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Evidence for a Universal Pathway of Abscisic Acid Biosynthesis in Higher Plants from ^{18}O Incorporation Patterns[1]

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ABSTRACT

Previous labeling studies of abscisic acid (ABA) with $^{18}O_2$ have been mainly conducted with water-stressed leaves. In this study, ^{18}O incorporation into ABA of stressed leaves of various species was compared with ^{18}O labeling of ABA of turgid leaves and of fruit tissue in different stages of ripening. In stressed leaves of all six species investigated, avocado (*Persea americana*), barley (*Hordeum vulgare*), bean (*Phaseolus vulgaris*), cocklebur (*Xanthium strumarium*), spinach (*Spinacia oleracea*), and tobacco (*Nicotiana tabacum*), ^{18}O was most abundant in the carboxyl group, whereas incorporation of a second and third ^{18}O in the oxygen atoms on the ring of ABA was much less prominent after 24 h in $^{18}O_2$. ABA from turgid bean leaves showed significant ^{18}O incorporation, again with highest ^{18}O enrichment in the carboxyl group. The ^{18}O-labeling pattern of ABA from unripe avocado mesocarp was similar to that of stressed leaves, but in ripe fruits there was, besides high ^{18}O enrichment in the carboxyl group, also much additional ^{18}O incorporation in the ring. In ripening apple fruit tissue (*Malus domestica*), singly labeled ABA was most abundant with more ^{18}O incorporated in the tertiary hydroxyl group than in the carboxyl group of ABA. Smaller quantities of this monolabeled product (C-1′-^{18}OH) were also detected in the stressed leaves of barley, bean, and tobacco, and in avocado fruits. It is postulated that a large precursor molecule yields an aldehyde cleavage product that is, in some tissues, rapidly converted to ABA with retention of ^{18}O in the carboxyl group, whereas in ripening fruits and in the stressed leaves of some species the biosynthesis of ABA occurs at a slower rate, allowing this intermediate to exchange ^{18}O with water. On the basis of ^{18}O-labeling patterns observed in ABA from different tissues it is concluded that, despite variations in precursor pool sizes and intermediate turnover rates, there is a universal pathway of ABA biosynthesis in higher plants which involves cleavage of a larger precursor molecule, presumably an oxygenated carotenoid.

In earlier work (3), it was shown that in stressed cocklebur leaves three of the four oxygen atoms of ABA are derived from molecular oxygen. It was determined by ^{18}O labeling experiments that one ^{18}O atom is rapidly incorporated in the carboxyl group, while smaller amounts of ^{18}O are incorporated over extended periods in the ring oxygens of ABA. The fourth oxygen atom, in the carboxyl group, is derived from water (3). These results were interpreted to indicate that stress-induced ABA is derived from a large precursor pool which already contains two of the four oxygens present in ABA. As the primary precursor is depleted over time, other precursors containing fewer oxygen atoms feed into the pathway. The high degree of isotope enrichment in the carboxyl group suggests oxidative cleavage of a larger molecule, probably yielding an aldehyde. This intermediate would be further oxidized by a dehydrogenase and incorporate an oxygen atom from water into the carboxyl group of ABA. This idea is supported by the recent finding that ABA-aldehyde is the likely immediate precursor of ABA in higher plants (16, 17).

The objective of the present studies was to compare the biosynthesis of ABA that was either stress-induced or developmentally regulated. For this purpose, ^{18}O labeling experiments were performed with different systems, such as turgid and water-stressed leaves, and ripening fruits. In the latter case, ABA production is developmentally regulated. In the mesocarp of ripening avocado fruits large increases in ABA levels have been reported (1, 10).

With the recent interpretation of the fragmentation pattern of Me-ABA obtained by NCI-MS[2] (11), ^{18}O atoms can be readily assigned to the ring or side chain of Me-ABA. This technique, as well as NCI MS-MS, were used to quantitatively determine isotopic enrichment at the various positions of ^{18}O-labeled Me-ABA from different tissues. These experiments demonstrated that although different labeling patterns were obtained in different species and plant parts, the results could all be interpreted in terms of a single biosynthetic pathway.

A preliminary account of some of this work has been presented earlier (20).

MATERIALS AND METHODS

Plant Material

Cocklebur (*Xanthium strumarium* L. Chicago strain) and tobacco (*Nicotiana tabacum* L. cv Kentucky No. 14) were grown in a greenhouse as before (19). The youngest fully

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[2] Abbreviations: NCI, negative chemical ionization; a.m.u., atomic mass unit; CI, chemical ionization; EI, electron impact; DPA, dihydrophaseic acid; M^-, odd electron negative molecular ion; m/z, mass/charge; Me-, methyl ester; PA, phaseic acid.

expanded leaves without midribs were used for wilting experiments.

Barley (*Hordeum vulgare* L. cv Robusta) and bean (*Phaseolus vulgaris* L. cv Stringless Black Valentine) were grown in a growth chamber under the conditions described (21). The first leaves of barley seedlings and the primary leaves of bean were used for stress experiments when the leaves were fully expanded.

