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Abscisic Acid Biosynthesis in Leaves and Roots of *Xanthium strumarium*[1]

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ABSTRACT

Research on the biosynthesis of abscisic acid (ABA) has focused primarily on two pathways: (a) the direct pathway from farnesyl pyrophosphate, and (b) the indirect pathway involving a carotenoid precursor. We have investigated which biosynthetic pathway is operating in turgid and stressed *Xanthium* leaves, and in stressed *Xanthium* roots using long-term incubations in $^{18}O_2$. It was found that in stressed leaves three atoms of ^{18}O from $^{18}O_2$ are incorporated into the ABA molecule, and that the amount of ^{18}O incorporated increases with time. One ^{18}O atom is incorporated rapidly into the carboxyl group of ABA, whereas the other two atoms are very slowly incorporated into the ring oxygens. The fourth oxygen atom in the carboxyl group of ABA is derived from water. ABA from stressed roots of *Xanthium* incubated in $^{18}O_2$ shows a labeling pattern similar to that of ABA in stressed leaves, but with incorporation of more ^{18}O into the tertiary hydroxyl group at C-1′ after 6 and 12 hours than found in ABA from stressed leaves. It is proposed that the precursors to stress-induced ABA are xanthophylls, and that a xanthophyll lacking an oxygen function at C-6 (carotenoid numbering scheme) plays a crucial role in ABA biosynthesis in *Xanthium* roots. In turgid *Xanthium* leaves, ^{18}O is incorporated into ABA to a much lesser extent than it is in stressed leaves, whereas exogenously applied ^{14}C-ABA is completely catabolized within 48 hours. This suggests that ABA in turgid leaves is either (a) made via a biosynthetic pathway which is different from the one in stressed leaves, or (b) has a half-life on the order of days as compared with a half-life of 15.5 hours in water-stressed *Xanthium* leaves. Phaseic acid showed a labeling pattern similar to that of ABA, but with an additional ^{18}O incorporated during 8′-hydroxylation of ABA to phaseic acid.

Research on the biosynthesis of ABA has focused on two pathways: (a) the direct pathway from mevalonic acid via farnesyl pyrophosphate, and (b) the indirect pathway involving a carotenoid precursor, with xanthoxin as an intermediate in the pathway (17, 26). Creelman and Zeevaart (6) presented evidence that one atom of ^{18}O was incorporated into the carboxyl group of ABA isolated from stressed *Xanthium strumarium* and *Phaseolus vulgaris* leaves incubated in the presence of $^{18}O_2$. This supported the hypothesis that ABA is derived from a larger precursor, presumably a carotenoid, by oxidative cleavage of the central polyene chain. In addition, it was shown that conversion of ABA to PA[3] required molecular oxygen, with one atom of ^{18}O being incorporated into the 8′-hydroxyl group of PA (6). Since these experiments were performed using relatively short incubation periods (6 h), stressed *Xanthium* leaves have now been incubated with $^{18}O_2$ for 12 and 24 h to determine if the pattern of ^{18}O incorporation differs under these conditions.

When roots of *Xanthium* are detached and water stressed, they also accumulate ABA, although the levels are lower than in stressed leaves (4). Furthermore, the carotenoid content of roots is much lower than that of leaves: 0.3 and 300 μg/g fresh weight, respectively (5). Roots provide, therefore, an interesting system to further examine the possible role of carotenoids in ABA biosynthesis.

In the present experiments, stressed *Xanthium* leaves and roots were incubated in atmospheres containing 20% $^{18}O_2$ for various times after which the ^{18}O labeling patterns of ABA and PA were determined. Furthermore, $H_2{}^{18}O$ was fed to a *Xanthium* leaf to determine if any of the oxygen atoms in ABA are derived from water.

Turgid *Xanthium* leaves contain low levels of ABA (29). It is not known whether ABA in turgid and stressed leaves is synthesized along the same or different biosynthetic pathways. To answer this question, turgid leaves were incubated in an atmosphere containing $^{18}O_2$ for up to 72 h.

MATERIALS AND METHODS

Plant Material. *Xanthium strumarium* L., Chicago strain, was grown as described (29). The youngest, fully expanded leaf was used in all experiments. For stress experiments, leaves were wilted until they had lost 13% of their fresh weight. They were then placed in an atmosphere of 80% N_2 and 20% $^{18}O_2$ as described (6). Leaves were incubated for 6, 8, 12, and 24 h. In experiments with turgid material, leaves were detached from plants and immediately placed in darkness in an atmosphere of 80% N_2 and 20% $^{18}O_2$ (with their petioles placed in distilled water) for 24, 48, and 72 h.

To determine if any of the oxygen atoms in ABA are derived from water, detached turgid leaves were allowed to take up $H_2{}^{18}O$ via the transpiration stream. They were then stressed and a small leaf disc (3 mm diameter) was removed from each leaf for determination of the isotopic composition of the water present

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[3] Abbreviations: PA, phaseic acid; ABA-GE, β-D-glucopyranosyl abscisate; CI, chemical ionization; DP, direct probe; EI, electron impact; GLC-TCD, gas liquid chromatography-thermal conductivity detector; M^+, positive molecular ion; M^-, negative molecular ion; m/z, mass/charge; Me-ABA, methyl ester of ABA; Me-PA, methyl ester of phaseic acid; NCI, negative chemical ionization.

in the leaf. Each disc was sealed in a small glass tube which had been drawn out into a capillary at the bottom. The tube was heated in a heating block at 80°C. The water that condensed on the glass wall was collected by centrifugation in the capillary for analysis by MS (see below).

[^{14}C]-(+)-ABA (85,000 cpm, 3.2 μg) was dissolved in 0.05 ml ethanol to which 0.45 ml distilled water as added. This solution was fed via the transpiration stream to a turgid *Xanthium* leaf. When the uptake was complete, distilled water was added to the vial and the leaf (with vial) was placed inside a sealed flask under the same conditions as leaves incubated in the presence of $^{18}O_2$.

Roots of *X. strumarium* were grown in solution culture as follows. Burs were soaked in water for 2 d and embryos were removed and placed on moist filter paper in Petri dishes. Two d later, the young seedlings were placed on a bed of soil, and vermiculite was layered over them. After approximately 1 week of growth, the young plants were removed from the soil/vermiculite and suspended from perforated boards over trays containing half-strength Hoagland nutrient solution. The plants were grown in a growth chamber for 2 to 3 weeks, with a change of the nutrient solution every week.

