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Sensitivity of Stomata to Abscisic Acid[1]

An Effect of the Mesophyll

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The effects of added abscisic acid (ABA) on the stomatal behavior of *Commelina communis* L. were tested using three different systems. ABA was applied to isolated epidermis or to leaf pieces incubated in the light in bathing solutions perfused with CO_2-free air. ABA was also fed to detached leaves in a transpiration bioassay. The apparent sensitivity of stomata to ABA was highly dependent on the method used to feed ABA. Stomata of isolated epidermis were apparently most sensitive to ABA, such that a concentration of 1 μM caused almost complete stomatal closure. When pieces of whole leaves were floated on solutions of ABA of the same concentration, the stomata were almost completely open. The same concentration of ABA fed through the midrib of transpiring detached leaves caused an intermediate response. These differences in stomatal sensitivity to added ABA were found to be a function of differences in the ABA concentration in the epidermes. Comparison of the three application systems suggested that, when leaf pieces were incubated in ABA or fed with ABA through the midrib, accumulation of ABA in the epidermes was limited by the presence of the mesophyll. Even bare mesophyll incubated in ABA solution did not accumulate ABA. Accumulation of radioactivity by leaf pieces floated on [^{3}H]ABA confirmed ABA uptake in this system. Experiments with tetcyclacis, an inhibitor of phaseic acid formation, suggested that rapid metabolism of ABA in mesophyll can have a controlling influence on ABA concentration in both the mesophyll and the epidermis. Inhibition of ABA catabolism with tetcyclacis allows ABA accumulation and increases the apparent sensitivity of stomata to applied ABA. The results are discussed in the context of an important role for ABA metabolism in the regulation of stomatal behavior.

It is now widely accepted that when plants experience soil drying, ABA concentrations in the bulk-leaf tissue or in the transpiration stream can increase several times above their normal endogenous values. These changes are often accompanied by closure of stomata (Wright and Hiron, 1969; Hoad, 1975; Ackerson, 1980; Pierce and Raschke, 1981; Raschke, 1982; Zeevaart and Boyer, 1984; Neales et al., 1989; Zhang and Davies, 1989, 1990a). Evidence of this kind has been used to support the argument that ABA has a crucial regulatory role in modifying stomatal behavior of drought-treated plants. More recently, it has been suggested that an ABA signal in the xylem stream of plants growing in drying soil can provide the shoots with a means of measuring the water status of the soil in the root zone (Tardieu and Davies, 1992).

Numerous experimental approaches have been used to demonstrate a role for ABA in the physiological process leading to stomatal closure: spraying ABA onto leaves (Mittelheuser and Van Steveninck, 1969; Jones and Mansfield, 1970; Davies, 1978), feeding ABA through the transpiration stream (Mittelheuser and Van Steveninck, 1969; Davies, 1978; Ackerson, 1980; Zhang and Davies, 1990b), and floating epidermal strips on an ABA solution (Ogunkanmi et al., 1973).

All of these methods have shown that synthetic ABA elicits stomatal closure. On the other hand, careful analysis of this response has shown that the reduction in stomatal aperture (conductance or rate of water loss through transpiration) due to ABA is extremely variable and seems to be highly dependent on the method used for the analysis of the response. The ionic composition and the solute potential of the incubation medium, the environmental conditions (e.g. temperature and CO_2 concentration), and the nutritional balance of the plants can influence the apparent sensitivity of stomata to ABA (Wilson et al., 1978; Weyers and Hillman, 1979a; Jarvis and Mansfield, 1980; Radin et al., 1982; Rodriguez and Davies, 1982; Cornic and Ghashghaie, 1991). Increases in ABA concentration in the transpiration stream in intact plants generated as a result of soil drying also have a highly variable effect on stomatal closure (Burschka et al., 1983; Gollan et al., 1992; Schurr et al., 1992; Tardieu et al., 1992).

