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Xylem Sap pH Increase: A Drought Signal Received at the Apoplastic Face of the Guard Cell That Involves the Suppression of Saturable Abscisic Acid Uptake by the Epidermal Symplast[1]

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Drought increased the pH of *Commelina communis* xylem sap from 6.1 to 6.7. Conductances of transpiring leaves were 50% lower in pH 7.0 than in pH 6.0 buffers, but bulk leaf abscisic acid (ABA) concentration and shoot water status were unaffected by pH. Stomatal apertures of isolated abaxial epidermis incubated on simple buffers increased with external pH, so in vivo this must be overridden by alternative pH effects. Reductions in leaf transpiration rate at pH 7.0 were dependent on the presence of 10^{-8} mol dm^{-3} ABA in the xylem stream. We inferred that at pH 7.0 leaf apoplastic ABA concentrations increased: pH did not affect distributions of ABA among leaf tissues, but isolated epidermis and mesophyll tissue took up more ^{3}H-ABA from pH 6.0 than from pH 7.0 buffers. The apoplastic ABA increase at pH 7.0 may result from reduced symplastic sequestration. A portion of ^{3}H-ABA uptake by the epidermis was saturable at pH 6.0 but not at pH 7.0. An ABA uptake carrier may contribute to ABA sequestration by the leaf symplast of well-watered plants, and its inactivity at pH 7.0 may favor apoplastic ABA accumulation in droughted plants. Effects of external pH on stomatal apertures in the isolated epidermis indicate that published data supporting a role for internal guard cell ABA receptors should be reassessed.

In droughted plants an increase in the ABA concentration of the apoplastic compartment of the leaf (Vanrensburg et al., 1996) is an important determinant of stomatal behavior (Gowing et al., 1993), and there is some evidence that a group of ABA receptors is located at the external surface of the plasmalemma of the guard cell (Hartung 1983; Hornberg and Weiler, 1984). When bound, the receptors induce changes in membrane ion transport and reduce the osmolarity of the guard cell such that it loses turgor pressure, which leads to stomatal closure (see Assmann, 1993).

ABA carried by the transpiration stream from the xylem vessels arrives in the leaf apoplast as a dominant source (Weyers and Hillman, 1979), giving rise to a concentration sufficient to close stomata (Loveys, 1984). There is much evidence that some of the ABA carried by the xylem stream is synthesized in roots that are in contact with drying soil (Davies and Zhang, 1991). The concentration of ABA in the xylem sap of well-watered sunflower plants collected by using techniques that match the transpirational flow rate of intact plants is between 1.0 and 15.0 nmol dm^{-3} (Schurr et al., 1992). In water-stressed sunflower plants the concentration of ABA can reach 3.0 μmol dm^{-3}. Applications of similar concentrations of synthetic ABA to the transpiration stream of detached leaves with normal water potentials commonly reduce water loss by approximately 50%. An ABA concentration of 15.0 nmol dm^{-3} does not induce stomatal closure when applied in the same way to *Commelina communis* leaves (e.g. Trejo et al., 1993), but Trejo et al. (1993, 1995) have shown that nmol dm^{-3} concentrations of ABA supplied directly to isolated epidermal strips of the same species were sufficient to close stomata. These results demonstrate that the leaf itself influences the response of the stomata in the epidermis to the constituents of the transpiration stream. Trejo et al. (1993) concluded that, in whole leaves, the accumulation of ABA by the epidermis was limited by the presence of the mesophyll. Cells of this tissue rapidly metabolized ABA (see also Gowing et al., 1993), thus maintaining a concentration gradient for continuous ABA sequestration into the symplast. Daeter and Hartung (1995) demonstrated that the epidermal symplast of barley leaves is an even better sink for apoplastic ABA than the mesophyll, because it has a catabolic rate that is 5-fold greater.

A physicochemical basis for ABA distribution between different leaf compartments has also been suggested to influence the local concentration of ABA available to the guard cell receptors. ABA is a weak acid that will preferentially accumulate in the more alkaline compartments of the leaf. At an unstressed apoplastic pH (5.2 to 6.5; Hartung and Slovik, 1991) more ABA will be present in its lipophilic undissociated form (ABAH), which readily diffuses across the plasma membrane into the more alkaline cytoplasm of mesophyll or epidermis cells (pH 7.2 to 7.4; Hartung and Slovik, 1991). Here it dissociates to lipophobic ABA^{-} (and H^{+}), which becomes trapped inside the cell (e.g. Heilmann

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Abbreviations: AS, artificial sap; DW, distilled water; pH_{ext}, external pH; RIA, radioimmunoassay; RWC, relative water content; SA, stomatal aperture; SWP, shoot water potential.

et al., 1980; Kaiser and Hartung, 1981). The combined effect of the sequestration of ABA into the more alkaline symplast and the continuous rapid catabolism of cytoplasmic ABA keeps its concentration in the apoplast low enough to prevent accumulation at the stomatal apparatus so that the leaf can respond to further stress signals.

The leaf can also influence stomatal behavior in response to dehydration without invoking the import of extra ABA from the xylem. Turgid leaves contain enough ABA to cause stomatal closure if it were uniformly distributed (Raschke, 1975). Stomata are able to close in response to water stress signals before an increase in bulk leaf ABA is observed (e.g. Loveys, 1977; Dörffling and Tietz, 1984; Cornish and Zeevaart, 1985). These authors suggested that dehydration of detached leaves in air can release ABA sequestered in the symplast so that it accumulates in the apoplast in the vicinity of the guard cells. The water stress signal that may mediate a release of ABA from the symplast may be an increase in apoplastic pH. Hartung et al. (1988) and Hartung and Radin (1989) pressure-dehydrated detached cotton leaves and detected a concomitant increase of apoplastic sap pH (from 6.3 to 7.3) and ABA concentration (from 0.1 to 1.3 μmol dm^{-3}). They concluded that dehydration increased the pH of the leaf apoplast by reducing H^+-ATPase activity. A mathematical model has been described, based on these results and on other leaf properties, which predicts that an increase in apoplastic pH induced by drought can bring about rapid stomatal closure in intact leaves through the symplastic release of ABA before the initiation of its synthesis (Slovik and Hartung, 1992a, 1992b).

More recently, the pH of the xylem sap itself has been studied in conjunction with soil water availability. Hartung and Radin (1989) found that the pH of xylem sap from water-stressed *Phaseolus vulgaris* roots increased from control levels of 6.3 to 7.2. The ABA concentration in the sap increased from 2.5 to 15.0 nmol dm^{-3}. A study of the effects of drought on the ionic content of the xylem sap of intact sunflower plants was carried out by Gollan et al. (1992), who found that the pH of xylem sap removed from well-watered plants was always between 5.8 and 6.6. At soil water contents below 0.13 g g^{-1} the pH increased to 7.1, leaf conductance started to decrease, increases in sap ABA concentration became significant, buffering capacity was reduced, and the cation to anion ratio increased. The authors proposed that the increased pH of the sap could function as an early signal of reduced soil water availability to the leaf, mediated by ABA. We have investigated this possibility in whole turgid leaves of *Commelina communis* L. The data available to date offer only correlations between increases in both xylem sap pH, or what was assumed to be apoplastic sap pH, and ABA concentrations in response to drought. Alternatively, conclusions have been based on models that use individual cell and tissue data to suggest what may be occurring in a whole, intact leaf. An increase in xylem sap pH could have several effects in an intact leaf that would all lead to stomatal closure:

1. It could increase the bulk-leaf ABA concentration by (a) increasing de novo ABA synthesis, (b) reducing catabolic degradation or conjugation of endogenous ABA, or (c) reducing transport of ABA from the leaf in the phloem.
2. It could change the water status of the leaf, which might (a) directly affect guard cell turgor pressure or (b) increase guard cell sensitivity to an unchanged, local ABA concentration (Tardieu and Davies, 1992, 1993).
3. It could have a direct effect on guard cell activity and SA, perhaps by changing membrane ion fluxes.
4. It could change the distribution of endogenous ABA within the leaf to increase the concentration in the vicinity of the guard cells by (a) changing the distribution of ABA between different leaf tissues, perhaps by inducing movement from the mesophyll to the epidermis; by (b) causing the release of ABA from symplastic stores (mesophyll, epidermis, or even guard cells themselves), as predicted in the model described by Slovik and Hartung (1992b); or by (c) causing an accumulation of ABA in the apoplast through inhibition of the normal modes of sequestration into the symplast, bearing in mind that ABA will still arrive from the veins of the leaf.

The present paper reports on a study designed to investigate these various possibilities.

MATERIALS AND METHODS

Seeds of *Commelina communis* L. were sown in John Innes No. 2 compost. After emergence, seedlings were transplanted 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, was used as a source of experimental material.

Chemicals and Radiochemicals

[G-^{3}H](±)-ABA, specific activity 2.0 TBq mmol^{-1}, was obtained from Amersham. This was diluted with PBS giving stock concentrations of (a) 8.9 nmol dm^{-3} (for RIA) and (b) 2 × 10^{-7} mol dm^{-3} (for ABA transport experiments). These were stored at −20°C in the dark.