In all experiments, roots were analyzed that had little secondary thickening. For stress experiments, the roots were detached from the plants, cut into approximately 2-cm sections, and stressed by allowing them to lose 50 to 60% of their fresh weight (4). They were placed in an atmosphere of 80% N_2 and 20% $^{18}O_2$ for 6 and 12 h as described above for leaves.

Extraction and Purification of Abscisic Acid and Catabolites. In initial experiments, ABA and PA were extracted, purified, and measured as described (3, 4, 6). In later experiments, particularly those involving roots and turgid leaves with relatively low levels of ABA, an additional purification step was added. The first purification step involved semipreparative reverse phase HPLC of the lyophilized crude extracts on a μBondapak C_{18}, 30 × 0.78 cm column (Waters Associates). Elution was by means of a linear gradient from 10 to 50% ethanol in water with 1% acetic acid in 25 min at a flow rate of 2.5 ml/min. PA and ABA-GE were collected together (22–23.5 min). The fraction containing ABA (28.5–30 min) was dried and methylated with ethereal diazomethane. After drying, ABA-GE was hydrolyzed in 2 N NH_4OH at 60°C for 2 h. The aqueous phase was evaporated, and ABA released from ABA-GE was separated from PA by reverse phase HPLC with a μBondapak C_{18}, 30 × 0.4 cm column. Elution was with a linear gradient from 10 to 60% methanol in water containing 1% acetic acid in 25 min at a flow rate of 2 ml/min. The fractions containing ABA (25.75–27.25 min) and PA (20.0–21.5 min) were collected, dried, and methylated. Me-ABA and Me-PA were further purified by normal phase HPLC with a μPorasil 30 × 0.4 cm column (Waters Associates). Elution was with a gradient of hexane in ethyl acetate from 10 to 60% for Me-ABA (12–13 min), and 20 to 70% for Me-PA (12–13 min), both in 10 min, at 2 ml/min. Quantification of Me-ABA and Me-PA was as described (3).

HPLC-Radiocounting. The crude extract from a *Xanthium* leaf fed [^{14}C]ABA was fractionated via a semipreparative μBondapak C_{18} column (see above) and the effluent was passed through a radioactive flow detector (Flo-One model, RadioAnalytic, Inc., Tampa, FL), using a solid scintillator flow cell.

Oxygen Measurements. To ensure that depletion of O_2 did not occur during the incubations due to respiration, the O_2 concentration was monitored every few hours, and replenished as necessary. O_2 content was measured by GLC-TCD using a Varian 3700 gas chromatograph. Analysis of the gas mixture was performed with a molecular sieve column (5A, 45–60 mesh, 2 m × 1/8 inch stainless steel). Quantification was performed with standards of 0, 2, 20, and 100% O_2 (balance, if any, was N_2). GLC conditions were: oven temperature 65°C, injector temperature 100°C, detector temperature 120°C, detector current 108 mamp, He carrier flow 26 ml/min.

Mass Spectrometry. The isotopic composition of water was determined with a Hewlett-Packard 5985 quadrupole mass spectrometer connected to a 5840A gas chromatograph. GLC conditions were: Tenax-GC 60/80 mesh column (Alltech Associates, Inc., Deerfield, IL) run isothermally at 185°C with the CI valve open (normally closed) and no jet separator used. The pressure in the source was 2×10^{-4} torr (normally $2–3 \times 10^{-6}$). The numbers of ions at m/z 18 and m/z 20 were quantified by selected ion monitoring.

GC-NCI and GC-EI of Me-ABA and Me-PA were performed using a JEOL-HX-110HF double focusing mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph (single stage jet separator), with He as the carrier gas (flow rate 12 ml/min). For GC-NCI, methane was used as the reagent gas (pressure in the source 5×10^{-6} torr). The column used was a DB-1 (15 m × 0.53 mm; J&W Scientific, Inc. Rancho Cordova, CA). GLC conditions were: oven temperature programmed from 180 to 250°C at 5°C/min for Me-ABA, or at 15°C/min for Me-PA.

DP-NCI was performed using a JEOL-HX-110HF double focusing mass spectrometer with a JMA-DA5000 data system using methane as the reagent gas (source pressure 5×10^{-6} torr). The ion source temperature was 115°C with a filament emission current of 300 μA and an ionizing voltage of 70 eV. An aliquot of each sample dissolved in approximately 2 μl of ethyl acetate was applied to the probe which was then inserted into the source and heated from 50 to 250°C at 32°C/min. Sensitivity of the GC/DP-NCI procedure was 1 to 5 ng of Me-ABA or Me-PA. MS-MS analysis of daughter-ions formed from the M^-s of (labeled and unlabeled) Me-ABA was performed on the JEOL HX-110HF double focusing mass spectrometer using constant magnetic field to electric field (B/E) linked scan method with He as the collision gas.

Chemicals. $^{18}O_2$ (99%) was purchased from Stohler Isotope Chemicals, Inc. (Waltham, MA), or Cambridge Isotopes (Woburn, MA). $H_2{}^{18}O$ (50.5%) was purchased from Cambridge Isotopes. [2-^{14}C]-(+)-ABA (4 mCi/mmol) was a gift from Dr. D. C. Walton, SUNY, Syracuse, NY. 4′-[^{18}O]ABA was prepared as described (12).

RESULTS

Labeling Pattern of Abscisic Acid and Catabolites in Stressed Leaves. After incubation in the presence of $^{18}O_2$ for 6 h, the majority of the ABA was labeled with a single ^{18}O atom which was located in the carboxyl group (location determined by the intensity of the ion at m/z 127 with GC-EI [6, 12]; data not shown). In addition, small but significant amounts of ^{18}O were found in both ring oxygens of ABA (Table Ia). With longer incubations, there was a relative increase in M^- 282 and 284 (two and three atoms of ^{18}O incorporated, respectively; Table Ia; Fig. 1). However, M^- 280 remained the base peak at all time points.

