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Movement of Abscisic Acid into the Apoplast in Response to Water Stress in *Xanthium strumarium* L.[1]

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ABSTRACT

The effect of water stress on the redistribution of abcisic acid (ABA) in mature leaves of *Xanthium strumarium* L. was investigated using a pressure dehydration technique. In both turgid and stressed leaves, the ABA in the xylem exudate, the 'apoplastic' ABA, increased before 'bulk leaf' stress-induced ABA accumulation began. In the initially turgid leaves, the ABA level remained constant in both the apoplast and the leaf as a whole until wilting symptoms appeared. Following turgor loss, sufficient quantities of ABA moved into the apoplast to stimulate stomatal closure. Thus, the initial increase of apoplastic ABA may be relevant to the rapid stomatal closure seen in stressed leaves before their bulk leaf ABA levels rise.

Following recovery from water stress, elevated levels of ABA remained in the apoplast after the bulk leaf contents had returned to their prestress values. This apoplastic ABA may retard stomatal reopening during the initial recovery period.

ABA is thought to be involved in the regulation of stomatal behavior during water stress (20, 26). Application of ABA to intact leaves or epidermal strips induces stomatal closure (8, 20) and wilted leaves accumulate large amounts of endogenous ABA (9, 29). There is now convincing evidence, however, that the decline in stomatal conductance, as water stress takes effect, often precedes the stress-induced increase in 'bulk leaf' ABA (Fig. 1; 4, 10, 15). Nevertheless, ABA may still be the causal agent of stomatal closure if the stress alters the internal distribution of the ABA already present in the unstressed leaves (18). A physicochemical basis for ABA redistribution has been suggested, based on the relative pH of the various leaf compartments (7, 12–14). Turgid leaves contain adequate amounts of ABA, if it were uniformly distributed and thus available to the guard cells, to cause stomatal closure (21). Since mature guard cells do not have plasmodesmata (23, 28), symplastic transport of ABA released from the mesophyll to the guard cells is improbable. However, ABA could easily be transported passively by the transpiration stream, via the apoplast, to the guard cells. If this occurs in leaves, it should be possible to detect a stress-induced increase in apoplastic ABA before bulk leaf ABA levels rise.

The available evidence relating to this problem is controversial so far. Ackerson (2) and Weiler *et al.* (27) concluded that the initial stress-induced stomatal response did not involve ABA transported to the guard cells from the mesophyll. Hartung *et al.* (13) presented evidence in support of the opposite viewpoint.

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It has also been observed that, during recovery from water stress, stomata fully reopen only after bulk leaf ABA levels have declined (Fig. 1; 3, 4, 16). The ABA present at high concentrations in the apoplast of stressed leaves (2) may not be quickly catabolized or reabsorbed by the mesophyll when the leaves regain turgor. If this is the case, then apoplastic ABA could still influence the guard cells and cause the lag in stomatal opening.

In this paper, we present data on the effect of water stress on the redistribution of ABA in leaves and relate this to bulk leaf stress-induced ABA accumulation. We also investigated the relevance of apoplastic ABA to the lack of correlation between stomatal conductance and the decline in bulk leaf ABA during recovery from stress.

Xanthium strumarium was chosen for these experiments because mature leaves of this plant are large, have a high ABA content when turgid, and are suitable for manipulation in a pressure chamber.

MATERIALS AND METHODS

Culture of Plant Material. Mature leaves (laminae 13–16 cm long) were harvested from greenhouse-grown plants of *Xanthium strumarium* L., Chicago strain, grown as before (32). The percentage of dry weight of turgid leaves, excluding the midrib and major veins, was 18.7%. The xylem volume of the 3 cm of petiole adjacent to the lamina was estimated as 3.4 μl, by direct measurement of vessel diameters at several points along the petiole, using a microscope graticule.

