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The Compartmentation of Abscisic Acid and β-D-Glucopyranosyl Abscisate in Mesophyll Cells[1]

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ABSTRACT

β-D-Glucopyranosyl abscisate (ABA-GE) is synthesized in *Xanthium strumarium* L. leaves during water stress. Following recovery from stress, the amount of ABA-GE does not decline. These observations led to the hypothesis that ABA-GE is sequestered in the vacuole where it is metabolically inert. The localization of abscisic acid (ABA) and ABA-GE was investigated by a dimethyl sulfoxide (DMSO) compartmentation method and by direct isolation of vacuoles.

With the DMSO compartmentation method it was shown that in *Xanthium* mesophyll cells ABA was in a compartment not accessible to DMSO, presumably the chloroplast, whereas ABA-GE was in a compartment accessible to DMSO, presumably the vacuole. Neutral red, which accumulates in the vacuoles, showed a similar DMSO concentration dependence for its release from the cells as ABA-GE.

Vacuoles isolated from *Vicia faba* L. leaf protoplasts contained 22% of the total ABA and 91% of the ABA-GE. Some of the ABA in the vacuole preparations was probably due to cytoplasmic contamination. These findings indicate that ABA-GE is sequestered in the vacuoles of mesophyll cells where the conjugated form of ABA is removed from the active ABA pool.

The plant growth substance ABA is metabolized via the unstable intermediate, 6′-hydroxymethyl-ABA, to PA[3] and DPA, or by conjugation of ABA to glucose, forming ABA-GE. PA and DPA may also be conjugated to glucose (17). The physiological role of these metabolites is not known. It has been postulated that ABA-GE is a storage form of ABA. At times when the level of ABA in the plant increases rapidly, such as during water stress, ABA would be hydrolyzed, releasing free ABA. However, the level of ABA-GE did not decrease in water-stressed leaves of *Xanthium strumarium*, but actually increased. After the stress had been relieved, the amount of ABA-GE remained constant for at least 34 d (20). During aging, the level of ABA-GE in leaves also increased (18, 21). In addition, when ABA-GE was applied to plant tissues, it was rapidly hydrolyzed to free ABA (20, 21). As a whole, these observations support the hypothesis that ABA-GE is localized in a cellular compartment where net hydrolysis of ABA-GE to free ABA does not occur. Conjugation of ABA and sequestration could serve as an inactivation mechanism to remove some of the ABA from the pool of physiologically active ABA. In this paper we present evidence that the bulk of ABA-GE is sequestered in the vacuoles of mesophyll cells.

MATERIALS AND METHODS

Plant Material. Plants of *Xanthium strumarium* L., Chicago strain, were grown from seeds in a greenhouse (19), and were well watered. The two most recently fully expanded leaves were harvested from plants grown for 2 to 3 months.

Vicia faba L. was grown in a growth chamber under a 20-h photoperiod as described (20).

DMSO Compartmentation Studies with *Xanthium* Mesophyll Cells. *Xanthium* mesophyll cell suspensions were prepared according to the method of Sharkey and Raschke (15). Isolated cells were treated with 0, 2.5, or 20% DMSO for 20 min. The cell suspensions were centrifuged at the end of the treatment and the amount of ABA and ABA-GE was measured in the supernatant and in the cells. Neutral red was preloaded into the vacuoles to determine the amount of DMSO necessary to disrupt the tonoplast (7). The cells were washed several times, after which DMSO was applied at various concentrations in 0.1 M Hepes buffer at pH 7.0. The amount of neutral red in the supernatant was determined spectrophotometrically at 530 nm. The cells were then boiled to release the remainder of the neutral red, centrifuged, and the amount of neutral red in the supernatant was determined.

Vacuole Isolation. Protoplasts were isolated from *V. faba* leaves as described (9) with slight modifications. The lower epidermis was removed and the leaves, with exposed mesophyll down, were floated on a solution containing 0.6 M mannitol, 1% cellulysin, and 0.5% macerase at pH 5.6 in plastic Petri dishes. The leaves were left to digest at 27°C for 2 to 4 h. The medium was then filtered through Miracloth to remove debris. The protoplasts were washed 3 times by low speed centrifugation (100 *g*). The medium was gradually changed during the washes into vacuole buffer which contained: 0.55 M mannitol, 0.2 mM KH_2PO_4, 1 mM KNO_3, 1 mM $MgSO_4$, 10 mM $CaCl_2$, 20 mM Mes, and 20 mM Hepes adjusted with 1 N KOH to pH 7.2. The protoplasts were then layered on top of a discontinuous gradient of 7.5 and 12% Ficoll (9). The gradients were centrifuged in a SW50.1 rotor of an L8-55M Beckman ultracentrifuge for 30 min at 300,000*g* (50,000 rpm). The vacuoles were collected from both interfaces of the gradient. The vacuoles were diluted with vacuole buffer 1:1 and washed by low speed centrifugation 3 times. The number of vacuoles and protoplasts was determined under a microscope with the aid of a hemacytometer.