Incorporation of ^{18}O into PA was also detected (Table Ic; Fig. 2). In the procedure used here, a sudden accumulation of PA was not induced by a stress-rehydration cycle (6, 29, 30). Thus, any PA formed represents turnover of ABA in the dehydrated leaves (29). It is for this reason that no large $M^- + 2$ was observed (6). Instead, both $M^- + 2$ and $M^- + 4$ gradually increased until after 24 h M^- 298 became the base peak which represents the incorporation of two ^{18}O atoms (Fig. 2; Table Ic). One of these ^{18}O atoms was present in the carboxyl group of PA and was originally incorporated during the synthesis of ABA, while the second one was introduced during 8′-hydroxylation of ABA to PA (6).

Low levels of ^{18}O incorporation were also detected in ABA-

Table I. *Incorporation of ^{18}O into Abscisic Acid, the Glucose Ester of Abscisic Acid, and Phaseic Acid in Stressed Xanthium Leaves*

Detached, stressed *Xanthium* leaves were incubated in darkness in the presence of $^{18}O_2$ for the times indicated. The data shown were obtained by DP-NCI (a), or GC-NCI ([b] and [c]). ABA and ABA-GE were analyzed as Me-ABA and PA as Me-PA. A value of 100% indicates that it was the most prominent ion (base peak). All other ions are relative to the base peak.

Incubation Time	Relative Abundance				
h	*m/z*				
(a) ABA	*278*	*280*		*282*	*284*
0	100	1.8		0	0
6	78.6	100		5.5	1.1
12	35.3	100		7.0	2.0
24	27.0	100		10.3	5.4
(b) ABA-GE					
8	100	23.5		0.8	0
(c) PA	*294*	*296*	*298*	*300*	*302*
0	100	2.1	0	0	0
6	100	31.2	21.6	2.1	1.2
12	100	55.8	92.3	7.2	1.9
24	36.2	34.9	100	9.8	1.2

GE (Table Ib), another catabolite of ABA. Approximately 19% of the total ABA-GA contained a single ^{18}O atom, which was located in the carboxyl group (data not shown). ABA-GE accumulates at a low rate in *Xanthium* leaves during water stress (29, 30), so that little incorporation of ^{18}O-labeled ABA into ABA-GE is to be expected. The present results indicate that this newly synthesized ABA-GE is derived from stress-induced ABA rather than from unlabeled ABA already present prior to the onset of stress.

Labeling Pattern of Abscisic Acid and Phaseic Acid in Stressed Roots. In stressed roots of *Xanthium* over 31% of the ABA contained two atoms of ^{18}O after 6 h incubation in the presence of $^{18}O_2$. This increased to 34% after 12 h (Fig. 1; Table IIa), indicating that little ABA synthesis took place after 6 h. After 12 h, less than 3% of the ABA contained three atoms of ^{18}O. One atom of ^{18}O was located in the carboxyl group. The position of the second ^{18}O atom as determined by tandem MS (MS-MS) using constant magnetic field to electric field (B/E) linked scan analysis of the parent M^- at m/z 282 (Me-ABA containing two atoms of ^{18}O). This technique allows the determination of fragment ions that are formed from a selected ion, in this case M^- at m/z 282. Loss of 20 mass units from this ion (M^--$H_2{}^{18}O$) indicated that the second ^{18}O was located in the hydroxyl group at C-1′ (12). Gray *et al.* (12) suggested that the oxygen atom at the 4′-keto group can also be lost as water. However, when 4′-^{18}O-Me-ABA was analyzed by GC-NCI, only M^--18 was observed which establishes conclusively that dehydration originates from C-1′ only, and not from C-4′. Thus, in Figure 1c, m/e 260 represents dehydrated unlabeled Me-ABA (278-18), whereas m/e 262 originates from Me-ABA labeled in the carboxyl group only (280-18), as well as from Me-ABA labeled in both the carboxyl and 1′-hydroxyl groups (282-20).

PA isolated from stressed roots incubated in the presence of $^{18}O_2$ showed a labeling pattern similar to that found in stressed leaves (Table IIb; Fig. 2), *i.e.* one more ^{18}O was present in PA than in the corresponding ABA. However, in PA from roots M^- + 4 was already the base peak after 6 h incubation in $^{18}O_2$ (Table IIb), whereas in leaves this was not observed until after 24 h (Table Ic). This suggests that the initial pool of PA was considerably smaller in roots than in leaves.

Incorporation of ^{18}O from $H_2{}^{18}O$ into Abscisic Acid during Water Stress. Analysis of the water in small discs taken from a leaf that had absorbed $H_2{}^{18}O$, indicated an ^{18}O enrichment of 21%. Analysis of ABA by GC-NCI isolated from the same leaf following water stress, showed that 17.5% of the total ABA contained one atom of ^{18}O (Table IIIa). It was further determined by GC-EI analysis that the labeled oxygen atom was located in the carboxyl group (Table IIIb). In the leaf labeled with both $H_2{}^{18}O$ and $^{18}O_2$, the ^{18}O enrichment of the water was 19%. Analysis of ABA extracted from this leaf indicated that 75.2% contained one and 13.6% two ^{18}O atoms in the carboxyl group (Table IIIb). When comparing these results with those obtained after labeling with either $H_2{}^{18}O$ or $^{18}O_2$, it follows that in the double-labeling experiment the first ^{18}O atom was derived from $^{18}O_2$ and the second one from $H_2{}^{18}O$ (Table IIIb).

