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SYNTHESIS, METABOLISM AND BIOLOGICAL ACTIVITY OF A DEUTERATED ANALOGUE OF THE PLANT HORMONE *S*-(+)-ABSCISIC ACID*

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Key Word Index—Abscisic acid; ABA; phaseic acid; deuterium labelled analogue; synthesis; metabolism; isotope effect; germination inhibition; *Zea mays*; *Lepidium sativum*; Gramineae; Cruciferеae.

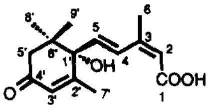
Abstract—The synthesis of a deuterated analogue of the plant hormone *S*-(+)-abscisic acid (ABA), *S*-(+)-[7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-nonadeuteroabscisic acid [(+)-d_9-ABA], is described. The biological activity of (+)-d_9-ABA was compared to that of (+)-ABA in two assays. Analogous to the metabolism of (+)-ABA to (−)-phaseic acid (PA) by maize (*Zea mays* L. cv Black Mexican Sweet) cell suspension cultures, (+)-d_9-ABA was oxidized but at a slower rate to d_8-PA at 25°. At low concentrations the deuterated analogue was a more effective inhibitor of cress seed (*Lepidium sativum* L.) germination than the natural hormone.

INTRODUCTION

The plant hormone *S*-(+)-abscisic acid (*S*-(+)-ABA, **1**), which regulates germination, transpiration and responses to abiotic stress, is metabolized to (−)-phaseic acid ((−)-PA, **3**) via 8′-hydroxyabscisic acid (**2**) by plants and cultured plant cells [1–3]. Our laboratories have been investigating the biological activity induced by **1** and **3** in various physiological processes [4, 5]. In cress seed (*Lepidium sativum* L) germination assays, ABA is a germination inhibitor, while phaseic acid (supplied as the racemic mixture) is inactive [5]. In maize (*Zea mays* L. cv Black Mexican Sweet) cell suspension cultures, **1** inhibits cell growth and causes a transitory shift in the pH of the medium, while **3** is inactive in both assays [4].

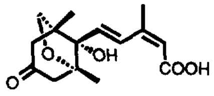
We were interested in studying the effect of deuterium-labelling the ring methyl groups of ABA on metabolism and biological activity of the molecule. It is expected that the rate of oxidation of the C–D bond at the C-8′ position would be slower than that of the C–H bond (primary isotope effect) [6] and that biological activity of the ABA analogue could be greater if persistence of the hormone was the limiting factor.

This paper reports the synthesis of *S*-(+)-[7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-nonadeutero-ABA, (+)-d_9-ABA (**4**), an analogue which has deuterium atoms at the C-8′ position where oxidation to 8′-hydroxyabscisic acid occurs. We have determined the relative rates of metabolism of *S*-(+)-ABA (**1**) and **4** in maize cell suspension cultures, and the effect of these two compounds on the growth of maize cells and in the germination of cress seeds.



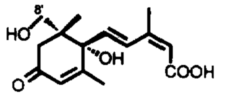
S-(+)-abscisic acid

1



(-)-phaseic acid

3



8′-hydroxyabscisic acid

2

RESULTS AND DISCUSSION

Synthesis of S-(+)-[7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-*nonadeutero-ABA* (**4**)

The desired nonadeuterated compound was synthesized through a route which we have utilized to generate several ABA analogues [7, 8]. This involves initial

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functionalization of the ring system followed by the addition of the side chain and subsequent functional group manipulation (see Scheme 1). However, some interesting new chemistry was developed to generate compound **8**, the deuterated version of the mono-ethyleneketal of oxoisophorone.

