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Abscisic Acid Accumulation by *in Situ* and Isolated Guard Cells of *Pisum sativum* L. and *Vicia faba* L. in Relation to Water Stress[1]

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ABSTRACT

Isolated guard cells, prepared by sonication of epidermal peels, were used to investigate the endogenous level of abscisic acid (ABA) in the guard cells of turgid and stressed leaves of *Vicia faba* L. and the *argenteum* (*arg*) mutant of *Pisum sativum* L. The guard cells of *V. faba* and *arg* were found to contain 18 and 8 times more ABA, respectively, when isolated from stressed leaves than from turgid leaves. Isolated guard cells of *V. faba* were also directly stressed with the osmoticum Aquacide III. These guard cells were capable of producing stress-induced ABA to at least 3 times their ABA level when non-stressed.

ABA is thought to be involved in the regulation of stomatal behavior during water stress as application of ABA to intact leaves or epidermal strips induces stomatal closure (7, 8, 23), and wilted leaves accumulate large amounts of endogenous ABA (8, 23, 27). Even the rapid stomatal closure observed during the onset of stress, before bulk leaf ABA levels rise in response to the stress (2, 15), appears to be induced by a redistribution of the mesophyll ABA (4, 14). However, there have been no direct measurements so far of the endogenous ABA content of guard cells.

In this paper, isolated guard cells, prepared by the sonication of epidermal peels (10, 21), were used to investigate directly the endogenous ABA levels in the guard cells of turgid and stressed leaves.

Guard cells prepared by sonication appear to be fully functional in many respects; for example in stomatal opening (21), photochemical activity (11), response to ABA (22), K^+ uptake (26), malate formation in the light (21), and catabolism of ABA (12). The guard cells should be relatively little affected by their isolation from other leaf cells, at least in the short term, since the mature guard cells do not have plasmodesmata (25, 29).

Guard cell preparations from two species were compared. The *argenteum* mutant of *Pisum sativum* (20) was used as it has a high stomatal density and the epidermis is only loosely attached to the underlying mesophyll (16, 18). *Vicia faba* was investigated as sonicated strips of this species have previously been used in a number of studies (11, 12, 21, 22, 26). The ability of *V. faba* guard cells to accumulate stress-induced ABA while isolated was also investigated.

MATERIALS AND METHODS

Culture of Plant Material. *Pisum sativum* L., mutant *argenteum* (*arg*) was grown in a controlled environment chamber under the same conditions as described (30). *Vicia faba* L. cv 'Long Pod' (W. Atlee Burpee Co., Warminster, PA) was grown in a soil:sphagnum (1:1) mixture at approximately 25°C with cool-white fluorescent illumination (175 μE m^{-2} s^{-1}) for 16 h daily. Recently expanded leaves of both species were used throughout the experiments. The stomatal density of both epidermises of *arg* and of the lower epidermis of *V. faba* was determined using a haemocytometer. The dry weight per unit area of sonicated strips from both species was also determined.

Guard Cell Preparation. The epidermis was peeled from both adaxial and abaxial leaf surfaces of *arg*, and with as little adhering mesophyll as possible. The upper and lower epidermises were stored separately in 0.1% Tween 20 in deionized H_2O at O°C. The strips were then sonicated with a Branson Sonic Power Sonicator (Branson Instrument Inc., Danbury, CT), equipped with 1.27 cm horn, to remove any adhering mesophyll cells and to break and remove the contents from the epidermal cells. The strips were sonicated up to 5 times in fresh ice-cold aqueous Tween 20. Each sonication period did not exceed 1 min. The upper and lower epidermises of *arg* were sonicated separately as the cells of the upper epidermis were found to be more fragile than those of the lower epidermis. Upper epidermis was initially sonicated at frequency setting 2, followed by a period at 3; lower epidermis was initially sonicated at 2, then at 3, followed by a period of 3 interspersed with 5 s bursts of 4. The peels were examined microscopically to determine whether further sonication was necessary; the final sonication treatment was repeated as required. The two epidermis samples were pooled following the sonication procedure.

