Plant Physiol. (1988) 88, 703–708
0032-0889/88/88/0703/06/$01.00/0

Abscisic Acid Is Not the Only Stomatal Inhibitor in the Transpiration Stream of Wheat Plants

Received for publication February 2, 1988 and in revised form April 28, 1988

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ABSTRACT

Xylem sap was collected from the transpiration stream of wheat (*Triticum aestivum* L.) plants and assayed for the presence of an inhibitor of transpiration using leaves detached from well-watered plants. Transpiration of detached leaves was reduced by nearly 60% by sap collected from plants in drying soil, and to a lesser extent (about 25%) by sap from plants in well-watered soil. As the soil dried the abscisic acid (ABA) concentration in the sap increased by about 50 times to 5×10^{-8} molar. However, the ABA in the sap did not cause its inhibitory activity. Synthetic ABA of one hundred times this concentration was needed to reduce transpiration rates of detached leaves to the same extent. Furthermore, inhibitory activity of the sap was retained after its passage through an immunoaffinity column to remove ABA. Xylem sap was also collected by applying pressure to the roots of plants whose leaf water status was kept high as the soil dried. Sap collected from these plants reduced transpiration to a lesser extent than sap from nonpressurised plants. This suggests that the inhibitory activity was triggered partly by leaf water deficit and partly by root water deficit.

Although stomatal closure of plants in drying soil usually coincides with leaf water deficit, several experiments have indicated that a decrease in stomatal conductance can occur without a change in leaf water status (2, 3, 18). Recent work in this laboratory also indicated that stomatal conductance can depend directly on soil water status; stomata started to close as the soil water content decreased even though the leaf water status was maintained at a high level (6). This suggests that a message from the roots can control stomatal conductance independently of the leaf water status. To test this hypothesis, sap was tapped from the transpiration stream of wheat plants while the soil dried, and its ability to affect stomatal conductance was assayed by feeding it to detached wheat leaves and measuring its effect on their transpiration rate. To distinguish whether it was leaf or root water deficit that caused the appearance of a message from the roots, xylem sap was also collected from plants whose leaf water status was kept high while the soil dried. To keep the leaf water potential close to zero, just enough pressure was applied to the soil to keep xylem sap at the tip of one of the leaves on the verge of bleeding, *i.e.* the hydrostatic pressure of the xylem of that leaf was zero. (The water potential of the leaves could be a little lower than zero during periods of rapid transpiration as it is likely that there is a difference in water potential between xylem and epidermis of a transpiring leaf). This pressurization technique as described in Passioura and Munns (14) does not affect the root water status, so it distinguishes between leaf water deficit and root water deficit as the trigger for a putative message from the roots.

MATERIALS AND METHODS

Growth Conditions. Wheat seedlings (*Triticum aestivum* L. cv Kite and Egret) were planted in pots holding 1600 g soil, which could fit inside pressure chambers. The soil was initially watered to about 0.18 g H_2O g^{-1} soil (corresponding to a soil water suction of about 10 kPa). Plants were grown in a controlled environment cabinet with a photosynthetic photon flux density of 400 to 500 μmol m^{-2} s^{-1} and a temperature of 22°C d (10 h) and 15°C night. For the well-watered treatment, the soil water content of pots was maintained at 0.15 g g^{-1}. For the drying treatment, the soil was never watered again; after about 4 weeks the soil water content dropped to about 0.07 g g^{-1} by which time there were 5 to 6 leaves on the main shoot. Transpiration and leaf expansion rates decreased at this time. During the previous week some plants were maintained at 'balancing pressure,' which involved applying a pressure to the pot in which the plant was growing so that the hydrostatic pressure of the xylem sap at the tip of a given leaf was always on the verge of bleeding, *i.e.* it was maintained at zero (atmospheric) pressure. This procedure is detailed in Passioura and Munns (14). The leaf selected for this was the leaf at highest water potential, usually leaf 2, so that no other leaves were bleeding. Thus, there were four treatments in total: plants with or without pressure in well-watered or drying soil.

