Deuterium-labeled Phaseic Acid and Dihydrophaseic Acids for Internal Standards

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The concentration of abscisic acid in plants is regulated not only by biosynthesis, but also by metabolism. Abscisic acid is metabolized to phaseic acid via 8′-hydroxyabscisic acid, and phaseic acid is then converted to dihydrophaseic acid and its epimer. A quantitative analysis of these metabolites is important as well as that of abscisic acid to understand changes in the concentration of abscisic acid in plants. However, no internal standards of the metabolites suitable for quantitative analysis have been reported. We prepared 7′-deuterium-labeled phaseic acid with a deuterium content of 86%, using the equilibrium reaction between phaseic acid and 8′-hydroxyabscisic acid. 7′-Deuterium-labeled dihydrophaseic acids were obtained by reducing 7′-deuterium-labeled phaseic acid. The levels of the metabolites in plant organs were determined by using the deuterated metabolites as internal standards.

Key words: deuterium labeling; abscisic acid; 8′-hydroxyabscisic acid; phaseic acid; dihydrophaseic acid

Abscisic acid (ABA, 1) regulates the physiological adaptation to environmental stress such as water deficiency, osmotic stress and low temperature, and embryonic development.1,2 The concentration of ABA in plant tissues change under stress and during physiological processes.3,4 The change is regulated not only by biosynthesis, but also by metabolism. The metabolism of ABA is mainly classified into two types, ring modification and conjugation.4,5 The former can intrinsically inactivate ABA, while the latter may be involved in the transportation or storage of ABA. In the ring-modification pathway, C-8′ of ABA is hydroxylated by 8′-hydroxylase to form 8′-hydroxy-ABA (2) (Fig. 1).6 8′-Hydroxy-ABA is easily isomerized to phaseic acid (PA, 3) by the intramolecular conjugated addition of 8′-oxygen to C-2′. This isomerization is probably catalyzed by an enzyme in plants,6 but occurs spontaneously in vitro. The isomerizing process has been precisely examined by Todoroki et al.7 Isomerization is an equilibrium reaction, and the final ratio of 8′-hydroxy-ABA to PA is 2:98 at 25°C, indicating a difference in free energy of 2.3 kcal/mole. Isomerization proceeds faster under alkaline conditions than acidic conditions, and the activation energy is 24.5 kcal/mole at 25°C and at pH 3. PA is metabolized to dihydrophaseic acid (DPA, 4) and epi-DPA (5) by reduction of the 4′-carbonyl group. Further metabolism after DPA and epi-DPA is unknown. DPA and epi-DPA may be end products of ABA, although the conjugation of DPA and epi-DPA with glucose occurs.

A quantitative analysis of these metabolites is necessary to understand the changes in the concentration of ABA in plants upon environmental stress and during physiological processes. Internal standards are essential for precise quantification.8,9 [G-3H]PA and [G-3H]DPA, which had been isolated from

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Abbreviations: ABA, abscisic acid; DPA, dihydrophaseic acid; PA, phaseic acid
plants fed with [G-3H]ABA, have been used as internal standards of PA and DPA to calculate the percentage recovery of the metabolites. However, using radioisotopic metabolites as internal standards is not practical. Preparation of the [2,6,6,6-2H4]PA methyl ester for an isotope dilution analysis has been reported by Willows et al., but the recovery and deuterium content were not high. There are therefore no deuterated derivatives suitable as internal standards of the metabolites, and remaining at 270°C.

Deuterium-labeled Phaseic Acid and Dihydrophaseic Acids

Materials and Methods

General experimental procedures. D2O (99.96% deuterium content), 40% NaOD in D2O (99.9% deuterium content), 37% DCl in D2O (99.5% deuterium content) and H218O (99% 18O content) were purchased from Aldrich Chemical Co (Milwaukee, WI, USA). Etheral CH4N2 was prepared from N-methyl-N-nitroso-p-toluenesulfonamide. (+)-ABA was purchased from BAL Planning Corporation (Ichinomiya, Aichi, Japan). NMR spectra were recorded with a Bruker ARX500 instrument (500 MHz for 1H, and 125 MHz for 13C), using TMS as the internal standard. Direct EIMS measurements were carried out with a Jeol JMS-600H mass spectrometer set at an electron potential of 70 eV, the temperature of the direct probe being increased from 30°C to 450°C at a rate of 128°C/min. Quantification of ABA, PA, DPA, and epi-DPA methyl esters was performed with a Shimadzu QP 5000 GC-MS instrument set at an electron potential of 70 eV, with a linear He flow of 50.2 cm/sec. The column temperature of the step gradient was 60°C for 2 min, this being increased from 60°C to 270°C at 10°C/min and remaining at 270°C for 35 min. The column was a CP-Si 5CB type (Shimadzu Chrompack, 25 m length × 0.25 mm i.d., 0.25 μm film thickness). Quantitative analyses were performed in the selected ion monitoring mode (SIM).