Synthetic racemic (±)-ABA was obtained from Lancaster Synthesis Ltd. (Morecambe, Lancashire, UK). Working solutions were made up as required by dilution from stock solutions in degassed DW, which were refrigerated in darkness. The monoclonal antibody AFRC MAC 252 used in RIA was specific for (+)-ABA and was generously provided by Dr. S. A. Quarrie (Institute of Plant Science Research, Norwich, UK). General reagents used in experiments were all BDH Analar grade from Sigma (Poole, Dorset, UK).

Experiments with Whole Leaves

Transpiration Bioassay and RIA

Leaves were removed from the plants, and the base was recut under degassed DW to avoid embolism and imme-

diately placed into plastic vials of 5.0 cm^3 volume. The vials contained 4.0 cm^3 of AS: 1.0 mmol dm^{-3} KH_2PO_4, 1.0 mmol dm^{-3} K_2HPO_4, 1.0 mmol dm^{-3} $CaCl_2$, 0.1 mmol dm^{-3} $MgSO_4$, 3.0 mmol dm^{-3} KNO_3, 0.1 mmol dm^{-3} $MnSO_4$; the sap was buffered to pH 6.0 with HCl unless otherwise stated. The ionic content of AS approximates that which was detected in well-watered sunflower xylem sap by Gollan et al. (1992), in which the buffer system was found to be H_2PO_4/HPO_4-. The vials were covered with aluminum foil to reduce evaporation, randomized in a growth cabinet (PPFD 350 μmol m^{-2} s^{-1}), and weighed every 30 min for 3 h to establish an initial transpiration rate. Leaves were then rapidly transferred to similar vials containing AS ± specified additions, such as ABA. Vials were again weighed every 30 min for 3 h (unless otherwise stated), after which time the leaf area above the foil was measured in a planimeter (Paton Industries, Pty. Ltd., South Australia). The transpirational rate of water loss was expressed per unit leaf area (single surface). In some experiments the leaves were weighed, transferred to vials containing 4.0 cm^3 of DW, and left in the dark overnight to reach full turgor before reweighing. They were then freeze-dried and reweighed to measure the RWC.

At the end of some experiments whole leaves were immediately frozen in liquid nitrogen, or they were quickly divided into abaxial epidermis, adaxial epidermis, mesophyll, and midrib so that these tissues could be frozen individually and then freeze-dried for 48 h. The tissue (10–15 mg minimum; approximately 12 leaves required for tissue separation studies) was then ground and extracted overnight at 5°C with deionized DW, using different extraction ratios (leaf dry weight:solvent) depending on the concentration of ABA fed (e.g. bulk leaf endogenous ABA = 1:25; epidermis from ABA-fed leaves = 1:150). ABA concentration was determined using RIA with the AFRC MAC 252 antibody (for protocol see Quarrie et al., 1988).

Experiments with De-Rooted Plants

Measurement of Transpiration, Conductance, Water Potential, and Xylem Sap pH

Plants were cut just above the root system and recut under degassed DW. They were immediately placed in 30-cm^3 plastic vials containing 20 cm^3 of AS, and transpiration rates were measured as above. Total leaf area was measured in a planimeter. In some experiments stomatal conductance was measured at 30-min intervals on the abaxial and adaxial surfaces of the fourth leaf using a diffusion porometer (MK III, Delta-T Devices Ltd., Cambridge, UK).

At the end of some experiments plant water potential was measured using a pressure bomb (Soil Moisture Equipment, Santa Barbara, CA). Upon removal from the vial the shoot base was blotted dry and quickly sealed into the bomb using silicone rubber bungs. The chamber was gradually pressurized with compressed nitrogen gas until the meniscus of the xylem sap became visible at the cut tissue surface and the pressure was noted.

Xylem sap pH was compared in fully watered and 6-d droughted plants that were either 4.5 weeks old or 6 weeks old at the outset of the treatment, and that had been grown in a 1:1 mixture of John Innes No. 2 compost and sand. The shoot was cut with a razor blade just above the roots and sealed into the pressure bomb as above. Pressure was applied above the balancing pressure (equal to the SWP) to express the xylem sap. Initial tests proved that the pH of the first drop of sap exuded was not representative of that in the rest of the plant (being more acidic). This may have been due to an accumulation of sap above the cutting point or to contamination from cut cells. This first drop of exudate was blotted away. The shoot was then submitted to over-pressures. Sap pH was measured by placing an Orion combination needle pH electrode (Orme Scientific Ltd., Manchester, UK) into the droplet.

Experiments on Individual Leaf Tissues

Measurement of SA

The abaxial epidermis was carefully removed from either side of the midrib of several leaves following the technique described by Weyers and Meidner (1990). The uncut strips were pooled and floated mesophyll-side down for 1 h in large plastic Petri dishes containing 25 cm^3 of 50 mmol dm^{-3} KCl in a growth cabinet (PPFD = 350 μmol m^{-2} s^{-1}) to open the stomatal pores. From this homogenous pool of tissue, strips were randomly transferred to Petri dishes containing 25 cm^3 of solution buffered to different pHs with HCl or KOH ± ABA, and incubated for 2 h. Three different buffer systems were used: (a) AS supplemented with 50 mmol dm^{-3} KCl, (b) 2.0 mmol dm^{-3} KH_2PO_4 and K_2HPO_4 supplemented with KCl, and (c) 10 mmol dm^{-3} Mes or Hepes buffer supplemented with KCl. The epidermal pieces were then transferred with a few drops of the appropriate incubation medium to slides, and pore widths of 30 stomata from each piece (usually 3 replicates) were immediately measured under a projection microscope.

Uptake of ^{3}H-ABA by Epidermis and Mesophyll Tissue

Time Course for Uptake. Abaxial epidermis or mesophyll tissue was isolated from several leaves and pooled on solutions of buffer B adjusted to pH 7.25 in Petri dishes in the growth cabinet (PPFD as above). Strips were cut into 0.5- × 1.0-cm squares and incubated for 2 h. It was assumed that any endogenous ABA left in the wall spaces would diffuse out. Approximately 40 squares were rapidly washed in KCl solution and transferred to Petri dishes of 25 cm^3 of buffer B containing 5×10^{-10} mol dm^{-3} ^{3}H-ABA adjusted to the appropriate pH. Transfer to each dish was usually staggered. Three squares were removed from each dish at 15-min intervals for 3 h. These were washed twice in unbuffered KCl so that only radioactivity taken up into the symplast remained, transferred to preweighed plastic scintillation vials, and weighed again. Liquid nitrogen was used to kill the cells. When this had evaporated, 5.0 cm^3 of scintillation fluid (Ecoscint H, Mensura Technology Ltd., Wigan, UK) was added and the contents were shaken overnight at 5°C. The radioactivity was measured in a scintillation counter (Packard Tri-Carbon 300) and expressed on a per-unit-tissue-weight basis. Adjustments

were made for the reduced counting efficiency of mesophyll samples, from which chlorophyll was leached by the scintillant.

Static Uptake. Abaxial epidermis and mesophyll tissue was treated as above. After 2 h of pretreatment on pH 7.25 buffer in the growth cabinet, four squares of tissue were washed and transferred to 10 cm^3 of uptake medium in 50-mm diameter Petri dishes. Strips were incubated for 1.5 h in buffer containing ^{3}H-ABA at 5×10^{-10} mol dm^{-3} ± unlabeled ABA at 10^{-6} mol dm^{-3}, adjusted to the required pH. At the end of the incubation period the tissue was treated as above, and radioactive content was measured.

Efflux of ^{3}H-ABA from Preloaded Epidermis and Mesophyll Tissue

Abaxial epidermis and mesophyll tissue was treated as above. Pretreatment was for 2 h in buffer B adjusted to pH 7.25 in the growth cabinet. Approximately 40 squares of tissue were washed and transferred to Petri dishes in the growth cabinet containing 25 cm^3 of uptake medium: pH 5.0 buffer containing 10^{-9} mol dm^{-3} ^{3}H-ABA. Loading of activity was carried out for 1.5 h. At the end of this period four squares were removed from each Petri dish, washed in pH 5.0 buffer, weighed, and treated as above to obtain an initial level of activity in the tissue. The remaining tissue was washed and transferred very rapidly to large Petri dishes in the growth cabinet containing 25 cm^3 of ABA-free efflux buffer adjusted to the required pH. At 10-min intervals over a 2.5-h time course, the efflux medium was mixed gently with a pipette, and 0.25 cm^3 of liquid was removed and placed in a scintillation vial with 5.0 cm^3 of Ecoscint H. At the end of the efflux period the remaining tissue was washed in pH 5.0 buffer, weighed, and placed in scintillant. The activity in the tissue and in the efflux medium was counted as above. Efflux was expressed as activity remaining in the tissue (on a per-unit-weight basis) at each time interval as a percentage of its total initial activity.

RESULTS

Effects of Drought on Xylem Sap pH

Figure 1 shows the results of an investigation of the effects of drought on the pH of xylem sap expressed from shoots of 4.5- and 6-week-old *C. communis* plants using a pressure bomb. The pH of the sap from the droughted plants was significantly higher than that expressed from the well-watered plants 6 d after the onset of treatments, corresponding to water potentials of −0.1 ± .03 MPa (control) and −0.36 ± .01 MPa (droughted) in 6-week-old plants; and −0.2 ± .04 MPa (control) and −0.26 ± .08 MPa (droughted) in 4.5-week-old plants. It is also evident that the pH of sap from the 4.5-week-old plants was much more alkaline than that of plants just 1.5 weeks older (Fig. 1). Because the younger plants contained minimal volumes of sap, fewer replicate measurements were obtained from these than from the older ones.