ABA Redistribution during the Onset of Water Stress. Petioles of detached leaves were placed in deionized H_2O to equilibrate for at least 1 h (see below, Fig. 2). The petioles were trimmed to 3 cm and the water potential (ψ) of the leaves was determined using a pressure chamber (25) lined with damp sponge. Evaporative water loss from the leaves in the pressure chamber was negligible and accounted for less than 2% of the weight lost during exudate collection. Prepurified nitrogen (Airco, Montvale, NJ) was used as the pressurizing gas. A 2-cm piece of amber latex tubing (5-mm diameter, Reichhold Chemicals, Cuyahoga Falls, OH) was then placed over the petiole, and the pressure was slowly increased so that the apoplastic solution was forced out of the leaf into the tube. Aliquots of exudate (10–120 μl) were collected with 100- and 250-μl syringes (Hamilton, Reno, NV) and analyzed for ABA content. The first aliquot from a leaf was collected at a pressure about 3 bars above the initial balancing pressure. Subsequent aliquots were collected by increasing the pressure by approximately 1 bar each time. A series of 9-mm discs, two per harvest, was punched from the central region of the same leaves (the midrib and major veins were avoided), and analyzed to provide a parallel time course for bulk leaf ABA accumulation. Repeated cutting of discs in this way has no discernible effect on ABA accumulation in either stressed or turgid leaves (6). To standardize conditions, and to minimize possible effects of the low partial pressure of O_2, leaves were

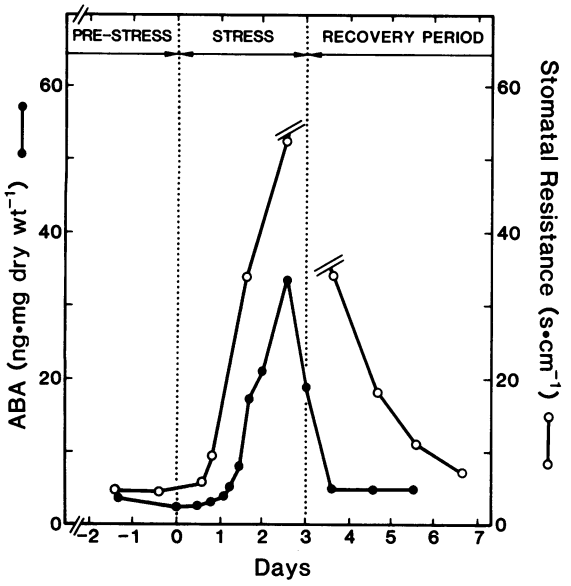

FIG. 1. Stomatal resistance and ABA accumulation and degradation in mature *Xanthium* leaves throughout a stress-recovery cycle. Data combined from Figures 2 and 6 (6).

repeatedly removed from the pressure chamber for 2 min, after a maximum of 15 min under pressure, throughout the sampling procedure. Pressure was increased at a rate not exceeding 4 bars·min^{-1} and decreased at no more than 6 bars·min^{-1}.

The behavior of five leaves from each of two treatments was compared: turgid leaves, and leaves stressed to a 12% loss of fresh weight immediately before they were placed in the pressure chamber. Stress was imposed with a stream of warm air. A 12% loss of fresh weight in *Xanthium* causes accumulation of stress-induced ABA (6).

ABA Levels during Stress Recovery. Intact plants were placed in a controlled environment chamber (32) and well watered for a week before the stress period. The plants were stressed by withholding water, and rewatered after the mature leaves had fully wilted. Stomatal resistance was measured throughout the prestress, stress, and recovery periods with a Li-Cor LI-65 autoporometer (Lambda). The autoporometer actually measures the total leaf resistance which includes both stomatal resistance and a minor cuticular component. Stomatal resistance of the upper epidermis only was monitored as this had previously been found to be more closely correlated with ψ in *Xanthium* than was the stomatal resistance of the lower epidermis (K. Cornish and J. A. D. Zeevaart, unpublished results). Following rewatering, mature leaves were harvested at various times after turgor had been regained and placed in the pressure chamber. Stomatal resistance was measured just before harvest. Single aliquots (80–120 μl) of exudate were collected from each leaf, (see above) to determine the apoplastic ABA level. Preliminary experiments on recovered leaves had demonstrated that consecutive exudate samples collected from single leaves contained a constant level of ABA until leaf wilt occurred in the pressure chamber. Leaf discs (two per harvest) were punched from the leaves before the plants were stressed, immediately before rewatering while fully wilted, and before and after the exudate was collected. All leaf and exudate samples were analyzed for ABA content.

Maintenance of Membrane Integrity: Conductivity. Consecutive 100-μl aliquots of exudate were collected (see above) from leaves treated as follows. (a) Leaves were placed in the pressure chamber immediately after removal from the plant. (b) Leaves were placed in the pressure chamber after their petioles had been in deionized H_2O for 1 h. (c) Leaf laminae were first frozen in liquid N_2 and thawed before sampling. Distilled deionized water was added to each aliquot to give a volume of 1 ml. The conductivity of each sample was measured with an ElectroMark Analyzer (Markson Science, Del Mar, CA).