Collection of Xylem Sap. Xylem sap was collected from leaf 2 by excising the blade near its base. In plants which were already under balancing pressure, this caused xylem sap to bleed at the cut, at a rate similar to that at which the leaf was previously transpiring; thus, the sap collected should have been identical to the transpiration stream. Plants which were not previously pressurized had a balancing pressure applied in order to collect sap. The rate of collection was 100 to 200 μL h^{-1} for plants in wet soil, and about 50 μL h^{-1} for plants in drying soil. Every 30 min the collected sap was removed to a freezer at −20°C.

Bioassay for Stomatal Inhibitor (Transpiration of Detached Leaves). Leaf 2 was detached at midmorning from glasshouse-grown, well-watered wheat seedlings of the same variety as those from which the sap was collected. The leaf was recut immediately under water 80 mm from the tip, placed in distilled water in plastic vials (400 μL capacity) in a controlled environment cabinet, and left for 1 h to recover from fluctuations in transpiration caused by the detachment. There were three to four replicate leaves per treatment. The water uptake of each leaf was then measured by 1 h by sequential weighings (15–30 min intervals) of the vial containing the leaf. When the rate of water uptake was constant, leaves were transferred to vials containing xylem sap, ABA or distilled water. Water uptake was measured for 1 to 2 h, at 15 to 25 min intervals or until the rate was constant, then

the leaf areas were measured with a planimeter, and the transpiration rates (water loss per unit leaf area) were calculated after allowing for the weight loss from blank vials (containing water and leaf-shaped plastic strips). The mean and standard error of the mean (SE) of the three to four replicates was calculated.

The effect of xylem sap (or ABA) was expressed by calculating the ratio of the transpiration rates before and after feeding xylem sap (or ABA), then dividing this by the ratio of the transpiration rates of leaves fed distilled water over the same time periods. This approach took account of any change with time of detachment in the transpiration rate of leaves fed distilled water throughout. The transpiration rate of detached leaves was found to be identical with those on the intact plant for the first few hours after detachment, then it usually declined slowly. This decline could not be prevented by feeding the detached leaves nutrients at a similar concentration as in xylem sap, instead of distilled water. The two varieties (Kite and Egret) gave identical results.

ABA Experiments. When synthetic (±)-ABA (Sigma Chemicals, St. Louis, MO) was fed to detached leaves it was either dissolved in water, to a maximum concentration of 2×10^{-5} M (+)-ABA, or in KOH which was then adjusted to a pH of 5 to 6 with HNO_3. The latter preparation resulted in KNO_3 concentrations of about 10 mM in ABA solutions of 1×10^{-4} M (+)-ABA. Identical effects on transpiration rates of detached leaves were obtained with the two methods for dissolving ABA. The synthetic (±)-ABA fed to detached leaves was assumed to have equal amounts of (+) and (−) enantiomers. All concentrations of synthetic ABA reported here are adjusted to assumed amounts of (+)-ABA.

Xylem sap was assayed for ions by chromatography (Waters ILC-2 Liquid Chromatograph) and for ABA by an indirect ELISA assay according to Walker-Simmons and Sessing (19) using a monoclonal antibody to ABA obtained from Idetek (San Bruno, CA). An authentic (+)-ABA standard obtained from Sigma was used in the xylem sap ELISA assays. This also gave independent confirmation of the content of (+)-ABA in the (±)-ABA fed to detached leaves. The accuracy of the ELISA assay was confirmed by independent blind assays on common samples of xylem sap and of synthetic ABA carried out using ELISA in this laboratory, radioimmunoassay (IE Henson, Perth, W.A.) and by GC.MS (BR Loveys, Adelaide, S.A.). Also, there was no evidence of nonspecific interference with the ELISA assay for dilutions of xylem sap over a 30-fold range and/or with addition of synthetic ABA. Stability of ABA in the xylem sap during collection was confirmed by spiking a collecting vial with ^3H(±)-ABA. Subsequent TLC on Silica gel plates showed no degradation by xylem sap of the added radioactive ABA.

