Plant Physiol. (1972) 49, 842–847

Abscisic Acid and Stomatal Regulation[1]

Received for publication July 9, 1971

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ABSTRACT

The closure of stomata by abscisic acid was examined in several species of plants through measurements of CO_2 and H_2O exchange by the leaf. The onset of closure was very rapid, beginning at 3 minutes from the time of abscisic acid application to the cut base of the leaf of corn, or at 8 or 9 minutes for bean, *Rumex* and sugarbeet; rose leaves were relatively slow at 32 minutes. The timing and the concentration of abscisic acid needed to cause closure were related to the amounts of endogenous abscisic acid in the leaf. Closure was obtained in bean leaves with 8.9 picomoles/cm^2. (+)-Abscisic acid had approximately twice the activity of the racemic material. The methyl ester of abscisic acid was inactive, and *trans*-abscisic acid was likewise inactive. The effects of stress on levels of endogenous abscisic acid, and the ability of very small amounts of abscisic acid to cause rapid closure suggests that stomatal control is a regulatory function of this hormone.

The reports that ABA could reduce transpiration (13, 16) and that this reduction is apparently due to stomatal closure (6, 21) suggest the possibility that this action might have important regulatory functions in the water relations of plants. The possibility became more impressive when Wright and Hiron (22) found that water stress could bring about large increases in the amounts of ABA in leaves, and when Imber and Tal (7) found that a wilty mutant of tomato could be brought into more normal turgor condition by the application of ABA. Subsequent experiments have shown that the ABA regulation of stomata was effective at rather low concentrations and worked quite quickly (9, 17). The experiments reported here will show that the stomatal response is very rapid indeed, and that the quantity and specificity of ABA needed for stomatal control are consistent with the concept of hormonal control of stomatal closure.

MATERIALS AND METHODS

Plant materials were grown in pots of fertilized soil within the greenhouse and included: *Phaseolus vulgaris* L., *Rumex obtusifolius* L., *Beta vulgaris* L., *Xanthium pennsylvanicum* Wall., *Zea mays* L., and *Rosa* spp. Mature leaves were excised in the greenhouse and petioles (or base of lamina for corn) were immediately recut underwater to insure H_2O continuity to the lamina. A fixed area of these single leaves was then enclosed in a thermostated and aerated leaf chamber for the measurement of H_2O and CO_2 exchange.

The leaf held within the chamber was illuminated by a single 150 w incandescent lamp (General Electric cool beam) which provided 2,000 ft-c within the chamber. The lamina of the experimental leaf was held normal to the incident light with its petiole immersed in distilled water or ABA solution (50 μM routinely). The exchange of H_2O and CO_2 by upper and lower leaf surface was monitored continuously on a Hydrodynamics relative humidity sensor and Beckman IRGA respectively. The signals from these two instruments, plus that from a YSI Kettering telethermometer, were transmitted to a Leeds and Northrup recorder.

Gas Circuit. Air was drawn from outside the laboratory with a diaphragm pump and passed through a surge tank (0.5 m^3 in volume) to stabilize CO_2 concentration and H_2O vapor pressure before entering the leaf chamber. During gas exchange measurements, the air stream leaving the chamber first passed over the relative humidity sensor (commonly operating at 23 to 25 C) and then to drying towers ($CaCl_2$ and $CaSO_4$) before entering the CO_2 analyzer (Beckman IRGA Differential Model 315A). A reference air stream, similarly dried, was supplied from the surge tank.

(±)-ABA was a gift from Shell Development Company, Modesto, California. 2-^{14}C-(±)-ABA was obtained from Dr. O. E. Smith, Riverside, California. ABA solutions were prepared by dissolving the weighed material in 1 N KOH and adjusting the final solution to pH 5.8. The ^{14}C-ABA (26 ± 2.6 mc/mmole) was diluted with unlabeled material to give the desired final concentration. Methylation of ABA was done according to the method of Schlenk and Gellerman (19).