Incorporation of Molecular Oxygen into Abscisic Acid in Turgid Leaves. Analysis by GC-NCI of ABA extracted from turgid leaves incubated in the presence of $^{18}O_2$ indicated that

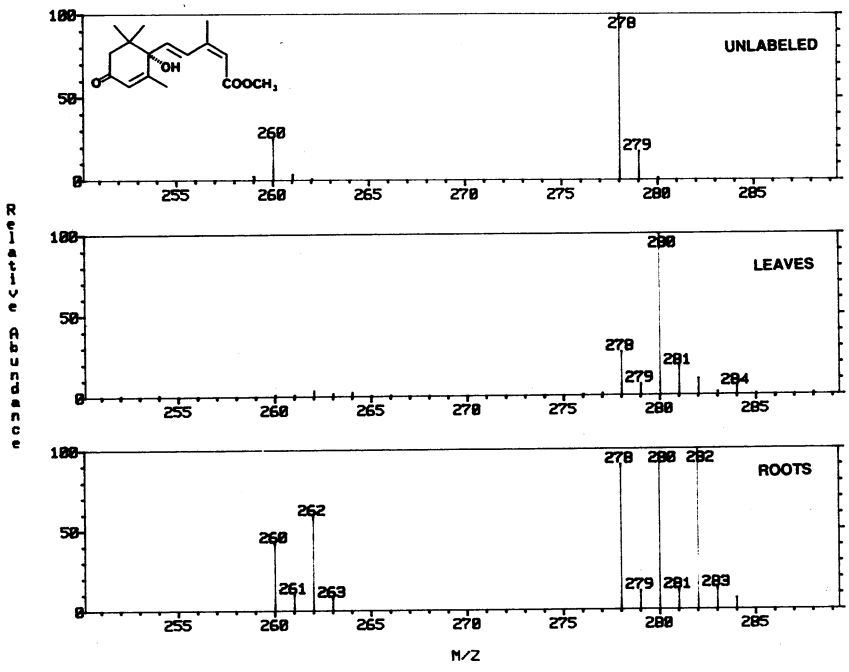

FIG. 1. Mass spectra of methyl abscisate analyzed by negative chemical ionization. Shown are spectra of unlabeled Me-ABA analyzed by GC-NCI (top), Me-ABA (DP-NCI) isolated from stressed *Xanthium* leaves incubated in the presence of $^{18}O_2$ for 24 h (middle), and Me-ABA (GC-NCI) isolated from stressed *Xanthium* roots incubated in the presence of $^{18}O_2$ for 12 h (bottom).

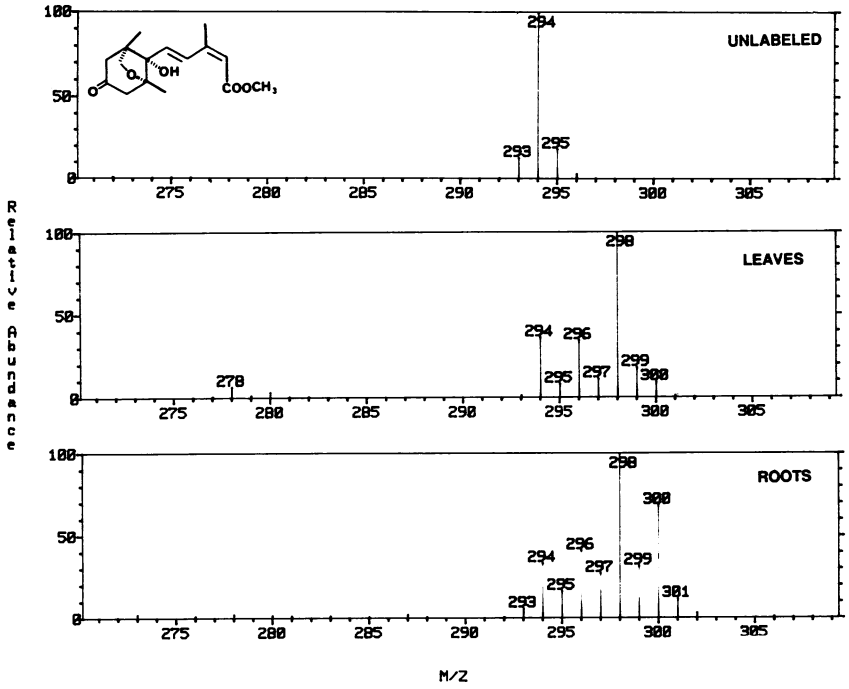

FIG. 2. Mass spectra of methyl phaseate analyzed by negative chemical ionization. Shown are spectra of unlabeled Me-PA analyzed by GC-NCI (top), Me-PA (DP-NCI) isolated from stressed *Xanthium* leaves incubated in the presence of $^{18}O_2$ for 24 h (middle), and Me-PA (GC-NCI) isolated from stressed *Xanthium* roots incubated in the presence of $^{18}O_2$ for 12 h (bottom).

Table II. *Incorporation of ^{18}O into Abscisic Acid and Phaseic Acid in Stressed Xanthium Roots*

Detached, stressed *Xanthium* roots were incubated in darkness in the presence of $^{18}O_2$ for the times indicated. The data shown were obtained by GC-NCI. ABA was analyzed as Me-ABA and PA as Me-PA. A value of 100% indicates that it was the most prominent ion (base peak). All other ions are relative to the base peak.

Incubation Time	Relative Abundance				
h	*m/z*				
(a) ABA	*278*	*280*		*282*	*284*
0	100	1.8		0	0
6	81.5	100		86.1	4.3
12	93.0	95.2		100	7.4
(b) PA	*294*	*296*	*298*	*300*	*302*
0	100	2.1	0	0	0
6	63.7	49.8	100	43.9	1.6
12	33.8	41.4	100	68.7	3.4

very small amounts of ^{18}O were incorporated into ABA and PA (Table IVa). For example, in the case of ABA, the relative intensity of $M^- + 2$ was 3.1% after 24 h, and 5.8% after 72 h. These results suggest that ABA turnover in the turgid leaves under our experimental conditions was very slow. On the other hand, results of a parallel experiment with [^{14}C]ABA contradict this conclusion. When fed via the petiole, [^{14}C]ABA was completely catabolized to PA and conjugates after 48 h (data not shown).

The ABA content of turgid leaves incubated in the presence of $^{18}O_2$ decreased during the first 24 h incubation period from 120 to 75 ng ABA/g fresh weight. This may explain the presence of one ^{18}O in 15% of the PA after 24 h (Table IVb). Since the ^{18}O content of PA was less after 48 h than after 24 h, the newly synthesized PA was apparently further catabolized. Similar results were observed in two separate experiments. No incorporation of ^{18}O into ABA-GE could be detected in turgid leaves at any time.