The lower epidermis of *V. faba* leaves was treated in a similar manner to that described above. A higher sonication frequency was required than for *arg*: an initial period at frequency setting 3, followed by one at 4, then a period of 4 interspersed with 5 s bursts of 5, this last repeated as required. It was not possible to peel both leaf surfaces of this species.

Sonication was halted when examination of the strips under the microscope revealed no contaminating epidermal or mesophyll cells. At this point, up to 4% of the guard cells had also been damaged as detected by the absence of cytoplasm or the inclusion of Evans blue. The viability of the guard cells was determined using the stains Evans blue and neutral red, and by examining the stomatal response to light and darkness in the presence of 25 mM KCl.

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ABA in Guard Cells of Sonicated Strips. In a series of experiments on both species, turgid leaves and stressed leaves were compared. Stressed leaves were dehydrated to 12% loss of fresh weight and incubated in plastic bags for 6 h in darkness at room temperature. Isolated guard cells were prepared from the turgid and stressed leaves as described above. The sonicated strips were then frozen in liquid N_2, lyophilized, weighed, and analyzed for their ABA content ("Extraction and Purification Procedures"). Samples of the intact leaves were also harvested and analyzed for ABA.

ABA Binding to Epidermal Cell Walls. Epidermal cell walls were obtained from the *arg* pea mutant by preparing sonicated strips, as described above, which were then exhaustively extracted with acetone and lyophilized. A small column was made by loosely packing about 40 mg of these cell walls into a 1 cm^3 plastic Luer-lok syringe with a 100 μm nylon filter at the tip. Two more syringes were placed in series on top of the column, the lower of the two also with a 100 μm filter at its tip to enclose the cell walls. The junctions between the syringes were sealed with silicone rubber. A 2.5 cm needle (26 G) was attached to the base of the column. The column was hydrated in aqueous Tween 20 and rinsed several times. A sample of 50,000 cpm of [^{3}H]ABA (16.0 Ci·$mmol^{-1}$) in aqueous Tween 20 was then aplied to the top of the column. A succession of solvents, 3 cm^3 of each, was then passed slowly through the column. Consecutive 0.5 cm^3 fractions were collected and their [^{3}H] content determined with a liquid scintillation counter. The solvents and their flow rates through the column were as follows: (a) aqueous Tween 20, 0.05 $cm^3 \cdot min^{-1}$, (b) acetone extractant, 0.03 $cm^3 \cdot min^{-1}$; (c) methanol, 0.3 $cm^3 \cdot min^{-1}$; (d) 1% aqueous acetic acid, 0.07 $cm^3 \cdot min^{-1}$.

ABA Levels in Sonicated Strips with Intact or Broken Guard Cells. Sonicated strips were prepared from stressed leaves of both species. The strips were divided into two portions and frozen in liquid N_2. One sample was then thawed and sonicated three times, at a high frequency, in ice-cold aqueous Tween 20. The freeze-thaw cycle was used to rupture the guard cell plasmalemmas and to allow the ABA contained to move into the medium during the subsequent sonication. Some strips were stained as described above and examined under the microscope. The strips and the sonication media were analyzed separately for ABA content ("Extraction and Purification Procedures").

Stress-Induced ABA Accumulation by Isolated Guard Cells. Solutions of the osmoticum Aquacide III (Calbiochem-Behring Corp., La Jolla, CA) were made using half-strength Hoagland solution (the basal medium) as solvent. The solute potential (ψ_s) of the different solutions was determined with a Wescor HR-33T dew point microvoltmeter equipped with C-52 sample chambers (Wescor). Sonicated strips were prepared from turgid *V. faba* leaves as described above. Single samples of strips were immersed in 5 cm^3 of medium in silanized 20 cm^3 scintillation vials. Each sample was incubated for 6 h in darkness and was stirred throughout to provide good aeration. The strips were then removed from the media, rinsed twice with basal medium, and frozen in liquid N_2, lyophilized, and weighed. The rinses were pooled with the medium for each sample. Both strips and media were analyzed for their ABA content ("Extraction and Purification Procedures").