Preparation of PA. The β-hydroxy-β-methyl-glutaryl ester of 8′-hydroxy-ABA was isolated from immature seeds of Robinia pseudo-acacia L. according to the method of Hirai et al. The ester (43 mg) was dissolved in a mixture of 1 ml of MeOH and 2 ml of a 2 M NaOH aqueous solution, and left at room temperature for 5 h. The solution was diluted with 20 ml of H2O, and partitioned with 10 ml of EtOAc four times at pH 2. The organic layer was washed with H2O, dried over Na2SO4, and filtered. The filtrate was concentrated and then subjected to silica gel (13 g) chromatography, using different mixtures of toluene and EtOAc as the eluent. The materials eluted with 50% and 60% EtOAc were combined and concentrated to give PA (31 mg). NMR δH (CD3OD): 1.01 (3H, s, H-9′), 1.21 (3H, s, H-7′), 2.07 (3H, d, J = 1.0 Hz, H-6), 2.38 (1H, dd, J = 18.0 and 2.4 Hz, H-5′pro-R), 2.47 (1H, dd, J = 17.9 and 2.4 Hz, H-3′pro-S), 2.71 (1H, dd, J = 18.0 and 2.8 Hz, H-5′pro-S), 2.80 (1H, d, J = 17.9 Hz, H-3′pro-R), 3.66 (1H, d, J = 7.6 Hz, H-8′pro-S), 3.94 (1H, dd, J = 7.6 and 2.8 Hz, H-8′pro-R), 5.78 (1H, brs, H-2′), 6.46 (1H, d, J = 15.9 Hz, H-5), 8.10 (1H, d, J = 15.9 Hz, H-4). NMR δC (CD3OD): 15.8 (C-9′), 19.4 (C-7′), 21.2 (C-6), 49.6 (C-6′), 53.2 (C-5′), 54.0 (C-3′), 78.6 (C-8′), 83.0 (C-1′), 87.8 (C-2′), 119.8 (C-2), 132.9 (C-4), 133.6 (C-5), 151.2 (C-3), 169.5 (C-1), 210.9 (C-4′). Assignment of the protons and carbons of PA was first confirmed by using the HMQC and HMBE spectra.

Preparation of [3′,3′,5′,5′-2H4]PA (PA-d2). PA (17 mg) was dissolved in 1.8 ml of 0.1 M NaOD and then kept at room temperature for 4 h in darkness. After acidification to pH 2 with 1 M DCl, the solution was diluted with D2O to 5 ml, and partitioned with 3 ml of dry EtOAc four times. The organic layers were combined, washed with 0.2 ml of D2O, dried over Na2SO4, filtered, and concentrated to give a pale yellow powder (19 mg). This powder was chromatographed on silica gel (3.8 g), using different mixtures of toluene and EtOAc as the eluent. The materials eluted with 50% and 70% EtOAc were combined and concentrated to give PA-d2 (15 mg). NMR δH (CD3OD): 1.01 (3H, s), 1.21 (3H, s), 2.07 (3H, d, J = 1.0 Hz), 3.66 (1H, d, J = 7.6 Hz), 3.93 (1H, d, J = 7.6 Hz), 5.78 (1H, brs), 6.47 (1H, d, J = 15.9 Hz), 8.10 (1H, d, J = 15.9 Hz). The deuterium contents at C-3′ and 5′ calculated from the signal integral were 99%. Five milliliters of etheral CH2N2 was added to 1 ml of MeOH containing PA-d2 (10 mg). After 2 h at room temperature, the solution was concentrated to give the methyl ester of PA (10 mg) as colorless oil. NMR δH (CDCl3): 1.04 (3H, s), 1.25 (3H, s), 2.02 (3H, d, J = 1.0 Hz), 2.48 (1H, dd, J = 18.4 and 1.6 Hz), 2.56 (1H, dd, J = 18.4 and 2.6 Hz), 2.62 (1H, d, J = 18.4 Hz), 2.66 (1H, dd, J = 18.4 and 1.6 Hz), 3.73 (3H, s), 3.78 (1H, d, J = 18.1 Hz), 3.98 (1H, dd, J = 18.1 and 2.6 Hz), 5.80 (1H, brs), 6.24 (1H, d, J = 15.9 Hz), 8.17 (1H, d, J = 15.9 Hz). EIMS m/z (rel. int.): 294 [M]+ (32), 276
[M-H₂O]⁺ (22), 263 (16), 244 (13), 233 (12), 219 (12), 204 (11), 177 (27), 167 (36), 163 (37), 154 (40), 139 (43), 135 (37), 125 (100), 122 (90), 121 (69).