If we are to sustain an argument that an ABA signal in the xylem can provide the plant with a way to measure soil water status (Tardieu et al., 1992), it is necessary to examine shoot responses to ABA to try to understand the basis of any apparent differences in the sensitivity of the response. In this paper we evaluate three methods of ABA application that apparently result in substantially different stomatal sensitivity to the hormone. ABA was applied to epidermal strips and to pieces of leaf incubated in solution in the light. A transpiration bioassay was also used to feed ABA to detached leaves. We also report the amounts of ABA in epidermis and mesophyll of leaves used with the different methods.

MATERIALS AND METHODS

Plant Material

Seeds of *Commelina communis* L. were sown in John Innes No. 2 compost. After emergence, seedlings were transplanted

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into 90- × 90-mm pots and grown in a controlled environment cabinet with a day/night temperature of 24 and 12°C and a photoperiod of 14 h with a PPFD of 320 μmol m^{-2} s^{-1}. The pots were watered daily to the drip point, and once a week they were watered with a full-strength modified Hoagland nutrient solution (Epstein, 1972). When the plants were 4 or 5 weeks old, the fourth leaf, which was the youngest fully expanded leaf at that time, was used as a source of experimental material.

Experiment with Epidermal Strips

Epidermis was carefully removed from the abaxial surface of *Commelina* leaves and cut into 5- × 10-mm pieces following the technique described by Weyers and Meidner (1990). Before incubation, the strips were floated all together for about 30 min in an 85-mm-diameter plastic Petri dish containing 10 mM Mes buffer (Sigma Chemical Co.) and 50 mM KCl (BDH Chemical, Ltd., Poole, England) adjusted to pH 6.15 with KOH. Most of the stomata were closed at this stage. From this "homogeneous" pool of epidermes, strips were picked at random and incubated on a water bath for 3 h at 25 ± 1°C under a PPFD of 280 μmol m^{-2} s^{-1} in 50-mm-diameter Petri dishes containing 15 mL of Mes buffer and KCl (at the same concentration as above) or Mes, KCl, and ABA at the following concentrations: 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mM. The synthetic (±)-ABA (Sigma) was supplied in the racemic form, but the concentration given in each treatment was calculated for the active (+)-enantiomer.

CO_2-free air (which was obtained by passing ambient air through a column of soda lime [BDH]) was supplied to each Petri dish at a rate of 100 mL min^{-1} through needles dipping into the medium. After incubation, the epidermal pieces were examined under a projection microscope, and pore widths of individual stomata were measured. Fifteen randomly selected stomata were measured in each strip of epidermis, and for each treatment five or six strips were examined.

Experiment with Pieces of Leaf

The leaf was cut into 5- × 20-mm segments parallel to the veins, using a razor blade and avoiding the main central vein. Before incubation, all of the leaf segments were also floated, abaxial surface up, for about 30 min in an 85-mm-diameter plastic Petri dish containing Mes and KCl. After this period, the leaf segments were picked at random, and the effects of the solutions described above were assessed during 3 h (in the same environmental conditions as for epidermis strips) by floating the leaf segments in 50-mm Petri dishes with the abaxial surface up. After the 3-h incubation, the abaxial epidermis of the leaf segments was detached, and the stomatal aperture was determined in the same manner as for epidermal strips.

Transpiration Bioassay Using Detached Leaves

Leaves were removed from the plants under degassed distilled water and immediately placed into plastic vials of 23 mm diameter (5-mL volume) filled with distilled and degassed water. Vials were covered with aluminum foil to reduce evapotranspiration. After the leaves were placed in the vials, they were left in darkness for 12 h to establish full turgor. After the dark period, the leaves were transferred to similar vials containing distilled and degassed water or ABA solutions at the same concentrations used for epidermal strips and leaf segments. The plastic vials were transferred to a growth cabinet (21°C, RH 35%, and a PPFD of 250 μmol m^{-2} s^{-1}) and weighed every 30 min to determine the transpiration rate. Measurements were carried out for 4 h, then the leaf area above the aluminum foil was determined in a planimeter, and the transpiration rate was expressed per unit leaf area.

