n*-hexane) nm: 265 (shoulder), 275, 285; GC–EI-MS (R_t 12.9 min) 70 eV *m*/*z* (rel. int.): 204 $[M]^+$ (29), 135 (100), 120 (6), 119 (7), 107 (64), 105 (15), 93 (32), 91 (20), 79 (10), 77 (10), 69 (6), 55 (9); GC–HR-EI-MS 70 eV: *m*/*z* 204.1881 ($C_{15}H_{24}$ requires *m*/*z* 204.1878); ^1H NMR ($CDCl_3$): δ 1.61 (3H, *s*, H-13), 1.68 (3H, *s*, H-12), 1.74 (3H, *d*, $J = 7.0$ Hz, H-1), 1.78 (3H, *s*, H-15), 1.79 (3H, *s*, H-14), 2.09 (4H, *m*, H-8, 9), 5.12 (1H, *m*, H-10), 5.54 (1H, *q*, $J = 7.0$ Hz, H-2), 5.88 (1H, *d*, $J = 10.7$ Hz, H-6), 6.17 (1H, *d*, $J = 15.3$ Hz, H-4), 6.33 (1H, *dd*, $J = 10.7$, 15.3 Hz, H-5). For spectral data of **10**, see Miyaura et al. (1982).

*3.9. Synthesis of **10** and **11***

At room temperature, a 1.0 M THF solution of potassium *tert*-butoxide (1.2 ml, 1.2 mmol) was added dropwise to a stirred suspension of ethyl triphenylphosphonium bromide (464 mg, 1.3 mmol) in THF (10 ml) under N_2. After 30 min stirring, pseudo-ionone (120 mg, 0.63 mmol) was added to the orange solution. The reactant mixture was then stirred for 40 min at room temperature under N_2. To the mixture, distilled H_2O (0.5 ml) was added and the solution was stirred for 1 h at room temperature. After addition of distilled H_2O (50 ml) to the mixture, the solution was partitioned with Et_2O (3 × 20 ml). The organic layers were then combined, washed with brine and distilled H_2O, dried (Na_2SO_4), filtered and concentrated in vacuo to give a yellow oil (592 mg). This oil was purified by $AgNO_3$–silica gel (1:20, 10 g) CC with mixtures of *n*-hexane and toluene as eluants. Materials eluted with 20% and 30% toluene in *n*-hexane were concentrated in vacuo to give compounds **10** (36 mg, 28% yield) and **11** (41 mg, 32% yield), respectively, as colorless oils. Compound **10**. ^{13}C NMR ($CDCl_3$): δ 13.1 (C-1), 16.9 (C-14), 17.7 (C-13), 20.5 (C-15), 25.7 (C-12), 26.7 (C-9), 40.2 (C-8), 124.0 (C-10), 124.2 (C-2), 125.1 (C-5), 125.7 (C-6), 127.5 (C-4), 131.7 (C-11), 133.3 (C-3), 139.0 (C-7). This is the first report of the ^{13}C NMR spectral data of **10**. Compound **11**. ^{13}C NMR ($CDCl_3$): δ 12.1 (C-15), 14.0 (C-1), 16.8 (C-14), 17.7 (C-13), 25.7 (C-12), 26.7 (C-9), 40.1 (C-8), 122.5 (C-5), 124.1 (C-10), 125.4 (C-6), 126.1 (C-2), 131.6 (C-11), 135.1 (C-3), 135.5 (C-4), 137.9 (C-7). This is the first report of the ^{13}C NMR spectral data of **11**. For other spectral data, see **11** isolated from *B. cinerea*.