Preparation of the [4⁻¹⁸O]PA methyl ester. The methyl ester (1 mg) of PA was dissolved in 20 µl of acetic anhydride, and 100 µl of H₂¹⁸O was added to the solution. The solution was heated at 45°C in a sealed flask. After 4 h, the solution was concentrated to give PA as a white solid.

Preparation of [7⁻¹⁸O]PA methyl ester. (1 mg) was dissolved in 1 ml of 0.2M NaOH and then the solution was acidified to pH 2 with 1M HCl, diluted acetic anhydride, and 100 µl of toluene to give the [4⁻¹⁸O]PA methyl ester (1 mg). EIMS m/z (rel. int.): 296 [M⁺ + 2]⁻ (33), 294 [M⁺]⁻ (1), 278 (8), 276 (23), 265 (16), 177 (28), 169 (38), 163 (47), 154 (45), 141 (53), 135 (45), 127 (92), 125 (62), 122 (100), 121 (82). The ¹⁸O content, calculated from the relative intensity of the [M⁺]⁻ and [M⁺]⁺ ions, was 96%. The [4⁻¹⁸O]PA methyl ester (1 mg) was dissolved in 1 ml of 0.2M NaOH and left at room temperature for 30 min. The organic layers were filtered, washed with a small amount of H₂O, dried over Na₂SO₄, and concentrated to give PA as a white solid. Etheral CH₃N₂ (1 ml) was added to 0.5 ml of MeOH solution of the solid to give the PA methyl ester.

Preparation of [7⁻¹⁸O,7⁻²H]JP A (PA-d₃). PA (10 mg) dissolved in 1.0 ml of 0.1 M NaOD was put into an NMR tube and kept at room temperature for 26 days in darkness. ¹H-NMR spectra of the solution were measured at the time intervals shown in Fig. 3. The deuterium contents calculated from the signal integral were 45% for C-3 pro-R, 97% for C-3 pro-S, 98% for C-5 pro-R, 97% for C-5 pro-S, and 87% for C-7⁻. The solution was acidified to pH 2 with 1 M HCl, diluted to 8 ml with H₂O, and partitioned four times with 4 ml of EtOAc. The organic layers were combined, washed with a small amount of H₂O, dried over Na₂SO₄, filtered, and concentrated to give PA-d₃ as a colorless solid (3 mg). NMR δH (CD₂OD): 1.01 (3H, s), 1.18, 1.19 and 1.21 (0.43 H in total, brs for 7⁻D₃H, brs for 7⁻DH₂ and s for 7⁻H, respectively), 2.07 (3H, s), 2.38 (1H, dd, J = 18.0 and 2.2 Hz), 2.46 (1H, dd, J = 18.0 and 2.2 Hz), 2.71 (1H, brd, J = 18.0 Hz), 2.75 (1H, d, J = 18.0 Hz), 3.66 (1H, d, J = 7.6 Hz), 3.94 (1H, dd, J = 7.6 and 2.7 Hz), 5.94 (1H, brs), 6.46 (1H, d, J = 15.9 Hz), 8.10 (1H, d, J = 15.9 Hz). The deuterium content at C-7⁻ calculated from the signal integral was 86%. PA-d₃ (5.5 mg) was dissolved in 0.5 ml of MeOH, and 2 ml of ethereal CH₃N₂ was then added to the solution. The solution was left at room temperature for 10 min, and concentrated to give the methyl ester of PA-d₃ as colorless oil (5.5 mg). NMR δH (CDCl₃): 1.05 (3H, s), 1.15, 1.17 and 1.17 (0.43H in total, brs for 7⁻DH₂, brs for 7⁻DH₂ and s for 7⁻H, respectively), 2.02 (3H, d, J = 1.2 Hz), 2.48 (1H, dd, J = 18.5 and 1.7 Hz), 2.56 (1H, dd, J = 18.5 and 2.7 Hz), 2.61 (1H, d, J = 18.5 Hz), 2.66 (1H, dd, J = 18.5 and 1.7 Hz), 3.73 (3H, s), 3.78 (1H, d, J = 18.1 Hz), 3.97 (1H, dd, J = 18.1 and 2.7 Hz), 5.80 (1H, brs), 6.23 (1H, d, J = 15.9 Hz), 8.17 (1H, d, J = 15.9 Hz). EIMS m/z (rel. int.): 297 [M+1]⁺ (37), 296 [M+1]⁺ (15), 295 [M+1]⁺ (2), 294 [M+1]⁺ (1), 279 [M+2-H₂O]⁺ (26), 265 (18), 247 (15), 233 (15), 219 (20), 204 (16), 177 (32), 170 (32), 163 (42), 154 (46), 142 (46), 135 (39), 125 (63), 122 (100), 121 (74). The deuterium content at C-7⁻ calculated from the ¹H-NMR spectrum and the total deuterium content calculated from the mass spectrum were both 86%.