Avocado (*Persea americana* Mill.) seedlings were grown from seeds of the cv Booth 7 that had been open pollinated. The seedlings were grown in a growth chamber under the same conditions as barley and bean seedlings.

Spinach (*Spinacia oleracea* L. cv Savoy Hybrid 612) was exposed to 8 LD under the same conditions as described (18). Fully expanded leaves were used for wilting experiments.

For stress experiments, leaves were wilted until they had lost 12 to 15% of their fresh weight. Tobacco leaves were more resistant to wilting and lost only 9.5% of their fresh weight. Stressed leaves were incubated for 6, 12, and 24 h in an atmosphere of 80% N_2 and 20% $^{18}O_2$ as before (3). In experiments with turgid material, the leaves were detached, briefly submerged in distilled water, and then placed in darkness in 80% N_2 and 20% $^{18}O_2$ with their petioles in distilled water. During the incubations, O_2 content was monitored as before and replenished when necessary (3).

Apple fruits (*Malus domestica* Borkh. cv Granny Smith) were obtained in a local store and kept at 4°C until use. Their previous history was unknown. Avocado fruits (*Persea americana* Mill. cvs Booth 7, Harris, and Lula) were shipped by airmail from Florida by Dr. T. L. Davenport, University of Florida, TREC, Homestead, FL. Thus, experiments could be started soon after the fruits had been detached from the trees which in most avocado varieties initiates the fruit ripening processes (9). When extended storage was necessary, avocado fruits were kept at 10°C.

For labeling experiments with $^{18}O_2$, slices or cylinders of mesocarp from the equator of a single avocado fruit were incubated as described for stressed leaves. Apple fruits were peeled and slices of the cortical tissue were used for incubation in $^{18}O_2$. In initial experiments 5 μL/L ethylene was added to incubated tissues to hasten ripening, but this was discontinued when it was found that incubated fruit slices of both apple and avocado produced ethylene at high rates. Measurement of ethylene was as described (6).

Extraction and Purification of ABA, PA, and DPA

ABA, and in turgid bean leaf samples also PA and DPA, was extracted with acetone as described (2). After removal of the acetone on a rotary evaporator, lipids in extracts from avocado mesocarp were removed by partitioning the aqueous concentrate three times with hexane. The next purification step involved semipreparative reverse phase HPLC of the lyophilized crude extracts on a μBondapak C_{18}, 30 × 0.78 cm column (Waters Associates), using a convex gradient (curve 5) from 0 to 50% ethanol in aqueous 1% acetic acid in 25 min. The flow rate was 2.5 mL/min. DPA was collected from 17.5 to 19.0 min, PA from 19.0 to 20.5 min, and ABA from 25 to 26.5 min. The fractions were dried and methylated with ethereal diazomethane. The final purification step involved 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, 20 to 70% for Me-PA, and 50 to 100% for Me-DPA, all in 10 min, and at 2 mL/min. Quantification of Me-ABA was as described (2). Me-PA and Me-DPA were not quantified in the present experiments.

Mass Spectrometry

GC-NCI-MS of Me-ABA and Me-DPA was performed on a JEOL HX-110 HF double focusing mass spectrometer equipped with a Hewlett-Packard 5890 gas chromatograph (single stage jet separator). The column used was a DB-1 megabore (15 m × 0.53 mm; J&W Scientific, Inc., Rancho Cordova, CA) with He as the carrier gas (flow rate 10 mL/min). GLC conditions were: oven temperature programmed from 190 to 260°C at 10°C/min. Methane was used as the buffering reagent gas.

GC-NCI-MS of Me-PA was performed on a Finnigan TSQ 70 equipped with a Varian 3400 gas chromatograph. The column used was a DB-5 capillary column (30 m × 0.25 mm, film thickness 0.25 μm; J&W Scientific, Inc.) with He as the carrier gas (flow rate 1 mL/min). GLC conditions were: oven temperature programmed from 60 to 210°C at 50°C/min, and from 210 to 280°C at 12°C/min. Methane or ammonia was used as the buffering reagent gas. The same conditions were used in MS-MS experiments to produce daughter ions of the M^- of ^{18}O-labeled Me-ABA. The parent ion underwent collisionally activated dissociation with O_2 as collision gas. The MS-MS spectra were recorded at a collision energy of 5 eV and O_2 pressure of 0.7 mtorr in the second quadrupole collision chamber.

Chemicals

$^{18}O_2$ (97–98%) was purchased from Cambridge Isotopes Laboratories (Woburn, MA).

RESULTS

Incorporation of ^{18}O into Abscisic Acid in Stressed Leaves

After incubation of stressed cocklebur leaves in $^{18}O_2$, the majority of ABA was labeled with one ^{18}O atom which was located in the carboxyl group. This conclusion was based on a combination of NCI- and EI-MS (3). It is now possible to locate ^{18}O atoms in Me-ABA solely on the basis of NCI spectra (11). Unlabeled Me-ABA gives an NCI spectrum with few fragment ions present (Fig. 1A). The M^- is the base peak at m/z 278. The following features of the NCI spectrum are important for assignment of incorporated ^{18}O atoms to various positions of the Me-ABA molecule. Loss of water from the tertiary hydroxyl group gives rise to the ion at m/z 260, and subsequent loss of one of the C-6′ methyl groups, or loss of methanol from the $[M-H]^-$ ion, yields m/z 245; the latter ion also involves loss of the 1′-hydroxyl group. Another fragment at m/z 152 contains the ring of Me-ABA with both O atoms, whereas m/z 141 contains the side chain with the two carboxyl oxygen atoms (11).

