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Isolation and Quantitation of β-D-Glucopyranosyl Abscisate from Leaves of *Xanthium* and Spinach[1, 2]

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ABSTRACT

From previous work (Zeevaart 1980 Plant Physiol 66: 672–678) *Xanthium* leaves are known to contain a high level of alkali-hydrolyzable conjugated abscisic acid. This abscisic acid conjugate has been isolated and identified by mass spectrometry, nuclear magnetic resonance, and chemical and enzymic degradation techniques, as the glucosyl ester of abscisic acid, β-D-glucopyranosyl abscisate. The glucosyl ester of abscisic acid was the only abscisic acid conjugate found in *Xanthium* leaves. It was also isolated from spinach leaves.

An insignificant amount of the glucosyl ester of abscisic acid partitioned into diethyl ether, whereas 12% partitioned into ethyl acetate. Consequently, removal of abscisic acid by partitioning with ethyl acetate will result in considerable losses of the glucosyl ester of abscisic acid from the aqueous phase. Diethyl ether is, therefore, recommended for separation of abscisic acid and the glucosyl ester of abscisic acid by solvent partitioning.

A method for quantitation of the glucosyl ester of abscisic acid as the tetraacetate derivative by gas-liquid chromatography with an electron capture detector was developed. The level of β-D-glycopyranosyl abscisate in *Xanthium* leaves increased from 3.6 nanomoles per gram fresh weight in turgid leaves to 22.9 nanomoles in leaves from plants subjected to seven wilting-recovery cycles. β-D-glycopyranosyl abscisate in *Xanthium* leaves may be a stable end product of abscisic acid metabolism.

The plant growth substance ABA is metabolized via the unstable intermediate, 6′-hydroxymethyl-ABA to PA[4] and DPA (15). Furthermore, ABA also forms water-soluble conjugates, so-called 'bound' ABA. A general, albeit indirect, procedure for measuring conjugated ABA is to remove free ABA by partitioning with organic solvents, such as diethyl ether or ethyl acetate, followed by alkaline hydrolysis of the conjugated ABA left in the aqueous phase and measurement of the liberated ABA (*e.g.* 3, 4, 10–12, 17, 19). The amount of conjugated ABA thus obtained is usually around one-tenth of the free ABA (12), but much higher values have been reported (4, 10, 19).

Whereas PA and DPA have been identified and measured in many plants, there are few examples of rigorous identification of ABA conjugates. ABA-GE was first identified by Koshimizu *et al.* (5) in immature fruits of yellow lupin. It was also found in the pseudocarp of the field rose (11). A neutral growth inhibitor in dormant apple embryos yielded ABA and glucose upon alkaline hydrolysis (2). After feeding (±)-[^{14}C]ABA to tomato shoots, ABA-GE was identified as a radioactive metabolite (11). When apple seedlings were fed (±)-ABA, an ABA-glucose complex was formed that was hydrolyzed at pH 10 (14). More recently, Loveys (8) observed that after feeding (±)-[^{14}C]ABA to tomato plants, an alkaline labile conjugate was formed which was different from ABA-GE. Milborrow (13) reported the formation of the 1′-*O*-α- and β-glucosides of ABA after feeding (±)-[^{14}C]ABA to apple seeds at 4°C and stated that the latter compound also occurs in tomato shoots. It appears, therefore, that so-called bound ABA may consist of more than one compound.

Previous work in this laboratory (19) has established the occurrence of conjugated ABA in turgid *Xanthium* leaves in levels higher than those of free ABA. During water stress, conjugated ABA increased at a low rate. We report in this paper the identification of this ABA conjugate as ABA-GE. Furthermore, a GLC method for quantitation of ABA-GE as the tetraacetate derivative is described.

MATERIALS AND METHODS

Plant Material. *Xanthium strumarium* L. Chicago strain was grown in a greenhouse as reported previously (19). To increase the level of conjugated ABA, the plants used for bulk extraction were subjected to five stress cycles prior to harvest. Water was withheld until all the leaves had become flaccid. The plants were then watered and the mature leaves were harvested 5 h later when all leaves had regained turgor.

Spinach plants (*Spinacia oleracea* L. cv. Savoy Hybrid 612, Harris Seed Co., Rochester, NY) were initially grown in a growth chamber under short-day conditions and subsequently transferred to long days (9). Different batches of plants were subjected to eight to 12 water stress cycles before the leaves were harvested.