ABA-like inhibitors were extracted as follows. The leaf tissue was selected for uniformity and similarity to that used for the gas exchange measurements. An outline drawing of each leaf was made for the estimation of leaf area. Each leaf was then washed in distilled water, blotted dry, and cut into small pieces into a weighed volume of ice-cold 80% (v/v) aqueous ethanol. After reweighing the container and contents, the tissue was homogenized in the ethanol. All subsequent steps were carried out using minimum possible illumination. The homogenized tissue was extracted three times with ethanol at 1 to 2 C over a period of 24 hr, the alcohol was removed by evaporation *in vacuo* at 25 to 30 C, and the aqueous extract was frozen, thawed and, centrifuged at 1600*g* for 30 min to remove suspended material (12). The supernatant was adjusted to pH 2.8 with 1 N HCl and partitioned three times with diethyl ether. The combined ether fractions were extracted alternately four times with small volumes of 5% (w/v) aqueous sodium bicarbonate and water. The combined aqueous fractions were adjusted to pH 2.8 and again extracted with diethyl ether. This ether fraction was dried over anhydrous sodium sulfate, reduced in volume, and chromato-

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graphed on sheets of Whatman No. 3 MM chromatography paper with isopropanol-ammonia (specific gravity 0.88)-water, (10:1:1, v/v). The zone corresponding to a marker spot of ABA was eluted with methanol and rechromatographed on a thin layer of Silica Gel G_{254} with benzene-ethyl acetate-acetic acid (50:5:2, v/v). The solvent was removed from the plate by evaporation in a stream of cool air. The band corresponding to an ABA marker spot, together with the two adjacent strips, were removed, eluted with methanol, and assayed with the *Avena* coleoptile straight-growth test. The amount of ABA-like activity in the extract was estimated by reference to known concentrations of synthetic ABA. It was assumed that only the (+)-enantiomer was active in this test (2, 3).

To determine the amount of ABA required to initiate stomatal closure ^{14}C-ABA (50 μM) was supplied to a detached leaf. As soon as the closing reaction had been initiated, the leaf was quickly removed from the chamber and cut into 1-cm squares. The label was then extracted from the tissue in a scintillation vial with 5% sodium bicarbonate. It was found that by alternately freezing and thawing the tissue, more than 95% of the radioactivity could be extracted. After removal of the tissue, the aqueous extract was dried down and resuspended in 1 ml of methanol. Ten ml of scintillation fluid (5 g/liter PPO + 0.3 g/liter POPOP in toluene) were then added. The amount of exogenous (+)-ABA was then calculated after correction for counting efficiency by the addition of a ^{14}C-toluene internal standard.

A sample of (+)-ABA was isolated from a can of frozen orange juice. The thawed juice was mixed with an equal volume of methanol, which precipitated a great deal of unwanted material. The precipitate was removed by centrifugation at 6,000*g* for 2 hr at 1 to 2 C. The methanol was removed *in vacuo* at 25 to 30 C. The aqueous solution was then purified in the same manner as the leaf extracts. Approximately 240 μg of crystalline material, crystallized from chloroform-hexane, was obtained from each 12 oz. can of juice. Although it was not positively identified as ABA, its biological activity and ultraviolet absorption spectrum strongly suggest that the sample was in fact ABA.

RESULTS

The data presented are drawn from a total of 27 separate experiments with detached leaves. The use of detached leaves was adopted because the ABA or other chemical could be applied while the stomatal behavior was being monitored. Attached bean leaves were found to respond to ABA similarly to detached leaves; this was achieved by enclosing a terminal trifoliate bean leaf in the chamber, excising the lamina along the midrib of the two remaining lateral leaflets, and immersing their cut tips in an ABA solution.

Time Course of Stomatal Response. Application of ABA to the petiole of a detached leaf causes abrupt and virtually complete closure of stomata of bean (Fig. 1), *Rumex* (Fig. 2), corn (Fig. 3), rose (Fig. 4), and sugarbeet (Fig. 5). The data in these figures illustrate the variations in lag time and subsequent speed of responses between species, with bean being the most rapid and complete responder, and rose being the slowest.

In order to examine the pattern of stomatal closure when the continuity of transpiration stream was interrupted, bean leaves were deprived of their water supply and the stomatal performance is recorded in Figure 6. It can be seen that interference with the transpiration stream causes an initial opening of the stomata, followed by closure. Similar hydropassive opening and closure of stomata was observed after cutting the petiole above the water supply, or after the application of grease to the cut end of the petiole. The stomatal closure fol-