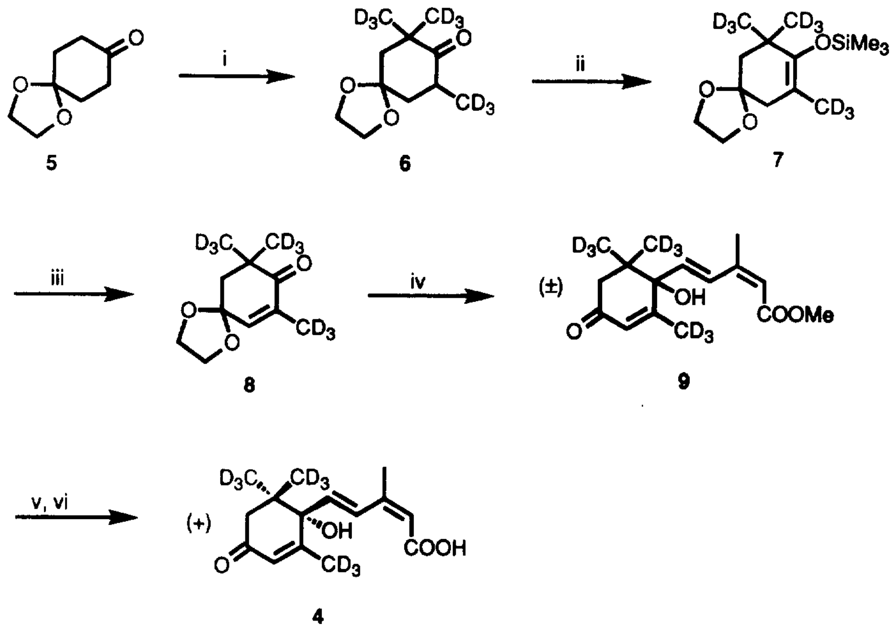
The three trideuterated methyl groups were introduced through an alkylation of ketone **5** with iodomethane-d_3. This afforded a rather complex mixture of di-, tri- and tetra-alkylated products. However, the proportion of the desired trialkylated product **6** can be optimized by careful regulation of the reaction conditions. Introducing the double bond proved to be a challenge, as typical procedures to introduce a phenylselenyl or bromo leaving group were unsuccessful. The C-2 position was activated by generating the trimethylsilyl enol ether **7**, which then permitted the synthesis of the enone system in a one pot reaction. Enol ether **7** was first titrated with bromine at $-78°$ followed by the elimination of the bromide *in situ* through addition of excess 1,8-diazobicyclo[5,4,0]undec-7-ene (DBU). This introduced the double bond very cleanly to give compound **8** in 97% yield.

Further elaboration to the nonadeuterated analogue of methyl abscisate (**9**) was carried out as previously reported [7, 8]. The two enantiomers of methyl ester **10** were separated on a preparative scale chiral HPLC column [9]. The (+)-methyl ester was hydrolysed to the desired acid **4** in 92% yield using pig liver esterase. Mass spectral analysis indicated the presence of nine deuterium atoms in the ABA molecule. Use of the enzyme catalysed reaction avoided exchange of the C-7′ deuterium atoms, which occurred under the conditions utilized for base catalysed hydrolysis.

Metabolism of (+)-d_9-*ABA in maize cell suspension cultures*

Maize cells (*Zea mays* L. cv Black Mexican Sweet) were incubated at 25° in culture medium containing 0, 25, or 100 μM of either (+)-ABA (**1**) or (+)-d_9-ABA (**4**) as previously described [4]. Both compounds were stable in the cell-free blanks, and no ABA or metabolites were detected in the medium of the control culture over the time course of the experiment. Figure 1 shows the change in ABA concentrations in the media of the cell cultures incubated with 100 μM **1** or **4**. A comparison of the initial rates showed that (+)-ABA was depleted from the culture medium approximately twice as fast as the deuterated analogue. Similar results were observed for cultures containing lower initial concentrations of ABA (data not shown).

The metabolism of the natural hormone to PA (**3**) was faster than the corresponding oxidation of the deuterated analogue. Figure 2 shows the variation in concentrations of PA in the media of the cell cultures incubated with 100 μM (+)-ABA and (+)-d_9-ABA. Over the 10–45 hr time period, the rate of conversion of **1** to PA was approximately 1.6-fold higher than the rate of conversion of **4** to the corresponding PA. Similar results were again observed with lower initial concentrations of ABA (data now shown). At the end of the culture period the unmetabolized ABA and the metabolite PA were isolated from the media of cultures incubated with **4** by a previously described chromatographic procedure [4, 10]. LC/CFSIMS analysis [11] showed that all nine deuterium atoms were retained in the unmetabolized ABA and that the PA had eight deuterium atoms as expected.



