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METABOLISM AND BIOLOGICAL ACTIVITY OF (+)- AND (−)-*C*-1′-*O*-METHYL ABA IN MAIZE SUSPENSION-CELL CULTURES*

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Key Word Index—*Zea mays* L. Black Mexican Sweet; Gramineae; abscisic acid analogues; growth inhibition; *C*-1′-methyl ether; metabolism; phaseic acid; electrospray.

Abstract—(+)-*C*-1′-*O*-Methyl abscisic acid is rapidly metabolized by suspension-cultured maize cells (*Zea mays* L. Black Mexican Sweet) to (+)-*C*-1′-*O*-methyl phaseic acid in an analogous process as seen for ABA. The presence of a methyl ether on the 1′-position of ABA does not interfere with enzymic oxidation at the 8′-carbon. The metabolite is demethylated to yield phaseic acid. A small amount of abscisic acid is also produced by direct demethylation. The (+)-*C*-1′-*O*-methyl ABA exhibits stronger growth inhibitory activity of the maize cells than (+)-ABA, suggesting that a free *C*-1′-hydroxyl group is not essential for biological activity of ABA in maize. The (−)-*C*-1′-*O*-methyl ABA is metabolized to (−)-ABA and to 7′-hydroxyABA and the corresponding *C*-1′-*O*-methyl-7′-hydroxy ABA.

INTRODUCTION

The synthesis and biological activity of optically pure abscisic acid (ABA) analogues, **1** and **2**, compounds which have a methyl group replacing the *C*-1′-hydroxyl hydrogen, have been recently described [1]. In an assay which measured their anti-transpirant activity in wheat seedlings, the methyl ethers were weakly active initially, but displayed ABA-like activity over time. This led us to consider the possibility that these analogues were being metabolized to more active substances in the plant. We therefore decided to investigate the metabolic fate of both (+)- and (−)-*C*-1′-*O*-methyl ABA. The objectives were to test the stability of the analogues in a well defined experimental system and to determine the products of metabolism, especially to monitor for the conversion of the analogues into the optical isomers of ABA, **3** and/or **4**.

We have studied the metabolism of (+)- and (−)-*C*-1′-*O*-methyl ABA in maize (*Zea mays* L. Black Mexican Sweet) suspension-cultured cells [2], a system we had previously used to investigate the metabolism of (+)- and (−)-ABA. Methyl ether **1** and natural ABA were also compared in a growth inhibition assay in the maize system to probe the involvement of the free hydroxyl group in recognition of natural ABA.

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RESULTS AND DISCUSSION

Metabolism of (+)-C-1′-O-*methyl ABA* (**1**) *in suspension cultured maize cells*

The metabolism of methyl ether **1** was followed using the procedure developed for analysing ABA metabolism in maize cell cultures [2], where the UV-absorbing components present in the culture filtrates were measured by HPLC. We had observed that natural ABA (**3**) was rapidly converted by the maize cells into phaseic acid (PA) (**5**). Methyl ether **1** was supplied at 120 μM to maize cell cultures and aliquots of the medium sampled at intervals over three days. By 24 hr, the methyl ether was completely depleted from the medium and one major, new unsymmetrical peak was seen by HPLC. Between 24 and 72 hr, the concentration of the putative metabolite decreased in the medium. Two other peaks were also detected, one transiently which had the same retention time as ABA, and one which slowly increased over time which had the same retention time as PA.