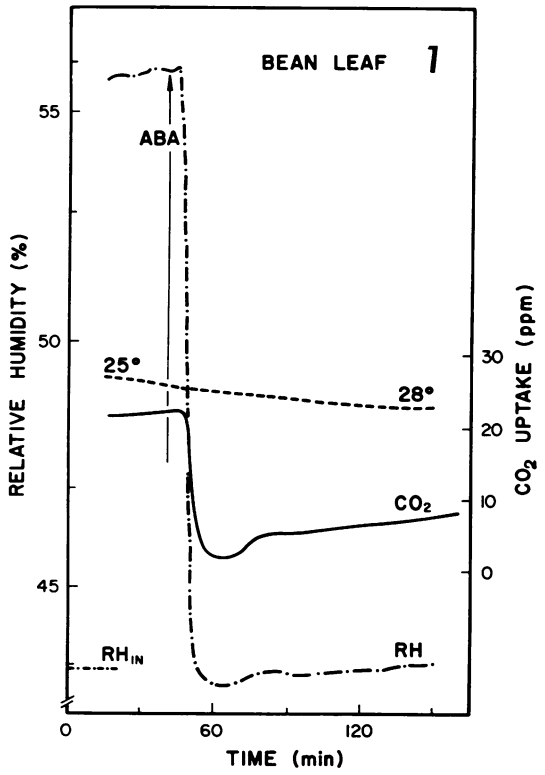


FIG. 1. Effect of ABA (50 μM) on CO_2 and water vapor exchange of an excised bean leaf. Arrow indicates time of ABA application. RH_{in} indicates relative humidity of air entering leaf chamber.

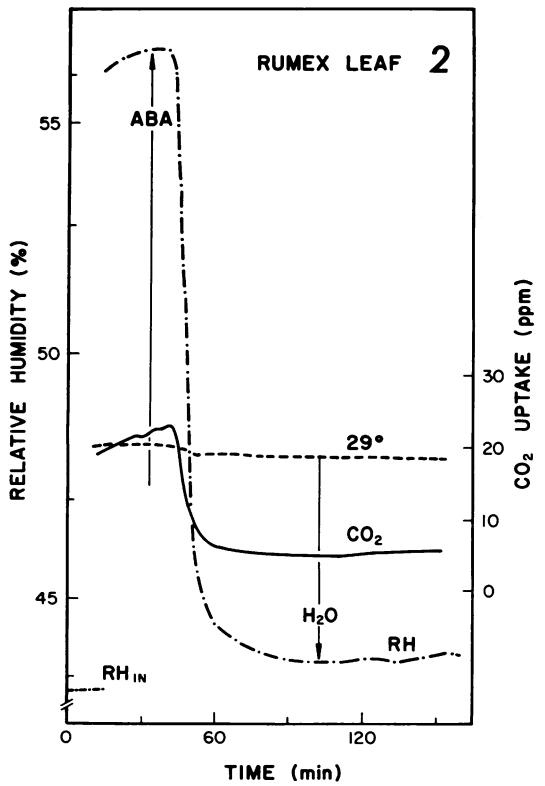


FIG. 2. Effect of ABA on CO_2 and water vapor exchange of an excised *Rumex* leaf.