*3.10. Synthesis of **12** and **13***

In the same manner as that described for **10** and **11**, α-ionone (120 mg, 0.63 mmol) was reacted with ethyl triphenylphosphonium bromide (464 mg, 1.3 mmol) and potassium *tert*-butoxide (1.2 ml of 1.0 M THF solution, 1.2 mmol) in THF (10 ml), and the reaction mixture was treated to give a yellow oil (504 mg). The latter was subjected to $AgNO_3$–silica gel (1:20, 10 g) CC using mixtures of *n*-hexane and toluene as eluants. Materials eluted with 0.5% and 1–3% toluene in *n*-hexane were concentrated in vacuo to give **12** (53 mg, 41% yield) and **13** (9.9 mg, 7.7% yield), respectively. Compound **12** (2*Z*,4*E*-5-(2′,6′,6′-trimethyl-2′-cyclohexene-1′-yl)-3-methyl-2,4-pentadiene). Colorless oil; UV: λ_{max} (*n*-hexane) nm (logε): 239 (4.41); IR: ν_{max} (KBr) cm^{-1}: 3030, 2960, 2915, 2855, 1470, 1455, 1435, 1375, 1365, 965, 820; GC–HR-EI-MS 70 eV: *m*/*z* 204.1875 ($C_{15}H_{24}$ requires *m*/*z* 204.1878); ^1H NMR ($CDCl_3$): δ 0.82 (3H, *s*, H-9′), 0.91 (3H, *s*, H-8′), 1.19 (1H, *dt*, *J* = 5.0, 13.1 Hz, H-5′), 1.46 (1H, *dt*, *J* = 7.9, 13.1 Hz, H-5′), 1.60 (3H, *d*, *J* = 1.4 Hz, H-7′), 1.73 (3H, *d*, *J* = 7.0 Hz, H-1), 1.79 (3H, *br.s*, H-6), 2.01 (2H, *br.s*, H-4′), 2.18 (1H, *d*, *J* = 9.5 Hz, H-1′), 5.33 (1H, *q*, *J* = 7.0 Hz, H-2), 5.41 (1H, *br.s*, H-3′), 5.45 (1H, *dd*, *J* = 9.5, 15.4 Hz, H-5), 6.41 (1H, *d*, *J* = 15.4 Hz, H-4); ^{13}C NMR ($CDCl_3$): δ 13.0 (C-1), 20.8 (C-6), 23.0 (C-7′), 23.1 (C-4′), 27.1 (C-8′), 27.6 (C-9′), 31.8 (C-5′), 32.2 (C-6′), 55.1 (C-1′), 120.6 (C-3′), 122.6 (C-2), 128.2 (C-4), 131.1 (C-5), 132.8 (C-3), 134.5 (C-2′). For GC–EI-MS, see **12** isolated from *B. cinerea*. Compound **13** (2*E*,4*E*-5-(2′,6′,6′-trimethyl-2′-cyclohexene-1′-yl)-3-methyl-2,4-pentadiene). Colorless oil; UV: λ_{max} (*n*-hexane) nm (logε): 236 (4.37); IR: ν_{max} (KBr) cm^{-1}: 3030, 2960, 2920, 2855, 1470, 1455, 1435, 1385, 1360, 965, 820; GC–HR-EI-MS 70 eV: *m*/*z* 204.1874 ($C_{15}H_{24}$ requires *m*/*z* 204.1878); ^1H NMR ($CDCl_3$): δ 0.81 (3H, *s*, H-9′), 0.89 (3H, *s*, H-8′), 1.17 (1H, *dt*, *J* = 4.9, 13.1 Hz, H-5′), 1.44 (1H, *dt*, *J* = 7.9, 13.1 Hz, H-5′), 1.60 (3H, *d*, *J* = 1.2 Hz, H-7′), 1.71 (3H, H-1, overlapped with H-6 signal), 1.72 (3H, H-6, overlapped with H-1 signal), 2.00 (2H, *br.s*, H-4′), 2.12 (1H, *d*, *J* = 9.5 Hz, H-1′), 5.33 (1H, *dd*, *J* = 9.5, 15.4 Hz, H-5), 5.38 (1H, *br.s*, H-3′), 5.47 (1H, *q*, *J* = 6.6 Hz, H-2), 6.02 (1H, *d*, *J* = 15.4 Hz, H-4); ^{13}C NMR ($CDCl_3$): δ 12.2 (C-6), 13.7 (C-1), 23.0 (C-7′), 23.1 (C-4′), 27.0 (C-8′), 27.6 (C-9′), 31.8 (C-5′), 32.3 (C-6′), 54.6 (C-1′), 120.5 (C-3′), 124.6 (C-2), 128.0 (C-5), 134.5 (C-3), 134.8 (C-2′), 136.0 (C-4). For GC–EI-MS, see **13** isolated from *B. cinerea*.