The structure of the major metabolite was determined by spectroscopic analysis of ethyl acetate extracts of the culture media and of the pure isolated product. Continuous flow secondary ion mass spectrometry (LC-MS) of an extract of the medium showed the major metabolite had a molecular ion at *m/z* 295 $[M + 1]^+$, a glycerol adduct at *m/z* 388 $[M + 94]^+$ and a fragment at *m/z* 263 $[M - MeOH + 1]^+$. As the molecular weight of the methyl ether is 278, these ions showed

that the metabolite contained an additional oxygen, giving it a M_r 294. This would be expected for **6**, *C*-1′-*O*-methyl phaseic acid; however, the possibility that the oxygen could have been added at a position other than the 8′-methyl group could not yet be discounted. The presence of **6** was confirmed by isolation of the metabolite from a large scale biotransformation experiment. The ^{1}H NMR spectrum for the metabolite was identical to that of PA, with the addition of a singlet at 3.39 ppm due to the C-1′ methyl. The concentrations of **1** and **6** present in the samples of the medium taken from the time course experiment (Fig. 1) were estimated using calibration curves for ABA and PA, which have the same chromophores and similar extinction coefficients as **1** and **6**, respectively.

The isolated product **6** gave a symmetrical peak on HPLC, contrasting with the asymmetrical peak observed in the culture medium. This is reminiscent of the behaviour of phaseic acid, which can exist in aqueous solution in equilibrium with the open from 8′-hydroxy-abscisic acid [3]. A similar equilibrium between 8′-hydroxy-*C*-1′-*O*-methyl ABA and the *C*-1′-*O*-methyl PA (**6**) would account for the asymmetrical peak observed in the HPLC of the culture filtrate, and increases the apparent concentration of metabolite as shown in Fig. 1, as the concentration calculation was based on a calibration curve for PA and the open form has been shown to have a higher extinction coefficient (Abrams *et al.*, in preparation).

Assuming the same enzyme is responsible for both the oxidation of (+)-ABA to (−)-PA and **1** to **6**, steric bulk in the region of the C-1′ centre of ABA does not interfere with the enzymic activity. Also, the hydroxyl group of ABA is not essential for substrate binding to the enzyme active site. The C-1′ centre is a potential site for attaching a photoreactive group, which could be used to label the enzyme covalently and, ultimately, assist in isolation of the enzyme.

When 120 μM **1** was supplied to the suspension culture, a peak which had the same retention time as ABA was detected in the medium between 3 and 24 hr and reached a maximum concentration of 7 μM (based on an ABA calibration curve). From 18 hr on, PA was also detected in the medium; its concentration increased gradually to 30 μM by 72 hr. The metabolic pathways of (+)-*C*-1′-*O*-methyl ABA are summarized in Fig. 2. The presence of PA was confirmed by LC-MS of the spent medium through comparison with a known standard. ABA and PA are likely formed from cleavage of their methyl ethers. Controls containing medium, but lacking maize cells, showed no formation of ABA from the methyl ether, or any other degradation products, suggesting the cleavage is an enzyme mediated process. It is possible that the formation of ABA from **1** could contribute to or be the sole cause of its biological activity in wheat [1]. A slow formation of ABA could also explain the delayed activity seen in the wheat seedling transpiration assay. However, the metabolism of these compounds has not been studied in wheat, and differences in uptake could also be involved in the whole plant. Therefore, to determine the importance of the *C*-1′-hydroxyl group of ABA, we wanted to relate our metabolism data to a physiological process in maize which was regulated by ABA. Thus, the following growth inhibition assay was carried out.

Natural ABA is a strong inhibitor of maize cell growth [2]. The activity of methyl ether **1** was compared with that of (+)-ABA in a four-day assay. The growth inhibition results in Fig. 3 show that **1** is equally or more active than ABA at all concentrations tested. The high activity of the (+)-*C*-1′-*O*-methyl ABA and the relatively low concentration of free ABA (Fig. 1) suggest that the methyl ether is recognized by the putative ABA receptor protein.