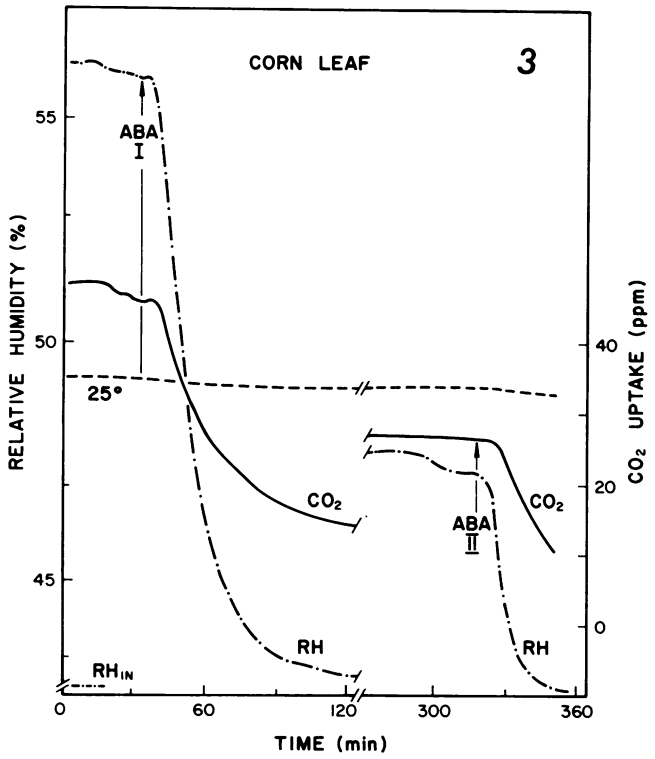
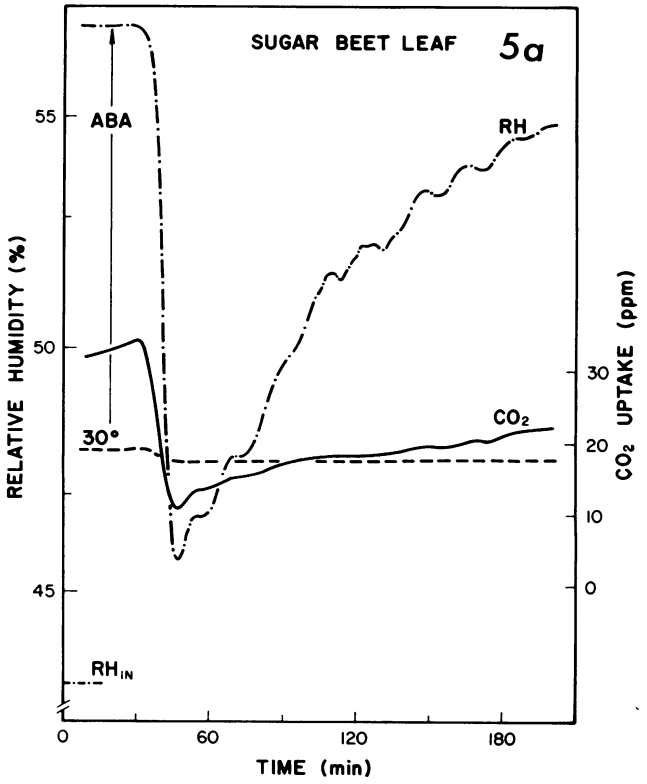


FIG. 3. Effect of two successive ABA applications on CO_2 and water vapor exchange of an excised corn leaf.



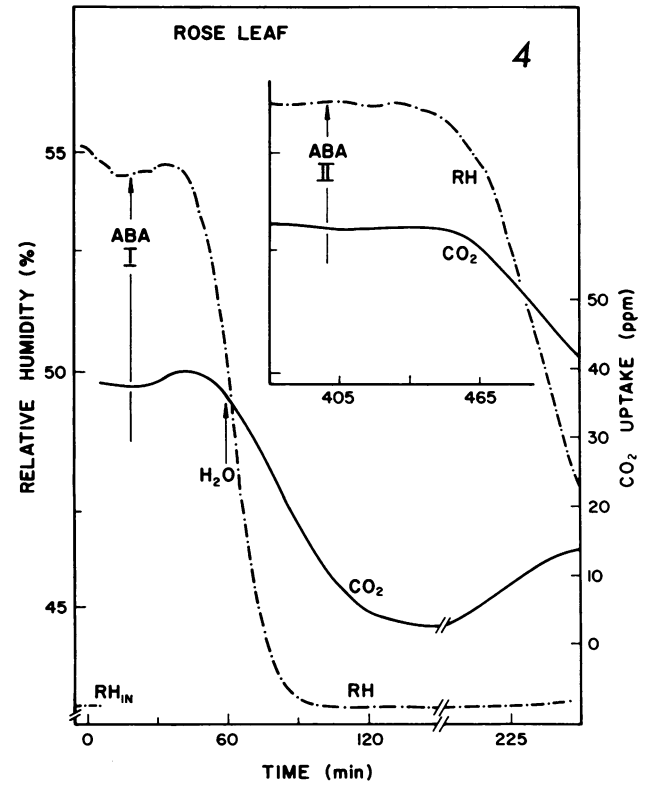


FIG. 4. Effect of two successive ABA applications on CO_2 and water vapor exchange of an excised rose leaf. Temperature was 25 C. Insert shows time change on the abscissa; ordinate scales are identical.