An immunoaffinity column of monoclonal antibody against (+)-*cis*, *trans*-ABA was used to remove ABA from xylem sap. The column provided by BR Loveys (Adelaide) was prepared according to the methods of Knox and Galfre (9). Elution of the xylem sap in water rather than in phosphate-buffered saline had no effect on the column's ability to retain (+)-ABA. No inhibitor of transpiration was released by the column; distilled water eluted through the column did not reduce transpiration of detached leaves.

RESULTS

Xylem sap was first collected from leaves of plants in soil dry enough to cause some stomatal closure (water content 0.07 g g^{-1}). The pressure applied for collection was about 1.5 MPa, which since transpiration rates were not high, approximated the leaf water potential. When this sap was fed to leaves detached from well-watered plants their transpiration rates decreased within 30 min reaching a low steady rate after 1 h. Figure 1a shows the results of one such assay. Transpiration rates of detached leaves largely recovered within 1 h of being transferred from sap to water (Fig. 1b). Sap collected from different plants in dry soils reduced the transpiration of detached leaves by values between 40 and 80%, the mean reduction being close to 60% (Fig. 2). In some assays the detached leaf was recut during the period of measurement of transpiration; this was to test the possibility that its transpiration was decreased merely because its xylem vessels were blocked (if air bubbles formed, or if large molecules or cell fragments were present in the collected sap or released from the cut leaf). Recutting leaves fed either distilled water, or xylem sap, did not alter their transpiration rate (Fig. 1b). Thus, the inhibitory effect of the sap appeared to be directly on stomata. For control leaves, transpiration rates were not affected by recutting and transferring to fresh water.

Surprisingly, xylem sap collected from plants in well-watered soil also had an inhibitory effect on transpiration of detached leaves (Fig. 1a). The extent of the inhibition varied widely from plant to plant (Fig. 2), *i.e.* the inhibitory effect was much more variable than that of xylem sap from plants in drying soil.

Xylem sap was analyzed for the major ions. The concentration of ions depended on the time of day at which the collections were made, sometimes falling two- to threefold during the day. When concentrations of individual ions were expressed as a proportion of the total ions, it became clear that three ions decreased relative to the others as the soil dried: HPO_4^{2-} decreased threefold, and Ca^{2+} and Mg^{2+} about twofold. The Ca^{2+}:Mg^{2+} ratio was 1:1. Table I shows data obtained for two plants of the cultivar Kite in well-watered and drying soil. This threefold decrease in HPO_4^{2-} was confirmed in a separate experiment with 10 plants of the cultivar Egret. The latter experiment also showed a small (30%) decrease in NO_3^-.

Synthetic ABA at a concentration of 5×10^{-6} M (+)-ABA reduced transpiration of detached leaves by 60% (Fig. 3), *i.e.* about the same proportion as did xylem sap from drying plants (Fig. 2). As synthetic ABA appeared to mimic the inhibitory effect of xylem sap, ABA concentrations in the sap were measured (see "Materials and Methods" for validation of these measurements). Xylem sap from well-watered plants contained about 1×10^{-9} M ABA (range 0.8–1.5 $\times 10^{-9}$ M), and sap from plants in drying soil contained about 5×10^{-8} M ABA (range 2–8 $\times 10^{-8}$ M), *i.e.* a 50-fold increase. However, concentrations of synthetic ABA of 5×10^{-8} M had little or no effect on the transpiration rate of detached leaves. In five separate assays, 5×10^{-8} M ABA gave transpiration rates of 83, 85, 88, 101, and 103% of leaves fed distilled water. Such low effectiveness of 5×10^{-8} M ABA on transpiration of detached whole leaves is not unique. For a number of other species it has been shown that stomatal closure only occurs for (+)-ABA concentrations higher than 5×10^{-6} M (10, 12, 13, 16), although Jackson and Hall (8) detected 27% inhibition with 5×10^{-8} M (+)-ABA in peas. Since 100 times more synthetic ABA was required to mimic the effect of xylem sap on detached leaves than was detected in the sap of wheat our results indicate that the stomatal inhibitor assayed in the sap is not ABA; the presence of ABA in the sap cannot explain the inhibitory effect of xylem sap from plants in drying soil. Neither can it explain the smaller but significant inhibitory effect of sap from well-watered plants, which contained 50 times less ABA.