Extraction and Purification Procedures. Samples of intact leaves were homogenized using a Polytron homogenizer (Brinkmann Instr., Westbury, NJ). All samples of leaf material, including the epidermal strips, were extracted with 80 and 100% acetone (acetone, 1% acetic acid, 0.01% 2,6-di-*tert*-butyl-*p*-cresol) in darkness, the leaves at 4°C and the strips shaken at room temperature, for at least 24 h. The leaf extracts were filtered and the tissue residue washed with acetone. The supernatant from the strip extracts was drawn off with a Pasteur pipette and more solvent added; this was repeated until the supernatant and the strips appeared colorless. The acetone was evaporated from the extracts and the remaining aqueous fractions were frozen in liquid N_2 and lyophilized. Before being lyophilized, extracts of strips that had been incubated in osmotica were partitioned three times with ethyl acetate and back-washed with aqueous 1% acetic acid to remove the remaining Aquacide III. Media samples were acidified to 1% acetic acid and partitioned as described. When the sonication medium was to be analyzed, the medium was acidified to 1% acetic acid and purified with C_{18} Sep-Pak cartridges (Waters Associates, Milford, MA) (30). All samples were then purified by semipreparative reverse phase HPLC as described (3) with some modification (4). The ABA content of the samples was quantified using a Hewlett-Packard 5840A gas chromatograph equipped with a ^{63}Ni-electron capture detector as described (3). Small amounts of (±)-[^{3}H]ABA (16.0 Ci·$mmol^{-1}$) were added to the samples to determine losses during the extraction and purification procedures. Overall recovery of [^{3}H]ABA added to the samples was 75 to 100% for the leaves and the sonicated strips, 70 to 85% for the sonication media, and was 70 to 90% for both strips and media from the experiments using Aquacide III. All data have been corrected for losses.

RESULTS AND DISCUSSION

Guard Cell Viability. Many mesophyll cells adhered to fresh epidermal peels of *V. faba* and some of the epidermal cells remained intact (Fig. 1A). Leaves of *arg* peeled more cleanly but again some of the epidermal cells were undamaged. Intact epidermal cells were clearly demarcated by their exclusion of the stain Evans blue (Fig. 1A). Guard cells prepared from epidermal strips of both species were found to exclude Evans blue (Fig. 1B) and take up the vital stain neutral red. The stomata also opened in the presence of light and K^+ and closed in darkness. Thus, the sonication procedure did not appear to damage the guard cells as was also concluded in previous investigations (11, 12, 21, 22, 26).

ABA Levels in Leaves and Guard Cells. The leaves of *arg* and *V. faba* accumulated stress-induced ABA to 11 and 30 times their turgid levels, respectively (Table I). These values are in the normal range for wild-type mesophytes. The *arg* mutation then, does not have obvious effects on ABA metabolism. The silvery cast of *arg* plants is due to extensive air spaces between the epidermis and mesophyll (16, 18, 20). This not only makes the leaves of this plant easy to peel, but also results in a relaxed control of stomatal distribution. The stomata are apparently randomly distributed in the mutant and as many as three guard cell pairs were observed adjacent to each other. *Arg* has nearly three times as many stomata per unit area as *V. faba* (Table II).