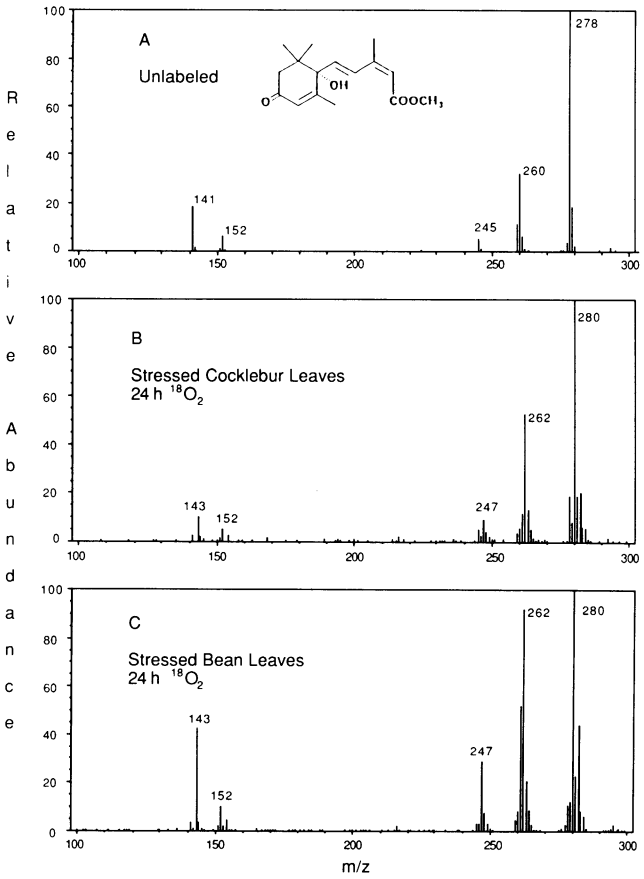

Figure 1. Mass spectra of Me-ABA analyzed by GC-NCI. Shown are spectra of A, unlabeled Me-ABA; B, Me-ABA isolated from stressed cocklebur leaves incubated in the presence of $^{18}O_2$ for 24 h; C, Me-ABA isolated from stressed bean leaves incubated in the presence of $^{18}O_2$ for 24 h.

As observed previously (3), in the spectrum of Me-ABA isolated from stressed cocklebur leaves that had been incubated in $^{18}O_2$, the majority of M^- was shifted from m/z 278 to 280 (Fig. 1B). The shift of m/z 141 to 143 indicates that the first ^{18}O atom incorporated was located in the side chain, *i.e.* in the carboxyl group. This is further supported by the presence of ions at m/z 247 and 262 rather than at m/z 245 and 260, respectively.

The ^{18}O labeling pattern of Me-ABA isolated from stressed cocklebur leaves was compared with the labeling pattern of ABA in stressed leaves of five other species. After incubation in $^{18}O_2$ for 6 h, the base peak had shifted from m/z 278 to 280 in Me-ABA from each species, indicating incorporation of a single ^{18}O atom in the carboxyl group (data not shown). With longer incubations, the abundance of m/z 282 and 284 (two and three ^{18}O atoms incorporated, respectively) gradually increased. As shown in Table I, the ^{18}O enrichment was very similar for all species after a 24-h labeling period, except that in barley and bean leaves a higher percentage of ABA molecules contained two ^{18}O atoms (Fig. 1C).

Incorporation of ^{18}O into Abscisic Acid in Turgid Leaves

In earlier experiments, very little incorporation of ^{18}O into ABA was found in turgid cocklebur leaves (3). However, in turgid primary leaves of bean, significant ^{18}O incorporation into ABA was observed (Table II). The labeling pattern was similar to that in stressed leaves, *i.e.* highest ^{18}O enrichment was found in the carboxyl group of Me-ABA (data not shown). This experiment was repeated twice with similar results. In each case, the ABA content decreased drastically during the incubation of detached bean leaves in $^{18}O_2$ in darkness. There was considerable conversion of ABA to PA, as evident from the ^{18}O incorporation into PA. However, very little ^{18}O was found in DPA (Table II). In the case of Me-ABA, m/z 280 (M^- +2) was the base peak due to incorporation of one ^{18}O atom into the carboxyl group, whereas in Me-PA m/z 298 (M^- +4) was the base peak, representing the incorporation of two ^{18}O atoms. One of these ^{18}O atoms was present in the carboxyl group and was originally incorporated during the synthesis of ABA, whereas the second one was introduced during 8′-hydroxylation of ABA to PA. This was confirmed

Table I. *Incorporation of ^{18}O into Abscisic Acid in Stressed Leaves of Different Species*

The detached, wilted leaves were incubated in darkness in $^{18}O_2$ for 24 h. A value of 100% indicates that it was the most prominent ion (base peak). All other ions are relative to the base peak. For unlabeled Me-ABA M^- = 278.