Several explanations for the discrepancy between the effects of synthetic ABA and endogenous ABA occurred to us. First we considered the possibility that the lack of effect of the synthetic ABA was related to the ion concentration of the solution fed to the detached leaf. It is well established that the effectiveness of ABA on epidermal strips depends on the concentrations of K^+ (17) and Ca^{2+} (5). However, the response of detached leaves to synthetic ABA was not enhanced by the presence of K^+ in the

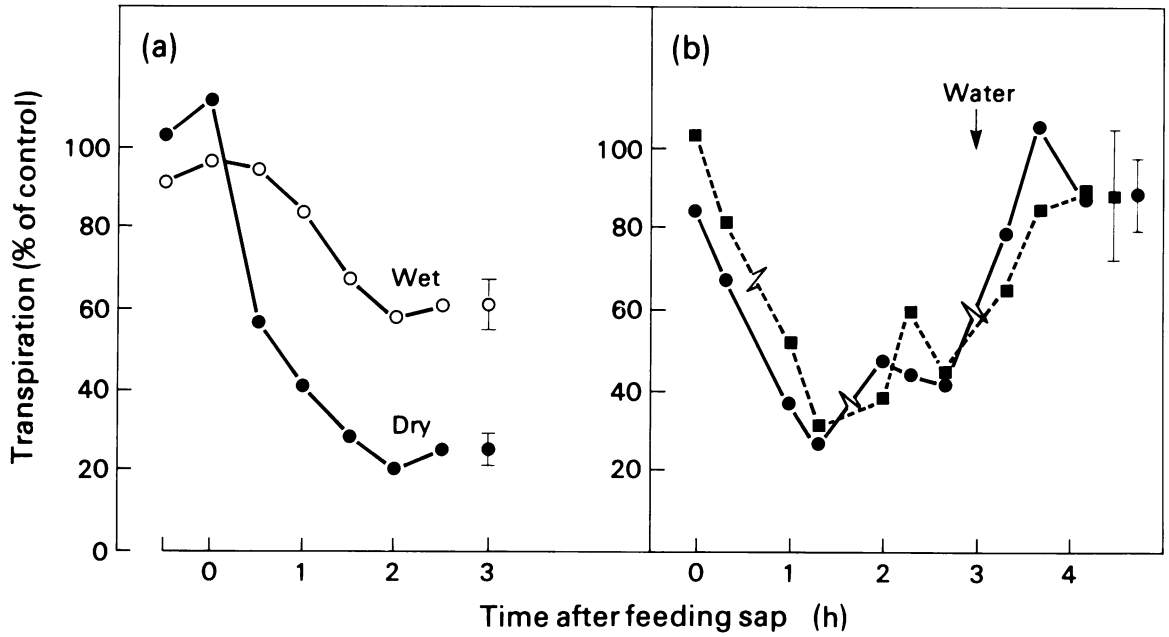

FIG. 1. Effect of xylem sap on the transpiration rate of detached leaves. a, Xylem sap from plants in well watered (○) or drying (●) soil; b, effect of recutting detached leaves fed xylem sap from plants in drying soil, and of transferring them back to water; (●) and (■) are two groups of leaves recut at different times. The break in the line (-∿-) indicates the time of recutting. The effect of the sap is expressed as percentage of the rate in distilled water; these control leaves were recut at 2.5 h, with no change in their transpiration rate. Bars show the average SE of the time intervals.