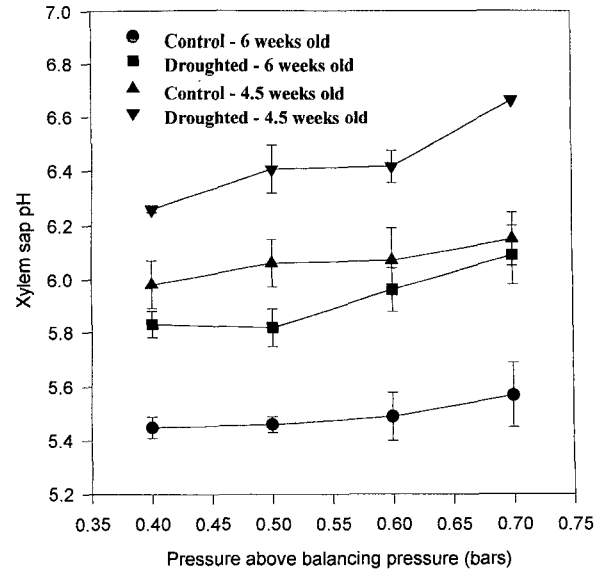

Figure 1. The effect of drought on the pH of xylem sap exuded from de-rooted *C. communis* shoots using a pressure bomb. The initial exudate was blotted away, and subsequent readings were taken from sap exuded at known over-pressures. Data are means of 6 replicates ± SE for 6-week-old plants, and of 3 to 5 replicates ± SE (except at over-pressures of 0.7 MPa) for 4.5-week-old plants.

Effects of ABA and pH on the Transpiration Rate of Whole, Detached Leaves

When ABA was introduced into the xylem stream of whole, detached leaves of *C. communis* at a pH representative of the well-watered sap of 4.5- to 5-week-old plants (pH 6.0), the transpiration rate was reduced in a concentration-dependent manner as expected (results not shown). An ABA concentration of 10^{-6} mol dm^{-3}, which is similar to that detected in sap from droughted plants, reduced transpiration by 38% from control levels, and 10^{-4} mol dm^{-3} ABA did so by 69%, both after 3 h.

Buffers (AS) adjusted to different pHs were supplied to the xylem stream of detached leaves, and the subsequent transpiration rates were measured for the first 3 h in the absence of 3×10^{-6} mol dm^{-3} ABA, and for a further 3 h with or without its inclusion in the uptake medium (Fig. 2, left and right). Figure 2, left, compares transpiration rates at pH 5.0 and 6.0. There was no difference in transpiration rate or the response to ABA between these two conditions. However, when leaves were placed into solutions buffered to pH 7.0 (Fig. 2, right, equivalent to the pH of sap from droughted plants; Fig. 1; Gollan et al., 1993), the transpiration rate was reduced to 40 to 50% of that at pH 6.0 in the absence of extra exogenous ABA. This reduction was rapid for the 1st h and reached a steady state after approximately 2 h. ABA did not reduce transpiration to rates lower than those detected in pH 7.0 buffer alone.

Effects of pH on ABA Concentrations in Whole Leaves

The ABA content of leaves that had transpired at pH 6.0 and pH 7.0 for 6 h was analyzed using RIA. On a dry weight basis the bulk ABA content of the leaves was not

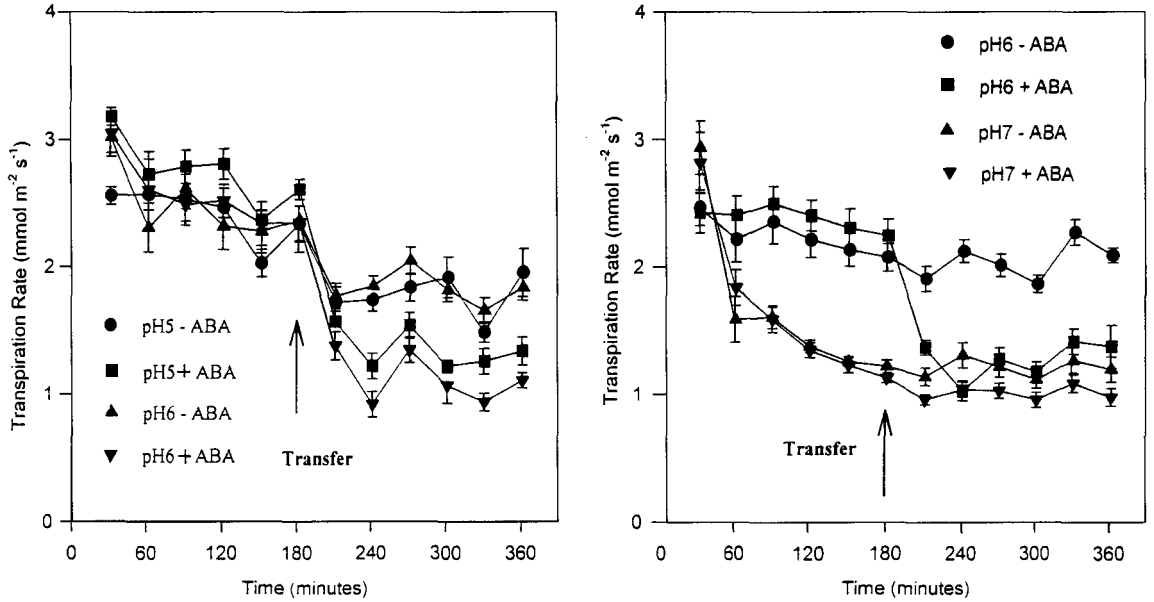

Figure 2. The effect of buffer pH 5.0 or 6.0 (left), or 6.0 or 7.0 (right) on the rate of transpiration of detached *C. communis* leaves in the light, in the presence and absence of 3×10^{-6} mol dm^{-3} ABA. An initial transpiration rate was established over 3 h in 4.0 cm^3 of AS buffered to the appropriate pH. Leaves were then transferred to identical solutions, ± ABA, and new transpiration rates were measured. Data are adjusted for leaf area and expressed as means ± SE (n = 6, left; n = 8, right).

significantly different between these treatments (Table I). When 3×10^{-6} mol dm^{-3} ABA was present in the uptake medium for the last 3 h of the bioassay, leaf ABA content was 57.6% greater at pH 6.0 and 69.4% greater at pH 7.0, however, the increase in ABA content only correlated with a reduction in transpiration rate at pH 6.0. The ABA content of the leaf tissue was significantly higher when 3×10^{-6} mol dm^{-3} ABA was supplied in the medium buffered to pH 7.0 than when it was supplied in that buffered to pH 6.0.

Effects of pH on Whole Shoot Transpiration and Water Relations

Whole shoots were divided from their roots and the effect of pH on their transpiration rate was measured. The reduction of transpiration was again seen when buffer was supplied at pH 7.0 as opposed to pH 6.0 (Fig. 3), indicating that the results described above were not merely a single-leaf phenomenon. After 3.5 h, pH 7.0 had reduced the transpiration rate to 24% of the control rate, however, the time lag before incipient reduction was longer than in a single leaf. At the end of the transpiration period (3.5 h) SWP was measured in a pressure bomb, and the fourth leaf of some of the shoots was detached to determine its RWC. There was no significant effect of pH on either of these parameters (Fig. 3).

Table I. *The effect of pH on the bulk ABA content of C. communis leaves after they have been treated as in Figure 2*

The tissue was freeze-dried, ground, and extracted at a dry weight: water ratio of 1:25. Eight leaves were pooled from each treatment and two aliquots of 50 mm^3 of the extracts were analyzed by RIA (Quarrie et al., 1988). Results from two separate experiments are expressed as mean ABA content (nmol g^{-1} dry wt) ± SE.

Treatment	Mean ABA Content	Significant Difference (from pH 6.0)
	nmol g^{-1} dry wt	
pH 6.0 − ABA	1.87 ± 0.38	na[a]
pH 6.0 + ABA	3.66 ± 0.18	na
pH 7.0 − ABA	1.82 ± 0.12	ns[b]
pH 7.0 + ABA	6.51 ± 0.30	ns

[a] na, Not applicable. [b] ns, Not significant.