Species	ABA Content		Relative Abundance			
	Turgid	Stressed				
	μg/g dry wt		m/z			
			278	280	282	284
Avocado	5.5	13.6[a]	44	100	8	1
Barley	0.04	3.2	19	100	35	8
Bean	0.2	8.4	6	100	37	3
Cocklebur	1.7	20.1	16	100	16	3
Spinach	0.3	4.2	11	100	19	3
Tobacco	0.3	7.1	28	100	14	2

[a] After 12 h incubation in $^{18}O_2$.

Table II. *Incorporation of ^{18}O into Abscisic Acid, Phaseic Acid, and Dihydrophaseic Acid in Turgid Bean Leaves*

Detached, turgid bean leaves, with their petioles in a vial containing distilled water, were incubated in darkness in the presence of $^{18}O_2$ for 29 h. For unlabeled Me-PA M^- = 294, for unlabeled Me-DPA M^- = 296.

	ABA Content	Relative Abundance				
	μg/g dry wt		m/z			
Me-ABA			278	280	282	284
0 h	0.2		100	3	0	0
29 h	0.03		96	100	45	5
Me-PA		294	296	298	300	302
0 h		100	3	0	0	0
29 h		71	63	100	32	1
Me-DPA		296	298	300	302	304
0 h		100	3	0	0	0
29 h		100	11	8	1	0

by the presence of fragment ions in the NCI spectrum of Me-PA analogous to those in the spectrum of Me-ABA representing the side chain (m/z 141 and 143) and the ring (m/z 168 and 170) (data not shown).

Incorporation of ^{18}O into Abscisic Acid in Avocado Fruits

When slices of mesocarp from unripe (hard texture) avocado fruits were incubated in $^{18}O_2$, the pattern of ^{18}O incorporation was very similar to that observed for ^{18}O-labeled ABA from stressed leaves (*cf.* Figs. 1, B and C, and 2A). There was high ^{18}O enrichment in the carboxyl group as evident from the shift of the ion at m/z 141 to 143 (Fig. 2A). ABA from both cultivars in early ripening showed the same labeling pattern (Table III). However, several differences between the cultivars were observed: Lula generally accumulated less ABA than Booth 7, but produced considerably more ethylene than Booth 7 (data not shown). Also, when $^{18}O_2$ labeling was performed after a 7-d storage period, the labeling patterns were quite different in ABA isolated from the two cultivars. In Lula, little ^{18}O was incorporated (Table III), presumably because ABA was synthesized at a low rate during the labeling period. On the other hand, ABA accumulation continued in

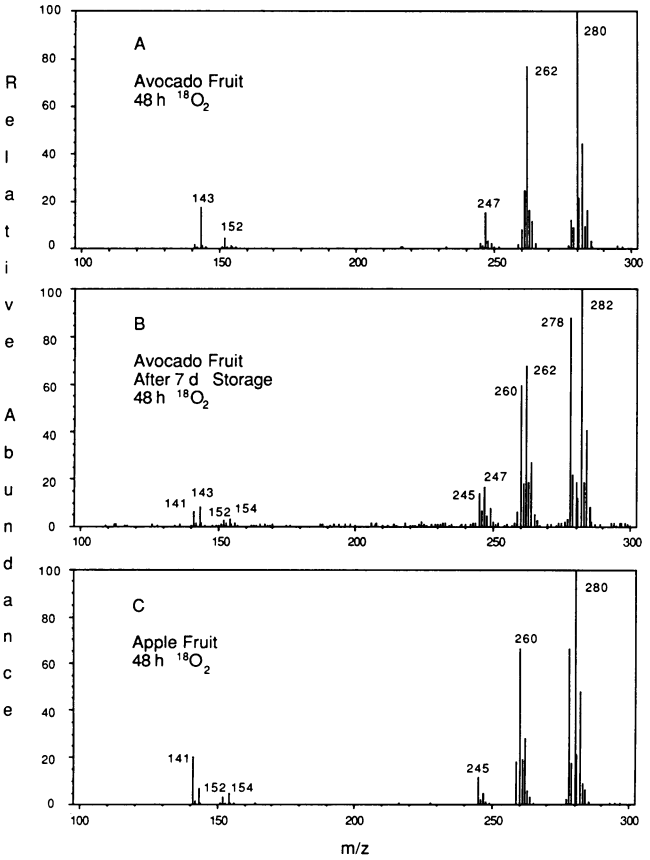

Figure 2. Mass spectra of Me-ABA analyzed by GC-NCI. Shown are spectra of A, Me-ABA isolated from unripe mesocarp of an avocado fruit, cv Booth 7, after incubation in $^{18}O_2$ for 48 h; B, Me-ABA from the same fruit as A following storage for 7 d at 10°C, after which mesocarp was incubated in $^{18}O_2$ for 48 h; C, Me-ABA isolated from apple fruit tissue, cv Granny Smith, incubated in $^{18}O_2$ for 48 h.