Scheme 1. Synthesis of (+)-[7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-nonadeutero-ABA, **4**: (i) NaH, CD_3I, THF; (ii) LDA, Et_3N, Me_3SiCl, THF; (iii) Br_2, CH_2Cl_2, then DBU; (iv) refs [7] and [8]; (v) resolution by HPLC; (vi) pig liver esterase, pH 7.5 potassium phosphate buffer, 1M KOH.

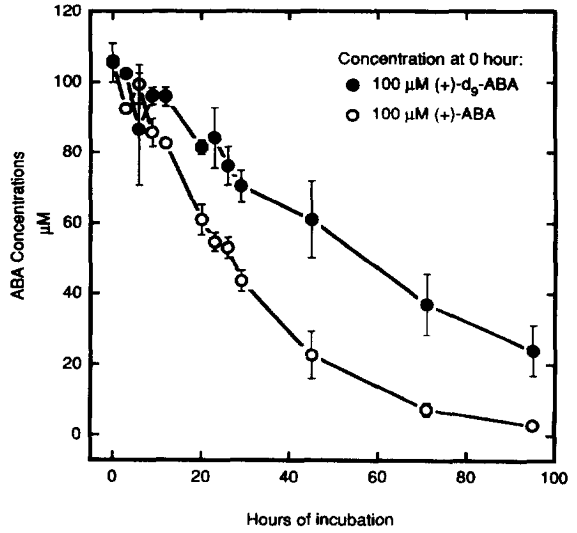


Fig. 1. Time course of variation in concentration of ABA in maize cell culture medium at 25°. Each data point represents the mean of four concentration determinations (two replicate cultures, concentration determination performed in duplicate for each culture). Vertical bars indicate standard deviation of the mean.

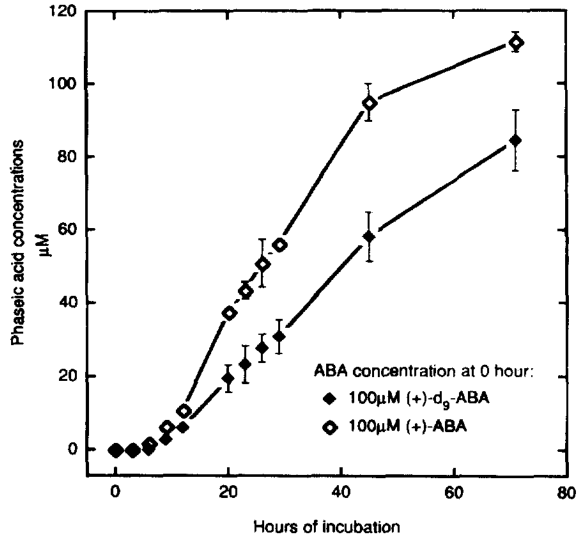


Fig. 2. Time course of variation in concentration of phaseic acid in maize cell culture medium at 25°. Each data point represents the mean of four concentration determinations (two replicate cultures, concentration determination performed in duplicate for each culture). Vertical bars indicate standard deviation of the mean.

Our results indicate that there was an 'observed isotope effect' [12, 13] of approximately 2 in the biotransformation of ABA to PA under our experimental conditions. This hydroxylation is catalysed by a cytochrome P_{450}-containing enzyme [14] and the observed isotope effect is consistent with results obtained with other enzymes of this type [15]. For these enzyme catalysed reactions, the isotope effect intrinsic to the hydroxylation step is in the range 10–13, but the catalytic rate is significantly determined by enzymatic steps other than the hydroxylation itself [15–17]. Some of these steps, as well as the conversion of the C–H bond to the C–OH bond, may also be sensitive to isotopic substitution and contribute to the observed isotope effect. Further experiments designed to investigate intramolecular isotope effects [15–17] using C-8′ monodeuterated ABA will be necessary to establish this.