Effects of pH on Abaxial and Adaxial Leaf Conductance

The effect of uptake buffer pH (AS) from 5.0 to 8.0 on the conductance of both surfaces of the fourth leaf of shoots separated from their root system was measured over 4 h using a porometer (Fig. 4, left and right). The effect of pH on both abaxial and adaxial leaf surfaces was identical, although control conductance (pH 6.0) of the adaxial surface was only approximately 15% of that at the abaxial surface. There was no significant difference in conductance between pH 5.0 and pH 6.0, but pH 7.0 reduced conductance by approximately 50% in comparison to that at pH 6.0 after 4 h. Conductances at pH 8.0 were extremely low, and it was observed that some of the leaves on the plants taking up pH 8.0 buffer became wilty. The RWC of the fourth leaf of half of the plants was measured and was found to be significantly lower only at pH 8.0 (n = 4): pH 5.0 = 99.4 ± 0.1%; pH 6.0 = 97.2 ± 1.0%; pH 7.0 = 98.3 ± 0.9%; pH 8.0 = 89.8 ± 1.0%. The bulk ABA content of the leaves was also analyzed, and it was found that at pH 8.0 the ABA content was 5-fold greater than at any other pH (n = 4): pH 5.0 = 1.11 nmol g^{-1} dry weight ± 0.18; pH 6.0 = 1.03 nmol g^{-1} dry weight ± 0.12; pH 7.0 = 1.00 nmol

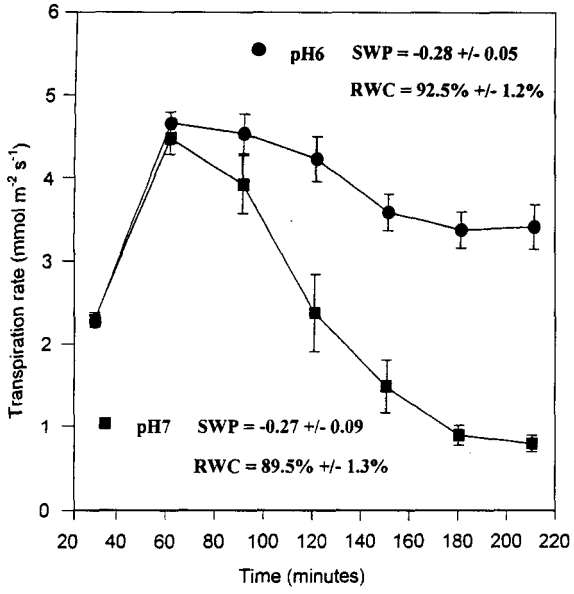

Figure 3. The effect of buffer pH on the rate of whole shoot transpiration of *C. communis* in the light over a 3.5-h period. Shoots separated from the root system were incubated in 20 cm^3 of AS (with 2.0 mmol dm^{-3} KH_2PO_4 and K_2HPO_4) buffered to the appropriate pH. Data are adjusted for total leaf area and expressed as means ± SE (n = 8). SWP (MPa) and RWC (% turgid water content) of the fourth leaf are also shown (means of seven plants).

g^{-1} dry weight ± 0.12; pH 8.0 = 5.36 nmol g^{-1} dry weight ± 0.71.

The Effect of pH on the SA of Whole Leaves

Figure 4, left, also shows SAs in abaxial epidermis peeled from whole leaves after a 3-h transpiration period in buffers (AS) of differing pH. These were not significantly different between pH 5.0 and pH 6.0, but those from leaves that had transpired at pH 7.0 and pH 8.0 were only 33% and 11%, respectively, of the size of those measured in epidermis peeled from leaves that had transpired at pH 6.0. Thus, an increase in buffer pH reduced the following variables in parallel: transpiration rate, abaxial and adaxial leaf conductance, and SAs in whole leaves.

The Effect of pH on SA in Isolated Epidermis

When isolated abaxial epidermis was preincubated in KCl in the light to open the stomata and transferred to solutions buffered to different pHs (buffer A, see "Materials and Methods"), there was no effect of pH on SA between 5.0 and 8.0 (Table II). This was very different from the effect of pH on SA seen in epidermis peeled from whole, detached leaves that had transpired for 3 h (see previous section). Buffer A is the AS that contains concentrations of ions equivalent to those detected in the xylem sap of well-watered sunflower plants (Gollan et al., 1992).

When the simple buffers B and C were used as incubation media for isolated epidermis tissue (phosphate or Mes and Hepes supplemented with KCl), pH had a very different effect on SA. Aperture increased with pH from 6.0 to 7.0 (Table III) or from 5.0 to 7.0 (Table IV). At the unphysiological pH of 8.0 the aperture was slightly lower than that at pH 7.0. In the presence of 10^{-7} mol dm^{-3} ABA, SA was decreased by approximately 60% in buffer B and by 30% in buffer C at both pH 6.0 and pH 7.0. There was an increase in SA with pH_{ext} from 6.0 to 8.0 in the presence of ABA. At pH 8.0, however, the stomata were no longer able to respond significantly to ABA. These results imply that the effect of pH on SA in epidermal strips was direct and was

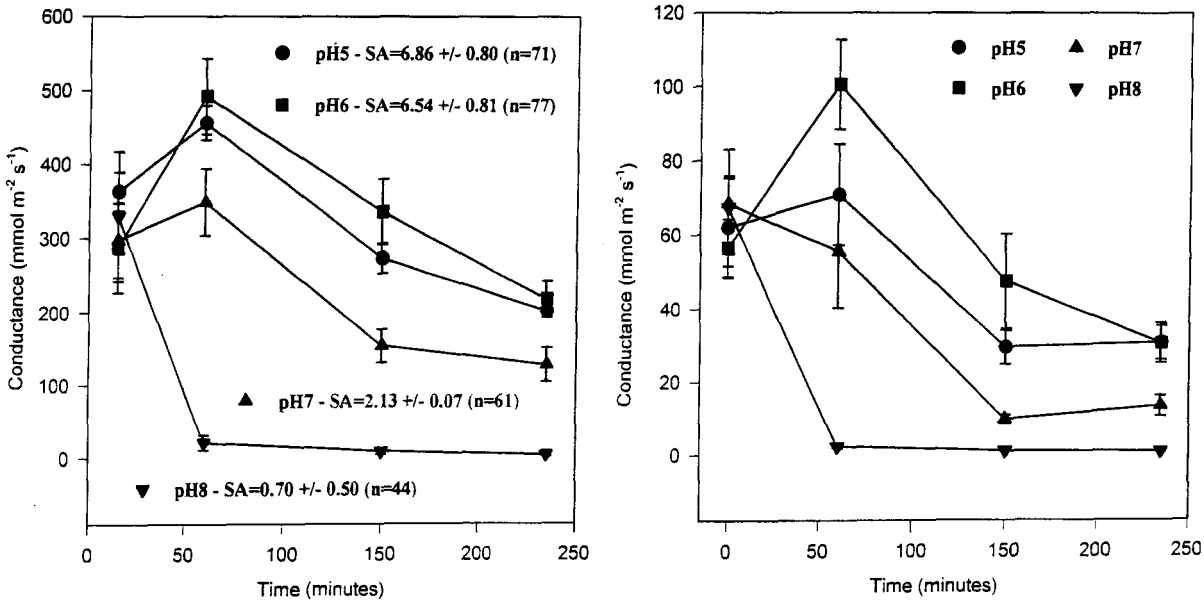

Figure 4. The effect of buffer pH on the conductance of the abaxial (left) and adaxial (right) surface of the fourth leaf of detached shoots over a 4-h period. Shoots separated from the root system were incubated in 20 cm^3 of AS buffered to the appropriate pH. Data are expressed as means ± SE (n = 7). Measurements of SA in abaxial epidermis stripped from leaves treated in the same way are also shown (mean μm ± SE).

Table II. *The effect of pH on stomatal aperture in isolated epidermis when incubated on buffer A for 2 h, after a 1-h preincubation in KCl in the light to elicit stomatal opening*

pH	Mean Aperture ± SE	n	Significance of Difference from pH 6.0
	μm		
5.0	6.54 ± 0.19	81	ns[a]
6.0	6.68 ± 0.21	38	–
7.0	6.18 ± 0.32	65	ns
8.0	6.14 ± 0.42	37	ns

Data are means ± SE (μm).

[a] ns, Not significant.

not mediated by ABA. If there is a tendency for this effect to occur in the intact leaf, it must be masked by alternative overriding effects of pH on some other variable that affects SA (see below).

The results described above show that the reduction of transpiration, conductance, and SA induced by pH 7.0 in whole, detached leaves was not the result of changes in shoot water relations or of a direct effect of pH on guard cells. Nor was it the result of increased ABA synthesis, reduced ABA metabolism, or reduced export of ABA from the leaf (in the phloem), because endogenous levels of bulk leaf ABA remained unaffected by pH. The following experiments demonstrated that the effect of pH 7.0 was, in fact, dependent on the presence of ABA in the xylem stream.

The Effect of Preincubation Medium Composition on Subsequent Responses of Transpiration to pH in Whole, Detached Leaves

Figure 5, left, shows that when intact, excised leaves were preincubated overnight in the dark in DW, the subsequent reduction of transpiration rate by buffers adjusted to pH 7.0, as compared with those adjusted to pH 6.0, was no longer seen. When leaves were detached from fresh plants and immediately placed in pH 7.0 buffer without preincubation in water, the reduction in transpiration rate occurred as usual (Figs. 2, right, 3, and 5). However, if 10^{-8} mol dm^{-3} ABA was included in the overnight preincubation medium, which itself was not a concentration high enough to reduce transpiration at control pH levels, the subsequent response to pH 7.0 was rescued (Fig. 5, right). When ABA was included in the pretreatment medium, the subsequent rate of transpiration at pH 7.0 was only 61% of the rate at pH 6.0, but in the absence of ABA during pretreatment, the subsequent transpiration rate at pH 7.0 was still 98% of that at pH 6.0 (Fig. 5, right). These data indicate that the pH 7.0 response required concentrations of ABA in the transpiration stream equivalent to those detected in well-watered plants.