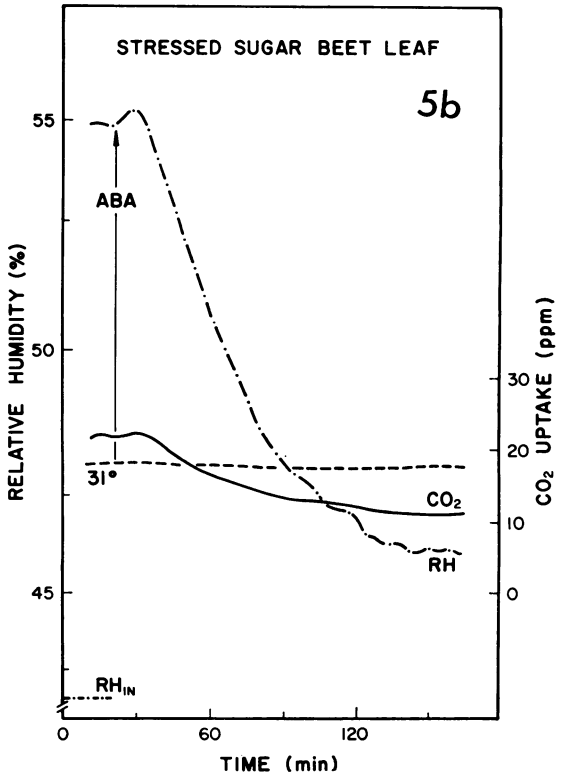


FIG. 5. a: Effect of ABA on CO_2 and water vapor exchange of an excised sugar beet leaf. b: Effect of ABA on CO_2 and water vapor exchange of a water-stressed sugar beet leaf. The leaf was placed in the leaf chamber, and the water supply to the petiole was removed for 135 min. The water was then replaced. The petiole was recut under water, and the leaf was allowed to recover. ABA was applied 240 min after the beginning of water stress.

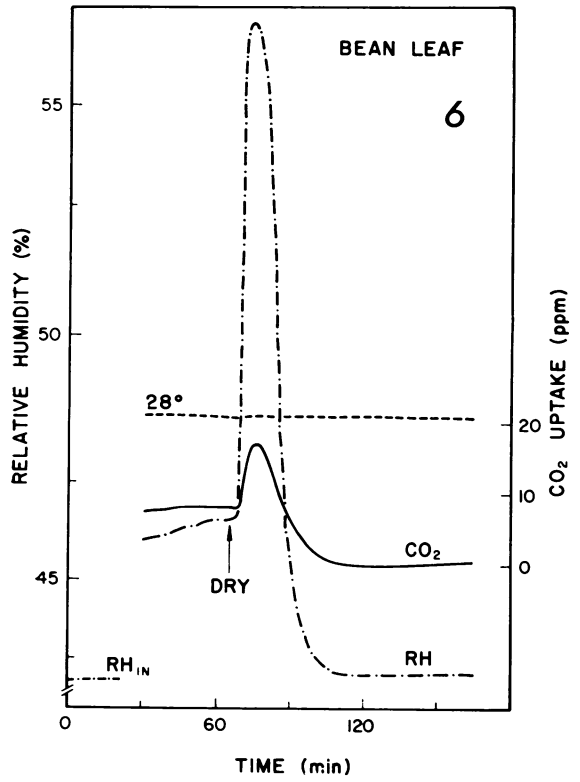


FIG. 6. Effect of removal of water supply on CO_2 and water vapor exchange of an excised bean leaf.

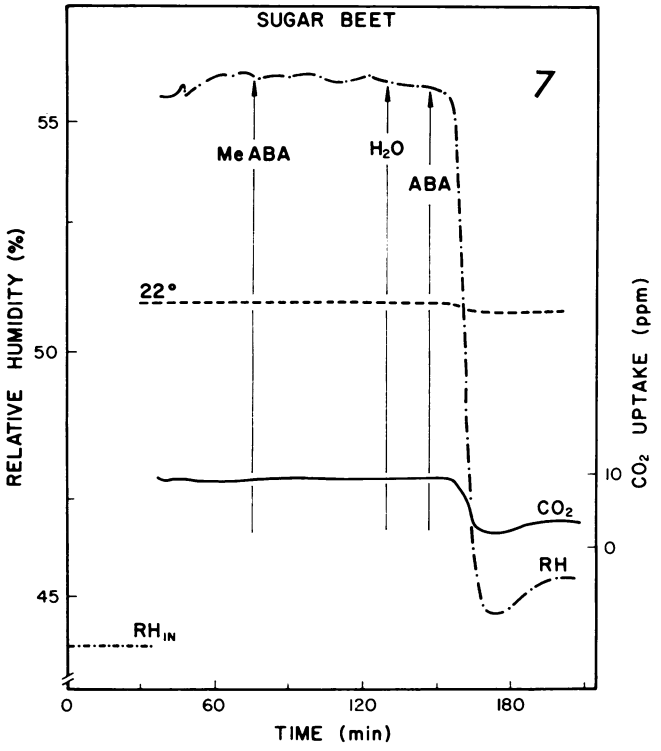


FIG. 7. Effect of ABA and methyl ABA on CO_2 and water vapor exchange of an excised sugar beet leaf.

lowing ABA application is of an entirely different pattern, and we conclude that the ABA experiments did not cause closure through an interruption of the transpirational stream.