We previously reported that (+)-ABA inhibits the growth of suspension cultured maize cells, whereas its metabolite (+)-PA is a much weaker inhibitor [4]. At concentrations of 1–100 μM, the inhibitory effect of (+)-d_9-ABA on the growth of maize cells were found to be statistically similar to that of the unlabelled compound (data not shown), whereas the activity of the metabolite d_8-PA was not determined. The isotope effect observed in the metabolism study was therefore not due to slower cell growth in the suspension cultures incubated with the deuterated analogue. The failure of (+)-d_9-ABA to exhibit enhanced growth inhibitory activity despite a two-fold reduction in its rate of conversion to d_8-PA, which is likely inactive, implies that the response of maize cells to ABA is relatively insensitive to small changes in rates of ABA metabolism.

Effect of (+)-ABA on the germination of cress seeds

Cress seeds (*Lepidium sativum* L.) were treated with 0–100 μM of either (+)-ABA or the deuterated analogue 4 as previously reported [5]. Root emergence was totally inhibited for 168 hr by either compound at 100 μM while shoot emergence was totally inhibited by either compound at 10 μM. At lower concentrations, compound 4 was a stronger inhibitor of root and shoot emergence than the natural hormone. This is illustrated in Fig. 3

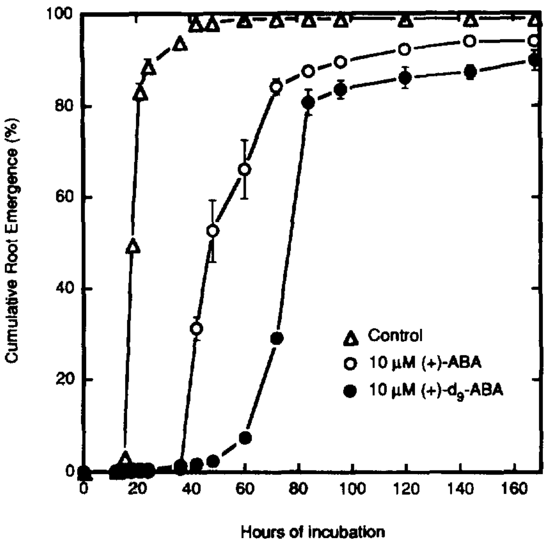


Fig. 3. Effect of (+)-ABA and (+)-d_9-ABA on root emergence of cress seeds at 25°. Vertical bars indicate standard deviation of the mean.

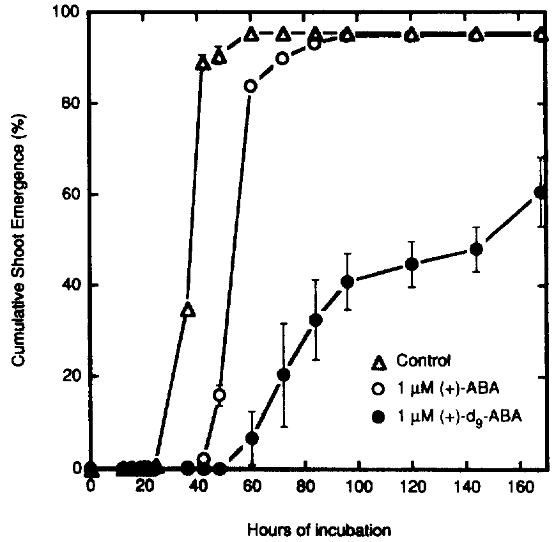


Fig. 4. Effect of (+)-ABA and (+)-d_9-ABA on shoot emergence of cress seeds at 25°. Vertical bars indicate standard error of the mean.

which shows the effect of 10 μM of either ABA or **4** on root emergence, and Fig. 4 which shows the effect of 1 μM of either compound on shoot emergence. These results indicate that **4** was a more persistent inhibitor than (+)-ABA on both root and shoot emergence. The increased activity is likely due to its slower rate of metabolism to inactive or weakly active products during the course of the experiment.

