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THE 1′,4′-*TRANS*-DIOL OF ABSCISIC ACID, A POSSIBLE PRECURSOR OF ABSCISIC ACID IN *BOTRYTIS CINEREA*

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Key Word Index—*Botrytis cinerea*; Hyphomycetes; abscisic acid; biosynthesis; 1′,4′-*trans*-diol of abscisic acid; 1′,4′-dihydroxy-α-ionylideneacetic acid.

Abstract—The 1′,4′-*trans*-diol of abscisic acid was isolated from cultures of *Botrytis cinerea*. The ^{2}H-labelled derivative was converted into abscisic acid by this fungus, but ^{2}H-labelled ABA was not converted into the diol. This suggests that the diol is not a metabolite of ABA but a possible precursor.

INTRODUCTION

Seven species of phyotopathogenic fungi have been found that produce abscisic acid (ABA, **1**) since Assante *et al.* first reported ABA production by *Cercospora rosicola* [1–4]. These fungi, especially *C. rosicola* and *C. cruenta*, have been used to study the biosynthesis of ABA, but an immediate precursor of ABA common to all fungi has not been found. Two compounds, 1′-deoxyabscisic acid and 1′,4′-dihydroxy-γ-ionylideneacetic acid, have been proposed as precursors in *C. rosicola* and *C. cruenta*, respectively [5–8]. We are interested in the biosynthesis of ABA by *Botrytis cinerea*, which was found by Marumo *et al.* to produce ABA [3]. While studying the effects of plant growth regulators on ABA production by *B. cinerea*, we found 1′,4′-*t*-diol of ABA (**3**) in the culture media. This paper describes its identification and conversion into ABA.

RESULTS AND DISCUSSION

We tested 95 strains of *B. cinerea*, seven strains of *B. allii*, five strains of *B. byssoidea*, three strains of *B. tulipae* and four strains of *B. squamosa* to find a strain producing large amounts of ABA. Only 33 strains of *B. cinerea* produced any ABA; none of the other *Botrytis* species produced it. Strain No. 39 of *B. cinerea* was selected for the following studies.

Botrytis cinerea No. 39 produced ABA at a yield of 3.5 mg/l on potato-dextrose medium by surface culture under fluorescent lights for 42 days. The onset of ABA production changed depending on the culture conditions. When cultured in the laboratory, production began 6 days after inoculation. This fungus did not produce ABA in shake culture in the dark or in the light. In surface culture, the mycelia were pale yellow in spring, but in autumn and winter, grey–green and black sclerotia formed.

We added plant growth regulators to the media to see if biosynthetic intermediates of ABA accumulated. Any ABA produced was assayed by HPLC, and metabolites were also looked for. Maleic hydrazide (2-chloroethyl)-trimethylammonium chloride (CCC) and zeatin inhibited ABA production (Table 1); 2′-*iso*-propyl-4′-(trimethylammonium chloride)-5′-methylphenylpiperidine-1′-carboxylate (AMO-1618) and 4-ethoxy-1-(*p*-tolyl)-*S*-triazine-2,6-(1H, 3H)-dione (TA) stimulated it (Table 1). AMO-1618 and CCC, metabolic inhibitors of gibberellin biosynthesis, have opposite effects on ABA production by *B. cinerea* [9]. Norman *et al.* found that both compounds inhibited ABA production by *C. rosicola* [10]. AMO-1618 stimulated the incorporation of [^{14}C]mevalonate into ABA by avocado mesocarp, and Milborrow interpreted this effect as a relative increase in availability of ^{14}C to ABA biosynthesis caused by inhibition of cytosolic sterol synthesis [11]. The response of *B. cinerea* to AMO-1618 and CCC seemed to resemble that of plants more than that of *C. rosicola*. Maleic hydrazide and zeatin also inhibit ABA production by *C. rosicola* [11, 12]. TA has a gibberellin-like effect on rice seedlings [13]. Although this action has been attributed to its inhibition of ABA biosynthesis, TA accelerated ABA accumulation by *B. cinerea*. The mechanism of action of TA may resemble that of AMO-1618.