Experiments with Tetcyclacis

Leaves were cut as before (pieces of leaf), and 5- × 20-mm segments were incubated for 5 h either in a solution of tetcyclacis (0.1 mM) and Mes buffer or in Mes buffer alone. After this incubation, pieces of leaf were transferred to a solution of Mes containing tetcyclacis at the same concentration but with ABA (1 μM) added. Leaves were incubated for 3, 4, and 5 h under the environmental conditions described for pieces of leaf, after which times epidermes were stripped and apertures of stomata in the abaxial epidermis were determined. Pieces of leaf preincubated in Mes for 5 h were also transferred to ABA and Mes or Mes plus tetcyclacis or were left in Mes as a control.

Measurements of ABA Concentration

ABA concentrations were determined in adaxial and in abaxial epidermis and in the mesophyll, with the following experimental conditions:

(a) Adaxial and abaxial epidermal strips and mesophyll pieces were floated separately for 3 h in Mes (control) or ABA (1 μM). After the 3-h incubation period, both epidermis and mesophyll tissues were collected, rinsed with distilled water, and immediately plunged into liquid nitrogen.

(b) Pieces of leaf were floated for 3 h in Mes (control) or ABA (1 μM). After the 3-h incubation period, the abaxial and adaxial epidermes were stripped from each piece of leaf. Epidermes and mesophyll were rinsed with distilled water and immediately plunged into liquid nitrogen.

(c) Whole leaves kept under the conditions described for the transpiration bioassay (see above) were fed with distilled, degassed water (control) or ABA (1 μM), and after a period of 3 h, the adaxial and abaxial epidermes were detached. Both epidermes and the bare mesophyll were immediately plunged into liquid nitrogen.

(d) Pieces of leaf were preincubated on tetcyclacis (0.1 mM) and Mes or on Mes alone and then transferred to solutions containing ABA (1 μM) with or without tetcyclacis (see above). After a 5-h incubation on these solutions, epidermes were stripped, and both epidermes and the bare mesophyll were rinsed with distilled water and immediately plunged into liquid nitrogen for ABA determination.

In all cases, the three tissues were freeze-dried separately during 48 h, ground, and extracted overnight (14 h) at 5°C with distilled, deionized water. Tissue from control treatments was extracted using a ratio of 70:1 (solvent vol-

ume:tissue dry weight), and tissue incubated in ABA was extracted using a ratio of 120:1. Water extraction for periods up to 48 h revealed no evidence of ABA metabolism after freeze-drying.

ABA concentration was determined using a radioimmunoassay. The monoclonal antibody used, which is specific for (+)-ABA (AFR MAC 62), was kindly provided by Dr. S.A. Quarrie. The protocol for radioimmunoassay was as described by Quarrie et al. (1988).

Uptake and Distribution of Radioactivity in Pieces of Leaf

Leaves were cut and treated in the manner described above, and then segments were incubated for 0.5, 1, 2, and 3 h in Mes buffer solution containing 0.13 μmol L^{-1} [G-^{3}H](±)-ABA (Amersham International; specific activity 2.0 TBq $mmol^{-1}$). At the end of the incubation period the pieces of leaf were rinsed twice for a few seconds in Mes buffer and blotted dry with a tissue. Adaxial and abaxial epidermis was then stripped, and the three tissues (abaxial and abaxial epidermis and bare mesophyll) were sealed into 2-mL Eppendorf tubes containing 250 μL of distilled water and were shaken overnight at 4°C. The tubes were centrifuged, a 200-μL aliquot was mixed with 1.5 mL of scintillation fluid (Ecocint H; National Diagnostic, Manville, NJ), and the radioactivity was measured in a scintillation counter (Packard Tri-Carbon 300).

RESULTS

When epidermal strips were incubated in ABA solutions, a marked stomatal closure resulted as the concentration increased (Fig. 1a). Reductions of 17, 59, 76, 79, and 87% in stomatal aperture in relation to the controls were observed as the concentration of ABA increased by orders of magnitude from 10^{-5} to 10^{-1} mM.