Bean leaves showed the most acute stomatal reaction to ABA, and on no occasion did we observe any recovery while the ABA solution was being supplied to detached bean leaves (attached leaves did however recover to a degree). Sugar beet leaves, on the other hand, showed a comparable sensitivity in their initial closure (Fig. 5a), but this was ordinarily followed by a gradual stomatal recovery. This recovery was hastened when the ABA solution was replaced with water; but on reapplying ABA, stomatal closure recurred. The second closure was less abrupt in sugar beet (Fig. 5b) and in other species where tested (see Figs. 3 and 4). This apparent reduction in stomatal sensitivity to ABA could be induced by either pretreatment with the hormone or by a brief period of moisture stress (Fig. 5b).

Some broad-scale comparisons between species with respect to their ABA sensitivity are made in Table I. Close comparison is rendered impossible due to differences in transpiration rate and leaf anatomy between the species tested; but bean leaves are obviously the most sensitive, whereas rose leaves show the slowest reaction. A difference of this magnitude cannot be attributed simply to unequal rates of transpiration, so these two contrasting species were selected for more detailed measurements on the critical internal concentration of ABA for stomatal closure.

Threshold Concentration Within the Leaf. The concentration of exogenous ABA that had to be attained within the leaf to trigger the stomatal reactions must have been extremely low for closure to occur within minutes of supplying ABA to the cut petiole. It is thus unlikely that extraction and bioassay would yield sufficiently accurate data on differences between exogenously supplied and endogenous ABA. Similarly, any attempt to compute the accumulated solute concentration from transpiration data was confounded by losses of ABA to the cells between the petiole and the part of the leaf blade being observed. Application of a tracer that moved with the ABA and which could be easily detected at low concentration might allow an estimate of the ABA concentration. Following some exploratory work, ^{32}P was discarded because it was found that this isotope did not move at the same rate as the ^{14}C-ABA. ^{14}C could not be detected *in vivo* with sufficient accuracy to allow nondestructive experiments, and so the amount of exogenously supplied ^{14}C-ABA was determined by extraction.

The results of these experiments are summarized in Table II and demonstrate the low concentration of ABA which is sufficient to trigger the stomatal reaction. Clearly, the rose leaf is less sensitive to ABA than bean, and this contrast parallels the differences in endogenous concentration. The data imply that stomatal closure may be initiated when the ABA

Table I. *Time Course of Stomatal Responses to Exogenous Racemic ABA (50 μM) in Different Species*

Values shown are based on mean data from a total of 16 separate experiments.

Species	Time Lag between Supplying ABA and the Onset of Closure	Time Elapsed from Onset to Completion of Closure
	min	
Phaseolus vulgaris	8	30
Rumex obtusifolis	8	35
Beta vulgaris	9	52
Zea mays	3	105
Rosa spp.	32	108

Table II. *Relationship between ABA Levels Which Cause Stomatal Closure and Endogenous ABA Levels in Leaves*

Leaves	Amount of Endogenous (+)-ABA Required to Initiate Stomatal Closure (amount of (±)-^{14}C-ABA × 0.5)	Amount of Endogenous ABA Concn in Leaves[1]
Bean	8.9 ± 0.6[2] pmoles cm^{-2} (N = 26)[3]	7.1[4] pmoles cm^{-2}
Rose	14.4 ± 0.5 pmoles cm^{-2} (N = 18)	19.8[4] pmoles cm^{-2}
Corn	34.9 ± 1.9 pmoles cm^{-2} (N = 10)	

[1] Estimated as amount of (+)-ABA and corrected for extraction loss. This material was harvested from the same plants used to initiate stomatal closure and was taken at about the same time of day.
[2] Standard error of the mean.
[3] Number of leaf segments assayed.
[4] Mean of two separate determinations.