No accumulated metabolites were found by HPLC even when ABA production was decreased by maleic hydrazide, CCC or zeatin. However, there was a UV-absorbing compound, the level of which had positive correlation with that of ABA, observed in the chromato-

Table 1. Effects of plant growth regulators on ABA biosynthesis by *B. cinerea*

Compounds	Concentration (μM)		
	500	100	10
Maleic hydrazide	25*	33*	—
CCC	42	94	—
AMO-1618	170	150	71*
TA	180	240	190
Zeatin	69	84	—

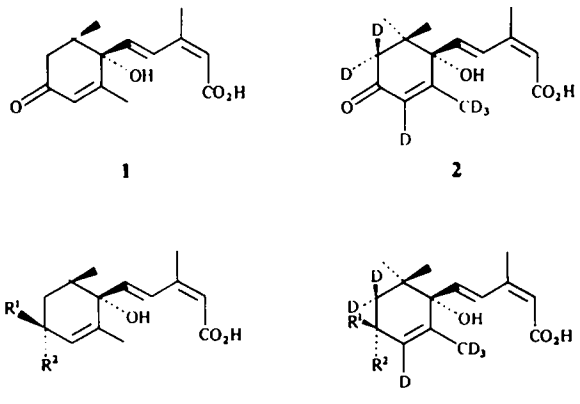
*Effects are expressed as the percentage of ABA in media when that of the untreated control is 100%. Values are means of three experiments.

grams. This compound was a normal metabolite for *B. cinerea*, because it was present in untreated medium.

Botrytis cinerea No. 39 cultured on a large scale yielded 1 mg of the metabolite, the R_f of which, on silica gel TLC, was lower than that of ABA.

The metabolite **3** had a UV absorption maximum at 262 nm and an intense positive Cotton effect in the ORD curve, which strongly suggested that it was related to ABA. The GC/MS of its methyl ester (3-Me) had $[M]^+$ at m/z 280 and $[M-H_2O]^+$ and $[M-2H_2O]^+$ ions at m/z 262 and 244 respectively. The base peak ion at m/z 125 was assigned to the 3-methylpentadienoic acid moiety of ABA. These results suggested that the metabolite was a dihydro-derivative of ABA having two hydroxyl groups in the ring system. Trimethylsilylation afforded a mono TMS ether, indicating that one hydroxyl group was tertiary. We concluded from the findings that the metabolite was a 4'-hydroxy-derivative of ABA: either the 1',4'-*trans*- or the 1',4'-*cis*-diol of ABA. The mass fragmentation pattern of the methyl ester of these diols coincided with that of 3-Me. Milborrow has reported that both epimeric diol ABA-Mes underwent dehydration to 4'-deoxyABA under the GC conditions used and did not give parent ions at m/z 280 [14]. However, we could separate the epimeric diol-ABA-Mes by GC. Furthermore, a small parent ion of variable intensity was observed in 10% of the samples tested. Since these epimeric diols could be separated by GC or HPLC, we compared chromatograms of **3** with those of authentic samples. Compound **3** and 1',4'-*t*-diol-ABA behaved in the same way on GC and HPLC. The possibility was that **3** might be 1',4'-dihydroxy-γ-ionylideneacetic acid, since Oritani *et al.* reported that these geometric isomers have very similar polarity and cannot be separated by silica gel TLC [7], was ruled out by measuring the ^1H NMR spectrum of 3-Me. In this experiment, the 2'-methyl group on the double bond and the 4'-allyl proton attached to a hydroxyl-bearing carbon were observed at δ1.70 and 4.25, respectively, and no exomethylene protons were seen. The absolute configuration at C-1' of **3** was found to be *S* since **3** had the same positive specific rotation as did the 1',4'-*t*-diol prepared from (+)-ABA. The fungal production of 1',4'-diol-ABA was established by this experiment.