The experiment was repeated to measure the endogenous ABA content and the RWC of the leaves at the end of the transpiration period. The RWC of the leaves was not significantly different between any of the treatments (not shown), and the bulk leaf ABA content was the same, whether or not ABA was present in the overnight pretreatment medium (not shown). The latter was surprising given published rates of metabolic degradation of ABA (e.g. Gowing et al., 1993, although these measurements were carried out in the light), and indicated that a general depletion of endogenous ABA from the leaves pretreated in the absence of exogenous ABA was probably not responsible for the lack of a subsequent effect of pH 7.0 on transpiration. Because all leaves contained the same final concentration of ABA, the pretreatment must somehow have altered the distribution of ABA in the leaf. One possibility is that in water-pretreated leaves, all endogenous ABA became partitioned into the symplast. The pH 7.0 response may require a continuous delivery of ABA from the transpiration stream to the apoplast (see below), rather than requiring ABA per se at a certain bulk leaf concentration. This possibility was tested further.

The Effect of pH on the Distribution of ABA between Leaf Tissues

Figure 6, left and right, shows that there was no change in the distribution of ABA between different leaf tissues (abaxial and adaxial epidermis, mesophyll, and midrib) when whole, detached leaves had transpired for 3.5 h in media buffered to either pH 6.0 or pH 7.0 before tissue separation, as measured by RIA. The same result was seen when exogenous ABA was supplied at a concentration of 3×10^{-6} mol dm^{-3}, which is equivalent to that in the

Table III. *The effect of pH on stomatal aperture in isolated epidermis, when incubated on buffer B for 2 h, in the presence and absence of 10^{-7} mol · dm^{-3} ABA, after a 1-h preincubation in KCl in the light to elicit stomatal opening*

Treatment	Mean Aperture	No. of Stomata (No. of Tissue Strips)	% ↓ by ABA	Significant Difference from pH 6.0
	μm			
pH 5.0	6.33 ± 0.20	141 (4)	–	ns[a]
pH 6.0	6.54 ± 0.55	230 (7)	–	–
pH 6.0 + ABA	1.38 ± 0.15	109 (3)	78.9% (s – 0.1%)[b]	–
pH 7.0	9.59 ± 0.44	219 (7)	–	s ↑ (0.1%)
pH 7.0 + ABA	4.37 ± 0.87	101 (3)	55% (s – 0.1%)	s ↑ (5%)
pH 8.0	7.89 ± 0.50	234 (7)	–	ns
pH 8.0 + ABA	6.12 ± 0.10	107 (3)	21% (ns)	s ↑ (0.1%)
KCl only (pH 5.7)	6.36 ± 0.57	177 (3)	–	ns

Data are means ± SE (μm) with significance levels (s) shown.

[a] ns, Not significant. [b] s, Significant ↓ by ABA.

Table IV. *The effect of pH on stomatal aperture in isolated epidermis, when incubated on buffer C for 2 h, in the presence and absence of 10^{-7} mol · dm^{-3} ABA, after a 1-h preincubation in KCl in the light to elicit stomatal opening*

Treatment	Mean Aperture	No. of Stomata (No. of Tissue Strips)	% ↓ by ABA	Significant Difference from pH 6.0
	μm			
pH 5.0	4.81 ± 0.16	152 (4)	–	ns ↓ [a]
pH 6.0	5.71 ± 0.53	259 (7)	–	–
pH 6.0 + ABA	3.83 ± 0.17	108 (3)	33.6% (s – 5%)	–
pH 7.0	7.70 ± 0.17	248 (7)	–	s ↑ (1%)
pH 7.0 + ABA	4.90 ± 0.20	104 (3)	36.3% (s – 0.1%)	s ↑ (5%)
pH 8.0	7.25 ± 0.70	239 (7)	–	ns ↑
pH 8.0 + ABA	5.87 ± 0.81	102 (3)	19.0% (ns)	ns ↑
KCl only (pH 5.7)	6.33 ± 0.42	99 (3)	–	ns

Data are means ± SE (μm) with significance levels (s) shown.

[a] ns, Not significant.

xylem sap of drought-stressed plants. Thus, an increase in the ABA content of the epidermis (by redistribution from the mesophyll) was not responsible for the closure of stomata in whole, detached leaves that had transpired at pH 7.0.

The ABA content of the adaxial epidermis was very high in comparison to bulk leaf values (see Table I) and to concentrations in the abaxial epidermis. This could be a result of different rates of ABA metabolism between the two epidermes.

The Effect of pH on the Uptake of ^{3}H-ABA by Isolated Mesophyll and Epidermis Tissue

Figure 7 shows the rate of uptake of 5×10^{-10} mol dm^{-3} ^{3}H-ABA from the external medium (buffer B) over a 3-h time course at pH_{ext} 6.0 and 7.0. ABA was taken up much more rapidly by the mesophyll symplast, and slightly faster by the epidermis symplast at pH 6.0 than at pH 7.0, i.e. more was sequestered away from the apoplast (external medium) at pH 6.0. This was as expected for the uptake of a weak acid into an alkaline compartment (the cell cytoplasm). After 1.5 h, uptake was approximately 45% greater from medium buffered to pH 6.0 than to 7.0. This was a suitable time period over which to conduct further studies.

The effect of unlabeled ABA on the uptake of ^{3}H-ABA from the external medium (buffer B) was assessed (Table V). In this study the uptake of labeled ABA by the epidermal and mesophyll symplast at pH 6.0 was also 40 to 50% greater than at pH 7.0. A saturable uptake component was detected in the epidermis but not in the mesophyll; 10^{-6}

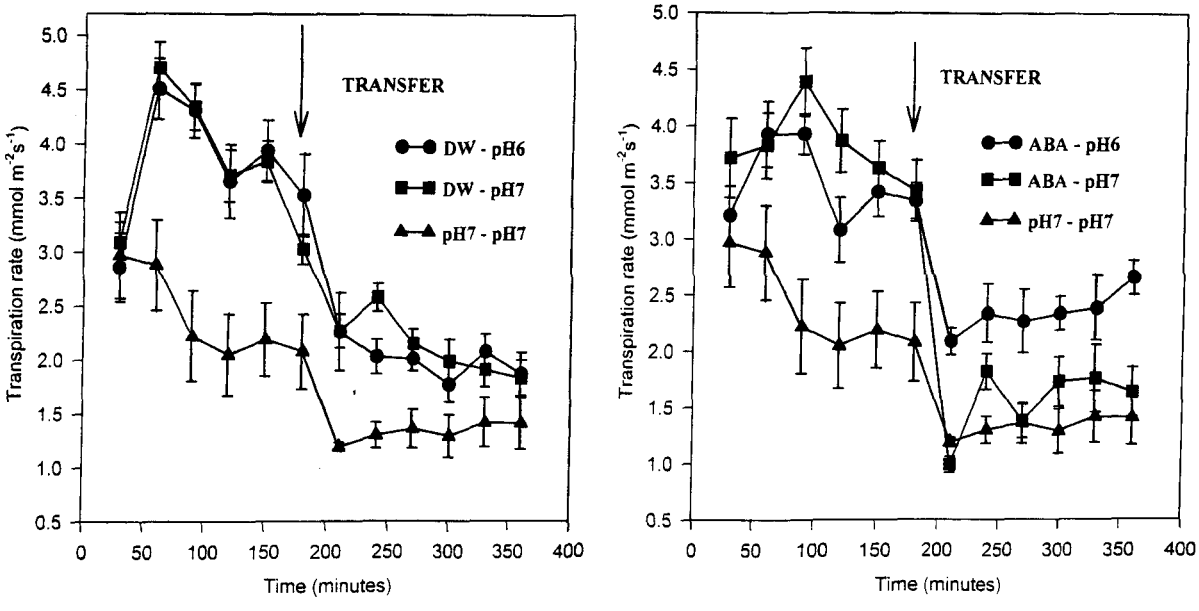

Figure 5. The effect of preincubation medium composition on the subsequent response of transpiration to pH in whole, detached *C. communis* leaves. Leaves were pretreated overnight in DW in the presence (right) and absence (left) of 10^{-8} mol dm^{-3} ABA in the dark. They were then transferred to vials containing 4.0 cm^3 of DW with (right) or without (left) ABA, or AS buffered to pH 7.0 (left and right) and an initial transpiration rate was established over 3 h. The second transfer was to AS buffer (supplemented with 3.0 mmol dm^{-3} phosphate salts) at pH 6.0 or pH 7.0. The final transpiration rate was calculated and expressed as a mean ± SE (n = 7).