Table III. *Incorporation of ^{18}O into Abscisic Acid in Avocado Mesocarp at Different Stages of Ripening*

Slices of mesocarp tissue were incubated in $^{18}O_2$ for 24 or 48 h. The initial $^{18}O_2$ labeling experiment was performed 2 d after the fruits had been detached from the trees. The fruits were then stored at 10°C and new slices of tissue were cut from the same fruits after 7 d for the second $^{18}O_2$ labeling experiment.

Incubation Time	ABA Content	Relative Abundance			
	μg/g fresh wt	m/z			
		278	280	282	284
cv Lula					
0 h	0.3	100	3	0	0
24 h	3.7	11	100	15	4
48 h	4.7	9	100	55	3
After storage for 7 d					
0 h	4.6	100	3	0	0
24 h	4.6	100	13	11	3
48 h	6.3	100	24	19	6
cv Booth 7					
0 h	1.0				
24 h	3.0	24	100	15	2
48 h	6.0	12	100	44	16
After storage for 7 d					
0 h	10.2	100	4	0	0
24 h	12.8	100	14	56	15
48 h	17.0	90	20	100	41

Booth 7 after the 7-d storage period at 10°C (Table III). In some samples, the base peak was shifted from m/z 278 to 282 (Fig. 2B), indicating high ^{18}O incorporation into two positions, with a substantial amount of triply labeled Me-ABA (m/z 284), and relatively little singly labeled ABA (m/z 280) formed. Again there was high ^{18}O enrichment in the carboxyl group (intensity of m/z 143 greater than 141). In view of the presence of ions at m/z 152, 154, and 156, there were ABA molecules with zero, one, or two ^{18}O atoms on the ring of Me-ABA. In the doubly labeled Me-ABA (M^- at m/z 282), the second ^{18}O was located in the tertiary hydroxyl group, as follows from the relative abundances of the ions at m/z 278 and 282 as compared to the ions m/z 260 and 262. Where the 1′-OH was unlabeled, loss of water from M^- =278 gave m/z 260. Likewise, loss of 20 a.m.u. (water incorporating 1′-^{18}OH) from m/z 282 yielded m/z 262, while m/z 264 was similarly derived from m/z 284 by loss of $H_2{}^{18}O$, again from the labeled tertiary hydroxyl. These assignments of ^{18}O atoms to the various positions were confirmed by NCI MS-MS analysis of individual isotopically labeled parent ions (m/z 280, 282, and 284) (see below). The third ^{18}O atom, in the 4′-keto position, was always considerably less abundant than the one in the 1′-hydroxyl. Assuming that ^{18}O incorporated in the 4′-keto position was exchanged to some degree with water (3), the percentage of ABA molecules with both ring oxygens labeled was underestimated. It appears, therefore, that the ripening stage at which this labeling pattern was observed, represents the time when the primary precursor, already containing two ring oxygens, was depleted, but the biochemical machinery for ABA biosynthesis was still highly active. Thus, the earlier precleavage intermediates lacking one or both ring oxygens, would incorporate ^{18}O into the ring positions during

the labeling period. An additional ^{18}O atom was then introduced into the side chain during the cleavage step to yield doubly or triply labeled ABA.

The shift in ^{18}O labeling pattern of ABA from that typical for stressed leaves to one where there is high ^{18}O enrichment in two and three O atoms, was also observed when a Booth 7 fruit was allowed to ripen at room temperature (Table IV). In this experiment, ethylene production was negligible at day 2, then started to increase and reached its maximum at 121 nL/g fresh weight/h at 5 d, and then declined, whereas ABA continued to increase throughout the experimental period. Thus, in agreement with previous work (1), the rise in ABA content followed an increase in the rate of ethylene production. The mesocarp which was initially hard had become soft by the fifth day. It should be noted that the ABA content approximately doubled after the fifth day (Table IV), although the fruit appeared fully ripe. A similar pattern of ^{18}O incorporation has also been observed in fruits of the cv Harris (data not shown).

Incorporation of ^{18}O into Abscisic Acid in Apple Fruits

The ABA content of apple fruit tissue was three orders of magnitude lower than that of avocado mesocarp, and did not significantly increase during the $^{18}O_2$ labeling period (Table V). Thus, increasing incorporation of ^{18}O into ABA with time was mainly due to turnover of ABA. The NCI spectrum of Me-ABA isolated from apple fruit tissue incubated in $^{18}O_2$ (Fig. 2C) exhibits features that are quite distinct from the spectra of ^{18}O labeled Me-ABA from stressed leaves and from avocado mesocarp (Figs. 1, B and C; 2, A and B). First of all, the ratios of m/z 141/143, and m/z 152/154 indicate that the highest ^{18}O incorporation was in the ring, not in the carboxyl group of Me-ABA. Second, the highest ^{18}O incorporation was in the tertiary hydroxyl group, as indicated by the relative abundances of the ions m/z 260, 262, and 264 as compared to m/z 278, 280, and 282. The high abundance of m/z 260 was due both to loss of 18 a.m.u. from m/z 278 and to loss of 20 a.m.u. (1'-^{18}OH) from m/z 280. This labeling pattern was confirmed by NCI MS-MS, using each individual M^- ion as a parent (see below). Thus, apple fruit tissue is the only exception so far to the rule that ^{18}O is always most abundantly incorporated into the carboxyl group of ABA.