Milborrow and Walton *et al.* found that 1',4'-*t*-diol-ABA fed to wheat or bean was converted into ABA [14–18]. Therefore, the 1',4'-*t*-diol produced by *B. cinerea* might be either a precursor or a metabolite of ABA. To examine these possibilities, feeding experiments with ^2H-labelled compounds were done. (+)-[2'-Me,3',5'-$^2H_6$]-ABA (**2**) was prepared by treating (+)-ABA with NaOD solution [19]. (+)-[2'-Me,3',4', 5'-$^2H_7$]-1',4'-*t*-diol-ABA (**5**) and its *cis*-diol isomer (**6**) were obtained by reduction of **2** with $NaBD_4$. The proportions of labelled ABA, the 1',4'-*t*-diol and the *cis*-diol of ABA were 88%, 94% and 94%, respectively, as estimated by GC/MS analysis. Mycelium of *B. cinerea* was added to a medium containing one of these deuterated compounds and incubated for 10 days. Ethyl acetate extracts were analysed by HPLC and methyl esters by GC/MS to calculate the percentage of 1',4'-*t*-diol-ABA and ABA biosynthesized from the labelled compounds. The relative intensities of parent ions of ABA-Me and of monodehydrated ions of 1',4'-*t*-diol-ABA-Me were employed in this quantitative assessment. The partial mass spectra of these compounds are shown in Fig. 1. The GC/MS of ABA-Me obtained from the culture medium after addition of [$^2H_7$]-1',4'-*t*-diol-ABA showed six parent ions assignable to [^1H]- and [1,2,4,5,6-$^2H_5$]-ABA-Me. The proportion of ^2H-labelled ABA-Me in the total amount of ABA was calculated to be 24% after correction for isotopic contributions and the ^2H of the [$^2H_7$]-1',4'-*t*-diol-ABA added. When [$^2H_7$]-1',4'-*cis*-diol-ABA was added to *B. cinerea*, the synthesized ABA also incorporated ^2H, and the proportion of ^2H-labelled ABA was 35%. These values are means of three experiments. The 1',4'-*t*-diol-ABA-Me recovered from the medium with added [$^2H_6$]-ABA showed only one monodehydrated ion at m/z 262 not assignable to labelled 1',4'-*t*-diol-ABA-Me and dehydrated ions due to ^2H-labelled 1',4'-*t*-diol-ABA-Me were not observed. 1',4'-*cis*-Diol-ABA was not detected in the medium. These results showed that both diol-ABAs were converted into ABA by *B. cinerea*, and that ABA was not converted into diol-ABA of either kind. Both diol-ABAs, especially 1',4'-*cis*-diol-ABA (**4**), seem to be unstable, being spontaneously oxidized to ABA [20]. However, on repeating the experiment with boiled fungus there was no incorporation of either [$^2H_7$]-diol-ABA into ABA, and the ^2H-label of the two [$^2H_7$]-diol-ABAs was not lost below pH 7.0. Since steric hindrance and isotopic effect of the deuterium atom



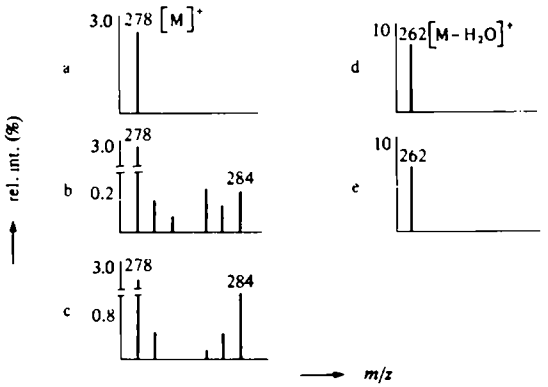


Fig. 1. Partial mass spectra of methyl esters of ABA and 1',4'-*t*-diol-ABA. a, [^1H]-ABA; b, ABA extracted from the culture medium after addition of [$^2H_7$]-1',4'-*t*-diol-ABA; c, ABA extracted from the culture medium after addition of [$^2H_7$]-1',4'-*cis*-diol-ABA; d, [^1H]-1',4'-*t*-diol-ABA; e, 1',4'-*t*-diol-ABA extracted from the culture medium after addition of [$^2H_6$]-ABA.

are slight, the conversion of these compounds to ABA are thus confirmed as characteristic of natural metabolism [21]. Therefore, we conclude that 1',4'-*t*-diol-ABA is a potent precursor of ABA in *B. cinerea*. 1',4'-*cis*-Diol-ABA also is a possible precursor, although its presence could not be detected in culture medium and mycelia of *B. cinerea* by GC-ECD. The *cis*-diol might be converted immediately to ABA and not accumulate. At present, we have no evidence whether the oxidation of the 4'-hydroxyl group is catalysed by one low-stereospecific oxidase or two stereospecific oxidases.