*3.11. Synthesis of [2-^{13}C]-**10**, **11**, **12** and **13***

Triphenylphosphine (1.8 g, 6.9 mmol) was suspended in [1-^{13}C]bromoethane (1.0 g, 9.2 mmol) and this suspension was heated at 120 °C for 12 h. The resulting colorless solid was washed with benzene and then dissolved in distilled H_2O. The aqueous solution was concentrated in vacuo and the remaining colorless solid was dried at 100 °C overnight to give [1-^{13}C]ethyl triphenylphosphonium bromide (2.4 g, 95% yield). In the same manner as that described for synthesis of **10**, **11**, **12** and **13**, α-ionone or pseudo-ionone were reacted with [1-^{13}C]ethyl triphenylphosphonium bromide and potassium *tert*-butoxide in THF to give [2-^{13}C]-**10**, **11**, **12** and **13**. The ^{13}C contents of these compounds were calculated from the relative intensities of the molecular ions in their mass spectra to be 99%. [2-^{13}C]-**10**: GC–EI-MS (R_t 12.3 min) 70 eV: *m*/*z* (rel. int.): 205 $[M]^+$ (30), 136 (100), 120 (7), 108 (51), 94 (24), 80 (6), 69 (8), 55 (5). [2-^{13}C]-**11**: GC–EI-MS (R_t 13.1 min) 70 eV: *m*/*z* (rel. int.): 205 $[M]^+$ (30), 136 (100), 120 (7), 108 (51), 94 (24), 80 (6), 69 (7), 55 (5). [2-^{13}C]-**12**: GC–EI-MS (R_t 6.8 min) 70 eV: *m*/*z* (rel. int.): 205 $[M]^+$ (21), 149 $[M - 56]^+$ (100), 134 (86), 120 (14), 107 (22), 92 (17), 70 (5), 55 (8). [2-^{13}C]-**13**: GC–EI-MS (R_t 7.6 min) 70 eV: *m*/*z* (rel. int.): 205 $[M]^+$ (21), 149 $[M - 56]^+$ (100), 134 (80), 120 (13), 107 (19), 92 (15), 70 (5), 55 (7).

*3.12. Administration of [2-^{13}C]-**10**, **11**, **12** and **13** to B. cinerea*

Botrytis cinerea was statically cultured in four flasks (300 ml), each containing potato-dextrose medium (50 ml) at 23 °C in the dark for 8 days. Following decantation of the medium, the mycelia were washed with sterile water (3 × 15 ml). Czapec-Dox medium (50 ml) and

[2-^{13}C]-**10** (0.5 mg in 50 μl EtOH) were then added to the mycelia in the flasks, and the culture was incubated at 23 °C in the dark for 7 days. The mycelia and medium were then separated by filtration, and the medium was acidified to pH 3.0 with 25% H_3PO_4, and partitioned with EtOAc (3 × 100 ml). The organic layers were combined, dried (Na_2SO_4), filtered and concentrated in vacuo to give an orange oil (75 mg). The latter was subjected to both silica gel CC and HPLC in the same manner as that described for the labeling experiment with $^{18}O_2$ to give ABA (6.4 mg) and **9** (0.5 mg). Each compound was next methylated and analyzed by ^{1}H, ^{13}C NMR and GC–EI-MS. The optical purity of ABA was calculated from peak areas in HPLC with a Daicel Chiralcel OD column (solvent, 80% *n*-hexane–20% 2-PrOH–0.1% HOAc; flow rate, 1.0 ml/min; detection, 254 nm). (+)- and (−)-ABA were eluted at R_ts 6.1 and 7.7 min, respectively. The mycelia (49 g) were then washed with distilled H_2O, homogenized in MeOH (30 ml) with sea sand, and extracted with EtOAc–MeOH (100 ml, 1:1). The extract was concentrated in vacuo and distilled H_2O (50 ml) was added to the residue. This solution was partitioned with EtOAc (3 × 20 ml), and the organic layers were combined, washed with distilled H_2O, dried (Na_2SO_4), filtered and concentrated in vacuo. The resulting brown oil (75 mg) was subjected to silica gel (5 g) CC with a mixture of *n*-hexane and toluene as eluants. Materials eluted with *n*-hexane and 5% toluene in *n*-hexane contained compounds **11**, which were analyzed by GC–EI-MS. The incorporation ratio was calculated using the following equation: Incorporation ratio (% of the metabolite biosynthesized from the administered substrate) = $PA/SB \times 100\%$; where P is the mole of the product isolated, A is % of ^{13}C-isotopic abundance of the product − 1.1 (% of natural abundance of ^{13}C), S is mole of the substrate administered, and B is the 99 (% of ^{13}C-isotopic abundance of the substrate). The ^{13}C-isotopic abundance of C-2 of ABA and **9** was evaluated by their ^{13}C NMR spectra (Hirai et al., 2000a), and those of **10**, **11**, **12** and **13** were calculated from their EI-MS.