It can be concluded that deuterium-labelling the ring methyl groups of (+)-ABA made the molecule more stable towards oxidation, in particular at the C-8′ position, by cultured maize cells and gave a more persistent hormone with activity comparable to or even greater than that of (+)-ABA in both bioassays described in this paper.

EXPERIMENTAL

Synthesis of S-(+)-[7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-*nonadeutero abscisic acid* (**4**)

4, 4-*Ethylenedioxy*-2,6,6-*tri*(d$_3$-*methyl*)*cyclohexanone* (**6**). A dry, 250 ml three-necked round-bottomed flask was equipped with a thermometer, a dropping funnel and a drying tube. NaH (16.2 g, 60% dispersion in mineral oil, 6 equiv.) was added, washed × 3 with hexane and then stirred with 20 ml THF. To this was slowly added a solution of 1,4-cyclohexanedione mono-ethyleneketal (**5**) (10 g, 0.064 mol) in 80 ml THF. The mixture was stirred for 1.25 hr at room temp., at which point iodomethane-d_3 (MSD isotopes, 99.5 at% D, 4 equiv., 16.3 ml) was slowly added over 0.5 hr, causing some increase in temp. The reaction mixt. was then heated at reflux overnight. After cooling, the reaction was quenched with saturated NH_4Cl. The organic layer was separated, and the aqueous layer washed with diethyl ether (3 ×). The combined organics were washed with brine, dried over Na_2SO_4 and concd. By GC (FID, DB5 column), this mixture contained 23% dialkylated, 49% trialkylated and 25% tetraalkylated product. Flash chromatography (10% ether in hexane) gave 2.8 g of pure trialkylated material (**6**) and 3.9 g of a 1 : 1 mixt. of tri- and tetraalkylated product (total of 4.7 g trialkylated ketone **6**, 36% yield). Both could be used in the following step. HREIMS: $[M]^+$ at *m/z* 207.1829 ($C_{11}H_9D_9O_3$ requires 207.1821): IR ν_{max} cm^{-1}: 2220 (C–D), 1710, (C=O); ^{1}H NMR (360 MHz, $CDCl_3$): δ4.04–3.80 (*m*, 4H, ketal), 3.10 (*dd*, 1H, J = 5.3, 14.0 Hz, H-2), 2.01 (*ddd*, 1H, J = 3.2, 5.4, 13.3 Hz, H-3eq), 1.87 (*dd*, 1H, J = 3.3, 14.0 Hz, H-5eq), 1.81 (*d*, 1H, J = 14.0 Hz, H-5ax), 1.66 (*t*, 1H, J = 13.6 Hz, H-3ax); ^{13}C NMR (90 MHz, $CDCl_3$): δ216.1 (C-1), 107.3 (C-4), 64.6 and 63.9 (CH_2O), 47.5, 46.0, 43.8, 43.3, 37.3.

4,4-*Ethylenedioxy*-2,6.6-*tri*(d$_3$-*methyl*)-1-*trimethylsilyloxy-cyclohex*-1-*ene* (**7**). Lithium diisopropylamide (LDA, 1.1 equiv., 1.25 ml of diisopropylamine, 3.4 ml of 2.6 M nBuLi) was generated at −78° in a dry, nitrogen-flushed flask containing 10 ml of THF. Ketone **6** (1.68 g, 0.0081 mol, in 5 ml THF) was added slowly and the mixture stirred for 5 min. Triethylamine (0.44 eq. 0.50 ml) and trimethylsilylchloride (1.7 eq, 1.75 ml) were mixed together in 5 ml THF and the solid ppt. removed before adding the mixture to the reaction vessel. Following addition, the reaction was stirred for 15 min, then quenched with saturated bicarbonate solution and extracted into CH_2Cl_2 (3 × 50 ml). The combined organics were washed with brine, dried over Na_2SO_4 and purified by flash chromatography (10% ether in hexane). This gave 0.91 g of the desired enol ether (**7**) (42%) and 0.77 g of recovered starting material (46%). The starting ketone can be recycled easily, and this also facilitates the use of mixtures of tri- and tetraalkylated products formed in the first reaction, as the separation of enol ether **7** and the starting material is very facile. HREIMS: $[M]^+$ at *m/z* 279.2200 ($C_{14}H_{17}D_9O_3Si$ requires 279.2216); IR ν_{max} cm^{-1}: 2220 (C–D); ^{1}H NMR (360 MHz, $CDCl_3$): δ3.89 (*s*, 4H, ketal), 2.23 (*s*, 2H, $CH_2CH=$), 1.68 (*s*, 2H, CH_2), 0.18 (*s*, 9H, $Si(CH_3)_3$).