DISCUSSION

Incorporation of ^{18}O from $^{18}O_2$ into Abscisic Acid during Water Stress. In previous experiments on incorporation of ^{18}O into ABA, isotope enrichment was determined by GC-MS using EI ionization (6). Since the M^+ of Me-ABA is an ion of low abundance (6, 12), this method is not sensitive to small amounts of isotope enrichment. With the availability of a JEOL HX-110HF mass spectrometer, incorporation of ^{18}O into ABA was analyzed by GC-MS using NCI. This method is very sensitive for electrophilic compounds and has the further advantage that M^- is the major ion in the mass spectrum of Me-ABA (21, 22). Thus, GC-MS-NCI is ideal for measuring the incorporation of heavy isotopes into ABA.

The results obtained with NCI confirm the EI data presented earlier for stress-induced ABA in leaves (6). The increased sensitivity of NCI over EI is the reason for observing more than one atom of ^{18}O incorporated into ABA after 6 h. The present results indicate that, in addition to the one atom of ^{18}O found in the

Table III. *Incorporation of ^{18}O from $^{18}O_2$ or $H_2{}^{18}O$, or from a Combination of the Two, into Abscisic Acid in Stressed Xanthium Leaves*

Detached, turgid *Xanthium* leaves were allowed to absorb 3 ml of $H_2{}^{18}O$ (40% enrichment) each. These leaves were then stressed and incubated in the presence of air, or of $^{18}O_2$, for 6 h. The data shown were obtained by GC-NCI (a), or by GC-EI (b). ABA was analyzed as Me-ABA. For NCI, a value of 100% indicates that it was the most prominent ion (base peak). All other ions are relative to the base peak. In the case of EI, the ion m/z = 125 represents the side chain with carboxyl group. The values are relative to the base peak $M^+ = 190$.

Form of ^{18}O Precursor	Relative Abundance				
	m/z				
(a) NCI	*278*	*280*	*282*	*284*	*286*
Unlabeled	100	1.5	0.3	0	0
$H_2{}^{18}O$	100	21.8	2.5	0.5	0
$^{18}O_2$	40.5	100	4.8	0.7	0
$H_2{}^{18}O + {}^{18}O_2$	16.1	100	23.4	5.7	2.0
(b) EI	*125*	*127*	*129*		
Unlabeled	24.5	0.9	0.2		
$H_2{}^{18}O$	22.4	4.3	0.7		
$^{18}O_2$	14.1	30.0	0.5		
$H_2{}^{18}O + {}^{18}O_2$	5.7	38.6	7.0		

Table IV. *Incorporation of ^{18}O into Abscisic Acid and Phaseic Acid in Turgid Xanthium Leaves*

Detached, turgid *Xanthium* leaves were incubated in darkness in the presence of $^{18}O_2$ for the times indicated. The data shown were obtained by GC-NCI. ABA was analyzed as Me-ABA and PA as Me-PA. A value of 100% indicates that it was the most prominent ion (base peak). All other ions are relative to the base peak.

Incubation Time	Relative Abundance			
h	*m/z*			
(a) ABA	*278*		*280*	*282*
0	100		1.8	0
24	100		3.1	1.0
48	100		3.5	1.6
72	100		5.8	2.6
(b) PA	*294*	*296*	*298*	*300*
0	100	2.1	0	0
24	100	18.4	5.0	0.3
48	100	5.4	2.3	0.5
72	100	8.3	2.1	0.8

carboxyl group, smaller amounts of ^{18}O were incorporated into the ring oxygens (Table Ia; Fig. 1). The position of the ^{18}O atoms is based on an EI spectrum of Me-ABA isolated from stressed *Xanthium* leaves incubated in $^{18}O_2$ for 24 h. The ion m/z 125 formed by the side chain (which contains the carboxyl group; Ref. 12) was shifted by two mass units, indicating that one atom of ^{18}O was present (see also Table IIIb). No enrichment of the ion at m/z 129 was detected which demonstrates that the second and third ^{18}O atoms incorporated were present in the ring positions. In addition, the ion at m/z 190, which contains the two ring oxygens and one of the carboxyl group oxygens (12), was shifted by up to six mass units (there were new ions at m/z 192, 194, and 196). Further support for ^{18}O being located in the ring oxygens comes from the isotope pattern of the ion at m/z 162, which contains only the ring oxygens (12). The GC-EI spectrum showed increased intensities of the isotope peaks at m/z 164 and 166. The relative intensities of the isotope-shifted ions at m/z 127, 164, and 166 confirmed that the predominant enrichment was in the carboxyl group.

The ^{18}O incorporation into ABA of water-stressed leaves suggests that there is one large, primary precursor pool which already contains two of the four oxygen atoms found in ABA (*i.e.* the two ring oxygens), with an additional oxygen atom being derived from water. This precursor gives rise to the large $M^- + 2$ in $^{18}O_2$ labeling experiments. With incubation periods longer than 6 h, more ^{18}O begins to appear in the ring oxygen atoms. This implies that there may be other compounds feeding into this large percursor pool which, during the conversion to this pool, incorporate ^{18}O into positions which ultimately form the ring oxygens of ABA. Alternatively, there could be three separate pathways to ABA. In this case, there would be three different precursors which incorporate one, two, or three atoms of ^{18}O, respectively, during their conversion to ABA. One of the precursors (one atom of ^{18}O incorporated in the carboxyl group) would be preferentially, and the other two more slowly, converted to ABA.

By analogy with the ^{18}O labeling pattern of ABA in roots, it is assumed that the ^{18}O enrichment in the C-4′ keto group of ABA in leaves was less than in the hydroxyl or carboxyl group. It is possible that this was, at least in part, due to exchange, since passing 4′-[^{18}O]ABA (86% enrichment) through the three HPLC columns used for ABA purification resulted in approximately 50% loss of the ^{18}O. This means that the relative abundances of $M^- + 6$ in the mass spectra of ABA are probably underestimated. However, the fact that $M^- + 6$ increased with time (Tables I, II), indicates that no complete exchange had taken place.

The ^{18}O labeling pattern of ABA in stressed roots differed from that in stressed leaves in that two oxygens rather than one showed a high degree of isotope enrichment (Table IIa; Fig. 1). One ^{18}O was located in the carboxyl group of ABA, while the second one was in the C-1′ hydroxyl group. These data suggest that a precursor lacking an oxygen function at C-1′ plays a more important role in the formation of stress-induced ABA in roots than in leaves.