Sugar Analysis. Exudate was collected from experimental leaves and from leaves the laminae of which were first frozen in liquid N_2 and thawed. The exudate was analyzed semi-quantitatively by TLC as described (30); sugar spots from samples and sugar standards were visualized by spraying with *p*-anisaldehyde:sulfuric acid:glacial acetic acid (1:2:100) and heating at 100°C (17).

Extraction and Purification Procedures. Exudate samples were purified with C_{18} Sep-Pak cartridges (Waters Associates, Milford, MA) (32). Leaf discs were extracted and purified as described (6), except that in the HPLC step the samples were eluted by means of a convex gradient from 0 to 50% ethanol containing 1% acetic acid in 25 min (the percentage ethanol at any point in the gradient after t min = 50 $[1 - (1 - t/25)^{-2}]$). Small amounts of (±)-[^{3}H]ABA (16.4 Ci·mmol^{-1}) were added to each sample to determine losses during the purification procedures. The ABA content of both exudate and leaf samples was quantified using a Hewlett-Packard 5840A gas chromatograph equipped with a ^{63}Ni-electron capture detector, as described (6). Overall recovery of [^{3}H]ABA added to the samples was 70 to 95% for both the exudates and the leaf discs.

RESULTS AND DISCUSSION

Maintenance of Membrane Integrity. Exudate collected from mature *Xanthium* leaves reached a steady conductivity after the leaves had been placed with their petioles in deionized H_2O for 1 h (Fig. 2). A similar constant conductivity was reached with exudate from just detached leaves after 400 μl of exudate had been collected (Fig. 2). Conductivity of the exudate then remained constant, even when 700 μl had been collected. This strongly suggests that the leaves were not damaged by the pressure chamber technique since slight damage would be detected as the solutes leaked into the apoplast. The conductivity of exudate from leaf laminae first frozen in liquid N_2 and then thawed was 2.17 ± 0.11 mSiemens, a 60- to 70-fold increase over the basal level (Fig. 2).

The test for sugars in the exudate also confirmed that very

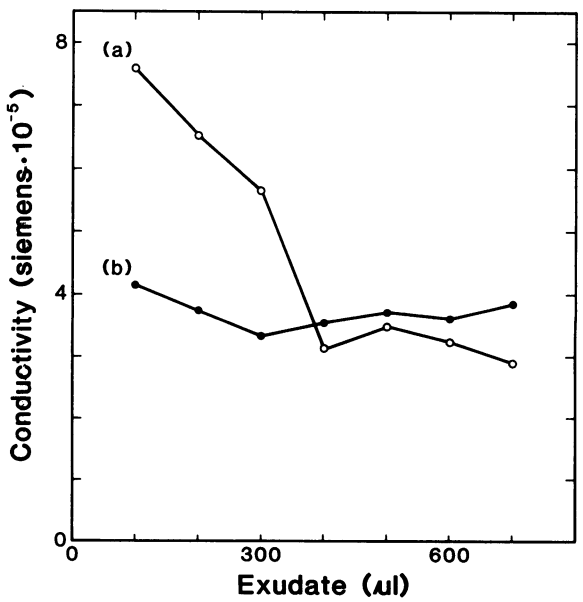

FIG. 2. Conductivity of exudate collected immediately after detaching a mature *Xanthium* leaf from the plant (a), and after the petiole of the detached leaf had been in deionized H_2O for 60 min (b).

little membrane damage was caused by the experimental procedures used. Exudate from the frozen-thawed leaves contained between 4 and 8 $\mu g \cdot \mu l^{-1}$ of both sucrose and glucose. In contrast, exudate from experimental leaves contained only 0.1 to 0.2 μg sucrose $\cdot \mu l^{-1}$, and a 20-μl sample had no detectable glucose; broken cells would undoubtedly release glucose into the apoplast. A 40- to 80-fold increase in sucrose levels in the exudate of frozen-thawed leaves was observed compared with that of the experimental leaves; this agrees closely with the 60- to 70-fold increase in conductivity of the exudate. The observed low level of sucrose in the experimental leaves and the absence of glucose is as expected, and does not indicate membrane damage; unlike glucose, sucrose is transported in the phloem and localized entry of sucrose into the apoplast occurs in leaves prior to phloem loading (11).