Extraction and Purification Procedures for ABA and Its Metabolites. *Xanthium* mesophyll cells, protoplasts, or vacuoles were extracted according to Zeevaart (19). The medium from the DMSO compartmentation experiments was filtered through a 0.45 μm nylon filter to remove the cells, and was then lyophilized.

To estimate losses during the extraction and purification pro-

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[3] Abbreviations: PA, phaseic acid; ABA-GE, β-D-glucopyranosyl abscisate; DPA, dihydrophaseic acid.

cedures, 2800 cpm (±) [^{3}H]ABA (16.4 Ci/mmol) and 7220 cpm of (±) [^{3}H]ABA-GE (149 mCi/mmol) were added to the samples. The average recovery of both compounds was 83%. During extraction of *V. faba* protoplasts and vacuoles, 700 cpm of [^{3}H] ABA and 950 to 1300 cpm of [^{3}H]ABA-GE were added. The average recovery of [^{3}H]ABA and [^{3}H]ABA-GE was 78 and 58%, respectively.

ABA and ABA-GE were purified by semi-preparative reverse phase HPLC on a 10-μm particle size μBondapak C_{18} column (30 × 0.78 cm, Waters Associates, Milford, MA). ABA and ABA-GE were eluted by means of a linear gradient from 0 to 50% ethanol in 1% acetic acid in 30 min at a flow rate of 2.5 ml/min. The fraction containing ABA-GE was hydrolyzed in 2 M NH_4OH at 60°C for 2 h. After drying, the ABA fractions were methylated with ethereal diazomethane. Quantification of ABA was performed by GLC-ECD as previously described (5), using the ethyl ester of ABA as internal standard. Each sample was dissolved in ethyl acetate containing 50 ng of ethylated ABA. Different amounts of methylated (±)-ABA for the standard curve were also dissolved in ethyl acetate containing ethylated ABA. The latter had a longer retention time than methylated ABA on the capillary column. The amount of ABA in each sample was determined by the ratio of methylated ABA to ethylated ABA. Therefore, injection errors were negated. The quantity of ABA in a sample thus calculated was corrected for recovery of [^{3}H] ABA in the sample.

Enzyme Assays. Glucose-6-P and P-glucomutase were assayed by following the reduction of $NADPH^+$ (16). For NADPH Cyt *c* reductase the reduction of Cytochrome *c* was followed (12). Cyt *c* oxidase assays were performed as above, except that the oxidation of reduced Cyt *c* was measured (12). The assay for NAD malic dehydrogenase followed the reduction of NAD^+ with L-malate (2). Vacuole enzyme markers could not be measured in *V. faba* due to interference in the α-mannosidase assay by endogenous compounds.

RESULTS

The cellular distribution of ABA and ABA-GE was investigated in mesophyll cells using two methods: (a) a DMSO compartmental analysis method, and (b) direct isolation of organelles from protoplasts.

DMSO Compartmental Analysis. This method exploits the differential sensitivity of the plasmalemma and the tonoplast to treatment with DMSO. The tonoplast is disrupted by a higher concentration of DMSO in the medium than the plasmalemma (7).

The optimal DMSO concentration required to release the vacuolar contents was determined after preloading *Xanthium* cells with neutral red (7). Observation of the cells with a light microscope indicated that the neutral red was localized within the vacuoles. Approximately 75% of the neutral red was released from the cells in 20% DMSO after 20 min, but only 10% was released with 2.5% DMSO.

Results of a preliminary experiment with isolated *Xanthium* mesophyll cells indicated that the release of ABA-GE increased with increasing DMSO concentrations in the medium (data not shown). In contrast, the release of free ABA from the *Xanthium* cells was not appreciably affected by the DMSO concentration.