Metabolism of (−)-C-1′-O-*methyl ABA* (**2**) *in suspension cultured maize cells*

The time course study of the metabolism of 100 μM **2** is shown in Fig. 4. The rate of metabolism of the (−)-form of the methyl ether is slower than the (+)-

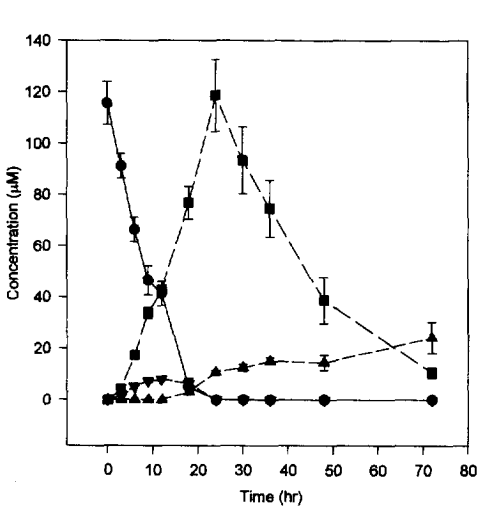

Fig. 1. Changes in levels of (+)-*C*-1′-*O*-methyl ABA and its metabolites during incubation with maize suspension-cell culture (● = **1**, ■ = **6**, ▲ = **5**, ▼ = **3**).

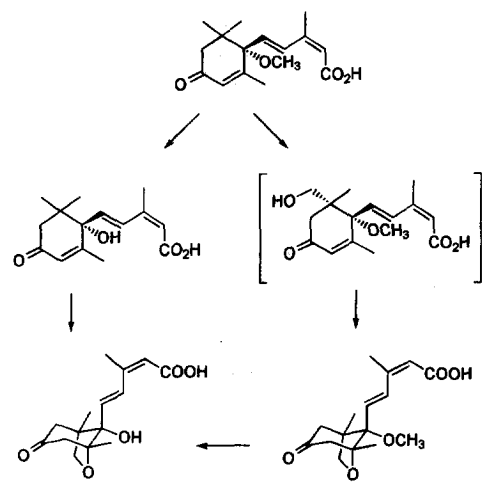

Fig. 2. Proposed pathways for metabolism (+)-*C*-1′-*O*-methyl ABA in maize suspension-cell culture.

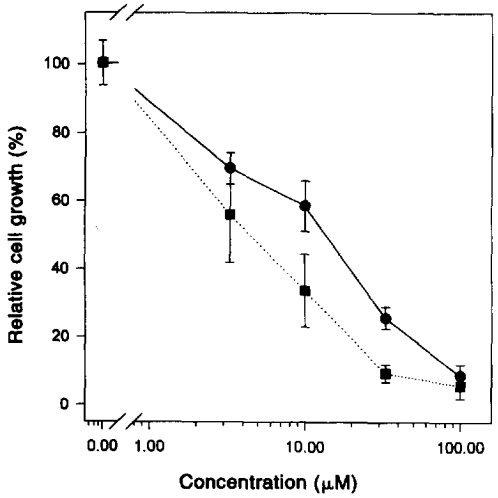

Fig. 3. Growth inhibition dose-response curve for (+)-ABA (●) and (+)-*C*-1′-*O*-Methyl ABA (■) in maize suspension-cell culture.

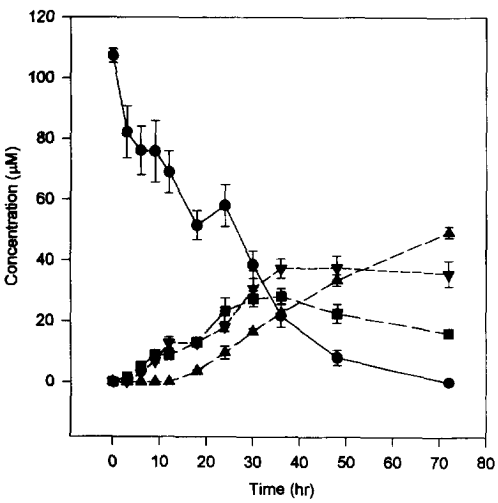

Fig. 4. Changes in the levels of (−)-*C*-1′-*O*-methyl ABA and its metabolites in maize suspension-cell culture (● = **2**, ■ = **4**, ▲ = **7**, ▼ = **8**).