Standard Compounds. (±)-ABA was purchased from Sigma. The tetraaceto-β-D-glucopyranoside ester of (±)-ABA was synthesized from (±)-ABA and α-D-bromoglucose tetraacetate (Sigma), with triethylamine as a catalyst (7). If required, the acetate groups were removed with a crude enzyme preparation from sunflower seeds (6) to give synthetic (±)-ABA-GE. (±)-[^{3}H]ABA-GE (149 mCi/mmol) was prepared by feeding (±)-[^{3}H]ABA (22.5 Ci/mmol, Amersham/Searle) via the transpiration stream to detached primary leaves of *Phaseolus vulgaris* and subsequently isolating the radioactive metabolite that co-chromatographed with (±)-ABA-GE.

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[2] The glucose ester of ABA can be named either as 1-*O*-abscisoyl-β-D-glucopyranose or β-D-glucopyranosyl abscisate. To emphasize the fact that it is an ester of ABA and to avoid confusion with 1′-*O*-abscisic acid-β-D-glucopyranoside, we have chosen the latter nomenclature.

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[4] Abbreviations: PA, phaseic acid; DPA, dihydrophaseic acid; ABA-GE, β-D-glucopyranosyl abscisate; Ac_4-ABA-GE, tetraacetate derivative of ABA-GE; GLC-ECD, gas-liquid chromatography with electron capture detector; GLC-FID, GLC with flame ionization detector; Me-ABA, methyl ester of ABA; *m/z*, mass/charge; TMSi, trimetylsilyl.

Extraction and Purification Procedures. The lyophilized plant material was extracted two times at 4°C with 80% acetone containing 1% (v/v) glacial acetic acid and 100 mg/l of the antioxidant 2,6-di-*tert*-butyl-4-methylphenol. The tissue residue was then homogenized in a Waring Blendor and stirred overnight in 80% acetone. A total of 300 g dry weight of *Xanthium* and 150 g of spinach leaves was extracted. The acetone was evaporated in a rotary evaporator. The remaining aqueous solution was transferred to separatory funnels and partitioned three times against diethyl ether to remove lipophilic material, including free ABA. The aqueous phase was adjusted to pH 3 with 6 N HCl and passed through charcoal-Celite columns, by using 1 g charcoal for every 6 g dry plant material extracted. After the columns were washed with water containing 1% acetic acid, they were eluted with 30% acetone. The acetone was removed with a rotary evaporator, and the aqueous phase frozen and lyophilized. The residue was fractionated via preparative reverse phase HPLC with a Bondapak C_{18}/Porasil B column (19). Conjugated ABA eluted between 16 and 22 min in a linear gradient of 95% ethanol (20 to 50% in 30 min) in 1% aqueous acetic acid at a flow rate of 9.9 ml/min. The collected fraction was reduced to the aqueous phase with a rotary evaporator, frozen, and lyophilized. The residue was dissolved in acetone and applied as a narrow band to 20 × 20 × 0.025 cm Silica gel 60 F-254 plates, (Scientific Products, McGaw Park, IL). The plates were developed in solvent system 1 (Table I). The quenching zone at $R_F = 0.36$ co-chromatographing with synthetic (±)-ABA-GE was scraped from the plates and eluted with acetone. The solvent was evaporated and the ABA-GE was further purified by analytical reverse phase HPLC with a μBondapak C_{18}, 30 × 0.4 cm column (Waters Associates, Milford, MA). The sample was eluted by means of a linear gradient from 10% to 50% methanol in 1% aqueous acetic acid in 40 min at a flow rate of 2 ml/min. ABA-GE eluted between 22 and 25 min. After lyophilization, the samples were acetylated with acetic anhydride-pyridine (1:1, v/v) overnight at room temperature. The reagents were removed *in vacuo* and Ac_4-ABA-GE was purified on Silica gel 60 F-254 plates by using solvent system 2 or 3 (Table I).