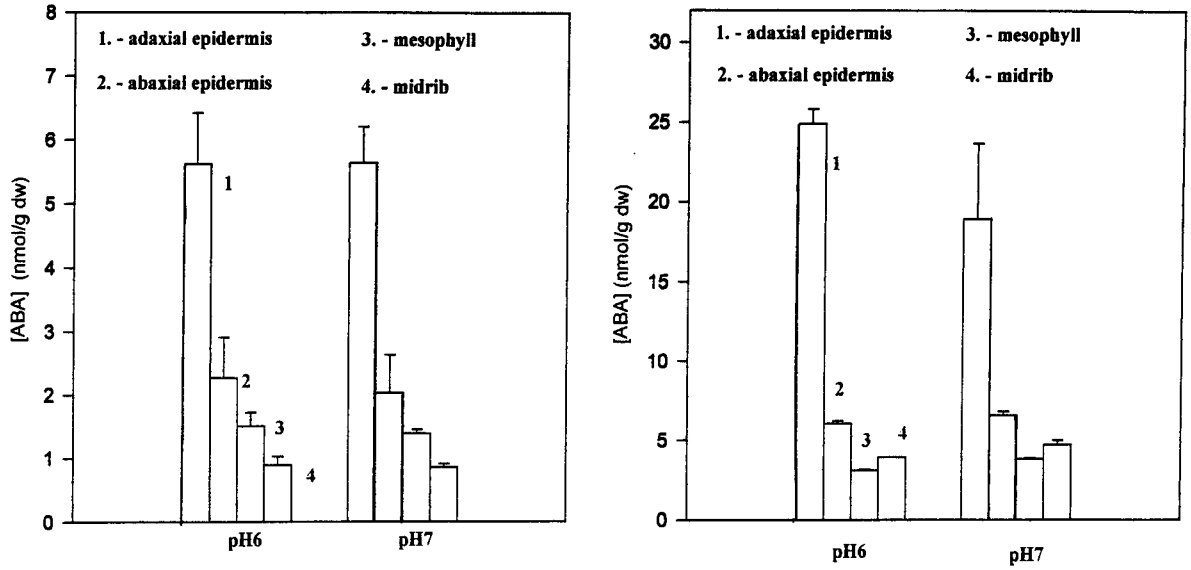

Figure 6. The effect of pH on the distribution of ABA between different tissues of whole, detached *C. communis* leaves after a transpiration period of 3.5 h, as measured by RIA. The distribution of endogenous ABA (left) and the distribution of ABA in leaves (right), which have been taking up 3×10^{-6} mol dm^{-3} ABA from the external medium, are shown. Data are the mean ABA contents of 3 aliquots of extract from 3 sets of 12 leaves, i.e. 9 readings, ± SE. Twelve leaves are required to strip enough of each individual tissue for the RIA analysis.

mol dm^{-3} unlabeled ABA in the external medium successfully competed with ^{3}H-ABA for uptake into the epidermal symplast. However, this only occurred at pH 6.0 and not at pH 7.0, indicating that the uptake component was only active at pHs characteristic of those in well-watered plant xylem sap. At pH 6.0 the remaining portion of uptake over and above that which occurred at pH 7.0 was presumably diffusive, because it was not affected by unlabeled ABA. In mesophyll tissue the difference between uptake at pH 6.0 and pH 7.0 was identical both in the presence and absence of unlabeled ABA, indicating that all uptake was diffusive. In summary, the symplast of epidermis and mesophyll tissue was able to sequester more ABA away from the apoplast (external medium) when this was buffered to pH 6.0 as opposed to 7.0, during the same time frame in which the effect of pH 7.0 could be detected in whole transpiring leaves. Two modes of symplastic ABA sequestration were reduced in the epidermis at pH 7.0: diffusive uptake and saturable, probably carrier-mediated, uptake.

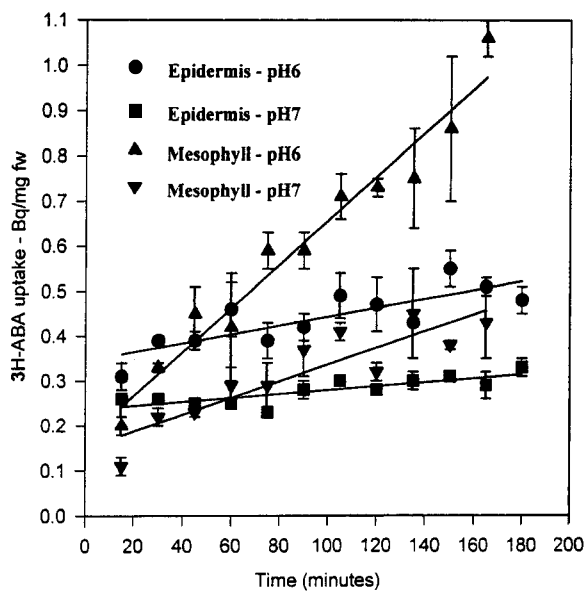

Figure 7. The effect of pH on a time course of the uptake, by isolated epidermis and mesophyll tissue, of 5×10^{-10} mol dm^{-3} ^{3}H-ABA from the external medium (buffer B) in the light, after a pretreatment of 2 h in pH 7.25 buffer. Data are means of three replicate treatments ± SE.

The Effect of pH on ^{3}H-ABA Efflux from Preloaded Epidermis and Mesophyll Tissue

Pieces of isolated abaxial epidermis and mesophyll tissue were pretreated in buffer B at pH 7.25 for 2 h in the light and then allowed to take up activity for 1.5 h. Initial activity was measured. The tissue was washed so that all activity was assumed to be symplastic, and transferred to ABA-free unlabeled buffers at either pH 6.0 or pH 7.0. The activity effluxed into the medium was measured over 2.5 h, and efflux was expressed as activity remaining in the tissue, as a percentage of the total initial activity (Fig. 8). The rate and amount of efflux from mesophyll tissue into media of either pH 6.0 or pH 7.0 was identical during the same time frame as that in which pH 7.0 reduced transpiration in whole, detached leaves. Rates of efflux from epidermal tissue were unaffected by pH, although slightly more ABA seemed to be released into pH 6.0 than into pH 7.0 buffers.

When we extrapolated these results to an intact leaf, we concluded that any differential partitioning of ABA that

Table V. *The effect of pH_{ext} on the uptake of 5×10^{-10} mol · dm^{-3} ^{3}H-ABA from buffer B by isolated epidermis or mesophyll, ± 10^{-6} mol · dm^{-3} unlabeled ABA*

Strips of tissue (0.5 × 1.0 cm) were preincubated for 2 h on pH 7.25 buffer and washed before transfer to uptake buffers. Uptake was for 1.5 h in the light. Activity is a mean of five separate experiments ± SE.

Treatment	Activity	% ↓ by pH 7.0	% ↓ by ABA
	mean Bq/mg fresh wt		
Epidermis			
pH 6.0	0.72 ± 0.05	–	–
pH 7.0	0.35 ± 0.03	−51% (s − 0.1%)	–
pH 6.0 + ABA	0.51 ± 0.03	–	−29% (s − 5%)
pH 7.0 + ABA	0.44 ± 0.04	−14% (ns)[a]	(+26%)
Mesophyll			
pH 6.0	0.68 ± 0.08	–	–
pH 7.0	0.40 ± 0.02	−41% (s − 5%)	–
pH 6.0 + ABA	0.67 ± 0.06	–	−2% (ns)
pH 7.0 + ABA	0.37 ± 0.03	−44% (s − 1%)	−6.3% (ns)

Data are means ± SE (μm) with significance levels (s) shown.

[a] ns, Not significant.

occurred between the symplast and the apoplast at pH 6.0 and pH 7.0 was wholly a result of effects of xylem sap and/or apoplastic pH on symplastic uptake from the apoplast, rather than on efflux of stored ABA from the symplast into the apoplast.

DISCUSSION

The pH of the xylem sap increased by 0.6 unit when water was withheld from *C. communis* plants for only 6 d, even though SWP was unaffected or only slightly reduced by soil drying (Fig. 1). Hartung and Radin (1989, *P. vulgaris*) and Gollan et al. (1992, sunflower) detected even greater effects of soil drying on xylem sap pH. A dehydration-dependent pH increase of midrib exudate, assumed to represent apoplastic sap, was also observed in detached leaves of a wide range of species (Hartung and Radin, 1989). These authors also showed that cotton leaf dehydration reduced the activity of outwardly rectifying proton-pumping ATPases at the plasma membrane and suggested that this caused the increased apoplastic pH. However, this mechanism was seemingly not involved in the alkalinization of xylem sap of plants in drying soil. Gollan et al. (1992) suggested that the reduced nitrate concentration and excess of cations that they detected in the xylem sap of sunflower plants in drying soil could be directly responsible for the increased pH: imbalances between strong cations and anions can control the pH of aqueous solutions (Stewart, 1983). We have shown here that an increase of xylem sap pH can be an early signal of reduced soil water availability to the leaves before the water relations of the shoot become affected by drought, and that this has the potential to act as a soil dehydration signal even before the xylem sap ABA concentration is increased. Once ABA is synthesized and transported into leaves, however, it can be envisaged that increased sap pH will enhance the ABA signal received at the guard cells. It is also important to note that the pH of sap expelled from older plants tends to be more acidic. Perhaps older plants respond less sensitively to xylem-sourced pH signals, or perhaps they may simply have a different range over which sap pH exerts its effects.