form, similar to the ABA case, where (−)-ABA is metabolized slowly by maize cells to (−)-7′-hydroxy-abscisic acid [(−)-7′-OHABA, **7**] [2]. By 72 hr, the methyl ether had been completely metabolized to a mixture of ABA, 7′-OHABA (as compared to known standards) and an unknown metabolite which by LC-MS contained one oxygen more than the substrate. In this instance, mass spectral analysis was carried using LC-MS with electrospray ionization. An ethyl acetate extract of the culture medium analysed by LC-MS confirmed the presence of ABA and 7′-OHABA. The unknown metabolite gave a molecular ion at *m/z* 295 $[M + 1]^+$, an ion at *m/z* 277 $[M - H_2O + 1]^+$ and a ion corresponding to the loss of methanol at 263 $[M - MeOH + 1]^+$. This suggested a M_r of 294 for the metabolite, which is one oxygen heavier than the methyl ether of ABA. Furthermore, an ion with *m/z* 239, corresponding to the loss of C_4H_8, was also detected. This fragmentation patterns occurs via a retro-Diels–Alder fragmentation of the ring (Fig. 5). The formation of this ion indicated that the new oxygen was not on the 8′- or 9′-carbon. By HPLC, the metabolite had a different retention time from *C*-1′-*O*-methyl PA and showed a UV spectrum (as collected through the diode array detector of the HPLC) which confirmed the presence of the conjugated enone system in the ring. We speculate that it is the methyl ether of 7′-OHABA (**8**). However, a large scale isolation was not carried out. As (−)-ABA is metabolized to (−)-7′-OHABA in maize culture, a similar metabolic fate for the (−)-methyl ether could be expected. Also, the increase in concentration of 7′-OHABA, the product of demethylation of **8**, is consistent with this structure. The high quantities of ABA being formed through metabolism could account for the biological activity of the analogue seen in wheat embryo germination inhibition and wheat seedling antitranspirant activity [1].

The chirality of the ABA produced in the metabolism of **2** was investigated to gain some insight into the mechanism of the methyl ether group cleavage. Breaking the O–CH_3 bond in the methyl ether by enzymic oxidation of the methyl group and hydrolysis of the resulting hemiacetal would leave the stereochemistry at C-1′ unchanged, forming (−)-ABA from (−)-*C*-1′-*O*-

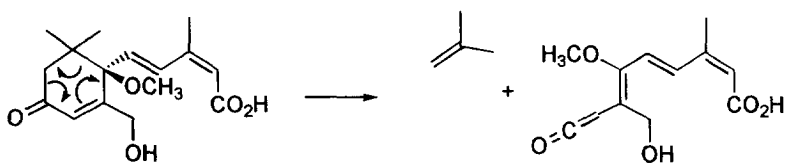

Fig. 5. Mass spectral fragmentation pattern for metabolite **8**.

methyl ABA (**2**). Hydrolysis of the (*C*-1′)–*O* bond could generate a carbocation intermediate which, upon quenching with water, would form both enantiomers of ABA. An ethyl acetate extract of the maize cell medium from the metabolism study of **2** at 72 hr was methylated with diazomethane and the methylABA produced was analysed by HPLC using a chiral column [4]. Of the two possible enantiomers of methylABA which could be present, only a peak corresponding to (−)-methylABA was observed. Thus, cleavage of the ether group occurs with retention of stereochemistry at the C-1′ position, suggesting that the cleavage is occurring through reaction at the methyl ether carbon.

Recognition of the free hydroxyl group of ABA. Both methyl ethers, **1** and **2**, were metabolized in the same manner as (+)- and (−)-ABA, respectively, in the maize cell culture. The enzyme(s) which oxidize either form of ABA can accommodate the added steric bulk at the 1′-hydroxyl group of ABA, and do not require the *C*-1′-hydroxyl group for binding to the enzyme. The change of lipophilicity of these analogues is not detrimental to metabolism, and may improve uptake of ABA analogues in plants. We have found a position on ABA which can be altered while keeping the activity of the oxidative enzyme high. A potential means of identifying the enzyme involved in ABA degradation is by use of photoaffinity labelling strategies via the C-1′ centre.