Carotenoids at Possible Precursors of Stress-Induced Abscisic Acid. The oxygen incorporation data support the hypothesis that carotenoids, specifically xanthophylls, are precursors of water-stress induced ABA. It is known that the hydroxyl groups of lutein and the epoxide groups of antheraxanthin and violaxanthin are derived from molecular oxygen (28). Further, the turnover of carotenoids in green leaves is low (10). Thus, the low incorporation of ^{18}O into sites which ultimately form the keto and hydroxyl groups of ABA could represent the biosynthesis of lutein, zeaxanthin, and antheraxanthin and subsequent conversion to violaxanthin. Since the violaxanthin pool is so large, and turnover is small, the amount of incorporation of ^{18}O into the ring positions of ABA would be small if ABA was derived from violaxanthin or a related xanthophyll.

The incorporation of ^{18}O into the carboxyl group of ABA is reminiscent of the conversion of β-carotene to vitamin A. Feeding experiments of rats with β-carotene in the presence of $^{18}O_2$ or $H_2{}^{18}O$ have shown that only $^{18}O_2$ is incorporated into vitamin A (25). Likewise, ^{18}O from $^{18}O_2$ is incorporated into β-cyclocitral during oxidative cleavage of β-carotene to form β-cyclocitral and crocetindial by a 7,8 (7′,8′) oxygenase in the cyanobacterium *Microcystis* (14). Such compounds, derived from C_{40} carotenoids by oxidative cleavage, have been called apocarotenoids (24).

Several other lines of evidence, in addition to the data presented here, have accumulated that suggest that stress-induced ABA is derived from carotenoids. Various corn mutants have been described which lack the ability to produce various carotenoids due to specific lesions in the carotenoid biosynthetic pathway (7). These mutants also have a greatly decreased capability to produce ABA (2, 19, 20). In addition, inhibitors of carotenoid biosynthesis, such as fluridone and norflurazon, also inhibit the accumulation of ABA (5, 7–9, 18). Walton *et al.* (27) described an experiment in which green bean leaves were pretreated with fluridone and labeled with $^{14}CO_2$ for 24 h, and then water-stressed for 14 h. ABA and several xanthophylls were isolated and total amounts and specific activities were determined. Fluridone did not inhibit the accumulation of ABA in water-stressed green leaves, but the specific activity was reduced to about the same extent as that of carotenoids. In another experiment (27) ^{18}O was introduced into the epoxide oxygen of violaxanthin by means of the xanthophyll cycle (28). Leaves were then stressed and violaxanthin and ABA analyzed by MS. Between 40 and 45% of the violaxanthin contained ^{18}O in the epoxide group. ABA that accumulated during water stress contained 10 to 15% ^{18}O in the ring oxygens, suggesting that a portion of the ABA was derived from violaxanthin. The possibility exists that part of the ABA was derived from violaxanthin that was not labeled with ^{18}O, since violaxanthin exists in two different pools (23).

The reduced level of carotenoids in roots relative to leaves provides a means to examine the role of these pigments in the biosynthesis of ABA in this organ. It is well established that roots contain carotenoids (11, 15). The major carotenoid in corn root caps is violaxanthin (16). In cultivated carrots, β-carotene predominates, while in wild carrots xanthophylls represent the majority of the small amount of carotenoids present (11). The ^{18}O incorporation into ABA from stressed *Xanthium* roots implies that there is a carotenoid precursor lacking an oxygen function at C-6 (C-1′ in ABA) that is important in roots, whereas in leaves this precursor plays only a minor role.

Incorporation of ^{18}O from $H_2{}^{18}O$ into Abscisic Acid during

Water Stress. The labeling experiments discussed so far have never demonstrated more than three atoms of ^{18}O incorporated into stress-induced ABA. This suggests that one of the oxygen atoms in ABA is derived from water, and presumably located in the carboxyl group. ABA isolated from leaves of *X. strumarium* fed $H_2{}^{18}O$ and subsequently stressed, showed incorporation of one atom of ^{18}O into the carboxyl group, whereas dual labeling with $H_2{}^{18}O$ and $^{18}O_2$ resulted in production of ABA with both oxygen atoms of the carboxyl group labeled. Exchange of ^{18}O from $H_2{}^{18}O$ into the carboxyl group of ABA is unlikely. In all $^{18}O_2$ labeling experiments, the highest ^{18}O enrichment was found in the carboxyl group (see above). Since the oxygens in a carboxyl group are equivalent, there would be little ^{18}O left, if exchange did occur.

These results, demonstrating incorporation of one oxygen atom from water into the carboxyl group of ABA, are also consistent with xanthophylls being precursors of stress-induced ABA. For example, cleavage of a xanthophyll, such as violaxanthin, by an oxygenase would give rise to xanthoxin with the oxygen atom in the side chain derived from molecular oxygen. Subsequent oxidation of xanthoxin hydrate by a dehydrogenase would incorporate an oxygen atom from water into the carboxyl group of ABA.

Abscisic Acid Biosynthesis in Turgid Leaves. While most work on the biosynthesis of ABA has focused on water-stress induced ABA, the biosynthetic pathway of ABA in turgid leaves and its turnover rate are unknown. Analysis of ABA isolated from turgid *Xanthium* leaves incubated in the presence of $^{18}O_2$, indicated that a very small amount of ^{18}O was incorporated into ABA (Table IV). These results suggest that the turnover rate of ABA in turgid *Xanthium* leaves is very low, on the order of several days. On the other hand, results of a parallel experiment with [^{14}C]ABA contradict those obtained by labeling with $^{18}O_2$. When fed via the petiole, [^{14}C]ABA was completely metabolized after 48 h (*cf.* 3). Since ABA levels in turgid *Xanthium* leaves remain fairly constant (29), the latter experiment implies that turnover must be quite rapid. There are two possible explanations for these conflicting observations. First, it is possible that the oxygen atoms in ABA found in turgid leaves do not originate from molecular oxygen. This would imply that the biosynthetic pathways for ABA in turgid and water-stressed leaves are different. Second, it is possible that exogenous ABA is metabolized differently from endogenous ABA. This implies that in turgid leaves ABA is normally isolated from catabolic enzymes, perhaps in chloroplasts. Exogenous ABA, on the other hand, would encounter these enzymes upon its entrance into the cytoplasm.