The release of ABA and ABA-GE from *Xanthium* mesophyll cells after treatment with DMSO is shown in Figure 1A. After 2 h, 90% of the ABA was left in the cells, regardless of the DMSO concentration. Likewise, 90% of the ABA-GE remained in the cells after treatment with 0 or 2.5% DMSO (Fig. 1B). However, 75% of the ABA-GE was released from the cells after incubation with 20% DMSO for 2 h (Fig. 1B). The time course for release of neutral red from *Xanthium* cells preloaded with this dye was also dependent on the DMSO concentration (Fig. 1C). With 20%

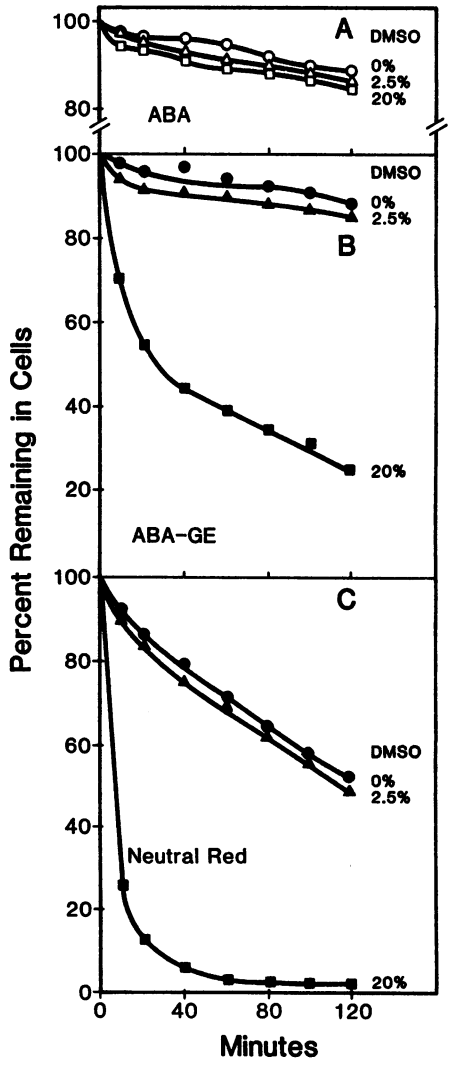

FIG. 1. Time course of the release of ABA (A), ABA-GE (B), and neutral red (C) from isolated *Xanthium* mesophyll cells as affected by the DMSO concentrations in the medium. ABA, ABA-GE, and neutral red were measured in the medium and in the cells over a 2-h period. The data are expressed as the percentages of the total ABA, ABA-GE, and neutral red that remained in the cells after the treatments. There was a total of 461 ± 41 ng of ABA and 1244 ± 13 ng of ABA-GE in each sample.

DMSO, more than 90% of the neutral red was released after 40 min. These results demonstrate that in 20% DMSO the vacuolar contents are released into the medium, and that ABA-GE is localized in a compartment accessible to 20% DMSO, presumably the vacuole. Free ABA appears to be localized within a compartment that is not disrupted by DMSO, presumably the chloroplast, since other workers have shown that a large proportion of ABA is localized in the chloroplasts (10, 11).

Isolation of Vacuoles. To demonstrate directly that ABA-GE is located in the vacuoles of mesophyll cells, vacuoles were isolated from protoplasts and the ABA-GE content of protoplasts and vacuoles was compared. This aspect of the work was carried out with *V. faba*, since *Xanthium* cell walls were not digested with any of the commercially available enzymes. Vacuoles were isolated from *V. faba* protolasts using an ultracentrifugation method (9) which was modified so that an enriched preparation containing 10^7 vacuoles could be obtained routinely. To determine the distribution of ABA and ABA-GE in the cell, the

amounts of both compounds present in an equal number of protoplasts and vacuoles were compared. The average of five experiments showed that 92% of the ABA-GE was localized in the vacuoles, whereas only 22% of the free ABA was in the enriched vacuole fraction (Table I). The cytoplasmic contamination of the vacuoles was estimated by assaying five cytoplasmic enzymes (Table II). On the average, 22% of the enzyme activity was associated with the vacuole fraction which indicates that some contamination with cytoplasm remained. The ultracentrifugation method for vacuole isolation was chosen for its reproducibility and high yield, although the method was clearly not optimal for elimination of cytoplasmic contamination.

