Abscisic Acid Movement into the Apoplastic Solution of Water-Stressed Cotton Leaves

ROLE OF APOPLASTIC pH

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ABSTRACT

Leaves of cotton (Gossypium hirsutum L.) were subjected to overpressures in a pressure chamber, and the exuded sap was collected and analyzed. The exudate contained low concentrations of solutes that were abundant in total leaf extracts, and photosynthetic rates and stomatal conductance were completely unaffected by a cycle of pressurization and rehydration. These criteria and others indicate that the experimental techniques inflicted no damage upon the leaf cells. The pH and abscisic acid (ABA) content of the apoplastic fluid both increased greatly with pressure-induced dehydration. Although ABA concentrations did not reach a steady state, the peak levels were above 1 micromolar, an order of magnitude greater than bulk ABA concentrations of the leaf blades. Treatment of leaves with fusocin decreased the K+ concentration, greatly reduced the pH rise, and completely eliminated the increase in ABA in the apoplast upon dehydration. When water-stressed leaves were pressurized, the pH of the exuded sap was increased by 0.2 units per 1 megapascal decrease in initial leaf water potential. Buffer capacity of the sap was least in the pH range of interest (6.5–7.5), allowing extremely small changes in H+ fluxes to create large changes in apoplastic pH. The data indicate that dehydration causes large changes in apoplastic pH, perhaps by effects on ATPases; the altered pH then enhances the release of ABA from mesophyll cells into the apoplastic fluid.

Stomatal closure during episodes of water stress is believed to be mediated by ABA (32, 36). Nonetheless, the chain of events linking ABA production by the mesophyll cells to the final event (loss of turgor in the guard cells caused by solute release) is unclear. Ignoring the question of ABA action at the guard cells, one must still account for several simultaneous processes, including ABA accumulation within the mesophyll cells, ABA release from the mesophyll cells to the apoplast, and ABA transport to and recognition by the guard cells. Changes in stomatal 'sensitivity' to ABA induced by water stress or other environmental stimuli (1, 8, 31) could result from effects on either release or recognition of ABA.

Recent studies have addressed the issue of ABA release from mesophyll cells to the apoplasm of leaves. Two reports indicated that water stress or osmotic stress increases ABA release (10, 15), whereas one report (2) indicated no such effect. Although methods differed among the various reports, there is no obvious explanation for the discrepancy. Thus, there is not yet agreement about the role of ABA release in water stress-induced stomatal closure.

Hartung et al. (14, 22) have obtained substantial evidence for a pH-dependent mechanism of ABA partitioning. High membrane permeability to the protonated, uncharged species ABA-H, but low permeability to the anionic species ABA-H, results in the accumulation of ABA in the most alkaline compartment of cells and its depletion in the most acidic compartment. Others (18) have shown that this explanation applies also to larger systems such as developing seeds, and is consistent with ABA transport and accumulation patterns between whole tissues.

In this paper we report experiments to isolate the apoplastic solution of cotton leaves. The objective was to determine the pH and the ABA content of that solution as the leaf underwent dehydration. The method involved subjecting leaves to overpressures in a pressure chamber. This technique, first applied by Ackerson (2) to the problem of ABA partitioning in leaves, has been studied to look for possible artifacts from experimental manipulations (2, 10). We have also characterized the procedure extensively to determine its usefulness, and we have probed the pH-regulation system of the leaves by application of FC.2

MATERIALS AND METHODS

Plant Material. Cotton (Gossypium hirsutum L. cv Deltapine 01) plants were grown in the field in Phoenix during the 1986 growing season. The plants were watered with a drip irrigation system to prevent the occurrence of water stress, and otherwise were grown with normal cultural practices. Once each week the plants were fertilized with urea (96 kg ha−1 N applied during the season) through the irrigation system; no other nutrients were added. Experiments reported here were performed on leaves from heavily fruited plants during August, September, and October of 1986. In well-watered field plants in this location, the Ψw for zero turgor is near −2.0 MPa (29).