Production of 1'-deoxyabscisic acid and 1',4'-dihydroxy-γ-ionylideneacetic acid by *B. cinerea* was not observed, and the presence of 1',4'-*t*-diol-ABA in cultures of *C. rosicola* and *C. cruenta* has not been reported. This variety of precursors of ABA might be caused by differences in culture conditions. We are not sure which compound is the actual precursor of ABA in higher plants.

EXPERIMENTAL

Screening for ABA-producing fungi. Each strain was cultured on 16 ml of a potato–dextrose–agar medium for a month in the dark, and then extracted with 30 ml Me_2CO. After filtration, the Me_2CO extract was dried and dissolved in 1 ml MeOH. A portion of the MeOH soln was analysed by HPLC [YMC-pack A311 ODS column (6 × 100 mm), 40% MeOH containing 0.1% HOAc, flow rate 2.0 ml/min (system A), detection UV_{254}].

Effects of plant growth regulators on ABA production of B. cinerea. *B. cinerea* No. 39 was cultured on 50 ml of potato–dextrose medium under fluorescent lights. After 35 days, the medium was replaced with a new medium containing a plant growth regulator at various concns, and incubated for 7 more days. An EtOAc extract from the culture medium was analysed by HPLC (system A).

Isolation of 1',4'-t-*diol-ABA. B. cinerea* No. 39 was cultured on 1.1 l. of new medium for 7 days after 35 days of culture. The EtOAc-soluble part (360 mg) of the culture medium was chromatographed on silica gel (Wako gel C-200, 27 g) eluted with a mixture of toluene and EtOAc containing 1% HOAc. The fraction eluted with 40% EtOAc was chromatographed on 12 g Celite 545 impregnated with 7.2 ml 1 M Pi buffer (pH 5.4), and eluted with a mixture of *n*-hexane and EtOAc. The fraction eluted with 40% EtOAc afforded a mixture of (6 mg) of ABA and *t*-ABA, which was identified by GC/MS after methylation. ABA-Me, EIMS (GC) 70 eV, *m/z* (rel. int.): 278 $[M]^+$ (1), 260 $[M-H_2O]^+$ (17), 190 (100), 162 (39), 134 (40), 125 (34), 112 (11), 91 (20). The fraction eluted with 60% EtOAc was purified by prep. HPLC (Cosmosil 5SL silica gel column 4.6 × 150 mm, $CHCl_3$–MeCN–HOAc, 99:1:1, flow rate 2.0 ml/min). The UV-absorbing peak at R_t 16.3 min was collected and concd to yield 1 mg 1',4'-*t*-diol-ABA as a colourless oil, $[\alpha]_D^{20}$ + 266° (EtOH; *c* 0.0297); ORD: $[\alpha]_{285}$ + 11 300°, $[\alpha]_{264}$ ±0°, $[\alpha]_{240}$ −16, 100° (EtOH; *c* 0.00168); UV λ_{max}^{EtOH} nm (ε): 262 (20 000). 1',4'-*t*-Diol-ABA-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 (GC) 70 eV *m/z* (rel. int.): 280 $[M]^+$ (1), 262 $[M-H_2O]^+$ (18), 244 $[M-2H_2O]^+$ (13), 230 (12), 206 (17), 192 (11), 189 (13), 187 (22), 175 (18), 174 (58), 159 (30), 147 (41), 146 (75), 145 (18), 133 (22), 125 (100), 121 (19), 119 (27), 112 (29), 111 (43), 105 (23). 1',4'-*t*-Diol-ABA-Me/TMS, EIMS (GC) 70 eV, *m/z* (rel. int): 352 $[M]^+$ (1), 334 (7), 319 (8), 287 (7), 262 (9), 230 (10), 188 (10), 187 (12), 174 (18), 173 (13), 171 (14), 161 (15), 159 (17), 147 (21), 146 (27), 145 (16), 133 (13), 125 (51), 123 (12), 105 (14), 73 (100).