Sonicated strips from stressed leaves of *arg* and *V. faba* contained 8 and 18 times the endogenous ABA level of turgid leaf strips, respectively (Table III). These are somewhat lower relative increases than those of 11 and 30 times seen in the stressed intact leaves (Table I). The values for the sonicated strips from the stressed *arg* leaves were quite variable, although elevated levels of at least three times the turgid level were always observed. This variability could partly be accounted for by variation in the amount of stress-induced ABA accumulated by the intact leaves (K Cornish, JAD Zeevaart, unpublished results). The levels obtained in all the sonicated strips may well underestimate the actual amount of ABA present *in vivo*, as ABA could move out of the strips into the medium during the sonication procedure. ABA efflux occurs quite readily from leaf slices (6) and roots (5), for example.

Although the ABA level in sonicated strips from turgid *arg* leaves was apparently much higher than in *V. faba*, the endogenous ABA level per guard cell pair (Table III) was similar in both species. However, as discussed above, *V. faba* guard cells appear to accumulate more ABA during stress than *arg* guard cells.

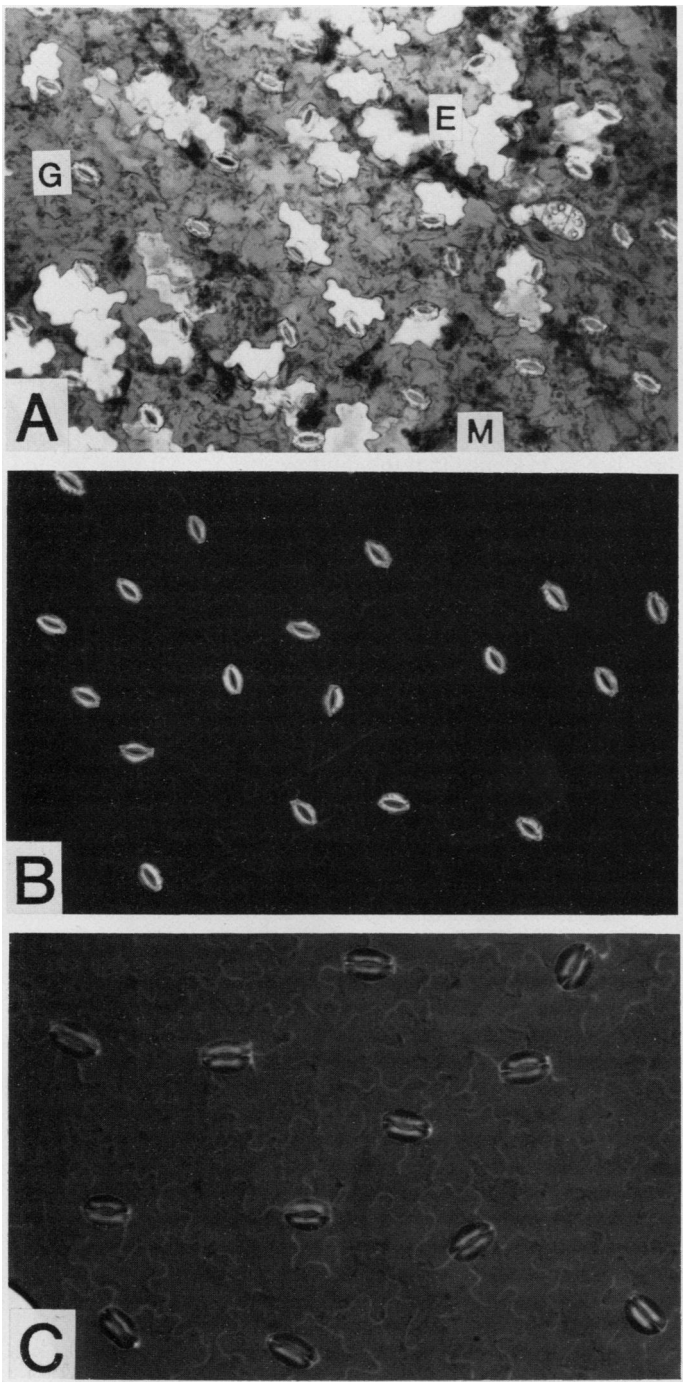

FIG. 1. Microphotographs of *V. faba* epidermal strips stained with Evans blue. A, Freshly peeled strips; M, mesophyll cells; E, intact epidermal cells; G, intact guard cells; B, after sonication; C, after the sonicated strips were frozen in liquid N_2, thawed, and sonicated again.