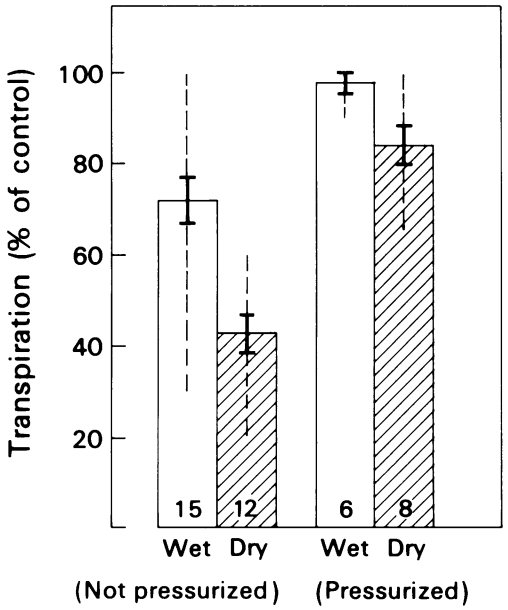

FIG. 2. Effect of xylem sap from pressurized and nonpressurized plants on the transpiration rate of detached leaves. (Leaf water potential in nonpressurized plants decreased as the soil dried; those of pressurized plants remained close to zero.) The effect of the sap is expressed as percentage of the rate in distilled water. Solid bars show the SE of all sap samples measured, dotted bars the range. The numbers at the base of the histograms are the number of sap samples measured.

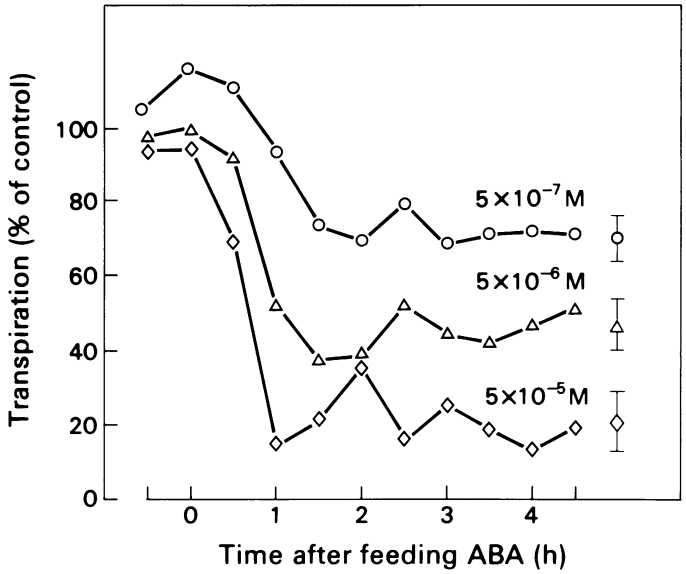

FIG. 3. Effect of synthetic ABA on transpiration of detached leaves; (○) 5×10^{-7} M; (△) 5×10^{-6} M; (◇) 5×10^{-5} M (+)-ABA. The effect is expressed as percentage of the rate in distilled water. Bars show the average SE of the time intervals.

range 0.1 to 10 mM; the effects were the same whether it was dissolved in pure water or in KOH. Furthermore, the concentrations of K^+ and Ca^{2+} in the transpiration stream (Table I) indicate that the leaves of our plants were not deficient in either of these ions. Also, the Ca^{2+} composition was lower in xylem sap from plants in dry than well-watered soil, which would, if anything, make the ABA in the xylem sap less effective (*cf.* 5). The second possibility considered was that leaves from plants in dry soil might be more sensitive to ABA than the test leaves, which were routinely from well-watered plants. There are several reports that water-stressed leaves have an increased sensitivity to ABA (1). However, leaves harvested from plants in soils of a similar dryness to those from which sap was collected showed the same sensitivity to synthetic ABA as did leaves from well-watered plants (Fig. 4). The third possibility considered was that there is something in the sap which activates the very low concentrations of ABA found there. This was tested by mixing xylem sap from plants in well-watered or drying soils with synthetic ABA. Mixing ABA with xylem sap before testing its effect on the transpiration

Table I. *Ion Concentrations (mM) in Xylem Sap Collected from Transpiring Plants in Well-Watered and Drying Soil*

Sap was collected from the base of the blade of leaf 2 at midday at balancing pressures of 0.6 and 1.5 MPa from two plants in well-watered and drying soils, respectively. Na^+ and Cl^- concentrations were about 1 mM each in plants from both soil conditions.