Quantitation of Conjugated ABA. Samples of *Xanthium*, each containing 10 leaves (26 to 33 g fresh weight) were extracted with acetone as described above. To each sample, 20,000 dpm of (±)-[^{3}H]ABA-GE was added to assess losses during extraction and purification. After evaporation of the acetone with a rotary evaporator, the aqueous residue was partitioned three times with diethyl ether. Three-fourths of the aqueous phase was then purified as described above for ABA-GE, and acetylated for analysis by GLC. The remaining one-fourth was adjusted to pH 12 to hydrolyze conjugated ABA. After 2 d, the pH was lowered to 3 and the solution was passed through a charcoal-Celite column to remove inorganic salts. ABA was eluted with 60% acetone. The dry residue after lyophilization was dissolved in a small volume of acetone, streaked on a Silica gel 60 F-254 plate, and developed in solvent system 4 (Table I). The zone co-chromatographing with authentic ABA was scraped off, eluted with acetone, and methylated with ethereal diazomethane. After measuring Ac_4-ABA-GE and Me-ABA by GLC, the radioactivity in each sample was determined as described (19). Recovery of radioactivity was between 49% and 56% for Ac_4-ABA-GE, and between 52% and 62% for the fractions measured as free ABA after alkaline hydrolysis. The data were corrected for recovery losses.

GLC. ABA-GE was measured as its tetraacetate derivative, Ac_4-ABA-GE, with a Hewlett-Packard 5840 gas chromatograph equipped with a ^{63}Ni electron capture detector. The samples were dissolved in ethyl acetate for chromatography. Routine analyses were done on a short column (46 × 0.2 cm), packed with 3% SE-30 on 100- to 120-mesh Gas Chrom Q (Supelco Inc., Bellefonte, PA). Conditions were as follows: column temperature 245°C, injection port 280°C, and detector at 300°C. Carrier gas was argon-methane (95:5) at a flow rate of 25 ml/min.

Free ABA was measured as Me-ABA after treatment with ethereal diazomethane by GLC-EC with a 1% XE-60 column as described (18), or with the same short 3% SE-30 column as used for Ac_4-ABA-GE, but with a column temperature of 155°C.

Free glucose was determined as its TMSi derivative with a 3% SE-30 column (180 × 0.2 cm) and FID detector. The temperature was programmed from 165 to 240°C at 1.5 C min^{-1} with N_2 at 25 ml/min as a carrier gas.

Mass Spectrometry. Mass spectra were routinely obtained with a Hewlett-Packard 5985 quadrupole mass spectrometer connected to a 5840A gas chromatograph. Samples were run through the short 3% SE-30 column (see above) at 240°C with helium (30 ml/min) as carrier gas. The ionizing potential was 70 ev. Inasmuch as it was difficult to obtain high m/z values due to discrimination by the quadrupole mass spectrometer, direct probe mass spectra were also obtained on an AEI MS-9 magnetic sector mass spectrometer with a probe temperature of 130°C.

NMR and UV Spectrometry. NMR spectra were taken at room temperature in acetone-d_6 with $Si(CH_3)_4$ as an internal standard by using a 270 MHz Bruker WH270 Fourier transform spectrometer.

UV spectra of ABA-GE and Ac_4-ABA-GE were taken in ethanol by using a Beckman DB spectrometer. For quantitation of Ac_4-ABA-GE $\epsilon = 20{,}000$ ($\lambda_{max} = 273$ nm) was used (5). Spectra of Me-ABA were measured in methanol and $\epsilon = 20{,}900$ ($\lambda_{max} = 265$ nm) was used for quantitation (18).

Enzymic Hydrolysis of ABA-GE. Samples were dissolved in the appropriate buffers and incubated at room temperature for 48 h with excess of the following enzymes: (a) α-glucosidase type I (Sigma) in 0.1 M (pH 7) phosphate buffer, (b) β-glucosidase from almonds (Worthington Biochemicals, Freehold, NJ) in 0.1 M (pH 4.6) acetate buffer. The enzymes showed a high degree of specificity for the substrates maltose and cellobiose, respectively. Progress of the reaction was monitored by the disappearence of ABA-

Table I. *Solvent Systems Used for Thin-Layer Chromatography with R_F* Values

Silica gel 60 F-254 plates, 0.25 mm thick, were developed to 12 cm. In the case of solvent system 4, the plates were developed to 6, 9, and 12 cm, successively.