Stability of the deuterium label of PA-d₃. PA-d₃ (0.1 mg) dissolved in 0.1 ml of MeOH was added to 2 ml each of a 50 mM buffer solution, NaOAc–HCl for pH 3 and pH 5, and Na₂HPO₄–NaH₂PO₄ for pH 7 and 8, before being kept at 25°C for 3 h. The solutions were partitioned with 2 ml of EtOAc three times at pH 3. The organic layers were washed with a small amount of H₂O, dried over Na₂SO₄, and filtered. The filtered solutions were concentrated and then dissolved in 0.1 ml of MeOH and 0.2 ml of ethereal CH₃N₂ to give methyl esters. The methyl esters were analyzed by EIMS to calculate the deuterium contents.

Preparation of [7⁻¹⁸O,7⁻²H]JPDPA (DA-d₃) and [7⁻¹⁸O,7⁻²H]epi-DPA (epi-DPA-d₃). The PA-d₃ methyl ester was reduced according to the method of Milborrow with a minor modification. The PA-d₃ methyl ester (5.5 mg) was dissolved in a mixture of 1.0 ml of MeOH and 0.5 ml of H₂O. The solution was cooled to 0°C, and 3 mg of NaBH₄ was added. After 40 min, the solution was diluted with 5 ml of H₂O and partitioned four times with 4 ml of EtOAc. The organic layers were combined, washed with a small amount of H₂O, dried over Na₂SO₄, and concentrated to give a colorless solid (5 mg). This solid was dissolved in 0.5 ml of MeOH, and 1.5 ml of 1 M NaOH was added to the solution. After 2 h at room temperature, the solution was acidified to pH 2, diluted with 6 ml of H₂O, and partitioned five times with 3 ml of EtOAc. The organic layers were combined, washed with a small amount of H₂O, dried over Na₂SO₄, filtered, and concentrated to give a colorless solid (4 mg). This solid was dissolved in 0.2 ml of MeOH and subjected to preparative HPLC under the
following conditions: column, YMC AQ-311 (ODS, 100 mm length × 6 mm i.d.); solvent, 40% MeOH in H2O containing 0.1% AcOH; flow rate, 1.0 ml/min; detection at 254 nm. The materials eluted at tR 4.9 min and 8.1 min were separately collected and concentrated to respectively give DPA-d6 (1.2 mg) and epi-DPA-d3 (2.3 mg). NMR of DPA-d6 δH (CD3OD): 0.93 (3H, s), 1.11, 1.13 and 1.15 (0.41H in total, brs for 7'-D2H, brs for 7'-D2H; and s for 7'-H), respectively, 1.66 (1H, brt, J = 13.6 Hz), 1.73 (1H, dd, J = 13.6 and 10.4 Hz), 1.81 (1H, dd, J = 13.6 and 7.0 Hz), 2.03 (1H, dd, J = 13.6 and 7.0 Hz), 2.07 (3H, s), 3.71 (1H, d, J = 7.4 Hz), 4.11 (1H, m), 5.77 (1H, brs), 6.48 (1H, d, J = 15.9 Hz), 7.95 (1H, d, J = 15.9 Hz). The deuterium content at C-7 was calculated from the signal integral and the total deuterium content calculated from the relative intensity of the molecular ions were 85% and 86%, respectively.