Origin of Sap	Ion Concentrations					
	K^+	Ca^{2+}	Mg^{2+}	NO_3^-	HPO_4^{2-}	SO_4^{2-}
	mM					
Wet	5.3	1.8	1.6	8.9	2.1	0.6
Dry	4.6	0.8	0.7	8.0	0.4	0.6

rate of detached leaves did not make the ABA more effective, *i.e.* there was no synergistic effect. For example, dilute concentrations of ABA did not become more effective when mixed with sap collected from plants in either well-watered or drying soil (Table II). Thus, there is nothing in the sap which activates synthetic ABA.

To confirm the conclusion that the ABA in the sap was not affecting stomatal conductance, ABA was removed from the sap by passing it through an immunoaffinity column. Sap collected from plants in drying soil was assayed for its effect on stomatal conductance of detached leaves before and after it was passed through the column. There was no decrease in its inhibitory activity (Table III).

Xylem sap was also collected from plants which had been growing under balancing pressure in well-watered or drying soils for the previous week. Eliminating leaf water deficit greatly reduced the inhibitor from the xylem sap from plants in both well-watered and drying soils (summarised in Fig. 2). ABA was analyzed in xylem sap from such plants, and for this purpose collections were made sequentially with time, initially every day, then every few hours after the leaf water potential started to fall. (Such a time sequence of collections is not possible with unpressurized plants as pressure is needed to collect sap.) ABA concentrations increased greatly as the soil dried to a critical stage (Fig. 5), which corresponded with the leaf water potential falling to about 1.4 MPa; this equates to the leaf water potential at which 50% stomatal closure is observed (6). The concentration of ABA in the sap from these pressurized plants was about the same as in nonpressurized plants at the same soil water content. This experiment shows that eliminating leaf water deficit has little effect on the transport of ABA from the roots, even though it reduced the amount of the unknown inhibitor.

DISCUSSION

This is the first report that xylem sap collected from transpiring plants contains a substance in quantities which inhibits transpiration. Others have found ABA in xylem sap in a wide range of species, *e.g.* willow (11), grape (12), apricot (13) other woody perennials (4), and sunflower (7), and increases in the ABA concentration in xylem sap of plants in drying soil have been reported, *e.g.* for sunflower (7) and *Ricinus communis* (20). However, the effect of the xylem sap itself on stomatal conductance has not been thoroughly tested. This is presumably due to the difficulty of obtaining a sufficient volume of xylem sap in a way which does not disturb the flow of the transpiration stream. Zhang *et al.* (21) present circumstantial evidence that ABA originating in roots in dry soil could control stomatal behavior, but our data show that the inhibitory effect of the xylem sap on stomatal conductance cannot be explained by the concentration of ABA in the sap. If ABA from the roots was controlling stomatal conductance, synthetic (+)-ABA at the same concentration should have reproduced the effect of the sap on detached leaves, but it did not. Synthetic ABA at 100 times the endogenous sap