Solvent	Composition	R_F Values			
		PA	ABA	ABA-GE	Ac_4-ABA-GE
1	Chloroform:methanol:water (75:22:3, v/v/v)	0.52	0.70	0.36	0.90
2	Chloroform:methanol (96:4, v/v)				0.53
3	Hexane:ethyl acetate (4:1, v/v)				0.15
4	Toluene:ethyl acetate:acetic acid (50:30:4, v/v/v)	0.33	0.49	0.0	0.45

GE on Silica gel 60 F-254 plates with solvent 1 (Table I), and the appearance of free ABA by GLC.

Determination of Partition Coefficients of ABA-GE. Samples of approximately 50 μg (±)-ABA-GE containing 25,000 dpm (±)-[^{3}H]ABA-GE were dissolved in 10 ml 0.1 M phosphate buffer at pH 4.5. The pH was adjusted to 3 with 6 N HCl, as is common practice for obtaining the acidic fraction by partitioning with organic solvents (19). The buffer was shaken vigorously with equal volumes of diethyl ether or ethyl acetate in 60-ml separatory funnels; each treatment was done in duplicate. After separation of the organic and aqueous phases, each phase was dried in a scintillation vial and the radioactivity determined by liquid scintillation spectrometry. Distribution of radioactivity between the two phases at 22°C was used to calculate the partition coefficient, K_d = radioactivity in aqueous phase/radioactivity in organic phase.

RESULTS AND DISCUSSION

Identification of ABA-GE in *Xanthium* and Spinach Leaves. ABA-GE was isolated from *Xanthium* and spinach leaves and identified by comparisons of its physical-chemical properties with those of synthetic material. The electron impact mass spectra of acetylated material from both *Xanthium* and spinach were identical with that of synthetic Ac_4-ABA-GE (Fig. 1), and were in agreement with published data (5, 7). The presence of a hexose is indicated by fragments at *m/z* 331 (76.4%), 271 (35.9%), 169 (100%), and 109 (80.9%) (1). Key fragments at *m/z* 594 (M^+, 0.3%), 576 (0.1%), 538 (1.0%), 441 (6.2%), 247 (51.1%), and 190 (61.0%) suggest an ABA substituent coupled through an ester linkage to C-1 of the hexose. This assignment is strengthened by the 270 MHz ^{1}H-NMR spectrum of the compound isolated from *Xanthium* which shows vinyl resonances (1 proton each) at 7.92 (*d*, J = 16 Hz), 6.45 (*d*, J = 16 Hz), 5.87 (s), and 5.74 (s) ppm downfield from $Si(CH_3)_4$. A single proton resonance, tentatively assigned to the anomeric hydrogen at C-1 of the hexose (5.52 ppm, *d*, J = 8.5 Hz) and the four methyl resonances (three protons each) at 2.11, 1.95, 1.10, and 1.04 ppm all agree with the NMR spectrum of ABA-GE from lupin seeds (5).

The nature and position of the hexose derivative was further confirmed by degradation studies. Both isolated and synthetic ABA-GE were unaffected (by TLC and GLC) by treatment with diazomethane, thus implying an ester linkage through C-1 of ABA. Hydrolysis with acid (2 N HCl), or base (KOH, pH 12) yielded 1 mole of ABA per mole glucose. Isolated and synthetic ABA-GE were both stable to α-glucosidase, but were rapidly hydrolyzed by β-glucosidase to give equimolar amounts of free ABA and glucose.

In summary, the evidence suggesting that the ABA conjugate isolated from *Xanthium* and spinach leaves is β-D-glucopyranosyl abscisate, is based on the following observations: (a) upon hydrolysis the conjugate yielded ABA and D-glucose; (b) it was hydrolyzed by β-, but not by α-glucosidase; and (c) the NMR and mass spectra, as well as chromatographic properties of the conjugate, were identical to those of synthetic (±)-ABA-GE.

In some plants, there are indications of the presence of ABA conjugates other than ABA-GE (8, 13, 16). The question arose, therefore, whether all conjugated ABA in *Xanthium* leaves can be accounted for by ABA-GE. This was investigated in the following experiment. Ten *Xanthium* leaves (24 g fresh weight) from plants subjected to three wilting-recovery cycles were extracted as usual. The residue of the 30% acetone fraction after charcoal purification was streaked on silica gel 60 F-254 plates. After development in system 1, six quenching zones were observed under a UV lamp. Each zone was scraped off and eluted. The eluates were subdivided: one-half was subjected to alkaline (pH 12) hydrolysis for 2 d, the other half was dissolved in 2 N HCl for 2 h. Each hydrolysate was tested for the presence of ABA. Only the zone at R_F = 0.36 which co-chromatographed with ABA-GE, yielded ABA after both alkaline and acidic hydrolysis. The zone at R_F = 0.52 consisted of free PA. These results suggest that only one ABA conjugate, *viz.* ABA-GE, is present in *Xanthium* leaves in significant quantity.