Incorporation of ^{18}O into Phaseic Acid. The incorporation of $^{18}O_2$ into PA was demonstrated in both leaves and roots (Fig. 2; Tables Ic and IIb). If the pool of ABA for the conversion to PA is homogeneous, then the incorporation of ^{18}O into PA may be predicted by knowledge of the extent of ^{18}O labeling of ABA, and the percentage of PA that contains no ^{18}O. This calculation is based on the fact that ABA molecules that contain zero, one, two, or three atoms of ^{18}O will give rise to PA molecules containing one, two, three, or four atoms of ^{18}O, respectively. For instance, one may calculate the fraction of PA containing two atoms of ^{18}O by multiplying the fraction of ABA containing one atom of ^{18}O by (1—fraction old PA [*i.e.* the fraction of PA that does not contain ^{18}O]), etc. In this calculation, it is assumed that there is no discrimination between ABA molecules (*i.e.* homogeneous distribution of labeled ABA in the pool which forms PA), and indeed one finds that the predicted values are very close to those actually observed (Table V).

The $^{18}O_2$ labeling technique can be used to estimate the half-life of ABA, as long as no isotopic discrimination or exchange occurs. ABA levels in stressed *Xanthium* leaves reach a new, steady state level after 6 h (29). This implies that the rate of formation of ABA is equal to its rate of degradation. One may distinguish between old (present before stress) and newly synthesized ABA (present after stress) because any newly synthesized ABA will contain ^{18}O. Hence, by following the disappearance of m/z 278 (ABA present before stress), one may calculate a half-life for stress ABA, assuming that the ABA pool for catabolism is homogeneous. That this assumption is correct, at least for the conversion of ABA to PA, is shown by the data in Table V. Assuming a steady state level of 17 μg ABA/g dry weight (4, 29), the calculated half-life is 15.5 h (see Appendix). This value may be compared with that derived from data in Zeevaart (29). During water stress ABA is primarily catabolized to PA, so that a half-life may be calculated based on the rate of accumulation of PA. If one assumes that ABA has reached a new, steady state level and that the conversion of ABA to ABA-GE is minimal during water-stress (29, 30), then one obtains a value of 16.6 h (data from Fig. 2 in Ref. 29 for the period from 6 to 31 h after onset of water stress) for the half-life of ABA in stressed *Xanthium* leaves. The difference between the two methods is that the ^{18}O method uses data on the disappearance of ABA, while the PA method measures just one aspect of ABA catabolism and neglects further catabolism of PA and conversion of ABA to ABA-GE. In turgid tomato shoots, radioactive ABA equal to approximately 10% of the endogenous pool, had a half-life of 7.4 h (21). This is in contrast to a value of 3 h reported for stressed bean leaves (13). It is possible that an elevated level of ABA results in increased turnover. This could explain the conflicting results obtained with turgid *Xanthium* leaves following ^{18}O labeling or ^{14}C-ABA feeding (see above). In the latter case, the ABA applied amounted to at least 6 times the endogenous content.

Table V. *Predicted and Actual Values for ^{18}O Incorporation into Phaseic Acid*

The data shown were obtained from the 12 h incubation in $^{18}O_2$ (Table I) and represent the contribution of each fraction to the total amounts of Me-ABA and Me-PA analyzed.

Ion	Actual Percent		Predicted Percent Me-PA
	Me-ABA	Me-PA	
M^-	24.5	38.9	
$M^- + 2$	69.3	21.7	15.0
$M^- + 4$	4.9	35.9	42.3
$M^- + 6$	1.4	2.8	3.0
$M^- + 8$		0.7	0.9

In conclusion, it has been shown that during long-term incubations (12 and 24 h) of stressed *Xanthium* leaves in $^{18}O_2$, up to three atoms of ^{18}O from $^{18}O_2$ are incorporated into ABA. One atom of ^{18}O is located in the carboxyl group of ABA and two atoms are found in the ring oxygen atoms. The fourth oxygen atom in the carboxyl group of ABA is derived from water. ABA purified from stressed roots of *Xanthium* incubated in $^{18}O_2$ showed a similar pattern, but with more incorporation into the tertiary hydroxyl group at C-1′ (ABA numbering scheme) at 6 and 12 h. We propose that ABA is an apocarotenoid, and that a xanthophyll lacking an oxygen function at C-6 (carotenoid numbering scheme) plays a crucial role in ABA biosynthesis in *Xanthium* roots. Both leaves and roots of *X. strumarium* contain sufficient amounts of carotenoids to account for the accumulation of ABA during water stress. ABA biosynthesis in turgid *Xanthium* leaves is either (a) made by a biosynthetic pathway that is different from the one for stress-induced ABA, or (b) has a half-life on the order of days (compared with a half-life of 15.5 h in water-stressed *Xanthium* leaves).

APPENDIX

In order to calculate the half-life of ABA in stressed leaves, three conditions must be met: (a) ABA levels are in a steady

state, (b) there is randomness of catabolism (old ABA is equivalent to newly made ABA), and (c) distribution of ABA is uniform within its compartment (1).

The steady state level of ABA in a stressed *Xanthium* leaf is approximately 17 $\mu g \cdot g^{-1}$ dry weight (4, 29). The average rate of ABA conversion, k, is to be calculated for the period of 6 to 24 h (t = 18 h) after onset of stress.

The differential describing the disappearance of a compound at steady state is (1)

$$\frac{dM^*}{dt} = \frac{-kM^*}{M},$$

where

M^* = amount of labeled compound
M = steady state level of compound

whose integral is

$$M^* = M_0^* \, e^{-kt/M}.$$

Therefore

$$\log \frac{M^*}{M_0^*} = \frac{kt}{2.3\,M}$$

for the data described above

$$\log M_1 - \log M_2 = \frac{-kt}{2.3\,M}$$

where

M_1 = amount of ABA unlabeled at 24 h (3.2 μg/dry weight) (fraction unlabeled at 24 h × steady state level)
M_2 = amount of ABA unlabeled at 6 h (7.2 μg/g dry weight) (fraction unlabeled at 6 h × steady state level)
M = steady state level of ABA (assume 17 μg/g dry weight)
t = 18 h

$$k = \frac{0.35}{18\text{ h}} \times 2.3 \times 17\ \mu g \cdot g^{-1}\ \text{weight}$$