Leaf Pressurization. Leaves were selected that were fully expanded and near the top of the canopy. Before excision from the plant, each leaf was shaded and enclosed in a humidified plastic bag. The leaf was then excised, the bag was sealed, and it was placed in a humidified insulated chest for transport to the laboratory. The elapsed time between excision and pressurization was 5 min or less.

Leaves were removed from the plastic bags and placed in a humidified pressure chamber, and the Ψw (xylem pressure potential) determined by standard procedures (31). After reaching the balancing pressure, the pressure was gradually increased to cause exudation. The excuded sap was collected through a small

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2Abbreviation: FC, fusocin; Ψw, water potential; p, osmotic pressure.
length of Tygon tubing fitted to the cut end of the petiole, and directed into a series of up to 20 tared 1.5-ml polyethylene microcentrifuge tubes. Each tube was intended to contain 10 µl of exudate (visual estimates of volume). The actual volume collected was determined immediately afterwards by the increase in tube weight.

In some other experiments only two fractions were collected. Fraction I was the exudate from a 0.6-MPa overpressure after reaching the initial balancing in pressure. Fraction II was the exudate from an additional 1-MPa overpressure. As a rule, each leaf produced about 10 µl of exudate for each 0.1-MPa increase in pressure. These fractions were pooled from many leaves, lyophilized, and later analyzed.

**Treatment with FC.** Ten µl of FC solution (0.42 mM) was injected into the petiole of an intact cotton leaf. Exudate was collected beginning 1 to 1.5 h after treatment. Injection has been routinely used as a nondestructive method to deliver ABA to leaves (8), and in these cotton leaves injection of only 2 µl of 0.5 mM ABA solution was sufficient to close stomata (data not shown).

**Exudate pH.** The pH of the exudate fractions was determined with an MI-410 combination pH microelectrode (Microelectrodes, Inc.) attached to a Beckman AlteX Φ 70 pH meter. In addition, samples of fractions I and II were brought to pH 10 with KOH, then titrated with 0.01 M HCl. The pH of all fractions was routinely determined prior to solute analysis (see below), and the reference electrode may have contributed to the K⁺ and Cl⁻ content of the solutions. During a 15-s measurement the transfer of KCl to the solution was estimated to be 7.5 pmol, several orders of magnitude less than amounts already present.

**Solute Analyses.** The lyophilized fractions were taken up in a solution of 10 mM CsCl and 50 mM Sr(NO₃)₂, and inorganic cations were determined with a Zeiss FMC-3 atomic absorption spectrometer. For anion determinations, samples were dissolved in water in a boiling water bath, centrifuged, and analyzed by HPLC using conductivity detection (Biotronik Anionenchromatograph). Total soluble sugars were assayed with an anthrone method (33) and are expressed as glucose-equivalents. Total amino acids were determined with ninhydrin (5) and are expressed as glycine-equivalents. Osmotic pressures were measured cryoscopically with a Knauer semimicroosmometer.

Analyses of lyophilized leaf material were carried out in similar fashion, except that the samples were digested in 16% (w/v) nitric acid at room temperature overnight before cation determination by atomic absorption. Sugars were analyzed by an enzymic method (16, 17). Osmotic pressures were determined by thermocouple psychrometry on frozen and thawed tissue samples (4).

It should be noted that the anion concentrations were too low to balance the cations in the exudates. Therefore unidentified anions (HCO₃⁻?) must account for a significant percentage of the solutes.

**ABA Analyses.** Lyophilized apoplastic fractions were dissolved in 100 µl of 50 mM tris buffer (pH 7.8), containing 150 mM CaCl₂ and 2 mM MgCl₂. The solution was assayed for ABA by ELISA using kits from Idetek Co. (San Bruno, CA). The tests were performed as described in the Idetek manual. The apoplastic solution was free of proteins and did not need to be purified before assay of ABA. Analysis of leaf ABA was by HPLC following the procedures of Guinn and Brummett (12). Sample size was 100 mg. Recovery of [³¹C]-ABA (added at the initial extraction) was 82%.