Partition Coefficients of ABA-GE. The partition coefficients of ABA-GE, K_d, in diethyl ether and ethyl acetate were 300 and 7.4, respectively. Only 0.33% of the radioactivity was present in diethyl ether, whereas 12% partitioned into ethyl acetate. After partitioning three times with equal volumes of ethyl acetate $(0.88)^3 = 0.68$ of the ABA-GE would still be left in the aqueous phase. This agrees with the earlier finding (Table I in Ref. 19) that after partitioning three times with ethyl acetate only 68% of the conjugated ABA remained in the aqueous phase. In some experiments (19) partitioning with ethyl acetate was performed six times; this would leave only $(0.88)^6 = 0.46$ of the ABA-GE in the aqueous phase. The implication of these data is that if separation of free ABA and ABA-GE is to be accomplished by partitioning, diethyl ether is the solvent of choice.

Quantitation of ABA-GE by GLC. Previous methods for measuring ABA-GE were based on analysis of free ABA released by alkaline hydrolysis. With the report that the 1′-*O*-glucoside of

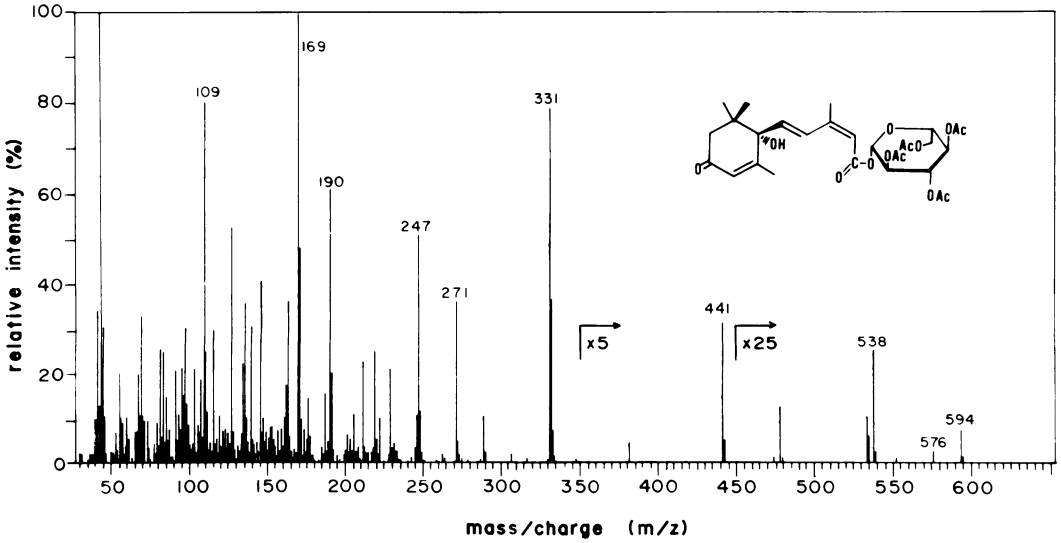

FIG. 1. Mass spectrum obtained with AEI MS-9 instrument of β-D-glucopyranosyl abscisate tetraacetate isolated from *Xanthium* leaves.