DISCUSSION

It has been assumed that only a small fraction of the cellular ABA is contained within the vacuole (4), although direct measurements of the amount of ABA in vacuoles have not previously been made. When chloroplasts were isolated from leaves, a large proportion of the leaf ABA was contained within these organelles (11, 14). Heilmann *et al.* (11) have shown that the distribution of ABA in the cell is determined by the pH gradients established among the different cellular compartments. The higher pH of the chloroplasts serves as an anionic trap for the weak acid, trapping more than 80% of the ABA in an illuminated leaf. The lower pH of the vacuole results in exclusion of ABA from the vacuole. Cowan *et al.* (4), using these rules for distribution of ABA within a cell and data on the pH of the various cellular compartments, calculated that 10 and 18.7% of the ABA in the cell would be in the vacuole in the light and dark, respectively. The amount of ABA in the vacuole is dependent upon the irradiance, because the pH of the chloroplast increases when it is illuminated (4). Results supporting these predictions were obtained with *Valerianella locusta* mesophyll protoplasts loaded with (±) [^{3}H]ABA. It was calculated that 13 and 28% of the [^{3}H] ABA in the cells was localized within the vacuole in the light and dark, respectively (10).

The amount of ABA in root vacuoles has also been estimated using compartmental analysis (3). The concentration of ABA in the cytoplasm was 3.63 μM, and in the vacuoles 0.39 μM. These workers assumed that the volume ratio of vacuoles to cytoplasm was 8 to 1. Based on this assumption, and recalculating these data, 46.2% of the total ABA in the cell was present in the vacuoles of the root cells.

The experiments reported here were not done in sufficiently strong light to sustain photosynthesis. Therefore, the ABA present in the vacuole fraction should be compared only to results of other experiments done in darkness. The direct measurement of 22% of the endogenous ABA present in the vacuoles of *V. faba* mesophyll cells (Table I), and the estimate of 16% of the ABA in the vacuoles of *Xanthium* (Fig. 1A), compare favorably with estimates and predictions made by other methods. Direct measurement of ABA in the vacuoles would be expected to give a slightly higher value than the actual amount of ABA present for several reasons. First, the vacuole preparations were contaminated with cytoplasmic components containing ABA. Second, the comparison was made between the vacuoles and the protoplasts, thereby neglecting the contribution made to the total ABA in the leaf by the free space. Consequently, the percentage of ABA in the vacuoles compared to that in the protoplasts would be higher than compared to that of the whole leaf.

Hartung *et al.* (10) found that in spinach leaves preloaded with radioactive ABA, 75 to 80% of the radiolabeled conjugated ABA was localized outside the chloroplasts. We found 91% of the ABA-GE in *V. faba* vacuoles and 80% in the vacuoles of *Xanthium* cells. Although knowledge of the location of ABA-GE in the cell does not necessarily elucidate its physiological role, these results suggest that ABA-GE is not physiologically active, and that conjugation is a means by which plants remove ABA from the active pool.

The glucose conjugates of other plant growth substances, such as GA_8 glucoside (8) and 2,4-D hydroxylated glycosides (6), are also localized in the vacuoles. Alibert *et al.* (1) observed that *o*-coumaric acid glucoside is synthesized in the cytoplasm and subsequently sequestered in the vacuole. We were unable to establish the formation of ABA-GE in the cytoplasm and its subsequent movement into the vacuole (E. A. Bray, unpublished results). The pH optimum for the ABA glucosyl transferase was around pH 5.0 (13) which has led to the suggestion that ABA-

Table I. *Compartmentation of ABA and ABA-GE in Mesophyll Cells of Vicia faba*

Vacuoles were isolated from mesophyll protoplasts. The amount of ABA and ABA-GE in a known number of protoplasts and vacuoles was analyzed in five separate experiments. The data are expressed as the percentages of these compounds found in the vacuoles with respect to the protoplasts.

	Content		% in Vacuoles
	Protoplasts	Vacuoles	
	ng per 10^6		
ABA	1.19 ± 0.21[a]	0.26 ± 0.07	22
ABA-GE	0.55 ± 0.14	0.50 ± 0.14	91

[a] Mean ± SE.

Table II. *Enzyme Markers in Mesophyll Cells of Vicia faba*

The extravacuolar contamination of the vacuole fraction was estimated by measuring the activity of the enzymes in a known number of protoplasts and vacuoles. The data are expressed as the percentage of activity in the vacuoles as compared with that in the protoplasts.