Determination of the Position of ^{18}O Enrichment by NCI MS-MS

MS-MS allows the determination of fragment (daughter) ions that are formed from a selected (parent) ion which in the present experiments was M^- of ^{18}O-labeled Me-ABA at m/z 278, 280, 282, or 284. In the case of the parent ion at m/z 278 (unlabeled Me-ABA), the daughter ions were as expected at m/z 141, 152, 245, and 260. When MS-MS was performed on m/z 280 of Me-ABA from water-stressed leaves (Table I), the daughter ions were m/z 143, 152, 247, and 262 (Fig. 3A), confirming that the first ^{18}O was incorporated into the carboxyl group of ABA. However, in the case of ABA from barley, bean, and tobacco leaves, up to 2% of the single ^{18}O incorporated was present in the tertiary hydroxyl group. In the other species of Table I this was less than 0.5%.

In contrast, MS-MS of m/z 280 of Me-ABA from avocado fruits gave varying results which turned out to be related to the ripening stage at which mesocarp was incubated in $^{18}O_2$ (Table IV). In unripe fruits, the pattern was the same as in Me-ABA from stressed leaves, *i.e.* the first ^{18}O atom was essentially all in the carboxyl group. However, as the fruit ripened, there was an increasing percentage of the first ^{18}O atom located in the tertiary hydroxyl group, with good agreement between the percentages present as m/z 141 and m/z 154 (Table IV).

MS-MS of m/z 280 of ^{18}O-labeled ABA from apple fruit indicated that a high percentage of ^{18}O was in the tertiary hydroxyl group and only a small amount in the carboxyl group. This is evident when comparing the relative abundances at m/z 141 and 260 with m/z 143 and 262 (Fig. 3C; Table V). Again, there was good agreement between ^{18}O enrichment in the ring (m/z 154) and the absence of ^{18}O in the carboxyl group (m/z 141). In a repetition of the experiment in Table V, 56 and 65% of the first ^{18}O atom were located in the ring of Me-ABA after 24 and 48 h incubation in $^{18}O_2$, respectively.

MS-MS of the ion at m/z 282 of Me-ABA from stressed

Table IV. *Incorporation of ^{18}O into Abscisic Acid in Avocado Mesocarp of cv Booth 7, during Different Stages of Ripening at Room Temperature, and Location of ^{18}O Atom by MS-MS of m/z 280*

Two cylinders (diameter 1 cm) of mesocarp tissue incubated in $^{18}O_2$ for 48 h in each treatment. Time of detachment of fruit from tree indicated as day 0.

Treatment	ABA Content	Relative Abundance				MS-MS of m/z 280	
						141/141 + 143	154/152 + 154
	μg/g fresh wt	m/z				%	
		278	280	282	284		
After 2 d	1.7	100	3	0	0		
$^{18}O_2$ 2→4 d	5.0	41	100	48	18	3	3
After 4 d	7.3						
$^{18}O_2$ 4→6 d	22.3	100	50	90	59	10	9
After 5 d	14.0						
$^{18}O_2$ 5→7 d	25.5	100	23	66	36	19	19
After 7 d	29.6						
$^{18}O_2$ 7→9 d	28.6	100	8	18	12	32	31

Table V. *Incorporation of ^{18}O into Abscisic Acid in Apple Fruit Tissue, and Location of ^{18}O Atom by MS-MS of m/z 280*

Slices of apple fruit tissue, cv Granny Smith, were incubated in $^{18}O_2$ for the times indicated.

Incubation Time	ABA Content	Relative Abundance				MS-MS of m/z 280	
						141/141 + 143	154/152 + 154
h	*ng/g fresh wt*	*m/z*				%	
		278	280	282	284		
0	11.6	100	3	0	0		
24	12.0	100	80	29	3	19	15
48	14.1	66	100	48	7	82	80