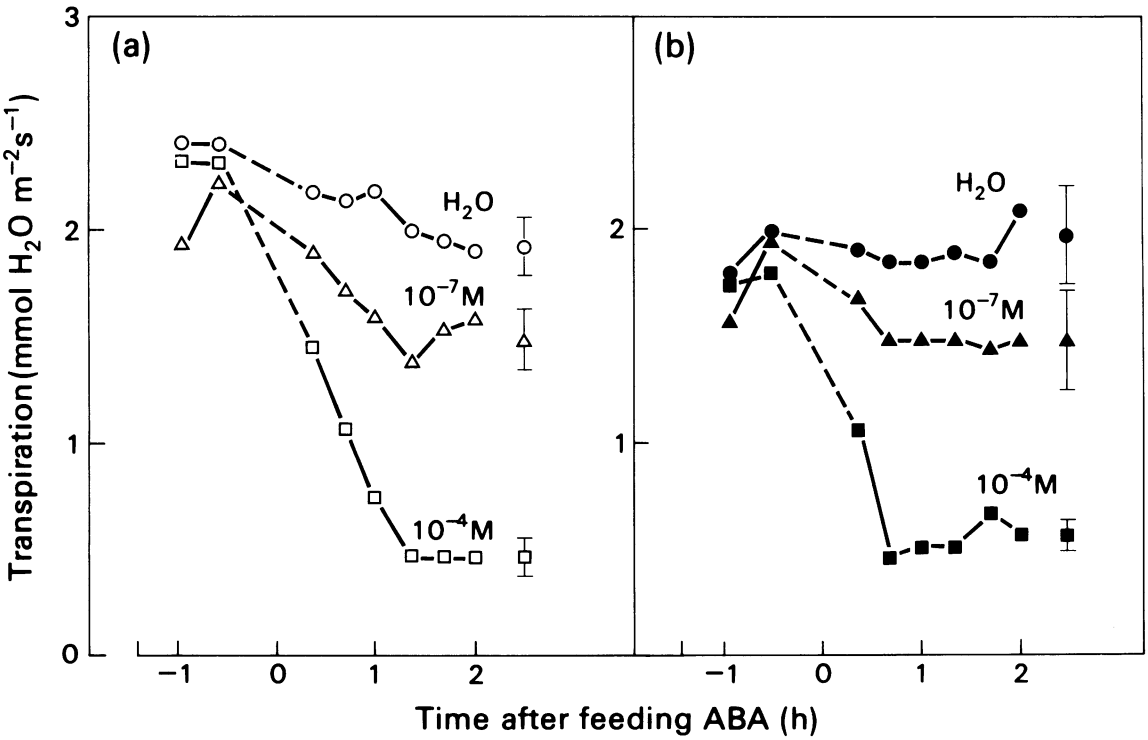

FIG. 4. Comparison between the effects of synthetic (+)-ABA on the transpiration rate of leaves detached from plants growing in (a) well-watered and (b) drying soil. Leaves were harvested from the drying soil when the elongation rates were reduced by 30%, which was a reduction similar to that observed in plants from which xylem sap was collected. Bars show the average SE of the time intervals.

Table II. *Effect of Mixtures of ABA and Xylem Sap on Transpiration Rate of Detached Leaves*

The effect of the sap is expressed as % of the rate in distilled water. The SE was less than 10% of the mean value. In experiment 1, sap was mixed in a 1:9 volume ratio with water or 10^{-3} or 10^{-6} ABA; in experiments 2 and 3, sap was mixed with an equal volume of water or 10^{-5} or 10^{-7} ABA.

Exp No.	Origin of Sap	Concentration of Synthetic ABA	Transpiration Rate (% control)		
			Sap/ water	Sap/ ABA	ABA/ water
		M			
1	Wet	1×10^{-4}	89	16	12
1	Wet	1×10^{-7}	89	88	80
2	Wet	5×10^{-6}	49	30	29
3	Dry	5×10^{-8}	41	44	101

Table III. *Effect of Removing ABA from Xylem Sap from Plants in Drying Soil on Transpiration Rate of Detached Leaves*

Assays were performed before and after stripping out the endogenous ABA using an immunoaffinity column.