The *C*-1′-*O*-methyl ether analogues are not ideal for determining the role of the hydroxyl group in ABA responses because the molecules are metabolized to ABA in plants. However, the results in maize that (+)-*C*-1′-*O*-methyl ABA has growth inhibitory activity suggests that the hydroxyl group in not critical for inducing ABA-like responses in maize.

EXPERIMENTAL

General. ^{1}H NMR spectra were recorded on a Bruker AMX-500 spectrometer. For clarity, the conventional ABA numbering system is employed in assignments of peaks in the ^{1}H NMR spectra. IR spectra were obtained with a Perkin Elmer Paragon 1000 FT-IR. Optical rotations were obtained from a Perkin-Elmer 141 Polarimeter and were carried out in MeOH. HREIMS were recorded with a VG 70-250SEQ double-focusing hybrid spectrometer. (+)-ABA and (−)-ABA were obtained by prep. HPLC resolution of (±)-methyl abscisate followed by hydrolysis of the resolved esters, as previously described [4]. The (+)- and (−)-*C*-1′-methyl ethers of ABA were synthesized as described in ref. [1].

Maintenance of cells. Suspension cultures of maize (*Z. mays* L. cv. Black Mexican Sweet) were maintained as described previously [5]. Cells were maintained at 25–28°.

Growth inhibition of **1**. Growth inhibition studies were carried out as previously described [2].

Metabolism studies of **1** *and* **2**. For each treatment, 2.0–2.2 g of cells were subcultured into each of three 100-ml flasks containing 50 ml medium. The following day, the analogues were introduced into the medium from a 50 mM ethanolic soln. The initial concn of **1** and **2** in each treatment was 100 μM. Blanks with analogues in cell-free medium were prepd to check for compound stability, as well as analogue free cells and medium, which were prepd to screen for formation of compounds which would interfere with the analysis of ABA-like compounds in the HPLC. The cultures were incubated on a rotary shaker at 25°. The medium from cultures treated with **1** and **2** were sampled for analysis every 3 hr during the first 12 hr, then at 6, 12 or 24 hr intervals for the next 3 days. At the end of the experiment, the cells were removed by filtration, and the filtrates for each analogue combined and frozen.

Analysis of culture medium. Media from cultures fed **1** and **2** were analysed by a previously reported HPLC method [2, 3]. Peaks in the chromatograms were identified by comparing their R_ts with those of authentic standards. For quantitation, calibration curves were constructed using solns containing an appropriate range of concns of (±)-ABA, (−)-PA and (±)-7′-HOABA.

The presence of known ABA metabolites was confirmed by LC-MS. The spent culture filtrate for each analogue was thawed, adjusted to pH 2.5, and extracted (4 × 30 ml) into EtOAc. The organic layer was washed with brine, dried over Na_2SO_4 and concd. The filtrate extract from feeding of **1** was analysed by LC-MS in the same manner as previously reported [6]. The filtrate extract from the feeding of **2** was analysed by continuous flow electrospray MS under the following conditions: 1 μl of sample dissolved in 50% MeCN–H_2O was injected on to a 0.3 × 200 mm fused silica column packed in this laboratory with CSC-inert-octyl (5 micron) reverse phase packing (SPE Ltd, Concord, Ont., Canada). The sample was eluted using an Applied Biosystems Model 140B solvent delivery system under gradient conditions at 4 μl min^{-1}. The gradient conditions were as follows: solvent A was 90% MeCN in H_2O containing 1% HOAC, and solvent B was 1% HOAC in H_2O. The proportion of solvent B was held at 90% for 10 min, then varied linearly to 50% after 15 min and to 30% after 20 min. The LC system was interfaced with a Fisons Instruments Trio 2000 quadrupole mass spectrometer (Manchester, U.K.) operating in the positive-ion electrospray mode. Repetitive scans were acquired from mass 100–900 every 3 sec. The capillary tip voltage was 4 kV and the sampling cone was held at 29 V. For confirmation of the presence of ABA, PA and 7′-HOABA in the metabolic mixt., MS data were compared to those of pure standards.