$$k = 0.76\ \mu g \cdot g^{-1}\ \text{dry weight} \cdot h^{-1}.$$

$$\text{Now half-life} = \frac{0.693\,M}{k} = \frac{0.693 \times 17\ \mu g \cdot g^{-1}\ \text{dry weight}}{0.76\ \mu g \cdot g^{-1}\ \text{dry weight} \cdot h^{-1}}$$

$$= 15.5\text{ h}.$$

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LITERATURE CITED

1. ARONOFF S 1967 Techniques of Radiobiochemistry. Hafner, New York, pp 79–80
2. BRENNER ML, B BURR, F BURR 1977 Correlation of genetic vivipary in corn with abscisic acid concentration. Plant Physiol 59: S-76
3. CORNISH K, JAD ZEEVAART 1984 Abscisic acid metabolism in relation to water stress and leaf age in *Xanthium strumarium*. Plant Physiol 76: 1029–1035
4. CORNISH K, JAD ZEEVAART 1985 Abscisic acid accumulation by roots of *Xanthium strumarium* L. and *Lycopersicon esculentum* Mill. in relation to water stress. Plant Physiol 79: 653–658
5. CREELMAN RA 1986 Stress-induced abscisic acid biosynthesis in higher plants. PhD thesis. Michigan State University, East Lansing
6. CREELMAN RA, JAD ZEEVAART 1984 Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. Plant Physiol 75: 166–169
7. FONG F, DE KOEHLER, JD SMITH 1983 Fluridone induction of vivipary during maize seed development. *In* JE Kruger, DE LaBerge, eds, Third Intern. Symp. on Pre-Harvest Sprouting in Cereals. Westview, Boulder, pp 188–196
8. FONG F, JD SMITH, DE KOEHLER 1983 Early events in maize seed development. Plant Physiol 73: 899–901
9. GAMBLE PE, JE MULLET 1986 Inhibition of carotenoid accumulation and abscisic acid biosynthesis in fluridone-treated dark-grown barley. Eur J Biochem 160: 117–121
10. GOODWIN TW 1980 The Biochemistry of Carotenoids, Ed 2, Vol 1. Chapman and Hall, New York
11. GOODWIN TW 1980 Carotenoids. *In* EA Bell, BV Charlwood, eds, Encyclopedia of Plant Physiology NS, Vol 8. Springer-Verlag, New York, pp 257–287
12. GRAY RT, R MALLABY, G RYBACK, VP WILLIAMS 1974 Mass spectra of methyl abscisate and isotopically labelled analogues. J Chem Soc Perkins Trans II, 919–924
13. HARRISON MA, DC WALTON 1975 Abscisic acid metabolism in water-stressed bean leaves. Plant Physiol 56: 250–254
14. JÜTTNER F, B HÖFLACHER 1985 Evidence of β-carotene 7,8(7′,8′)oxygenase (β-cyclocitral, crocetindial generating) in *Microcystis*. Arch Microbiol 141: 337–343
15. KARRER P, E JUCKER 1950 Carotenoids. Elsevier, New York
16. MAUDINAS B, J LEMATRE 1979 Violaxanthin, the major carotenoid pigment in *Zea mays* root cap during seed germination. Phytochemistry 18: 1815–1817
17. MILBORROW BV 1983 Pathways to and from abscisic acid. *In* FT Addicott, ed. Abscisic Acid. Praeger, New York, pp 79–111
18. MOORE R, JD SMITH 1984 Growth, graviresponsiveness and abscisic-acid content of *Zea mays* seedlings treated with fluridone. Planta 162: 342–344
19. MOORE R, JD SMITH 1984 Graviresponsiveness and abscisic-acid content of roots of carotenoid-deficient mutants of *Zea mays*. Planta 164: 126–128
20. NEILL SJ, R HORGAN AD PARRY 1986 The carotenoid and abscisic acid content of viviparous kernels and seedlings of *Zea mays* L. Planta 169: 87–96
21. NONHEBEL HM, BV MILBORROW 1986 Incorporation of ^2H from ^2H_2O into ABA in tomato shoots: evidence for a large pool of precursors. J Exp Bot 37: 1533–1541
22. RIVIER L, M SAUGY 1986 Chemical ionization mass spectrometry of indol-3yl-acetic acid and *cis*-abscisic acid: evaluation of negative ion detection and quantification of *cis*-abscisic acid in growing maize roots. J Plant Growth Regul 5: 1–16
23. SIEFERMANN-HARMS D 1980 The role of carotenoids in chloroplasts in higher plants. *In* P Mazlaik, P Benveniste, C Costes, R Douce, eds, Biogenesis and Function of Plant Lipids. Elsevier, New York, pp 331–340
24. SINGH H, HR CAMA 1975 Metabolism of carotenoids. J Sci Ind Res 34: 219–230
25. VARTAPETYAN BB, AA DMITROVSKII, DG ALKHAZOV, IK LEMBERG, AB GIRSHIN, GM GUSINSKII, NA STARIKOVA, NN EROFEEVA, IP BOGDANOVA 1966 A new approach to the study of the mechanism of biosynthesis of vitamin A from carotene by activation of oxygen O^{18} as a result of a nuclear reaction O^{18} (α, n, γ) by means of α-particles accelerated in a cyclotron. Transl Biokhimiya 31: 759–763
26. WALTON DC 1980 Biochemistry and physiology of abscisic acid. Annu Rev Plant Physiol 31: 453–489
27. WALTON DC, Y LI, SJ NEILL, R HORGAN 1985 Biosynthesis of abscisic acid: a progress report. *In* DG Randall, DG Blevins, RL Larson, eds, Current Topics in Plant Biochemistry and Physiology 1985, Vol 4. University of Missouri, Columbia, pp 111–117
28. YAMAMOTO HY, CO CHICHESTER 1965 Dark incorporation of ^{18}O into antheraxanthin by bean leaf. Biochim Biophys Acta 109: 303–305
29. ZEEVAART JAD 1980 Changes in the levels of abscisic acid and its metabolites in excised leaf blades of *Xanthium strumarium* during and after water stress. Plant Physiol 66: 672–678
30. ZEEVAART JAD 1983 Metabolism of abscisic acid and its regulation in *Xanthium* leaves during and after water stress. Plant Physiol 71: 477–481