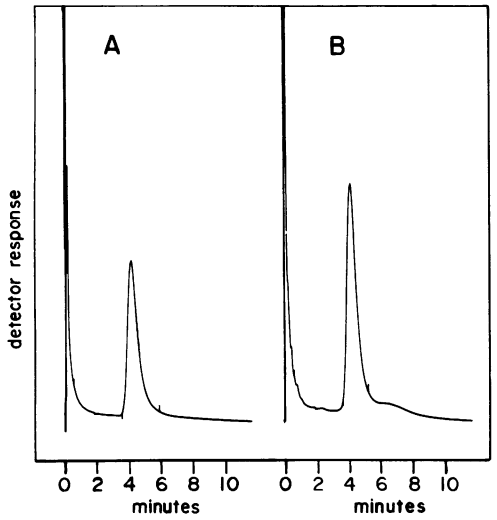

FIG. 2. Gas chromatograms of the tetraacetate derivative of β-D-glucopyranosyl abscisate. A, GLC profile after injection of 12.5 ng (0.021 nmol) synthetic (±)-AC_4-ABA-GE on 46-cm column packed with 3% SE-30 and run at 245°C with ECD. B, GLC profile after injection of aliquot that was equivalent to extract from 2 mg fresh weight of *Xanthium* leaves subjected to nine wilting-recovery cycles.

Table II. *Comparison of the Levels of Conjugated ABA in Leaves of Xanthium Plants Subjected to Different Numbers of Stress Cycles by Two Different Methods*

Conjugated ABA was determined by direct measurement of Ac_4-ABA-GE, or after alkaline hydrolysis and measurement of the ABA liberated as Me-ABA.

Treatment of Plants	Ac_4-ABA-GE	Free ABA after Alkaline Hydrolysis
	nmol/g fresh wt	
Turgid	3.6	3.1
One stress cycle	10.6	10.6
Seven stress cycles	22.9	22.6

Table III. *Comparison of the Levels of Free ABA and of Conjugated ABA Liberated by Alkaline Hydrolysis in Spinach Leaves That Were Turgid or Subjected to Several Stress Cycles*

Stressed plants were not watered until all leaves were clearly flaccid. Leaves harvested 4 h after watering.

Treatment	Free ABA	Conjugated ABA
	nmol/g fresh wt	
Experiment 1		
Turgid	0.05	0.10
Three stress cycles	0.28	0.41
Experiment 2		
Turgid	0.08	0.15
Nine stress cycles	0.26	0.50

ABA is a naturally occurring compound (13), it is now apparent that it is not necessary that all ABA released by alkaline hydrolysis comes from ABA-GE. For this reason, it was desirable to develop a procedure for the direct analysis of ABA-GE, or its derivative. For ease and sensitivity, the method of choice is GLC-ECD. Although ABA-GE can be silylated with Trisil (Pierce Chemical Co., Rockford, IL) to give the penta-TMSi derivative (M^+ at m/z = 786), this derivative was unstable and not suitable for quantitation. Acetylation with acetic anhydride and pyridine was complete in 5 h at room temperature, although the reaction could be allowed to go overnight with little or no decomposition. Because of the low volatility of Ac_4-ABA-GE, the acetic anhydride and pyridine can conveniently be removed *in vacuo* without loss of the acetylated derivative. The tetraacetate derivative also has the advantage that it can be easily synthesized (7) for use as a standard. The low volatility of Ac_4-ABA-GE necessitates the use of high temperatures for GLC. Although a 180-cm column with a light liquid phase coating (either 0.5% or 1% SE-30) is satisfactory, better results are obtained with a short (45 cm) column packed with 3% SE-30. With a column temperature of 245°C, the retention time of Ac_4-ABA-GE was 4.3 min (Fig. 2). The ECD was approximately half as sensitive to the Ac_4-ABA-GE as to Me-ABA on a molar basis.

A comparison of the amounts of ABA-GE in *Xanthium* leaves as determined by measurement of Ac_4-ABA-GE, or after hydrolysis, is shown in Table II. The good agreement between these two different methods is evident. These results strongly suggest that the ABA released by alkaline hydrolysis in *Xanthium* leaves can be quantitatively accounted for by the ABA-GE. It is clear from the data in Table II that the level of ABA-GE increased considerably with increasing number of wilting-recovery cycles. After seven cycles, the leaves contained 22.9 nmol ABA-GE/g fresh weight, or expressed as free ABA, 6 μg/g fresh weight. A considerable increase in the level of conjugated ABA after several water stress cycles has also been observed in spinach (Table III); it should be noted, however, that in this plant the levels of both ABA and conjugated ABA are much lower than in *Xanthium* (19). The ABA-GE that accumulates in *Xanthium* in response to water stress is quite stable (19; and J. A. D. Zeevaart, unpublished). The physiological role of this ABA-GE, if any, is not known at present. It may represent an inert end product of ABA metabolism which is sequestered in the cell.

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