Table III. *Comparison of the Racemic Mixture of (±)-ABA with (+)-ABA in the Closure of Stomata in Isolated Xanthium Leaves*

	Transpiration Rate	Time for Initiation of Closure
	mg min^{-1} cm^{-2}	*min*
(±)-ABA (0.1 mM)	0.61	10.90
(+)-ABA (0.1 mM)	0.56	5.00
(±)-ABA (0.2 mM)	0.57	4.70

level is approximately doubled over its endogenous concentration.

The data for the corn leaf shown in Table II emphasize the need to make direct assays of the critical ABA concentration rather than make interpolations from the time course of stomatal response (Table I and Fig. 3). Apparently the shorter lag in the corn leaf can be attributed to its higher rate of transpiration.

Specificity for ABA. We found that *trans*-ABA (50 μM) was without effect on *Xanthium* and sugarbeet in the dark in the presence of CO_2-free air. 2,4-Dinitrophenol (1 mM), coumarin (1 mM) and cycloheximide (5 μg/ml) had no effect on stomatal closure within 30 min of application. One anomaly remains, however. The methyl ester of ABA, which has been credited with growth inhibitory properties similar to ABA (11), was inactive. Experiments were conducted on dock, sugarbeet, and *Xanthium* using 50 μM methyl-ABA and no stomatal response was produced. Subsequent ABA application could elicit rapid closure (Fig. 7). *Xanthium* showed a lack of response to methyl-ABA concentration as high as 0.2 mM.

It was of interest to determine whether the unnatural (−) enantiomer of ABA was active in inducing stomatal closure. Since pure (−)-ABA is not available, a comparison was made between the effect of identical concentrations of (+)-ABA and (±)-ABA on the stomatal behavior of *Xanthium* leaves. Each treatment was duplicated. It can be seen in Table III that the racemic ABA is approximately half as effective as the same concentration of (+)-ABA. Doubling the concentration of the racemic mixture produced an effect indistinguishable from the (+)-ABA effect. This is consistent with the assumption that only the (+)-ABA is effective on stomata.

DISCUSSION

The high degree of specificity for the naturally occurring form of ABA and the extremely small amount needed to elicit stomatal closure make the control of this response an attractive candidate for a normal physiological function of ABA in leaves: Wright and Hiron (22) and Milborrow and Noddle (15) have shown that severe wilting can produce up to a 40-fold increase in levels of endogenous ABA within 4 hr. Our data indicate that a 2-fold increase is sufficient to initiate stomatal closure. This level would be reached soon after the onset of water stress, suggesting a means of fine control over water loss.

Antitranspirants such as phenyl mercuric acetate apparently act first on the photosynthetic system, causing a build-up of CO_2 in the tissues which results in stomatal closure (14). This cannot be the case with ABA-induced closure, as a rapid decrease in transpiration is observed in the dark if the stomata are opened with CO_2-free air (unpublished observation). Jones and Mansfield (9) reached the same conclusion after failing to reverse the ABA effect with CO_2-free air. We have also found that the gas exchange of a *Chlorella* culture is unaffected by the addition of ABA.

In contrast to Jones and Mansfield (9) who showed an effect of topical ABA application lasting up to 9 days in some cases, we have observed quite rapid recovery from ABA treatment in many experiments, *e.g.*, Figures 4 and 5, a and b. This may have been due to the fact that they applied approximately 10 times more hormone than would have been required to close the stomata; the excess may then have acted as a "reservoir." Stomatal recovery is not necessarily as protracted as Jones and Mansfield's (9) data suggest because bean leaves can show stomatal recovery in 1 to 2 days following water stress (5).

Koshimizu *et al.* (11) have reported that the methyl ester of ABA is active in growth inhibition tests; the contrasting evidence reported here of no activity in short term stomatal closure responses might be interpreted as a lack of activity for the methyl ester until it had been hydrolyzed to the free acid. This would assume that the longer growth tests would have allowed sufficient time for the regeneration of the free acid in the plant tissue.

The mechanism whereby ABA is able to regulate stomatal aperture remains unknown. A general permeability change is unlikely since stomata that have been closed in the dark in CO_2-free air by ABA readily reopen upon illumination (unpublished observation). Moreover, hydropassive stomatal opening was observed on bean leaves whose stomata had been previously closed with ABA. For such an opening reaction to have occurred the stomatal apparatus must have retained differential permeability with respect to surrounding epidermal cells despite the ABA treatment. A direct effect on the guard cells in terms of K^+ efflux is an attractive hypothesis but, at the present stage, must still be regarded as speculative.

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