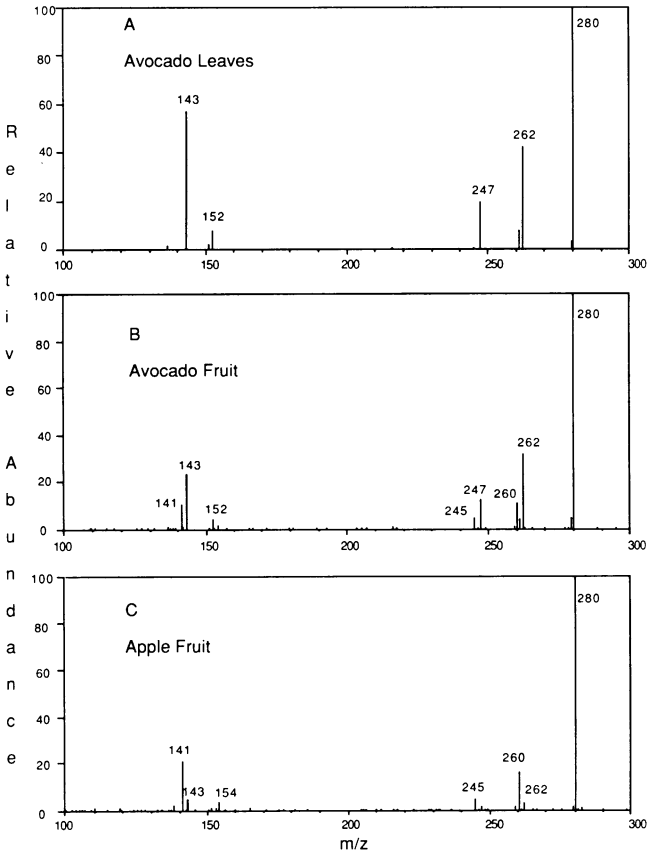

Figure 3. MS-MS of parent ion at m/z 280 of ^{18}O-labeled Me-ABA. Shown are A, daughter ions of m/z 280 of Me-ABA isolated from water-stressed avocado leaves; B, daughter ions of m/z 280 of Me-ABA isolated from ripe avocado mesocarp (see Fig. 2B); C, daughter ions of m/z 280 of Me-ABA isolated from apple fruit tissue (see Fig. 2C).

leaves (Table I) gave ions at m/z 143, 154, 247, and 262, indicating that the second ^{18}O atom was in the tertiary hydroxyl group. Unexpectedly, these spectra showed that a second molecular species of doubly labeled Me-ABA was present. There were two low abundance ions at m/z 145 and at m/z 152, indicating incorporation of the two ^{18}O atoms into the carboxyl group alone. This amounted to 12% of the total ions at m/z 143 and 145 in the case of stressed avocado and barley leaves, and 5% or less in ABA from the other species in Table I. The abundance of m/z 145 always corresponded with that of m/z 152, thus confirming that some of the doubly labeled ABA molecules (with two ^{18}O atoms in the carboxyl group) lacked ^{18}O in the ring. The presence of two ^{18}O atoms in the carboxyl group of Me-ABA derived from molecular oxygen is surprising, since it was demonstrated in earlier work with stressed cocklebur leaves that the second ^{18}O atom in the carboxyl group is derived from water (3). There is at present no explanation for this small, but significant incorporation of two ^{18}O atoms from $^{18}O_2$ into the carboxyl group. Since m/z 282 was much less abundant than m/z 280 in ABA from stressed leaves (Table I), and only a small fraction yielded the ion at m/z 145, this fragment was less than 0.5% of the base peak in NCI spectra and was, therefore, not observed in these spectra.

MS-MS of the parent at m/z 282 in Me-ABA from avocado mesocarp gave predominantly daughter ions at m/z 143, 154, 247, and 262, indicating one ^{18}O atom in the carboxyl group and the second one in the tertiary hydroxyl. Approximately 1.5% of the doubly labeled Me-ABA contained two ^{18}O atoms in the carboxyl (see above) in unripe fruits, and 0.2% or less in ripe fruits. In some Me-ABA samples from avocado mesocarp m/z 284 was abundant (Fig. 2B). MS-MS of this ion yielded daughter ions at m/z 143, 156, 249, and 264, confirming the presence of two ^{18}O atoms in the ring and one in the carboxyl group.

DISCUSSION

Comparison of ^{18}O incorporation into ABA in water-stressed leaves of a number of different species showed that in each case most ^{18}O was incorporated into the carboxyl group with much less ^{18}O enrichment in the oxygen atoms on the ring. In both barley and bean leaves, ^{18}O enrichment in the tertiary hydroxyl group was greater than in ABA from other species. This may be due to a smaller primary precursor pool in barley and bean leaves than in leaves of the other four species.

Water-stressed roots of cocklebur incorporate two ^{18}O atoms into ABA in high abundance (3). Analysis by MS-MS of a sample of ^{18}O-labeled ABA from stressed cocklebur roots indicated that the first ^{18}O atom was exclusively in the carboxyl group, and the second one in the tertiary hydroxyl group (TG Heath, JAD Zeevaart, unpublished observations). Thus, the ^{18}O labeling pattern of ABA from stressed cocklebur roots was identical to that of ABA from stressed leaves.

Significant incorporation of ^{18}O into ABA in turgid leaves was only observed in the case of bean (Table II). Apparently,

the turnover of ABA is much faster in bean than in cocklebur leaves. A similar difference between these two species was also observed when leaves were stressed for 5 h, then rehydrated for 1 h, followed by incubation in $^{18}O_2$ for 6 h. After recovery from stress for 1 h, the ABA content was rapidly decreasing (13, 19). In the case of cocklebur, no ^{18}O incorporation was observed, whereas in bean leaves significant enrichment was found with the relative abundances of m/z 278, 280, 282, and 284 at 100, 53, 34, and 5, respectively. During the labeling period the ABA content of the bean leaves decreased from 12.4 to 0.2 μg ABA/g dry weight. Thus, while ABA levels were decreasing rapidly in these bean leaves, synthesis of ABA was still taking place.