4,4-*Ethylenedioxy*-2.6.6-*tri*(d$_3$-*methyl*)*cyclohex*-2-*enone* (**8**). The silyl enol ether **7** (0.94 g, 0.0034 mol) was dissolved in 10 ml CH_2Cl_2 and cooled to −78°. The reaction mixt. was titrated with a freshly prepared solution of 10% Br_2 in CH_2Cl_2 until the bromine colour persisted. At this point, excess DBU (2 ml) was added and the mixt. was allowed to warm to room temp. and then was left stirring overnight. The reaction was quenched with 1 M HCl, and the organic layer removed. The aqueous layer was washed with CH_2Cl_2 (3 × 30 ml) and the combined organics were washed with brine soln and dried over Na_2SO_4. The crude mixt. was purified by washing it through a bed of silica gel with 50% ether in hexane to give 0.67 g (97%) of the product. HREIMS: $[M]^+$ at *m/z* 205.1666 ($C_{11}H_7D_9O_3$ requires 205.1664); IR ν_{max} cm^{-1}: 2220 (C–D), 1675 (C=O); ^{1}H NMR (360 MHz, $CDCl_3$): δ6.25 (*s*, 1H, =CH), 3.94 (*s*, 4H, ketal), 2.01 (*s*, 2H, CH_2); ^{13}C NMR (90 MHz, $CDCl_3$): δ204.1 (C=O, C-1), 139.6

(C=), 135.3 (=C), 103.7 ($C(OCH_2)_2$), 64.5 (OCH_2), 46.0 (CH_2, C-5), 37.2 (C-6).

Methyl [7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-*nonadeutero abscisate* (**9**). Following the experimental procedure previously published using similar ring system [7, 8], the monoketal of d_9-oxoisophorone (**8**) was elaborated to methyl [7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-d_9-abscisate (**9**). HRIEMS: $[M]^+$ at m/z 287.2073 ($C_{16}H_{13}D_9O_4$ requires 287.2083); mp = 94.5–96.5; IR ν_{max} cm^{-1}: 3600 (OH), 2220 (C–D), 1705 (C=O, ester), 1660 (C=O, enone); ^1H NMR (360 MHz, $CDCl_3$): δ7.85 (*d*, 1H, J = 16.0 Hz, C-5H), 6.13 (*d*, 1H, J = 16.0 Hz, C-4H), 5.92 (*s*, 1H, C-2H), 5.74 (*s*, 1H, C3′-H), 3.69 (*s*, 3H, $COOCH_3$), 2.45 (*d*, 1H, J = 17.1 Hz, CHH), 2.26 (*d*, 1H, J = 17.1 Hz, CHH), 1.99 (*d*, 3H, J = CCH_3), 1.57 (*s*, 1H, OH). The two enantiomers were separable on a preparative Chiracel OD column [9] and showed the following rotations: $[\alpha]_D^{20}$ = +400.1 [MeOH, *c* 1.86] and $[\alpha]_D^{20}$ = −406.0 [MeOH, *c* 1.82].