Changes in the deuterium content of PA-d3, DPA-d3, and epi-DPA-d3 after extraction, purification and methylation. The same method as that used for the quantitative analysis without a plant extract was applied. The method of the quantitative analysis is described later. Five micrograms each of PA-d3, DPA-d3, and epi-DPA-d3, and the experiment was conducted three times.

Stability of the deuterium label of ABA-d6. ABA-d6 was prepared according to the method of Netting et al. with some modification.19) (+)-ABA-d6 (12 mg) was dissolved in 1.2 ml of a 0.1 m NaOD D2O solution and kept at 25°C for 2 days. The solution was diluted with 4 ml of D2O and partitioned with EtOAc five times at pH 2. The organic layers were combined, washed, dried over Na2SO4, filtered, and concentrated to give (+)-ABA-d6 (11 mg). (+)-ABA-d6 (2 mg) was dissolved in 0.5 ml of MeOH and 3 ml of ethereal CH2N2 and then left at room temperature for 10 min. The solution was concentrated to give the methyl ester of (+)-ABA-d6 d3 (2 mg). (+)-ABA-d6 d3 (1 mg) dissolved in 0.1 ml of MeOH was added to buffer solutions at pH 3, 5, 7 and 8, and then treated by the same method as that used for PA-d3. The deuterium contents were determined from the 'H-NMR spectra.

Quantitative analysis of ABA and the ABA metabolites in plant organs. Three 10-year-old 'Ohrin' apple trees [Malus sylvestris (L.) Mill. var. domestica (Borkh.) Mansf.] were selected at random at Hiroshima Prefectural University, and samples of the fruit, leaves, and buds were collected on Nov. 6, 2002. Samples were also collected from three randomly selected 10-year-old 'Ishiji Unshu' satsuma mandarin trees [Citrus unshiu Marc.] on Nov. 6, 2002 at the Hiroshima Prefectural Agricultural Research Center. The fruit, leaves, and buds (10 g fresh weight) were soaked in 80% MeOH, and 0.2 μg each of ABA-d6, PA-d6, DPA-d3, and epi-DPA-d3 was added to the solution as an internal standard. The extract was passed through a glass fiber filter, and then concentrated to an aqueous solution in vacuo. After adjusting to pH 2.5 with 0.1 m H2PO4, the solution was extracted with CH2Cl2. The CH2Cl2 extract was dried in vacuo, and the residue was dissolved in 1 ml of 25% CH3CN before being subjected to preparative HPLC (column, ODS Mightysil RP-18, 250 mm length × 4.6 mm i.d.; solvent, 25% to 50% CH3CN in H2O containing 0.1% AcOH over a period of 30 min and then held at 50% for 5 min; flow rate, 1.5 ml/min; detection, 254 nm). The fractions containing ABA, PA, DPA,
and epi-DPA were collected, dried in vacuo and methylated with ethereal CH$_2$N$_2$ for 15 min. The methylated materials were dissolved in 30 μl of MeOH and then analyzed by GC-MS. The methyl esters of ABA and its metabolites had the following retention times: ABA methyl ester, 19.52 min; PA methyl ester, 20.25 min; DPA methyl ester, 20.75 min; epi-DPA methyl ester, 20.60 min. The ions were measured as follows: ABA-d$_4$ methyl ester/ABA-d$_4$, methyl ester as m/z 190, 260, 194, and 264; PA-d$_6$ methyl ester/PA-d$_6$ methyl ester as m/z 276, 294, 279, and 297; DPA-d$_6$ methyl ester/DPA-d$_6$ methyl ester as m/z 278, 296, 281, and 299; and epi-DPA-d$_6$ methyl ester/epi-DPA-d$_6$ methyl ester as m/z 278, 296, 281, and 299. The ABA concentrations were derived from the ratio of peak areas for m/z 190 (d$_4$) /194 (d$_3$), the PA concentrations were derived from the ratio of peak areas for m/z 276 (d$_6$)/279 (d$_5$), and the DPA and epi-DPA concentrations were derived from those for m/z 278 (d$_6$)/281 (d$_7$). Identification of the ABA, PA, DPA and epi-DPA methyl esters in the samples was achieved by comparing the fragmentation patterns with the standards in the total ion monitoring mode.