Stomata measured in epidermal strips that were incubated with the mesophyll attached (pieces of leaf) and stripped at the end of the incubation period behaved quite differently from those in the first experiment. Reductions of 7, 11, 13, 49, and 85% in stomatal aperture were obtained as the concentration of ABA increased by orders of magnitude from 10^{-5} to 10^{-1} mM (Fig. 1b).

In the transpiration bioassay (Fig. 1c) only small reductions in transpiration (3 and 7% in relation to the control) were obtained with the first two concentrations of ABA (10^{-5} and 10^{-4} mM, respectively). Higher concentrations provoked larger decreases in transpiration rate of 35% (10^{-3} mM ABA), 48% (10^{-2} mM ABA), and 59% (10^{-1} mM ABA).

ABA contents in adaxial and abaxial epidermes and mesophyll are shown in Figure 2. The distribution of ABA between the three tissues depended on the experimental method used to supply ABA. When strips of epidermis or bare mesophyll were incubated in Mes, the ABA contents of these tissues were very similar (Fig. 2a, Mes). On the contrary, when epidermal strips or bare mesophyll were incubated in ABA, the ABA content increased 26-fold in the adaxial epidermal tissue, increased 43-fold in the abaxial epidermis, but increased only 3-fold in the mesophyll relative to the corresponding controls (Fig. 2a, ABA).

In the case of incubated leaf pieces the ABA content of the three tissues was very similar whether tissues were incubated in ABA or Mes, with an average ABA content of only 0.3 mg g^{-1} dry weight (Fig. 2b).

When ABA was fed through the transpiration stream there was a 3-fold increase in ABA in the adaxial epidermis, a 4-fold increase in the abaxial epidermis, and a 4-fold increase in the mesophyll, in relation to the respective controls (Fig. 2c).

Although the reduction of stomatal aperture or the restriction of leaf water loss showed no consistent relationship with the concentration of ABA fed to the leaf (Fig. 1), there was a clear relationship between the percentage reduction in stomatal aperture or transpiration and the ABA concentration in the abaxial epidermis (Fig. 3).

In leaves preincubated in tetcylacis and then exposed to ABA with tetcyclacis, the apparent sensitivity of stomata to the ABA applied increased (Fig. 4). This was apparently a function of the extra ABA found in the epidermis as a result of the tetcyclacis treatment (Fig. 5).

When pieces of leaf were incubated in labeled ABA, radioactivity in epidermis and mesophyll increased with time.

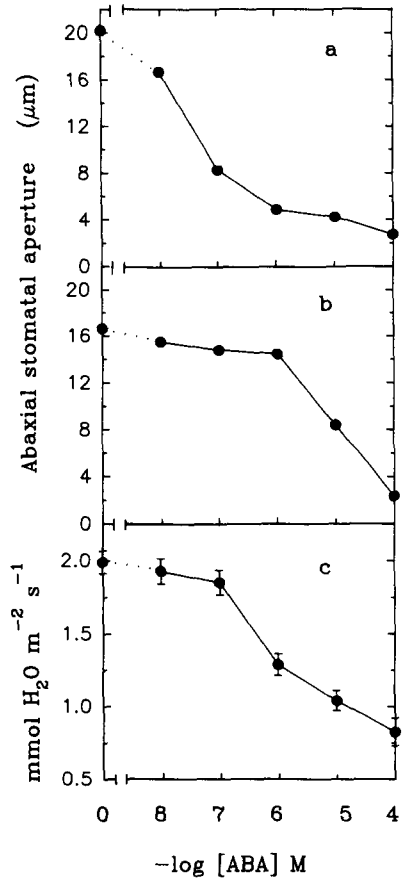

Figure 1. Stomatal aperture in epidermal strips (a) and epidermal strips from pieces of leaf (b) and transpiration of detached leaves (c) of *C. communis* L. floated on Mes or fed with H_2O and floated on or fed with different concentrations of ABA for 3 h. Each point represents the average ± SE of 60 measurements (a and b) and five replicates (c).