ABA Redistribution during the Onset of Stress. *Xanthium* leaves, even those from the same plant, showed considerable variation in their basal levels of apoplastic ABA (15 to 40 pg ABA $\cdot \mu l^{-1}$ exudate). The rate at which exudate was collected also varied between leaves. Thus, it was not possible to amalgamate data from different leaves. The results of two leaves that were turgid when placed in the pressure chamber are shown in Figure 3. The level of ABA in both the apoplast and the leaf as a whole remained constant until wilting symptoms appeared. The level of ABA in the exudate rose substantially within the next 10 to 15 min and then rapidly increased over the rest of the time-course. Stress-induced bulk leaf ABA accumulation occurred at least 10 to 15 min later (Fig. 3).

A very similar pattern was observed when stressed leaves were placed in the pressure chamber (Fig. 4). These leaves began to accumulate stress-induced ABA after approximately 50 min of stress, as has previously been reported for detached *Xanthium* leaves (31). ABA started to increase in the xylem exudate at least 20 min earlier (Fig. 4).

In all 10 leaves examined, the ABA in the xylem exudate, the 'apoplastic' ABA, invariably increased before bulk leaf ABA levels rose. This was completely independent of the rate of exudate collection and of the total volume of exudate collected.

It has been shown that guard cells accumulated ABA when epidermal strips were floated on radioactive ABA solutions (28). In intact leaves, apoplastic ABA would be readily available to the guard cells and so the initial increase in ABA reported here for *Xanthium* may be relevant to rapid stomatal closure in stressed leaves.

ABA continued to move into the apoplast to high levels after the leaves began to synthesize ABA, as has also been reported for cotton leaves (2). In the present study, stressed detached

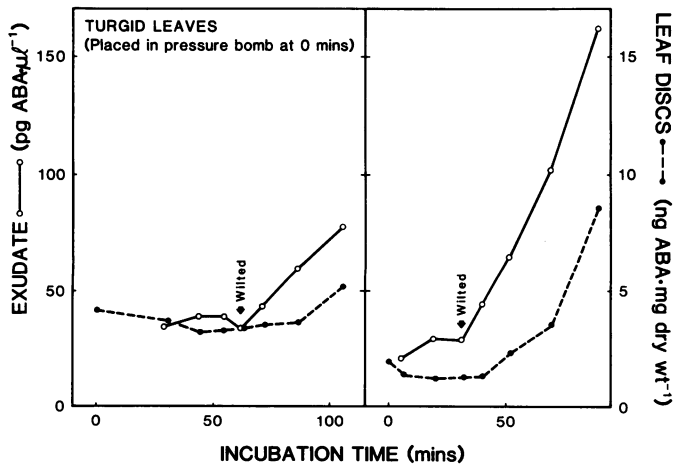

FIG. 3. Changes in ABA levels in xylem exudate and leaf tissue of two mature *Xanthium* leaves placed in the pressure chamber while turgid.

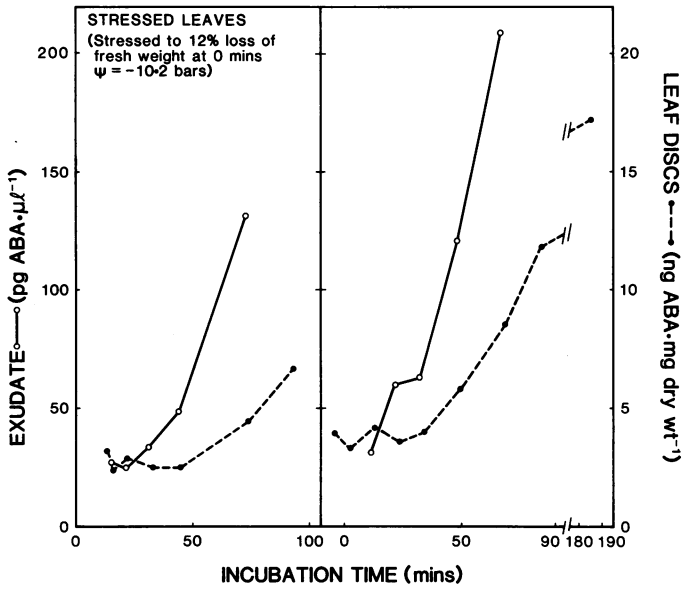

FIG. 4. Changes in ABA levels in xylem exudate and leaf tissue of two mature *Xanthium* leaves placed in the pressure chamber after loss of 12% of the fresh weight.