Raschke (24) estimated from various sources that less than one to a few fmol ABA·mm^{-2} of leaf area were usually required to initiate stomatal closure. When the ABA levels in the sonicated strips (Table III) were expressed in this way, it was found that the ABA level in strips from stressed leaves compared with that in the turgid leaves, increased 0.88 and 0.75 fmol·mm^{-2} in *arg* and *V. faba*, respectively. Thus, the ABA located in the guard cells of sonicated strips of stressed leaves does seem to represent a physiological concentration although, due to efflux (see above), the levels measured may considerably underestimate the *in vivo* ABA level.

Table I. *ABA Levels in Turgid and Stressed Intact Leaves*

Stressed leaves were dehydrated to a 12% loss of fresh weight before incubation. The number of values in each mean are given in parenthesis. Values for *V. faba* ± SE.

Species	ABA Content	
	Turgid	Stressed
	ng·mg^{-1} dry wt	
P. sativum, arg	0.94 (2)	10.3 (2)
V. faba	0.39 ± 0.02 (4)	11.6 ± 2.3 (3)

Table II. *Stomatal Density and the Relationship between Dry Weight and Area of Sonicated Epidermal Strips*

The values for *P. sativum, arg* represent the mean of the upper and lower epidermises, those for *V. faba* are for the lower epidermis alone.

Species	Stomatal Density	Weight of Sonicated Epidermis
	stomata·cm^{-2}	*ng dry wt·cm^{-2}*
P. sativum, arg	21,200	75.4
V. faba	7,920	130

ABA Binding to Epidermal Cell Walls. Only a relatively small proportion of the epidermis is comprised of guard cells and so it was necessary to determine whether or not the ABA extracted from the sonicated strips was actually located in the guard cells prior to its extraction. It is possible, for example, that ABA released from the broken mesophyll and epidermal cells during sonication could have bound to the epidermal cell walls and remained there while the strips were immersed in the aqueous Tween 20. The extraction procedure could destroy such nonspecific binding and account for the ABA levels then found in the extraction medium.

When [^{3}H]ABA was applied to the column of purified epidermal cell walls, most of the radiolabel was eluted with aqueous Tween 20, so no binding of ABA to the cell walls was observed. The radiolabel recovered in the fractions amounted to a total of 50,187 cpm, from the calculated 50,000 cpm applied. The actual amount of [^{3}H]ABA added to the column was about 22 ng·g^{-1} dry epidermal cell wall, or about half the ABA level in the sonicated strips from turgid *arg* leaves (Table III). As no binding of this small amount of ABA was detected, it can be concluded that the ABA extracted from the sonicated strips, of both turgid and stressed leaves, does not arise from nonspecific binding to the cell walls during sonication, and is, therefore, presumably from the only intact cells remaining, the guard cells.

ABA Levels in Sonicated Strips with Intact or Broken Guard Cells. The stain Evans blue could enter the guard cells after the strips were frozen in liquid N_2, thawed, and sonicated again (Fig. 1C), which indicates that the guard cell plasmalemmas had been ruptured by this treatment. Very little ABA remained in the strips while a concomitant increase of ABA was observed in the sonication medium (Table IV). Thus, it was established that the ABA extracted from sonicated strips was indeed located in the guard cells.

Stress-Induced ABA Accumulation by Isolated Guard Cells. When sonicated strips of *V. faba* were directly stressed with the osmoticum Aquacide III, ABA, in the combined strips and media, was produced to 3.2 times the level in the turgid strips (Table V). Thus, the guard cells of *V. faba* are capable of producing stress-induced ABA themselves.