**Rehydration experiments.** Leaves were excised in the field with their petioles under water, then brought into a growth chamber maintained at 30°C with a PAR of 450 µmol m⁻² s⁻¹. After 30 min, the photosynthetic rate was determined using a supplemental light source to increase PAR to 1500 µmol m⁻² s⁻¹. Both photosynthesis and stomatal conductance were determined with an ADC portable photosynthesis system (Analytical Development Co., Hoddesdon, England) equipped with a DL-2 datalogger. This is an open gas-exchange system; steady state rates were achieved within 20 s after the cuvette was clamped to the leaf. Because the CO₂ content of the air in the growth chamber rapidly increased when the investigators were present, the air intake for the gas-exchange system was maintained outside the chamber. The actual CO₂ concentration of the air varied from 440 to 510 µl L⁻¹ in different experiments but was constant during each set of measurements.

Immediately after measurement of photosynthesis, the leaves were brought into the laboratory and subjected to one of four treatments. Control leaves were left with their petioles in water. The other treatments involved dehydrating the leaves by (a) removing them from the water and allowing them to transpire until visibly wilted (final ψw = -2.3 ± 0.1 MPa); (b) subjecting them to a 1-MPa overpressure (final ψw = -1.6 ± 0.1 MPa); or (c) subjecting them to a 2-MPa overpressure (final ψw = -2.6 ± 0.05 MPa). The stressed leaves were then rehydrated by recutting the petioles under water and allowing 1 h for water uptake. Leaves of all four treatments were transferred to the growth chamber, allowed to acclimate for 30 min, and treated as before to measure photosynthesis and stomatal conductance.

**RESULTS**

**Solute of the Expressed Sap.** The sap isolated from leaves contained low concentrations of numerous solutes (Table I). The cation present in highest concentration was K⁺, at 4.5 mM in fraction I and 3.3 mM in fraction II. The total leaf extract contained about 270 mM K⁺ (Table I). The apoplastic fractions contained only very small quantities of malate or substances that co-chromatographed with malate (<0.1 mM malate-equivalents), whereas the leaf extract contained about 170 mM malate. Sugars were also present at very low concentrations (<0.4 mM glucose-
equivalents) in both apoplastic fractions. Further tests (17) revealed that about 75% of the sugar was sucrose, with the remainder divided between glucose and fructose. In the leaf extract, only about 20% of the sugar was sucrose (data not shown). The π of both apoplastic fractions was very low at 0.026 and 0.014 MPa, respectively (Table I).

Special note should be taken of the K⁺/Na⁺ ratios of the sap. This ratio was approximately 10:1 for both fractions I and II, but was greater than 20:1 for the leaf extract (Table I). Thus, the leaf cells were depleted of Na⁺ relative to the expressed sap, indicating a high K⁺/Na⁺ selectivity of the membranes of cotton leaf cells. Taken together, these characteristics strongly indicate that the expressed sap was carrying apoplastic solutes with minimal contamination from the leaf cells.

**Photosynthesis of Pressurized Leaves**. The effect of pressurization on photosynthetic activity was determined as a further check on possible side effects of the experimental procedures. When leaves were pressure-dehydrated and rehydrated, final photosynthetic rates and stomatal conductances were somewhat less than the initial values. However, the controls and the non-pressurized wilted-rehydrated leaves were equally affected (Table II). Across all four treatments, photosynthetic rates were diminished by 15 to 23%, slightly less than the reduction in conductance (24–33%). The similar behavior in all treatments indicates that neither a cycle of dehydration-rehydration, nor the pressurization itself, disrupted the biological functioning of the leaf. The small changes in photosynthesis may have resulted from excision of the leaf, which should, through obstruction of translocation, cause accumulation of ABA and partial stomatal closure (34).

**Exudate pH**. The pH of fraction I was 6.7, and that of fraction II was 7.3 (Table I). The source of the difference between fraction I and fraction II was found further by collecting expressed sap in a series of small fractions (about 10 μl each). The pH of the first fraction was lowest, typically near 6.3 (Fig. 1). Thereafter the pH rose with increasing expressed volume, rapidly at first but with the rate of increase soon slowing. The pH became nearly stable at a value near 7.5, typically when about 100 μl of solution had been expressed (Fig. 1). Pressure-volume curves of similar leaves (29) indicate that this degree of dehydration corresponds approximately to the point of zero turgor. In well-watered cotton plants, zero turgor is believed to trigger stress-dependent ABA accumulation (27, 28).