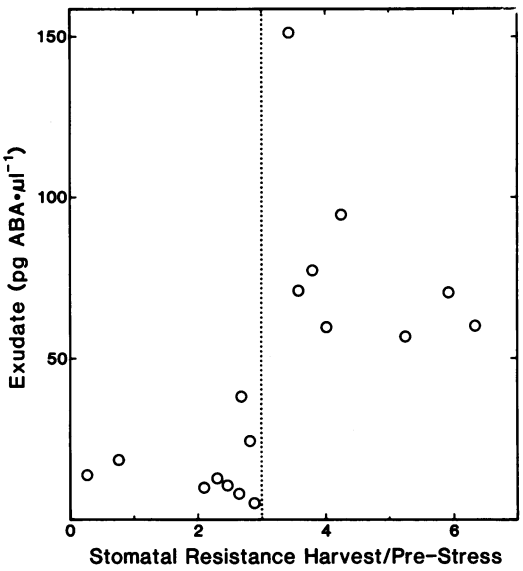

FIG. 5. ABA levels in the exudate of mature leaves from stressed and rewatered *Xanthium* plants plotted against the ratio of stomatal resistance at harvest to that at prestress. Bulk leaf ABA levels had returned to their prestress values in all leaves shown. Harvest/prestress ABA $\leq$ 1.9 derived from Figure 1.

Xanthium leaves contained up to 800 pg ABA $\cdot \mu l^{-1}$ exudate after 4 h incubation in the dark.

Turgid leaves placed in the pressure chamber began to accumulate bulk leaf ABA 20 to 30 min after visual wilting symptoms became apparent (Fig. 3). ABA accumulation in rapidly stressed detached *Xanthium* leaves would normally begin after about 50 min (Fig. 4; 6, 31). Such leaves pass from full turgor to visible wilting in less than 5 min which is much faster than the 20 to 30 min required by the leaves in the present experiments. Since a leaf is comprised of a heterogeneous population of cells, it is possible that a proportion of the mesophyll cells lost turgor and initiated stress-induced ABA synthesis some time before the entire lamina wilted.

The ABA released into the apoplast before bulk leaf ABA levels rose was in the right concentration range to stimulate

stomatal closure. Raschke (22) estimated from various sources that less than one (21) to a few (19, 28) fmol ABA·mm^{-2} of leaf area were usually required to initiate stomatal closure. In this investigation of mature *Xanthium* leaves, the concentration of ABA in the exudate commonly rose 20 to 30 pg·ul^{-1} before bulk leaf ABA began to increase (Figs. 3 and 4). This fraction of the exudate contained an amount of ABA equivalent to approximately 0.2% of the total leaf ABA. This corresponds to an increase of 0.5 to 0.75 fmol ABA·mm^{-2} of leaf area (average area of mature leaf laminae approximately 150 cm^2, fresh weight approximately 4 g). As shown in Figures 3 and 4, much greater quantities of ABA quickly became available to the guard cells as bulk leaf ABA began to increase.

ABA Levels during Stress Recovery. When wilted plants were rewatered, stomatal reopening did not coincide with the decline of bulk leaf ABA (Fig. 1). To determine the role of apoplastic ABA during stress-recovery, it was necessary to investigate leaves which had returned to their prestress bulk leaf ABA levels (harvest/prestress ABA $\leq$ 1.9; see Fig. 1), but which still had elevated stomatal resistances (Fig. 5). All leaves analyzed showed normal patterns of ABA accumulation and stomatal closure when the plants were water stressed (6). Leaves with stomatal resistances still at least 3 times higher than their prestress values had elevated apoplastic ABA contents (Fig. 5). These leaves contained sufficient apoplastic ABA to stimulate stomatal closure in nonstressed leaves (see above) and so a lag in stomatal opening may occur after recovery from stress (2). The slower decline of ABA in the apoplast compared with the leaf as a whole, is possibly due to slow uptake of ABA by the mesophyll cells and a dearth of extracellular catabolic enzymes. The high levels of the catabolite phaseic acid present during the lag period (6) may also restrain the guard cells (24), although this effect is probably slight (K. Raschke, personal communication). As suggested by Raschke (22), full stomatal recovery could correspond to a resynthesis of enzymes degraded during stress. It is also possible that full stomatal recovery was delayed due to increased sensitivity of the guard cells to ABA (1), and/or to lack of cytokinins (5). During the final phase of stomatal reopening in leaves with stomatal resistances <3-fold prestress values (Fig. 5), the apoplastic ABA was at basal level.

In conclusion, when mature *Xanthium* leaves wilt, ABA is released into the apoplast before stress-induced ABA accumulation begins. This rise in apoplastic ABA may be relevant to rapid stomatal closure in stressed leaves. Elevated apoplastic ABA levels may also delay stomatal reopening immediately following rewatering.

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