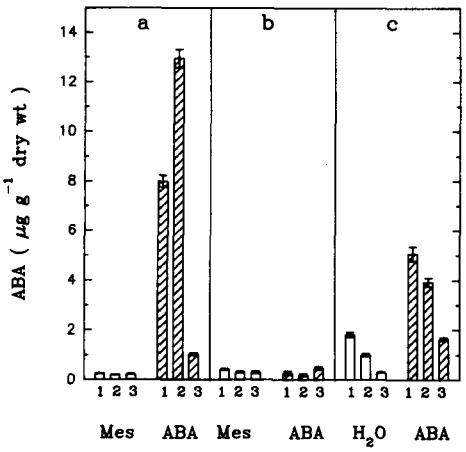

Figure 2. ABA concentration in adaxial epidermis (1), abaxial epidermis (2), and mesophyll (3) of *C. communis* L. floated on Mes or fed with H_2O and floated on or fed with ABA (10^{-3} mM). a, Epidermal strips; b, pieces of leaf; c, transpiration bioassay. Each column represents the average ± SE of five replicates.

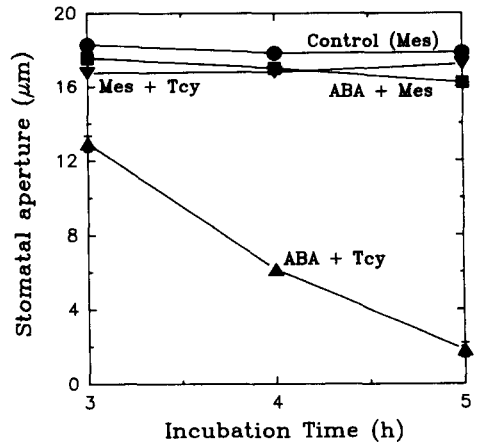

Figure 4. Stomatal aperture of epidermal strips of *C. communis* L. from pieces of leaf preincubated in Mes buffer or 10^{-1} mM tetcyclacis (tcy) for 5 h and after incubation for 3, 4, and 5 h in Mes buffer, 10^{-3} mM ABA + Mes, 10^{-1} mM tcy + Mes, and 10^{-3} mM ABA + 10^{-1} mM tcy. Each point represents the average of 30 measurements.

Substantial radioactivity accumulated in both the mesophyll and in the epidermis but preferentially in the mesophyll (Fig. 6), such that 50% of the ^3H taken up by the pieces of leaf by the end of the incubation period was found in the mesophyll, 29% in the abaxial epidermis, and 21% in the adaxial epidermis.

Using the specific activity of the [^3H]ABA applied at 0.13 μmol L^{-1}, we calculated the amount of radioactive ABA in abaxial epidermis (assuming that the radioactivity measured was still ABA). For a stomatal density of 50 stomata mm^{-2}, 37.6 amol ABA/stomatal complex were found. According to Weyers and Hillman (1979b), this amount of ABA should be enough to cause a significant reduction in stomatal aperture in *Commelina*. Based on these results, we predict that incubation of pieces of leaf in 1 μM ABA (a concentration almost 10 times higher than that of the radioactive ABA) would result in sufficient uptake of the growth regulator to cause substantial accumulation of ABA and closure of the stomata. This was not the case (Figs. 2 and 3).

DISCUSSION

It is clear that the apparent sensitivity of stomata to applied ABA is highly dependent on the method used to apply the growth regulator. In particular, there is a surprisingly large difference between the effects of ABA applied via an incubating medium to isolated epidermis or to pieces of leaf. Epidermal strips floated on ABA solutions take up a large amount of the growth regulator. Leaf pieces on the same