As one possible explanation of these results, the low pH of the initial fractions may have resulted from contamination by broken cells of the cut end of the petiole. If this were true, then the K⁺ concentration should have been very high, because the leaf cells contain extremely high levels of this cation (Table I). Analysis of the exudate revealed an apparent biphasic relationship between the K⁺ concentration and volume expressed. The K⁺ level was high in the first fraction, then decreased rapidly to a steady trend line during the first few fractions collected (Fig. 1). The Na⁺ concentration of exudate similarly implied that contamination by cut cells was short-lived, as it remained below 0.4 mM and displayed little or no trend with volume expressed (Fig. 1).

Fraction I exudate was titrated to determine its buffer capacity. Decreasing the pH from 7.3 to 6.7 (Table I) required 159 nmol H⁺·ml⁻¹·cm⁻² (Fig. 2). Each leaf released a volume of approximately 60 μl into fraction I, or 1 μl cm⁻². However, the apoplastic water content of cotton leaves is about 2 μl cm⁻² (30). To establish the observed pH change, the net flux of H⁺ into the apoplastic fluid would need to be 159 × 0.001 × 2 = 0.32 nmol H⁺ cm⁻². In addition, one must consider the buffering effects of the cell wall in vivo. Pfanz (25), using a fluorescence method of pH determination (26), estimated the buffer capacity of the cell wall of young cotton leaves to be 1.25 × the buffer capacity of the apoplastic fluid. Therefore, a pH change of 0.6 units in vivo presumably would require 0.32 + 0.40 = 0.72 nmol H⁺ cm⁻². Obviously, very small changes in proton fluxes would suffice to establish a large pH change in the apoplastic compartment. The buffering capacity of fraction II was even lower in this pH range (not shown).

The process governing apoplastic pH was investigated using FC as a probe. FC is known to stimulate uptake of K⁺ and extrusion of H⁺ from cells (23). Leaves dehydrated after treatment with FC (Fig. 3) did not show the pronounced increase in apoplastic pH typical of the untreated leaves (Fig. 1). The pH of the first fraction was 6.35, close to the value for untreated leaves, but the pH thereafter rose only to about 6.6 during pressurization. The K⁺ levels in the sap from FC-treated leaves were considerably lower than in the controls. This observation, typical of FC treatment (23), indicates that the effects of FC in this

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**Table II. Photosynthesis and Stomatal Conductance of Excised Cotton Leaves Subjected to Various Experimental Procedures**

Results are expressed as a percentage of the initial rate of CO₂ uptake or the initial stomatal conductance. The mean initial photosynthetic rate was 11.1 μmol CO₂ m⁻² s⁻¹ and the mean initial stomatal conductance was 0.21 mol H₂O m⁻² s⁻¹. Values shown are means of six determinations ± se.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Leaf Performance</th>
<th>Photosynthesis Conductance</th>
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<tbody>
<tr>
<td>Control (continuously in water)</td>
<td>85 ± 7</td>
<td>76 ± 8</td>
</tr>
<tr>
<td>Wilted in air, then rehydrated</td>
<td>82 ± 11</td>
<td>76 ± 8</td>
</tr>
<tr>
<td>1-MPa overpressure, then rehydrated</td>
<td>81 ± 6</td>
<td>72 ± 8</td>
</tr>
<tr>
<td>2-MPa overpressure, then rehydrated</td>
<td>77 ± 14</td>
<td>67 ± 8</td>
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proximately 0.1 μM. It increased steadily to about 1.2 μM as the pH rose, then decreased before again rising during the latter stages of dehydration. Other leaves displayed somewhat different patterns (not shown), but were all characterized by two important features: the ABA concentration increased during some portion of the pH rise, and the peak ABA concentration was between 0.8 and 1.8 μM. These peak concentrations can be compared to the bulk ABA concentration of 0.12 μM in the leaf blade (Table I). Thus, the expressed sap was enriched 7- to 15-fold in ABA over the bulk leaf concentration. It should be noted that leaves of field-grown cotton plants tend to have low bulk concentrations of ABA because high temperature decreases its accumulation (31). McMichael and Hanny (24) reported similar ABA concentrations in field-grown cotton leaves.