In unripe avocado fruits, the pattern of ^{18}O incorporation was very similar to that in stressed leaves. However, as the fruit ripened, quite different ^{18}O incorporation patterns were observed (Fig. 2, A and B). It appears that the increased incorporation of ^{18}O on the ring of ABA can best be interpreted by assuming that the primary precursor (a carotenoid with both ring oxygens incorporated prior to the labeling period) pool had been depleted, while the enzymes for ABA biosynthesis were still active.

With the application of MS-MS techniques, one major difference in the ^{18}O-labeling patterns observed in this study was the significant amount of Me-ABA labeled only in the tertiary hydroxyl group, *i.e.* singly labeled Me-ABA lacking the expected ^{18}O atom in the carboxyl group. Although the amount found of this molecular species was not detectable in stressed cocklebur and avocado leaves (Fig. 3A), and in unripe avocado fruits, it was present in small amounts in stressed barley, bean, and tobacco leaves. In ripe avocado fruits, on the other hand, tertiary hydroxyl labeled Me-ABA was a significant component of the monolabeled material (Fig. 3B). At the other extreme, singly labeled Me-ABA from apple fruits (in three out of four samples) contained more ^{18}O in the tertiary hydroxyl than in the carboxyl group (Fig. 3C; Table V). Thus, these various species and tissues produce monolabeled ABA that represents a continuum from ^{18}O incorporation into only the carboxyl group, through a mixture of carboxyl and tertiary hydroxyl labeled ABA, to predominantly tertiary hydroxyl labeled ABA.

These results showing ^{18}O incorporation into the tertiary hydroxyl group of ABA without label in the carboxyl are apparently in conflict with the hypothesis that ABA is derived from a larger precursor molecule by oxidative cleavage (3). On the basis of this hypothesis, ^{18}O enrichment in the tertiary hydroxyl group can at most be equal to, but not higher than in the carboxyl group. One possible explanation is postcleavage incorporation of ^{18}O at the 1′-position, but the only evidence for this to occur in higher plants is in *Vicia faba* (reviewed in ref. 22). Postcleavage oxidation at the 1′-position would also rule out xanthoxin as an intermediate in ABA biosynthesis (22). A second ABA biosynthetic pathway via an ionylidene intermediate is also unlikely, since this would mean that during fruit ripening there is a gradual switch from the indirect to the direct pathway (22). In view of the present results (Table IV, V; Fig. 3) this implies that of ABA synthesized via the direct pathway, neither oxygen atom in the carboxyl group is derived from molecular oxygen. However, this is not in agreement with previous $^{18}O_2$ labeling studies with *Cercospora rosicola* (see note in ref. 4) in which the direct pathway operates.

A more likely explanation is that the initial cleavage product, or some other aldehyde intermediate, is in some tissues relatively slowly converted to ABA, thus allowing ^{18}O in the aldehyde group to exchange with ^{16}O of water (7, 15) in the intracellular environment and thus be lost (the product could not be distinguished from unlabeled ABA). In tissues containing little or no tertiary hydroxyl monolabeled ABA, further oxidation of the cleavage product would be rapid, or occur in an environment that precludes ^{18}O exchange. Thus, in this case there would always be a very tight coupling between incorporation of ^{18}O into the initial cleavage product and its subsequent conversion to a carboxylic acid with retention of ^{18}O. In avocado fruits, there is apparently a gradual uncoupling as the fruit ripens, whereas in apple fruits at the ripening stage used in our experiments, most ^{18}O incorporated in the side chain of the cleavage product would be lost through exchange, because of slow conversion of the aldehyde intermediate. This implies that monolabeled Me-ABA containing ^{18}O in the C-1′ hydroxyl group (m/z 280) actually represents doubly labeled Me-ABA that has lost the aldehyde label prior to conversion to carboxylic acid. Evidence supporting ^{18}O loss from an intermediate prior to conversion to a carboxylic acid has been obtained with apple fruit tissue in which ABA-aldehyde showed an ^{18}O labeling pattern similar to that of ABA (14).

If the aberrant ^{18}O labeling patterns in ripening fruits are indeed due to exchange of ^{18}O from an intermediate in the pathway, then the ^{18}O-labeling is basically the same in all higher plant systems studied so far; stressed leaves and roots (3), stressed etiolated bean leaves (8), turgid leaves, maize embryos (5), and fruits. These results strongly suggest, therefore, that there is only one pathway for ABA biosynthesis in higher plants, although the regulation of ABA biosynthesis is undoubtedly different in the various tissues. A prediction following from this interpretation is that in tissues producing tertiary hydroxyl monolabeled ABA, the long residence time of the aldehyde intermediate may allow sufficient quantities of the aldehyde intermediate to be isolated for identification. Finally, it should be noted that all the tissues used in this study contain carotenoids, so that the dictum 'ABA biosynthesis only in the presence of carotenoids' still holds (22). By contrast, in fungi ABA is synthesized via ionylidene intermediates (12, 22).

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