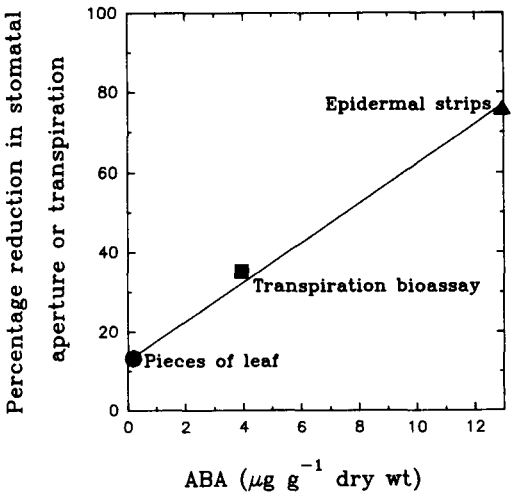

Figure 3. Reduction in stomatal aperture or transpiration as a function of the ABA concentration found in abaxial epidermis of *C. communis* L. after incubation or after feeding 10^{-3} mM ABA for 3 h, using three different experimental approaches.

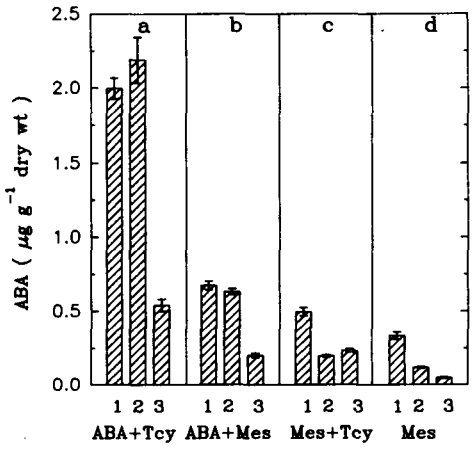

Figure 5. ABA concentration in adaxial epidermis (1), abaxial epidermis (2), and mesophyll (3) of *C. communis* L. floated on 10^{-3} mM ABA + 10^{-1} mM tetcyclacis (Tcy) (a), 10^{-3} mM ABA + Mes (b), Mes + 10^{-1} mM Tcy (c), and Mes (d). Each column represents the average ± SE of three replicates.

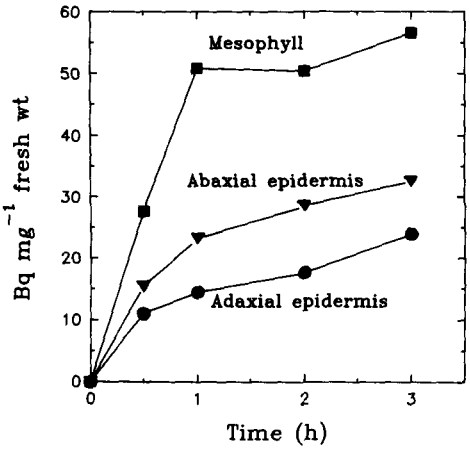

Figure 6. Uptake and distribution of radioactivity in epidermis and mesophyll of *C. communis* L. from pieces of leaf incubated for 3 h in a solution of 0.13 μmol L^{-1} [^{3}H]ABA.

solutions accumulated only very small amounts of ABA. Because the penetration of ABA into leaf pieces must be restricted by the cuticle, the growth regulator must enter the leaf pieces through the cut edges. This was shown by incubation of leaf pieces in [^{3}H]ABA. The amount of radioactivity found in the leaf increased with the time of incubation, with the ^{3}H accumulating mainly in the mesophyll (Fig. 6). Results show that there was substantial uptake of ABA by the leaf (50% of the total uptake after 3 h was in the mesophyll). The very low concentrations of ABA found in all tissues after leaf incubation (Fig. 2) suggest, therefore, that ABA metabolism can at least keep pace with ABA uptake in this system.

A further indication of the rapidity with which ABA can be metabolized in the mesophyll is provided by the low ABA content of bare mesophyll tissue incubated in ABA. We predict a rapid uptake of ABA by bare mesophyll cells in a solution at pH 6.15 (Kaiser and Hartung, 1981; Hartung and Slovik, 1991). Low ABA concentrations in these tissues again suggest that ABA metabolism has a controlling influence on ABA accumulation in leaf tissues.