FC-treated leaves displayed a pattern of ABA release very different from that of the untreated leaves. The initial concentration in the exudate was 0.1 to 0.2 μM (Fig. 3), similar to the concentration in exudate from untreated leaves (Fig. 1). In FC-treated leaves, though, exudate ABA levels rapidly decreased to zero during dehydration (Fig. 3). The ABA concentration was below the limits of detection during the latter stages of dehydration, and at no time did the exudate ABA level exceed the bulk concentration in the leaf. All replicates of the FC treatment behaved similarly. It is clear that FC greatly reduced the movement of ABA into the apoplast in conjunction with its stimulation of H+ extrusion. Interestingly, the results illustrate that FC effects on stomata may not be limited to the guard cells. The well-known stomatal opening response to FC (23) may be mediated in part by changes in apoplastic ABA concentrations.

**Water Stress and Exudate pH.** Leaves were excised from plants under varying degrees of water stress, and subjected to overpressures. As before, exudate was collected in a series of fractions from each leaf (Fig. 4). Again, the pH was lowest in the first fraction, rising rapidly until about five fractions had been expressed, and rising slowly thereafter (Fig. 4A). Superimposed upon that pattern was an increase in pH with decreasing initial leaf pH (Fig. 4A). Regression analysis revealed that in both early and late stages of pressurization, the pH rose about 0.2 units for a decline of 1 MPa in initial pH. The pH differential between different fractions was little affected by the initial degree of stress. There was substantial variability among leaves, and the correlation coefficient for fraction 20 was not significantly different from zero (P < 0.05). The pH values were converted to H+ concentrations and new regressions calculated (Fig. 4B). The slopes of these regressions were greater at high initial pH (lower FC) than at low initial pH (higher FC), indicating that the inhibition of proton-translocating ATPases was greatest in the early stages of stress. Again, the correlation coefficient for fraction 20 was not significantly different from zero.

**DISCUSSION**

Numerous methods have been applied to the problem of estimating apoplastic solute concentrations (20, 21, 35). All have some drawbacks (21). The pressure chamber method seems to be generally applicable to leaves, but it too has some obvious disadvantages. First, the cut end of the petiole can contaminate the sap, as can cells damaged by the applied pressure. The sap composition discounts this possibility for cotton leaves (Table I). Also, if there were damage, it did not affect photosynthesis or stomatal conductance after relief of the pressure (Table II). Ackerson (2) and Cornish and Zeevaart (10) earlier reached similar conclusions. Second, the leaf is incubated in darkness inside the chamber, and thus the data are not representative of a photosynthesizing leaf. In studies of ion or solute fluxes, this must be considered a serious limitation to the generality of the results. Third, the leaf is excised from the plant, and the excision can have long-term effects like the effects of girdling (34). Indeed,
excision may have had larger effects than pressurization or other manipulations (Table II). Fourth, the collected fluid may be a mixture of exudate from several leaf compartments (phloem, xylem vessels, xylem parenchyma, mesophyll parenchyma, etc.), and it is difficult to discern the precise origin of the exudate. In the case of ABA fluxes, this is an important point because ABA is known to accumulate in the phloem to high concentrations (11, 37). Fifth, imposition of an external pressure, although it causes dehydration, does not allow the pressure potential to decrease to zero (3). In this sense pressurization does not mimic a true water stress (2, 3). Differences in the nature of the dehydration, however, do not alter tissue response in terms of ABA production (2, 3, 10).