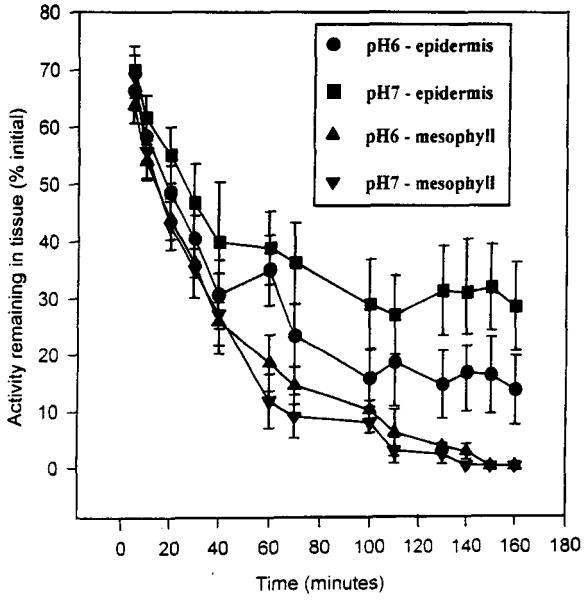

Figure 8. The effect of pH on the efflux of ^{3}H-ABA from preloaded epidermis and mesophyll tissue into unlabeled ABA-free buffer B in the light. Preincubation was for 2 h in pH 7.25 buffer, loading was for 1.5 h in 10^{-9} mol dm^{-3} ^{3}H-ABA in pH 5.0 buffer, and efflux was for 2.5 h in pH 6.0 or pH 7.0 buffer.

Applications of pH 7.0 buffer to whole, detached leaves of *C. communis* via the xylem stream reduced SA in comparison to controls supplied with pH 6.0 buffer (Fig. 4, left), thus reducing abaxial and adaxial leaf conductance (Fig. 4, left and right) and transpirational water loss (Figs. 2, left, 3, and 5, left and right). To our knowledge, this has not previously been demonstrated (although Behl and Hartung [1986] mentioned a similar effect in spinach leaves without providing supporting data), despite the wealth of informa-

tion predicting that an increase in apoplastic pH (of only 0.2 unit) is a strong enough signal to close stomata in a whole turgid leaf. This may be because previous attempts to carry out the experiment employed buffers that affect transpiration. For example, we found that 10 mmol dm^{-3} Mes buffer, commonly used in other bioassays, reduced transpiration by approximately 60% in comparison to DW (results not shown) and inhibited the response to exogenous ABA.

The conceivable ways in which increased xylem sap pH might reduce SA in whole leaves (and hence conductance and transpiration rate) were mentioned at the beginning of this article. We have shown that endogenous concentrations of ABA were not increased when leaves had transpired in media buffered to pH 7.0 as opposed to pH 6.0 (Table I, results described in reference to Figs. 4 and 6, left). However, in the presence of 3×10^{-6} mol dm^{-3} exogenous ABA, the bulk-leaf ABA content was greater after leaves had transpired in pH 7.0 as opposed to pH 6.0 buffer. This indicates that either reduced metabolism or reduced export of ABA from the leaf via the phloem may have had an effect on leaf ABA content in the presence of large concentrations of this compound, equivalent to those in the xylem sap of severely droughted plants. Effects of pH_{ext} on rates of ABA catabolism have not previously been measured, but rates have been compared in turgid and wilted leaves. Little difference was observed between those of wheat (Murphy, 1984) or *Xanthium strumarium* (Cornish and Zeevaart, 1984). Increased apoplastic pH may directly affect metabolic enzyme activity. Alternatively, the substrate (ABA) may no longer be compartmentalized alongside the necessary enzymes (which occur in the mesophyll or epidermal cytoplasm; Daeter and Hartung, 1995), as a result of effects of pH on ABA distribution (Fig. 7). In addition, because phloem loading of ABA from the leaf involves its uptake over lipid membranes into the more alkaline phloem sap, it is possible that increased apoplastic alkalinity will reduce such an ABA flux. These processes may occur to some extent in leaves of turgid plants, but because lower concentrations of ABA are involved, they do not have sufficient impact on total leaf ABA content to affect SA. It should also be noted that when leaves took up media buffered to pH 8.0 in the absence of exogenous ABA, we detected a 5-fold increase in their endogenous bulk ABA content after 4 h, as well as a significant reduction of leaf RWC and an extremely low rate of conductance. Thus, at pH 8.0 but at no other pH tested, a stimulation of ABA synthesis in the leaf may have been partially responsible for the abnormally large reduction of conductance.

Leaf and shoot water relations are not affected by pH 5.0 to 7.0 (inferred from Figs. 3 and 4). However, as noted above, at pH 8.0 (still commonly used in leaf bioassays), the RWC of leaves was significantly lower than at all the other pH values tested after 4 h, and the leaves appeared wilty. It is possible that pH 8.0 could indeed act as a hydraulic signal, although xylem sap never becomes this alkaline in vivo. The reduction of RWC seen at pH 8.0 might reduce guard cell turgor or increase the sensitivity of guard cells to ABA (Tardieu and Davies, 1992, 1993). The reduced RWC would also have stimulated ABA synthesis in the leaf. This was substantiated by the finding that ABA concentrations were 5-fold greater in leaves that transpired in pH 8.0 buffer. The effect of pH 8.0 buffer on RWC could indicate (magnified 10-fold) that pH 7.0 may be a 2-fold drought signal to detached leaves and shoots, one mediated by water relations and one by ABA, although we believe the latter to be the only important signal in the intact plant.

SA in the abaxial epidermis peeled from intact leaves that had transpired at different pHs tended to decrease as pH increased (Fig. 4, left). When epidermis was isolated from the leaf and then incubated in AS (plus KCl) buffered to different pHs, there was no effect on aperture of pH from 5.0 to 8.0 (Table II). However, when "simple" buffers were used, without the extra ions such as calcium found in the sap of well-watered plants, increasing pH_{ext} increased SA in both the presence and absence of 10^{-7} mol dm^{-3} ABA in the external medium (Tables III and IV), i.e. alkaline pH_{ext} can be an opening signal in isolated epidermis, although it is a closing signal in the intact leaf. Schwartz et al. (1994) showed the same effect of a range of pH on the stomata of *C. communis* but did not discuss this result.

In some recent papers, enhanced effects of ABA on stomatal closure at acidic pH_{ext} have been used to argue for internal guard cell binding of ABA. The fact that acidic pH itself reduces SA compared with the effect of a more alkaline pH treatment (this paper; Schwartz et al., 1994) must cast some doubt on the validity of this argument. The following authors all found that although pH per se had no effect on SA, ABA was much more effective at closing stomatal pores at a more acidic pH_{ext}: Ogunkanmi et al. (1973, *C. communis*); Kondo et al. (1980, *Vicia faba*); Kondo and Maruta (1987, *Vicia faba*); Paterson et al. (1988, *C. communis*); and Anderson et al. (1994, *C. communis*). Although pH itself did not affect SA in these studies, our data suggest the possibility that low pH may somehow predispose the guard cell to ABA, and that this may account for the extra ABA effect without arguing for intracellular ABA binding. This conclusion is consistent with the observation that internal microinjection of ABA does not always induce stomatal closure in the absence of external ABA (Anderson et al., 1994; but see Schwartz et al., 1994).

The predisposition of the guard cell to ABA may involve pH-stimulated modifications of guard cell ionic balance. The literature already contains some evidence that pH_{ext} itself is able to change guard cell ion concentration: Ilan et al. (1994) found that a reduction of pH_{ext} from 8.1 to 5.5 significantly reduced the magnitude of outward K^+ currents in patch-clamp experiments, and Blatt (1992) found that acidic pH_{ext} activated inward K^+ channels in intact *V. faba* guard cells, although these findings would tend to oppose stomatal opening at high pH_{ext}. MacRobbie (1995) proposed that a threshold guard cell ABA concentration was required for closing responses, such as K^+ efflux, to occur, monitored as transient efflux of radioactive Rb^+ from preloaded tissue. Transference of epidermal strips from a medium buffered to pH 6.0 to one buffered to pH 8.0 indeed resulted in a smaller ABA-induced stimulation

of Rb^+ efflux than that which occurred when epidermal strips were transferred to media of lower pH_{ext}. However, it was also demonstrated that transfer of strips from pH 6.0 to pH 8.0 solutions induced substantial Rb^+ efflux transients before the addition of ABA, i.e. increased pH_{ext} itself promoted cation efflux, although no explanation was given for this finding.

A good indication that low pH_{ext} primes the guard cell to respond more readily to closing stimuli was provided by Kondo and Maruta (1987). They found the osmotic potential of *V. faba* guard cells to be more negative at pH 6.0 than at pH 4.0, although aperture was unchanged. Final concentrations of K^+ and malate in the epidermal strips containing these guard cells were much higher at pH 6.0. At pH 4.0 ABA closed stomata without greatly affecting guard cell osmotic potential, but at pH 6.0 ABA-induced stomatal closure correlated with a much greater increase in osmotic potential. However, these authors interpreted their findings as differential effects of ABA on guard cell wall loosening at different pH_{ext}. It is not clear how high pH_{ext} decreases guard cell osmotic potential or even increases SA, but given that several authors have shown that it induces K^+ efflux from guard cells, the accumulation of some other solute such as malate may be important. An initial efflux of K^+ may not always indicate a closing response (see MacRobbie, 1995).