Enzyme	Activity per 10^6		Activity in Vacuolar Fraction
	Protoplasts	Vacuoles	
	nmol·min^{-1}		%
Glucose-6-P dehydrogenase (cytoplasm)	82	16	20
P-glucomutase (cytoplasm)	459	96	21
NAD malic dehydrogenase (cytoplasm, mitochondria, (chloroplasts)	295	70	24
Cyt c oxidase (mitochondria)	696	66	9
NADPH Cyt c reductase (mitochondria, ER)	319	121	38
			$\bar{x}$ = 22

GE is synthesized in the vacuole (10).

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LITERATURE CITED

1. ALIBERT G, AM BOUDET, P RATABOUL 1982 Transport of *o*-coumaric acid glucoside in isolated vacuoles of sweet clover. *In* D Marmé, E Marrè, R Hertel, eds, Plasmalemma and Tonoplast: Their Functions in the Plant Cell. Elsevier Biomedical, Amsterdam, pp 193–200
2. ASAHI T, M NISHIMURA 1973 Regulatory function of malate dehydrogenase isoenzymes in the cotyledons of mung bean. J Biochem 73: 217–225
3. BEHL R, WD JESCHKE, W HARTUNG 1982 A compartmental analysis of abscisic acid in roots of *Hordeum distichon*. J Exp Bot 32: 889–897
4. COWAN IR, JA RAVEN, W HARTUNG, GD FARQUHAR 1982 A possilbe role for abscisic acid in coupling stomatal conductance and photosynthetic carbon metabolism in leaves. Aust J Plant Physiol 9: 489–498
5. CREELMAN R, JAD ZEEVAART 1984 Incorporation of oxygen into abscisic acid and phaseic acid from molecular oxygen. Plant Physiol 75: 166–169
6. DAVIDONIS GH, RH HAMILTON, RO MUMMA 1982 Evidence for compartmentalization of conjugates of 2,4-dichlorophenoxyacetic acid in soybean callus tissue. Plant Physiol 70: 939–942
7. DELMER DP 1979 Dimethylsulfoxide as a potential tool for analysis of compartmentation in living plant cells. Plant Physiol 64: 623–629
8. GARCIA-MARTINEZ JL, JB OHLROGGE, L RAPPAPORT 1981 Differential compartmentation of gibberellin A_1 and its metabolites in vacuoles of cowpea and barley leaves. Plant Physiol 68: 865–867
9. GUY M, H KENDE 1984 Conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene by isolated vacuoles of *Pisum sativum* L. Planta 160: 281–287
10. HARTUNG W, H GIMMLER, B HEILMANN 1982 The compartmentation of abscisic acid (ABA), of ABA-biosynthesis, ABA-metabolism and ABA-conjugation. *In* PF Wareing, ed, Plant Growth Substances 1982. Academic Press, New York, pp 325–333
11. HEILMANN B, W HARTUNG, H GIMMLER 1980 The distribution of abscisic acid between chloroplasts and cytoplasm of leaf cells and the permeability of the chloroplast envelope for abscisic acid. Z Pflanzenphysiol 97: 67–78
12. HODGES TK, RT LEONARD 1974 Purification of a membrane-bound adenosine triphosphatase from plant roots. Methods Enzymol 32: 392–406
13. LEHMANN H, HR SCHÜTTE 1980 Purification and characterization of an abscisic acid glucosylating enzyme from cell suspension cultures of *Macleaya microcarpa*. Z Pflanzenphysiol 96: 277–280
14. LOVEYS BR 1977 The intracellular location of abscisic acid in stressed and non-stressed leaf tissue. Physiol Plant 40: 6–10
15. SHARKEY TD, K RASCHKE 1980 Effects of phaseic acid and dihydrophaseic acid on stomata and the photosynthetic apparatus. Plant Physiol 65: 291–297
16. SIMCOX PD, EE REID, DT CANVIN, DT DENNIS 1977 Enzymes of the glycolytic and pentose phosphate pathways in proplastids from the developing endosperm of *Ricinus communis* L. Plant Physiol 59: 1128–1132
17. WALTON DC 1980 Biochemistry and physiology of abscisic acid. Annu Rev Plant Physiol 31: 453–489
18. WEILER EW 1980 Radioimmunoassays for the differential and direct analysis of free and conjugated abscisic acid in plant extracts. Planta 148: 262–272
19. 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
20. ZEEVAART JAD 1983 Metabolism of abscisic acid and its regulation in *Xanthium* leaves during and after water stress. Plant Physiol 71: 477–481
21. ZEEVAART JAD, GL BOYER 1984 Accumulation and transport of abscisic acid and its metabolites in *Ricinus* and *Xanthium*. Plant Physiol 74: 934–939