(+)-[7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-*Nonadeuteroabscisic acid* (**4**). (+)-Methyl [7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′]-d_9-abscisate (**9**) (63 mg, 0.22 mmol) was dissolved in 25 drops of methanol. Phosphate buffer (potassium phosphate, 0.1 M, pH 7.5, 2.5 ml) was added followed by pig liver esterase (EC3-1-1, Sigma, suspension in 3.2 M $(NH_4)_2SO_4$, 0.3 ml) and 1 M KOH (3 drops). The mixture was stirred at room temp. for 24 hr, at which time the mixture was acidified with 10% HCl and extracted into ethyl acetate. The organics were washed with water, saturated NaCl, dried over Na_2SO_4 and concd. The crude product was purified by prep. TLC (silica gel 60 F254, 1 mm × 20 cm × 20 cm, tolune–ethyl acetate–acetic acid = 25:15:2) to give 55 mg (92% yield) or (+)-(7′, 7′, 7′, 8′, 8′, 8′, 9′, 9′, 9′-d_9)-abscisic acid (**4**). HREIMS: $[M]^+$ at m/z 273.1933 ($C_{15}H_{11}D_9O_4$ requires 273.1927); IR ν_{max} cm^{-1}: 3000 (*br*, OH), 2220 (C–D), 1670 (C=O); ^1H NMR (360 MHz, $CDCl_3$): δ7.77 (*d*, 1H, J = 16.3 Hz, C-5H), 6.24 (*d*, 1H, J = 16.0 Hz, C-4H), 5.91 (*s*, 1H, C-2H), 5.74 (*s*, 1H, C3′-H), 2.52 (*d*, 1H, J = 16.9 Hz, CHH), 2.17 (*d*, 1H, J = 16.8 Hz, CHH), 2.03 (*d*, 3H, J = 1 Hz, $=CCH_3$); $[\alpha]_D^{20}$ = +393.6 [MeOH, *c* 0.5%].

Metabolism experiment

Maize cell suspension cultures (*Zea mays* L. cv Black Mexican Sweet) were incubated at 25° in medium containing 0, 25, 50, or 100 μM (+)-ABA or (+)-d_9-ABA as previously described [4]. Cultures were set up in duplicate for each concentration, and the experiment using 100 μM solutions was performed three times. Cell-free blanks containing (+)-ABA or the deuterated analogue were included to check for compound stability. The culture media were sampled in duplicate at each indicated time point over 4 days and the compositions were analysed by HPLC (Hisep column) as previously described [4].

Identification of metabolites. A cartridge containing 1 g Amberlite XAD-2 resin (Alltech, Extract-clean) was conditioned by eluting sequentially with 3 column volumes of the following solvents: *i*-PrOH, *i*-PrOH–H_2O (3:1, v/v), *i*-PrOH–H_2O (1:1, v/v), *i*-PrOH–H_2O (1:3, v/v), and 1% q. HOAc. The filtrate (approx. 5 ml) from a culture incubated with 100 μM (+)-d_9-ABA for 4 days was run through the cartridge, which was then eluted with 1% aq. HOAc (3 column heights) followed by *i*-PrOH (5 column heights). The eluant was analysed by HPLC (Hisep column) for the presence of ABA-type compounds [4]. The fractions containing PA were combined and concentrated under aspirator pressure at 40°. The residue was dissolved in CH_3CN–1% aq. HOAc (1:3, v/v) (approx. 1 ml). HPLC showed there were about 90% PA and 10% ABA (by peak area). Analyses by LC/CFSIMS [11] indicated the PA had a molecular weight of 288 (i.e. d_8-PA) and the unmetabolized ABA had molecular weight of 273 (i.e. d_9-ABA).

Germination inhibition experiment

Cress seeds (*Lepidium sativum* L.) were treated with (+)-ABA and (+)-d_9-ABA 25° in the dark as previously reported [5]. The experiments were replicated three times, with 100 seeds per 9 cm Petri dish. The seeds were placed on two layers of Whatman no. 1 filter paper pre-wetted with 3 ml of aqueous solution containing 0, 0.01, 0.1, 1, 10 or 100 μM of either (+)-ABA or (+)-d_9-ABA. The compounds were initially dissolved in acetone and made up to volume with a final acetone concentration of 0.1%. The control also contained 0.1% acetone. The time taken for both radicle and shoot emergence was noted every 3 hr or as otherwise noted. Root emergence was defined as the presence of radicles equal to or greater than seed length exhibiting positive geotropism. Shoot emergence was defined as the stage at which the cotyledonary leaves had expanded and the testa was shed.

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