Isolation of metabolite from **1**. Cells (18 g) were subcultured into a 1-l flask containing 500 ml medium which had been autoclaved. The following day, 11.1 mg **1**, dissolved in 0.5 ml EtOH, was introduced into the medium. The initial concn of **1** was 80 μM. The culture was incubated at room temp. on a rotary shaker at 150 rpm for 24 hr at which time, HPLC analysis showed all **1** had been consumed. At the end of

the culture period, the cells were removed by filtration. The filtrate was frozen until processed for isolation.

The metabolite **6** was extracted from the culture filtrate by a chromatographic procedure [7]. A column of Supelco Amberlite XAD-2 resin was prepd as follows: XAD-2 resin (100 g) was soaked in MeOH for 15 min, the solvent was decanted followed by soaking in H_2O for 15 min, which was again decanted; H_2O was added and the slurry was packed into a column (3 cm diam.). The column was eluted successively with H_2O (3 column heights). The filtrate from the culture was thawed, acidified to pH 2.5 with 1 M HCl, and eluted slowly (1 hr for the 500 ml) through the XAD-2 column. The column was then eluted with 0.1 M HCl (150 ml) followed by *i*-PrOH (200 ml). ABA-type compounds were present in the *i*-PrOH. The eluate was analysed by HPLC (Hisep column) for the presence of metabolites. The frs containing the metabolites were combined and concd *in vacuo* to give 28 mg crude product, which was purified by prep. TLC [silica gel 60 GF_{254}, 20 cm × 20 cm × 1 mm, toluene–EtOAc–HOAc (25:15:2) as eluent], giving 3.4 mg metabolite **6**, which showed the following spectral properties. IR (neat) ν_{max} cm^{-1}: 3200 (*br*, OH), 1713 (C=O, ketone), 1685 (C=O, acid); HREIMS: [M] at *m/z* 294.1476 ($C_{16}H_{22}O_5$ requires 294.1467); ^1H NMR (500 MHz, CD_3OD): δ 8.18 (*d*, 1H, H4, $J_{4\text{-}5}$ = 17.0 Hz), 6.20 (*d*, 1H, H5, $J_{4\text{-}5}$ = 17.0 Hz), 5.85 (*s*, 1H, H2), 3.92 (*dd*, 1H, H8′a, $J_{8'a\text{-}5'ax}$ = 2.9 Hz, $J_{8'a\text{-}8'b}$ = 7.4 Hz), 3.61 (*d*, 1H, H8′b, $J_{8'a\text{-}8'b}$ = 7.4 Hz), 3.39 (*s*, 3H, H1′-OCH_3), 2.80 (*d*, 1H, H3′ax, $J_{3'ax\text{-}3'eq}$ = 18.0 Hz), 2.68 (*dd*, 1H, H5′ax, $J_{5'ax\text{-}8'a}$ = 2.4 Hz, $J_{5'ax\text{-}5'eq}$ = 18.2 Hz), 2.41 (*dd*, 1H, H3′eq, $J_{3'eq\text{-}5'eq}$ = 2.4 Hz, $J_{3'eq\text{-}3'ax}$ = 18.0 Hz), 2.34 (*dd*, 1H, H5′eq, $J_{5eq\text{-}3'eq}$ = 2.4 Hz, $J_{5'eq\text{-}5'ax}$ = 18.2 Hz), 2.09 (*d*, 3H, H6, *J* = 1 Hz), 1.36 (*s*, 3H, H7′), 1.16 (*s*, 3H, H9′). $[\alpha]_D^{20}$ = +23° (MeOH, *c* 0.28%).

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