Our studies indicate that the apoplastic pH of leaves can be determined through the use of pressure exudates. The exudate pH rose quickly in the first fractions collected, then tended to stabilize after a volume of about 100 μl had been collected. One interpretation is that the early fractions may represent changing contributions from the various compartments of the leaf, perhaps sequentially sampling apoplastic sap from the major veins, minor veins, and finally the mesophyll (21). (The volume of the xylem itself may not be significant in the behavior of this system, because reasonable assumptions about the size and number of vessels lead to volume estimates of less than 10 μl.) Another interpretation is that the increasing pH represents response to tissue dehydration. Because the exuded sap carries other solutes as well as H⁺ ions (Table I), the interpretation applied to the pH data must also apply to the other solutes. The first possibility, that pH increases because of changing contributions from different leaf compartments, seems incompatible with the rapid establishment of steady state levels of K⁺ and Na⁺ in the exudate (Figs. 1 and 3). The K⁺ concentration and especially the Na⁺ concentration stabilized considerably before the pH stabilized. Unless the apoplastic K⁺ and Na⁺ levels were identical in the various leaf compartments, the ‘sequential-sampling’ model (21) cannot fit these data. It seems more likely that at least part of the increase in pH reflects changing activities of the ultimate leaf compartment from which the exudate is derived. Because the mesophyll parenchyma are the dominant cell type of the leaf, we assume that the changes in the sap during pressurization reflect the effects of dehydration upon those cells.

Another check on the interpretation of the data is provided by comparison of the pressure-induced pH changes with those caused by stressing intact plants. As the initial pH of the leaves decreased, the pH of all fractions collected increased at the rate of about 0.2 pH units MPa⁻¹ (Fig. 4A). It is instructive to compare this slope with the relationship of pH to Vw of a single leaf during dehydration. Each fraction required a pressure increase of approximately 0.1 MPa; therefore the dPH/dVw, calculated from the vertical distances between the curves in Figure 4A, was much greater than 0.2 units MPa⁻¹ in the early stages of pressurization, decreasing to that rate after about 50 μl was expressed (Fig. 4). The rapid early changes in pH could be related to the possible participation of other leaf compartments in the release of water. This estimate of the volume of ‘mixed’ sap (50 μl) is similar to the earlier estimates based upon K⁺ levels (Fig. 1). The convergence of the effects of water stress and of pressurization after that point offers further evidence that the pH rise is in part a normal result of dehydration of the leaf.

The fluctuations in ABA concentration of the exudate (Fig. 1) are difficult to interpret. It is important to realize that the apoplastic ABA is transferred directly from the cytosol, a compartment of relatively small volume. Thus, any impediments to resupply of ABA to the cytosol during dehydration may well cause large transient changes in ABA fluxes to the apoplast. Other explanations are also possible, but data to distinguish among them are lacking. Nonetheless, the ABA concentration of exudate rises to extremely high values during the leaf dehydration process; similar concentrations applied exogenously cause substantial stomatal closure (31). The observation that FC completely prevents this rise is important. Because FC stimulates proton-translocating ATPases (23), the data strongly indicate that dehydration inhibits plasma membrane-bound ATPases. Preliminary experiments show that diethylstilbestrol and erythrosin B, two inhibitors of proton-translocating ATPases (6, 9), increase the pH of the expressed sap (data not shown). This result is also consistent with a role for ATPases in determining apoplastic pH.

Increasing pH of the mesophyll apoplast during leaf dehydration is an attractive hypothesis to explain the enhanced release of ABA during water stress (10, 15). In particular, it can account for the increased release of ABA before there is any de novo synthesis, and it can thus explain the initiation of stomatal closure before leaf ABA content increases (7, 19). It can also explain the inhibition of ABA export from mildly stressed leaves (13), because phloem loading from an alkaline apoplast is much slower (11). However, we have not proved directly that the pH gradient between cytosol and apoplast is altered by the changes in the apoplastic pH. Preliminary experiments with 31P-NMR yielded no evidence for dehydration-induced changes in cytosolic pH, but the results remain inconclusive.

In summary, we have found that pressure-induced dehydration increases the pH and ABA content of exuded sap from leaves; the sap appears to be mostly derived from the apoplast of the mesophyll; the effects of pressurization mimic those of water stress on the intact plant; and decreasing the apoplastic pH by
other means (FC treatment) greatly decreases the ABA content of the sap. Finally, the buffering capacity of the sap is least in the normal pH range, and therefore the sensitivity of the system to H⁺ fluxes is greatest. This evidence points to a highly efficient system to maximize stomatal responsiveness to water stress via enhanced release of ABA to the apoplasm.

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