	Before	After
ABA ($\times 10^9$ M)	27	<0.03
Transpiration (% control)	66 ± 10	43 ± 7

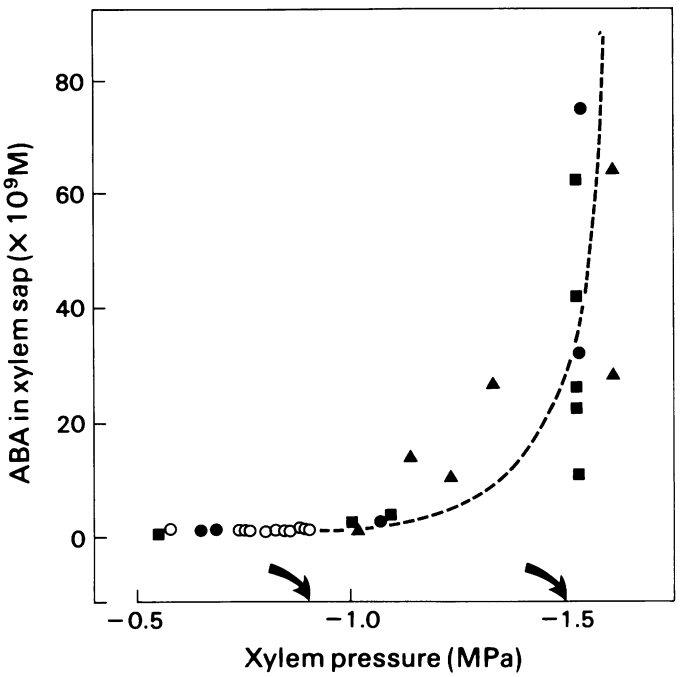

FIG. 5. ABA concentrations in xylem sap as a function of xylem hydrostatic pressure. This pressure is taken as being similar in value (opposite in sign) to the pressure applied to collect the sap. The closed symbols represent three different plants in drying soil; the open circles represent five different plants in well-watered soil. The arrows indicate, respectively, the xylem pressures at which stomatal conductance starts to decrease and then is decreased by 50% (values from Gollan *et al.* 6).

concentration was needed to produce the same response on detached leaves (the sap having 5×10^{-8} M ABA).

This is also the first report claiming that ABA is not the main inhibitor of transpiration present in xylem sap. Loveys *et al.* (13) working with apricot trees reported results consistent with ours. They found that ABA concentrations in the xylem sap were at least 20-fold less than required for stomatal closure when ABA was applied to detached leaves. For grapevines this difference was not as large (2–10-fold less ABA in xylem sap) (12). Our data, however, show not only this inconsistency between ABA in xylem sap and amounts required to close stomata but, also that there is a separate inhibitory activity in the sap and this remains even after ABA has been removed. ABA is therefore not the main stomatal inhibitor. Furthermore, these ABA removal experiments, and the mixing experiments with added low levels of synthetic ABA (Table II), indicate that there is no simple synergistic or antagonistic interaction between ABA and any other chemical in the xylem sap. There must be a compound other than ABA in xylem sap which inhibits stomatal conductance. Many compounds unrelated to ABA induce stomatal closure (15). However, a list of possible inhibitors must include precursors of ABA, although the rapidity of the effect (Fig. 1) makes it unlikely, and also compounds which activate ABA already in the test leaf, (*e.g.* releasing it from a compartment in which it is inactive).

Given the hypothesis that a message from the roots can control stomatal conductance, it was not surprising to find that xylem sap from plants in drying soils had a large inhibitory effect on transpiration but, the presence of an inhibitor in xylem sap from supposedly well-watered plants (Figs. 1 and 2) was surprising. Possibly during the day there were periods of low leaf water status in control plants and this explanation is made credible by the finding that the inhibition was almost eliminated when well-watered plants were maintained under pressure to remove any leaf water deficit (Fig. 2). However, only part of the inhibitory activity of sap from plants in drying soils could be eliminated by preventing leaf water deficit. This suggests that part of the inhibitory effect is triggered by leaf water deficit, and part by root water deficit. Thus, the origin of the inhibitory effect is complex, for it may involve messages from the leaves as well as the roots.

Acknowledgments—We thank Drs. M. L. Tonnet for ion chromatography of xylem sap, B. R. Loveys for the donation of the ABA-affinity column, and I. E. Henson and J. B. Passioura for continued support and criticism. Excellent technical assistance was given by S. Jabs, P. A. Gardner, and I. Licis.

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