At first sight, this conclusion seems to contradict earlier reports when isolated epidermis stressed separately from the mesophyll was unable to synthesize measurable quantities of ABA (9, 19). However, these investigators stressed intact epidermis where

Table III. *ABA Levels in Sonicated Epidermal Strips and Guard Cells from Turgid and Stressed Leaves*

The values (±SE) for *P. sativum, arg* represent the mean of the upper and lower epidermises; those for *V. faba* are for the lower epidermis alone. The number of values in each mean are given in parentheses. The ABA levels per guard cell pair are derived from the first two columns together with the data from Table II.

Species	ABA Content of Sonicated Epidermis		ABA Content of Guard Cells	
	Turgid	Stressed	Turgid	Stressed
	ng·mg⁻¹ dry wt		*fg·guard cell pair⁻¹*	
P. sativum, arg	0.044 ± 0.007 (4)	0.35 ± 0.25 (5)	0.15	1.24
V. faba	0.009 ± 0.003 (3)	0.16 ± 0.02 (3)	0.15	2.64

Table IV. *ABA Levels in Sonicated Strips with Intact or Broken Guard Cells*

Epidermis was peeled from stressed leaves. After sonication, half the tissue was frozen in liquid N_2 and thawed to rupture the guard cell plasmalemmas, and sonicated again before analysis. Each value for *P. sativum, arg* is the mean of two; the *V. faba* values are single determinations.

Species	ABA Content of Sonicated Epidermis		
	Sonicated epidermis	After guard cell breakage	
		Sonicated epidermis	Sonication wash
	ng·mg⁻¹ dry wt		
P. sativum, arg	0.57	0.03	0.68
V. faba	0.14	0.04	0.08

Table V. *ABA Levels in Sonicated Strips of V. faba and in the Incubation Media*

Samples were stirred in 5 cm^3 of medium for 6 h in darkness. The media consisted of half-Hoagland solution alone or with the addition of Aquacide III.

Incubation Medium ψ_s	ABA Content		
	Sonicated strips	Medium	Combined strips and medium (means)
bars		*ng·mg⁻¹ dry wt*	
−1.5	0.007	0.012	
	0.009	0.017	0.022
−7	0.011	0.044	
	0.013	0.061	
		0.069	0.070

guard cells are greatly outnumbered by epidermal cells. It seems probable that if the guard cells are the only cells in the epidermis that can produce stress-induced ABA, then the actual increases that may occur could be easily masked by the unchanging, but relatively large amount of ABA contained by the epidermal cells. Similarly, a study of isolated guard cell protoplasts showed only a small ABA increase of 30% at most in hyperosmotic treatment (28), but the untreated guard cell protoplasts were already stressed by the necessarily hyperosmotic isolation medium. Furthermore, protoplasts were incubated at 0°C at which temperature ABA accumulation would not be expected to occur.

The production of ABA by *V. faba* guard cells does not mean that this ABA is necessarily of great relevance to stomatal response to water stress. For example, the rapid closure of stomata in response to stress appears to be mediated by a movement of ABA from the mesophyll into the apoplast (4). ABA in the apoplast would be readily available to the guard cells and, as the leaves start to accumulate stress-induced ABA, much larger amounts of ABA appear in the free space (1, 4). Furthermore, there is now evidence that the binding sites for ABA are located on the external surface of the guard cell plasmalemma (13, 17), which again suggests that guard cell ABA production is not likely to be involved in stomatal closure.

Conclusions. The guard cells of the two species investigated were found to contain considerably higher levels of ABA when isolated from stressed leaves than from turgid leaves (*arg* 8 times, *V. faba* 18 times), although it is not possible to assess the relative contribution of mesophyll ABA to the guard cell ABA in these experiments. However, isolated *V. faba* guard cells were capable of producing at least three times their turgid ABA level when directly stressed with an osmoticum.

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