In contrast to simple buffers, increasing pH_{ext} no longer resulted in greater SA when AS buffer was used (Table II), possibly through some effect of the extra ions it contained, such as 1.0 mmol dm^{-3} $CaCl_2$. Hartung (1983) also found that pH itself had no direct effect on SA, and that ABA closed stomata to the same extent in epidermal strips of *Valerianella locusta* whatever the pH_{ext}. Similarly, Hornberg and Weiler (1984) found that ABA induced stomatal closure over a range of pH_{ext} in *V. faba*. One difference between the composition of the media used in these reports and those described earlier in which low pH_{ext} increased the effect of ABA, or actually reduced SA itself (Tables III and IV; Schwartz et al., 1994), is the external concentration of divalent cations, which is low or zero in the latter. High external concentrations of divalent cations (1.0 mmol dm^{-3} or above) may reduce the effect of pH_{ext} on the ionic status of the guard cell. The calcium concentration in the apoplastic sap surrounding *C. communis* guard cells will be much lower than this in vivo, because of its deposition as calcium oxalate crystals in leaf cells en route to the leaf surface (Ruiz and Mansfield, 1994). De Silva et al. (1985) found that concentrations of calcium above 0.1 mmol dm^{-3} actually reduced SA in isolated *C. communis* epidermis. The in vivo apoplastic calcium concentration is unknown, but it is likely that sap composition is something between the three types used here (Tables II–IV).

Our results with isolated epidermal material demonstrate that SA could potentially be increased directly by the increased pH of the transpiration stream in vivo, but because we found a reduction of aperture by high pH in intact leaves, some other effect of pH must override this. The reduction of transpiration by pH 7.0 buffer in intact leaves requires the presence of ABA in the xylem stream at 10^{-8} mol dm^{-3} (Fig. 5, left and right), a concentration equivalent to that of well-watered sunflower xylem sap (Gollan et al., 1992). Originally, pretreatment of leaves in DW was done to reduce bulk leaf ABA concentration because it was presumed that catabolic depletion would occur overnight: published half-lives of ABA range from 36 min in cherry to 3.2 h in *Hordeum vulgaris* (see Gowing et al., 1993). However, RIA data (not shown) indicated that bulk-leaf ABA concentrations were the same in fresh leaves as in those supplied with ABA, as well as in those held overnight in DW. This may have been because rates of ABA degradation are much lower in the dark (Loveys and Milborrow, 1984; Gowing et al., 1993). We presume that during pretreatment in DW normal sequestration processes caused ABA levels to increase in the symplast (see Fig. 7; Table V) and to decrease in the apoplast. This would determine the loss of response to pH 7.0.

There was no effect of pH on the distribution of endogenous ABA, or internalized exogenous ABA, between different leaf tissues (Fig. 6). This was surprising given reports in the literature of movements of ABA from the mesophyll to the epidermis upon dehydration of leaves in air (Singh et al. [1979], *C. communis*; Weiler et al. [1982], *V. faba*). Wilting induced the efflux of ^{14}C-ABA from the mesophyll of preloaded *V. locusta* leaf tissue to the lower epidermis (Hartung et al., 1983). During dehydration of detached leaves, ABA is synthesized in the mesophyll tissue, and it may simply be taken up more effectively by the epidermis (Daeter and Hartung, 1995). Even in unstressed leaves the epidermis contains a higher concentration of ABA than the mesophyll, especially at the adaxial surface (Fig. 6). Alternatively, ABA movement between leaf tissues may only occur during very severe drought.

The rate of symplastic uptake of ^{3}H-ABA by both isolated mesophyll and abaxial epidermal tissue is faster when the external medium is buffered to pH 6.0 than to pH 7.0 (Fig. 7). In an intact leaf this would cause ABA to accumulate in the apoplast at pH 7.0. Diffusion of ABA among cell compartments was discovered (Heilmann et al., 1980) to be a direct result of pH gradients and has since been found to be responsible for ABA uptake in all leaf cell types. Like Daeter and Hartung (1990), we found no evidence for a saturable component of ^{3}H-ABA uptake in mesophyll tissue. Uptake was independent of the addition of 10^{-6} mol dm^{-3} unlabeled ABA to the external medium (Table V). However, Table V also shows that a saturable component was responsible for a portion of the ABA taken up by the epidermal symplast at pH_{ext} 6.0, because unlabeled ABA was a successful competitor for this portion of total uptake. We can conclude that both carrier-mediated and diffusive uptake contribute to the efficiency of ABA sequestration by this tissue at a pH_{ext} equivalent to that of well-watered sap (Fig. 1).

The competition between labeled and unlabeled ABA was unlikely to be focused on external membrane binding sites because the saturable portion was dependent on the existence of a membrane pH gradient—it disappeared at

pH_{ext} 7.0—and ABA binding to isolated membranes of *V. faba* was found to be pH-independent (Hornberg and Weiler, 1984). We found epidermal uptake at pH_{ext} 7.0 to be much lower and without the involvement of a saturable component. There is already some evidence for the existence of ABA uptake carriers in plant tissues, which are more active at lower pH_{ext}. Astle and Rubery (1980, *P. vulgaris*; 1983, various species) detected a saturable ABA uptake component restricted to root apical tissue that was dependent on the membrane pH gradient, but not the electrical gradient. The authors suggested that the carrier was an electroneutral ABA^-/H^+ symport. Milborrow and Rubery (1985) detected a carrier in suspension cultures of root callus cells (see also Windsor et al. [1992], hopbush callus), which could only bind compounds with close steric similarities to ABA. They concluded that it may be responsible for the polar transport of ABA in roots by virtue of its graded distribution. Baier and Hartung (1988) demonstrated that a saturable transport mechanism was involved in the uptake of ABA by the guard cell vacuole of *V. locusta* protoplasts. Bianco-Colomas et al. (1991) detected carrier-mediated uptake of ABA in suspension-cultured *Amaranthus tricolor* hypocotyl callus, which was partially energized by the pH gradient and was specific for ABA. Perras et al. (1994) found that the uptake of ^{3}H-ABA by *H. vulgare* embryo callus cultures had a saturable component that was inactive at pH 7.0. The only report of carrier-mediated ABA uptake by leaf tissue (Daeter and Hartung, 1993), other than our own, demonstrated that ABA uptake into barley epidermal protoplasts was only saturable at pH 7.25 and not at more acidic pH_{ext}, in contrast to the reports described above. However, the concentrations of ABA used in the study by Daeter and Hartung (1993) were 10^{-3} and 7.5×10^{-2} mol dm^{-3} labeled and unlabeled ABA, respectively. It seems possible that these concentrations could themselves have affected the membrane pH gradient.

Our study implies a realistic function for an ABA uptake carrier in leaf tissue: we suggest that it contributes to the rapid sequestration of ABA away from the apoplastic compartment of the leaf, which prevents the closure of stomata in well-watered plants. The carrier becomes inactive at pH_{ext} 7.0 (similar to the apoplastic sap of a droughted plant, Fig. 1), when diffusive uptake is also much reduced, and ABA accumulates in the apoplastic compartment, causing stomatal closure in the intact leaf. Carrier activation at pH 7.25 (Daeter and Hartung, 1993) would oppose the effect of high pH_{ext}, to reduce symplastic ABA sequestration through diffusive uptake, and would therefore oppose the accumulation of apoplastic ABA to a concentration high enough to cause stomatal closure (although Daeter and Hartung suggest that just such a mechanism could fine-tune ABA levels in the apoplast).

We could find no evidence that increasing the pH_{ext} increased the rate of efflux of ABA from either epidermis or mesophyll tissue (Fig. 8). Examination of previous studies demonstrating ABA efflux reveals that it may only result from specific sets of conditions. Most of these involved dehydrating isolated cells or tissues in hyperosmotic media, whereas we found that pH exerted its effects in turgid leaves (Hartung et al. [1981], isolated spinach chloroplasts; Hartung et al. [1983], spinach leaf sections; Behl and Hartung [1986], *V. locusta* guard cells; Daeter and Hartung [1990], *V. locusta* leaf discs). However, it would seem that hyperosmotic treatment affects cytoplasmic pH and that this phenomenon, rather than a reduced membrane pH gradient per se, correlates with ABA efflux (Hartung et al., 1981; Hartung and Radin, 1989). Kaiser and Hartung (1981) demonstrated that the efflux of ABA from the cytoplasm of isolated mesophyll cells of *Papaver somniferum* increased with pH_{ext} in the presence of KNO_2, which acidified the cytoplasm, but they were unable to detect an effect of external pH, from 5.0 to 8.0, on ABA release in the absence of this salt.

To summarize, we have determined experimentally that an increase in the pH of the medium supplied to whole, detached *C. communis* leaves reduces the aperture of their stomatal pores in an ABA-dependent manner, most probably by increasing the ABA concentration of the apoplastic compartment. However, this is not the result of the efflux of ABA from symplastic stores, but of a cessation of the normal, rapid sequestration of ABA away from the apoplast. Reductions of both diffusive and carrier-mediated ABA uptake occurred in epidermal tissue. ABA in the apoplast is in contact with the guard cell receptors and evidence is provided here that data supporting a role for internal ABA receptors must be treated cautiously.

It seems clear that drought-induced variation in xylem sap pH may act as a root-sourced signal to the leaves. Such a signal may result in the closure of stomata even in the absence of a drought-induced increase in xylem sap ABA. Observations of this kind mean that care must be exercised when drawing conclusions over whether or not small increases in xylem-borne ABA concentrations are adequate to explain observed changes in stomatal behavior.

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