**Results and Discussion**

Preparation of PA-d$_6$, DPA-d$_6$ and epi-DPA-d$_6$

The chemical synthesis of PA has been reported by three research groups.\textsuperscript{19–22} Deuterium can be introduced at C-3\textsuperscript{o} of PA by a treatment with sodium deuteroxide. The chemical synthesis of PA has been reported possessing deuterium at C-3\textsuperscript{o} by a treatment with sodium deuteroxide. The alcoholic deuterium was completed within 30 min to give PA-d$_6$. The substitution of four hydrogens at C-3\textsuperscript{o} and -5' of PA with sodium deuteroxide. The substitution of four hydrogens at C-3\textsuperscript{o} and -5' with deuterium was completed within 30 min to give PA-d$_6$. However, most all of the deuterium of PA-d$_6$ was lost upon methylation by diazomethane. This loss of deuterium could be explained by the nucleophility of diazomethane. Diazomethane abstracted a deuterium ion at C-3\textsuperscript{o} or -5' of PA-d$_6$, and the resulting carbonium ion abstracted a water molecule from a hydroxyl group of methanol that was used as the solvent. A repeat of the reaction would substitute four deuteriums of PA-d$_6$ with hydrogen. Methylation by diazomethane is necessary for a quantitative analysis by GC-MS, meaning that PA-d$_6$ would not be suitable as an internal standard. The stability of deuteration at C-3\textsuperscript{o} and -5' of PA-d$_6$ upon methylation is described later. Labeling of the 4\textsuperscript{o}-oxygen of PA with $^{18}$O was next attempted. The methyl ester of [4\textsuperscript{o}-$^{18}$O]PA was obtained by a treatment with H$_2$O in the presence of acetic anhydride, but 4\textsuperscript{o}-$^{18}$O was easily substituted with $^{18}$O during alkaline hydrolysis in H$_2$O. These results suggested that a short-term treatment with labeling reagents resulted in easy loss of the labels.

Finally, the introduction of deuterium at C-7\textsuperscript{o} of PA was attempted (Fig. 2). A PA sample contains 8\textsuperscript{o}-hydroxy-ABA at 2\% of its weight due to equilibrium between PA and 8\textsuperscript{o}-hydroxy-ABA. 8\textsuperscript{o}-Hydroxy-ABA has an enone group as well as ABA, suggesting that deuteration could be introduced at C-3\textsuperscript{o}, -5', -7', -1'O and -8'O of 8\textsuperscript{o}-hydroxy-ABA by a treatment with sodium deuteroxide to give 8\textsuperscript{o}-hydroxy-ABA-d$_6$, 8\textsuperscript{o}-Hydroxy-ABA-d$_8$ would be isomerized to PA-d$_6$ possessing deuterium at C-3\textsuperscript{o}, -5', -7' and -1'O in a sodium deuteroxide solution. The alcohlic deuterium at 1'O would then be exchanged with hydrogen during extraction by partitioning between ethyl acetate and water to give PA-d$_6$. Short-term treatment of PA-d$_6$ with sodium hydroxide would substitute four deuteriums at C-3\textsuperscript{o} and -5' with hydrogen, and the remaining three deuteriums at C-7' to give PA-d$_5$. The substitution of three hydrogens at C-7' of PA with deuterium was expected to take a long time, since the amount of 8\textsuperscript{o}-hydroxy-ABA in a PA sample was very low. Figure 3 shows changes in the deuteration content at C-7' of PA dissolved in a sodium deuteroxide solution, as monitored by measurement of the $^1$H-NMR spectra. The deuteration
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Changes in Deuterium Contents at C-3', -5' and -7' of ABA-\(d_6\) after Methylation, and after Incubation at Various pH Values