Relatively high ABA concentrations result when epidermal strips without mesophyll are incubated in ABA solutions (Fig. 2a). Despite the fact that metabolism of ABA by epidermal strips and guard cells of *C. communis* L. has been observed (Singh et al., 1979; Grantz et al., 1985), these results suggest a relatively low rate of ABA metabolism in this tissue. It is important to note, however, that when mesophyll tissue is attached to epidermis, the mesophyll tissue exerts a controlling influence on epidermal ABA content. This means that, although there is no unique relationship between the stomatal response and the ABA concentration applied to "the stomata" in the three systems, there is a clear linear relationship between the ABA concentration in the epidermis and the stomatal response (Fig. 3).

Our hypothesis that metabolism of ABA in the mesophyll tissue can have a controlling influence on ABA concentration in the epidermis, and therefore upon apparent stomatal sensitivity to ABA applied to the leaf, is supported by the results obtained using tetcyclacis, an inhibitor of the metabolism of ABA to phaseic acid (Grossmann et al., 1987; Daeter and Hartung, 1990; Zeevaart et al., 1990). Tetcyclacis treatment resulted in increases in ABA accumulation in all tissues of the leaf (Fig. 5) and an increase in the apparent sensitivity of the stomata to applied ABA (Fig. 4). These data can be interpreted in terms of a limitation in ABA catabolism, a resulting build-up of ABA in the epidermis, and an increased stomatal response. It should be noted, however, that the stomatal responses in this experiment are greater than would be predicted from the relationship between stomatal response and ABA concentration in abaxial epidermis that has been compiled for the nontetcyclacis experiments (Fig. 3). It can only be assumed that the very long incubation times necessary to ensure uptake of tetcyclacis by the leaves have themselves resulted in a change in some other aspect of leaf physiology that has influenced sensitivity of stomata to ABA.

In much of our work on the drought responses of plants, we have attempted to relate stomatal behavior to changes in ABA concentrations in the xylem stream (Zhang and Davies, 1989; Trejo and Davies, 1991; Tardieu et al., 1992). Under controlled environment conditions, the relationship between these two variables is often remarkably conservative. In the field, however, we have detected changes in sensitivity to the ABA signal as a function of the time of day (Tardieu and Davies, 1992). Experiments with isolated epidermis have shown that an important component of this variation can be related to fundamental differences in the properties of guard cells themselves. Nevertheless, the results of the present work suggest that an important and probably highly variable component of the stomatal response to the ABA signal will be the effectiveness with which the mesophyll controls the ABA concentration in the epidermis. This will be a function of the activity of the enzymes involved in ABA metabolism, which can vary with changes in the water status of the leaf (Zeevaart and Creelman, 1988) and other environmental conditions. The relative influence of the mesophyll will vary with the flux of ABA into the leaf. For example, local concentrations may increase as flux increases (Gowing et al., 1993) unless the rate of metabolism is also linked to flux. Weyers and Hillman (1979b) fed radioactive ABA to a transpiring *Commelina* leaf and found approximately 12% of the total radioactivity in the lower epidermis after 1 h. It would be of interest to know how much of this radioactivity is ABA and whether the partitioning between compartments changes with flux.

In a recent analysis, Canny (1990) has showed that the mesophyll tissue in the leaf may modify the composition of the transpiration stream before it gets to the sites of ABA action on the guard cells. We provide here an example of this phenomenon that may be important in the regulation of growth and physiology in plants subjected to drought. In the analysis of plant performance in the field it will prove difficult to come to grips with the importance of ABA partitioning in the leaf, at least until we are able to measure ABA concentrations in the epidermis of leaves of more species. This may not necessarily be a problem for certain kinds of study, in which empirical relationships between xylem ABA concentrations and stomatal behavior have provided us with a useful framework of analysis (Tardieu and Davies, 1993). To aid in our mechanistic understanding of the effects of ABA signals

on stomatal behavior and growth we need some way of routinely assessing the pH-dependent partitioning of ABA between different leaf compartments (Hartung and Slovik, 1991) and the rates at which ABA is broken down to an inactive form.

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