Compounds [2-^{13}C]-**11**, **12** and **13** were added to the mycelia in four, eight and three flasks, respectively, and cultured in the same manner as that for [2-^{13}C]-**10**. ABA and compound **9**, and compounds **10**, **11**, **12** and **13** were purified from the media and mycelia, respectively, analyzed by ^{13}C NMR or GC–EI-MS, and the incorporation ratio was calculated as described above.

Acknowledgements

The authors thank Dr. Hirotaka Yamamoto of Japan NUS Co., Ltd. for valuable discussions and advice throughout the research, and Professor Takashi Maoka of Kyoto Pharmaceutical Science University for suggestions regarding the spectral analysis of carotenoids. This work was supported by a Grant-in-Aid, No. 14360065, from the Ministry of Education, Science, Sports and Culture of Japan, and in part by COE for Microbial-Process Development Pioneering Future Production Systems (COE program of the Ministry of Education, Culture, Sports, Science and Technology, Japan).

References

Bennett, R.D., Norman, S.M., Maier, V.P., 1984. Biosynthesis of abscisic acid from farnesol derivatives in *Cercospora rosicola*. Phytochemistry 23, 1913–1915.

Bennett, R.D., Norman, S.M., Maier, V.P., 1990. Intermediate steps in the biosynthesis of abscisic acid from farnesyl pyrophosphate in *Cercospora rosicola*. Phytochemistry 29, 3473–3477.

Biemann, K., 1962. Mass Spectrometry: Organic Chemical Applications. McGraw-Hill, New York.

Britton, G., Goodwin, T.W., Harriman, G.E., Lockley, W.J.S., 1977. Carotenoids of the ladybird beetle, *Coccinella septempunctata*. Insect Biochem. 7, 337–345.

Creelman, R.A., Zeevaart, J.A.D., 1984. Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. Plant Physiol. 75, 166–169.

Ebenezer, W.J., Pattenden, G., 1993. *cis*-Stereoisomer of β-carotene and its congeners in the alga *Dunaliella bardawil*, and their biogenetic interrelationships. J. Chem. Soc., Perkin Trans. I, 1869–1873.

Gray, R.T., Mallaby, R., Ryback, G., Williams, V.P., 1974. Mass spectra of methyl abscisate and isotopically labeled analogues. J. Chem. Soc., Perkin Trans. II, 919–924.

Hartung, W., Gimmler, H., 1994. VII. A stress physiological role for abscisic acid (ABA) in lower plants. Prog. Bot. 55, 157–173.

Hirai, N., Okamoto, M., Koshimizu, K., 1986. The 1′,4′-*trans*-diol of abscisic acid, a possible precursor of abscisic acid in *Botrytis cinerea*. Phytochemistry 25, 1865–1868.

Hirai, N., Yoshida, R., Todoroki, Y., Ohigashi, H., 2000a. Biosynthesis of abscisic acid by the non-mevalonate pathway in plants, and by the mevalonate pathway in fungi. Biosci. Biotechnol. Biochem. 64, 1448–1458.

Hirai, N., Okamoto, M., Fujimura, M., Ichiyama, T., Todoroki, Y., Ohigashi, H., Takeda, N., Yoshizumi, H., Tatematsu, A., 2000b. Mechanism of formation of dehydrated ions from abscisic acid and its 1′,4′-diol methyl esters in electron ionization mass spectrometry. J. Mass Spectrom. Soc. Jpn. 48, 8–22.

Holmberg-Betsholtz, I., Lund, E., Björkhem, I., Wikvall, K., 1993. Sterol 27-hydroxylase in bile acid biosynthesis. J. Biol. Chem. 268, 11079–11085.