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Deuterium content (%)</th>
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<tbody>
<tr>
<td>None</td>
<td>96 98 98 95 84</td>
</tr>
<tr>
<td>Methylation</td>
<td>96 96 96 95 84</td>
</tr>
<tr>
<td>pH 3</td>
<td>96 97 97 95 86</td>
</tr>
<tr>
<td>pH 5</td>
<td>96 96 97 95 85</td>
</tr>
<tr>
<td>pH 7</td>
<td>96 96 96 95 84</td>
</tr>
<tr>
<td>pH 8</td>
<td>96 96 96 95 84</td>
</tr>
</tbody>
</table>

**Stability of the deuterium label of ABA-\(d_6\)**

Loss of the deuterium labels of PA-\(d_4\) during methylation by diazomethane cast doubt on the stability of the deuterium labels of ABA-\(d_6\), although precise data on this stability have not been reported.\(^{20}\) Changes in the deuterium content at C-3', -5' and -7' of ABA-\(d_6\) were examined before and after methylation by diazomethane. The content at C-5' \(-d_pro-R\) and -7' \(-d_pro-S\) decreased from 98% to 96%, but that at C-3' and -7' did not change (Table 1). This result shows that methylation by diazomethane decreased the deuterium content at C-5', like the case of PA-\(d_4\). However, this decrease was much smaller than that of PA-\(d_4\) and would not affect the quantitative analysis. The stability of the 5'-deuterium of ABA-\(d_6\) was due to the presence of a 2'-double bond. This double bond would increase \(pK_a\) of 5'-hydrogen by reducing the electron-withdrawing effect of the 4'-carbonyl group through the delocalization of an electron. The stability of the deuterium labels of ABA-\(d_6\) in aqueous solutions at pH 3–8 was also examined. The decrease in deuterium content at C-5' was small, while the deuterium contents at C-3' and -7' were not affected. These results confirm that the deuterium labels of ABA-\(d_6\) had sufficient stability as an internal standard for the quantitative analysis.

**Quantitative analysis of ABA and the ABA metabolites in plants using the internal standards**

The contents of ABA and its metabolites in fruit, leaves, and buds of apples and satsuma mandarins sampled in November 2002 were assayed by using ABA-\(d_6\), PA-\(d_4\), DPA-\(d_3\), and epi-DPA-\(d_3\) as internal standards. The results are shown in Table 2.

In apple, the amount of ABA in the buds was higher than that in the fruits and leaves. The buds of apples, which come from deciduous fruit trees, exhibit deep dormancy in November.\(^{20}\) The high content of ABA in the buds is consistent with the involvement of ABA in dormancy.\(^{20}\) The level of PA was highest in the buds, as for ABA, suggesting that not only the biosynthesis but also the metabolism of ABA was active. The level of epi-DPA was higher in the three organs than that of DPA, and was higher in the fruits than the level of PA. This finding shows...
that, not DPA, but epi-DPA was the major metabolite of PA, although epi-DPA has been studied less than DPA. The variation in the metabolite contents of the three organs implies that the regulation of ABA metabolism was different among the organs.

In satsuma mandarins, the level of ABA was highest in the fruits. ABA in the fruits would be necessary for attracting the sugars formed by photosynthesis in the leaves. In non-climacteric fruit such as grape berries and satsuma mandarins, the ABA level increases toward harvest. The high fruit such as grape berries and satsuma mandarins, photosynthesis in the leaves. In non-climacteric highest in the fruits. ABA in the fruits would be of the three organs implies that the regulation of metabolism in plants.

The quantitative analysis of the ABA metabolites using trideuterated derivatives as internal standards would reveal the precise regulation of ABA metabolism in plants.

References


Table 2. Contents of ABA and Its Metabolites in Apple and Satsuma Mandarin (nmol/kg fresh weight)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Apple</th>
<th>Satsuma mandarin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fruit</td>
<td>Leaf</td>
</tr>
<tr>
<td>ABA</td>
<td>1.88±0.03</td>
<td>0.23±0.05</td>
</tr>
<tr>
<td>PA</td>
<td>0.05±0.01</td>
<td>0.32±0.07</td>
</tr>
<tr>
<td>DPA</td>
<td>0.01±0.00</td>
<td>0.09±0.01</td>
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<tr>
<td>epi-DPA</td>
<td>1.65±0.53</td>
<td>0.26±0.04</td>
</tr>
</tbody>
</table>
Deuterium-labeled Phaseic Acid and Dihydrophaseic Acids