Preparation of Me esters of 1',4'-t- *and* cis-*diol-ABA.* (+)-ABA-Me (40 mg) was dissolved in MeOH (3 ml) and H_2O (6 ml) and then $NaBH_4$ (80 mg) was added at 0°. After 7 hr, the soln was acidified and extracted with EtOAc. The EtOAc extract was purified on a silica gel (12 g) CC eluted with *n*-hexane–EtOAc (4:1) to afford 1',4'-*t*-diol-ABA-Me (12 mg) and 1',4'-*cis*-diol-ABA-Me (24 mg). 1',4'-*cis*-Diol-ABA-Me, $[\alpha]_D^{20}$ + 140° (EtOH; *c* 0.025); ^{1}H NMR (400 MHz, $CDCl_3$): δ0.94 (3H, *s*), 1.03 (3H, *s*), 1.68 (3H, *br s*), 1.69 (1H, *dd*, *J* = 13.7 and 7.0 Hz), 1.81 (1H, *dd*, *J* = 13.7 and 6.1 Hz), 2.00 (3H, *br s*), 3.71 (3H, *s*), 4.25 (1H, *m*), 5.67 (1H, *s*), 5.70 (1H, *s*), 6.04 (1H, *d*, *J* = 16.2 Hz), 7.77 (1H, *d*, *J* = 16.2 Hz); EIMS (GC) 70 eV, *m/z* (rel. int.): 280 $[M]^+$ (2), 262 $[M-H_2O]^+$ (19), 244 $[M-2H_2O]^+$ (10), 230 (16), 192 (19), 189 (11), 187 (25), 175 (28), 174 (91), 159 (42), 147 (76), 146 (96), 145 (31), 133 (35), 125 (100), 121 (30), 119 (69), 112 (33), 111 (61), 105 (31).

Preparation of ^{2}H-*labelled compounds.* (+)-[$^2H_6$]-ABA (60 mg) was dissolved in 8 ml CD_3OD and 4 ml D_2O, and 72 mg $NaBD_4$ was added at 0°. The reaction mixture was left at 0° for 17 hr and acidified with 1 N HCl. After dilution with 100 ml H_2O, the soln was extracted with EtOAc. The EtOAc extract was chromatographed on 7.5 g of ODS (YMC gel I-40/64) and eluted with 40% MeOH to give (+)-[$^2H_7$]-1',4'-*t*-diol-ABA (17 mg) and (+)-[$^2H_7$]-1',4'-*cis*-diol-ABA (29 mg). (+)-[$^2H_7$]-1',4'-*t*-Diol-ABA, colourless needles (EtOAc), mp 181–185°. (+)-[$^2H_7$]-1',4'-*cis*-Diol-ABA, colourless platelets (EtOAc), mp 153–154°.

Addition of ^{2}H-*labelled compounds. B. cinerea* No. 39 was cultured on 50 ml of medium in the laboratory for 7 days. After decantating the medium and washing (× 3) the mycelia with sterile water, 800 μg of ^{2}H-labelled compound in 50 μl MeOH was added together with 50 ml new medium and incubated for 10 days. The medium was filtered and extracted with EtOAc at pH 3. The EtOAc extract was concd, and ABA, 1',4'-*t*-diol-ABA and 1',4'-*cis*-diol-ABA were collected by HPLC (system A). Each compound was methylated and analysed by EIMS (GC), 70 eV.

Stability of ^{2}H-*labelled compounds.* Each ^{2}H-labelled compound (20 μg) was dissolved in buffers of pH 2.5, 3.0, 3.5, 4.5, 5.2, 6.3 and 7.5, and left under the same conditions as in the feeding expt for 10 days. The EtOAc extracts obtained from the buffer solns were analysed by EIMS (GC), 70 eV.

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