Hose, S., Zänglein, A., Berg, T.V.D., Schultze, W., Kubeczka, K.-H., Czygan, F.-C., 1997. Ontogenetic variation of the essential leaf oil of *Melissa officinalis* L. Pharmazie 52, 247–253.

Kato, T., Oritani, T., Yamashita, K., 1987. Metabolism of (2*Z*,4*E*)-γ-ionylideneethanol and (2*Z*,4*E*)-γ-ionylideneacetic acid in *Cercospora cruenta*. Agric. Biol. Chem. 51, 2695–2699.

Kettner, J., Dörffling, K., 1995. Biosynthesis and metabolism of abscisic acid in tomato leaves infected with *Botrytis cinerea*. Planta 196, 627–634.

Lanzon, A., Albi, T., Cert, A., Gracian, J., 1994. The hydrocarbon fraction of virgin olive oil and changes resulting from refining. J. Am. Oil. Chem. Soc. 71, 285–291.

Leung, J., Giraudat, J., 1998. Abscisic acid signal transduction. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 199–222.

Miyaura, N., Suginome, H., Suzuki, A., 1982. Stereospecific synthesis of (2*Z*,4*E*,6*E*)-3,7,11-trimethyl-2,4,6,10-dodecatetraene [*trans*(C_{10})-allofarnesene]. Bull. Chem. Soc. Jpn. 55, 2221–2223.

Neill, S.J., Horgan, R., Walton, D.C., Mercer, C.A.M., 1987. The metabolism of α-ionylidene compounds by *Cercospora rosicola*. Phytochemistry 26, 2515–2519.

Norman, S.M., 1991. β-Carotene from the abscisic acid producing strain of *Cercospora rosicola*. Plant Growth Regul. 10, 103–108.

Oritani, T., Niitsu, M., Kato, T., Yamashita, K., 1985. Isolation of (2*Z*,4*E*)-γ-ionylideneethanol from *Cercospora cruenta*, a fungus producing (+)-abscisic acid. Agric. Biol. Chem. 49, 2819–2822.

Sakai, T., Hirose, Y., 1969. Farnesenes isolated from the volatile oil of *Perilla frutscens f. viridis* Makino. Bull. Chem. Soc. Jpn. 42, 3615.

Schwartz, S.H., Qin, X., Zeevaart, J.A.D., 2003. Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. Plant Physiol. 131, 1591–1601.

Seo, M., Koiwai, H., Akaba, S., Komano, T., Oritani, T., Kamiya, Y., Koshiba, T., 2000a. Abscisic aldehyde oxidase in leaves of *Arabidopsis thaliana*. Plant J. 23, 481–488.

Seo, M., Peeters, A.J.M., Koiwai, H., Oritani, T., Marion-Poll, A., Zeevaart, J.A.D., Koornneef, M., Kamiya, Y., Koshiba, T., 2000b. The *Arabidopsis* aldehyde oxidase 3 (*AAO3*) gene product catalyzes the final step in abscisic acid biosynthesis in leaves. Proc. Natl. Acad. Sci. USA 97, 12908–12913.

Yamamoto, H., Oritani, T., 1997. Incorporation of farnesyl pyrophosphate derivatives into abscisic acid and its biosynthetic intermediates in *Cercospora cruenta*. Biosci. Biotechnol. Biochem. 61, 821–824.

Yamamoto, H., Inomata, M., Tsuchiya, S., Nakamura, M., Uchiyama, T., Oritani, T., 2000. Early biosynthetic pathway to abscisic acid in *Cercospora cruenta*. Biosci. Biotechnol. Biochem. 64, 2075–2082.

Yamamoto, H., Inomata, M., Uchiyama, T., Oritani, T., 2001. Identification of 2,3-dihydro-γ-ionylideneethanol in *Cercospora cruenta*. Biosci. Biotechnol. Biochem. 65, 810–816.

Yoshida, R., Takano, K., 1992. Abscisic acid produced by plant pathogenic fungi and symptom expression in diseased levels of flowers. In: Proceedings of 19th Annual Meeting of Plant Growth Regulator Society of America, San Francisco, CA, pp. 159–164.

Zeevaart, J.A.D., Heath, T.G., Gage, D.A., 1989. Evidence for a universal pathway of abscisic acid biosynthesis in higher plants from ^{18}O incorporation patterns. Plant Physiol. 91, 1594–1601.