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Synthesis and metabolism of abscisic acid in detached leaves of *Phaseolus vulgaris* L. after loss and recovery of turgor

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Abstract. Metabolism of abscisic acid (ABA) was studied after wilting and upon recovery from water stress in individual, detached leaves of *Phaseolus vulgaris* L. (red kidney bean). Loss of turgor was correlated with accumulation of ABA and its metabolites, resulting in a 10-fold increase in the level of phaseic acid (PA) and a doubling of the level of conjugated ABA. The level of conjugated ABA in turgid leaves was no higher than that of the free acid. These results indicate that accumulation of ABA in wilted leaves resulted from a stimulation of ABA synthesis, rather than from a release from a conjugated form or from inhibition of the metabolism of ABA. The rate of synthesis of ABA was at its maximum between 2.5 and 5 h after turgor was lost, and slackened thereafter. In wilted leaves, the rate of conversion of ABA to PA climbed steadly until it matched the rate of synthesis, after about 7.5 h. Upon rehydration of sections from wilted leaves, the rate of synthesis of ABA dropped close to zero within about 3 h, while the rate of conversion to PA accelerated. Formation of PA was two to four times faster than in sections maintained in the wilted condition; it reached a rate sufficient to convert almost one-half of the ABA present in the tissue to PA within 1 h. In contrast, the alternate route of metabolism of ABA, synthesis of conjugated ABA, was not stimulated by rehydration. The role of turgor in the stimulation of the conversion of ABA to PA was investigated. When leaves that had been wilted for 5 h were rehydrated to different degrees, the amount of ABA which disappeared, or that of PA which accumulated during the next 3 h, did not depend linearly on the water potential of the rehydrated leaf. Rather, re-establishment of the slightest positive turgor was sufficient to result in maximum stimulation of conversion of ABA to PA.

Key words: Abscisic acid metabolism – Leaves (ABA, water stress) – Phaseic acid – *Phaseolus* – Turgor – Water stress.

Introduction

In many plants, drought causes an accumulation of ABA, particularly in their leaves. Upon rehydration of a leaf its ABA content returns to a level typical of leaves of unstressed plants (for review, see Wright 1978). The level of ABA will depend on the relative rates of synthesis and metabolic removal of this hormone. Knowledge of the factors that affect accumulation and disappearance of ABA will contribute to an understanding of how plants cope with water stress and recover from it.

Harrison and Walton (1975) and Zeevaart and Milborrow (1976) showed that, in leaves of *Phaseolus vulgaris*, ABA was metabolized either by hydroxylation then rearrangement to phaseic acid (PA) followed by oxidation of PA to dihydrophaseic acid (DPA), or by formation of alkaline-hydrolyzable conjugated ABA. Of these two pathways, the former appeared to be the predominant one.

Harrison and Walton (1975) fed (+)-[2-^{14}C]ABA to leaves of *P. vulgaris* and found that metabolism of ABA continued during water stress. We have, therefore, examined how the pool sizes of the major metabolites of ABA change with time during and after water stress, in order to compare the rate of synthesis with the rate of metabolic removal of ABA and to estimate how much each of the two pathways for

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Abbreviations: ABA = abscisic acid; DPA = dihydrophaseic acid; PA = phaseic acid; ψ_{leaf} = leaf water potential; π = osmotic pressure

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the metabolism of ABA contributes to the reduction of the ABA level in leaves during recovery from water stress. Recently, Zeevaart (1980) presented data on this subject obtained with detached leaves of *Xanthium strumarium*. Among his results was the finding that much more ABA is converted to PA than is being conjugated, in both wilted and rehydrated leaves of *Xanthium strumarium*.

In a previous study (Pierce and Raschke 1980a) we provided evidence that turgor, rather than ψ_{leaf} or π, is the critical parameter of leaf water status that affects accumulation of ABA. We report here on the influence of turgor on the metabolism of ABA. Effects of changes in turgor were examined both with increasing severity of water stress and with increasing relief from water stress. A preliminary report of this work was presented at the 1980 Annual Meeting of the American Society of Plant Physiologists (Pierce and Raschke 1980b).

Material and methods

Plants. Plants of *Phaseolus vulgaris* L. cv. Mecosta (red kidney bean; seeds from Foundation Seed Co., East Lansing) were cultivated in a growth chamber which had a 16-h photoperiod at 85 W m^{-2} of light from General Electric (Cleveland, O., USA) cool-white fluorescent lamps. Day temperature was 27° C; night temperature was 21° C; relative humidity was 80%. The plants were kept well-supplied with water. The terminal leaflets of fully developed leaves were used for the experiments when the plants were 4.5–6 weeks old. For simplicity's sake, "leaf" is used throughout this paper instead of "leaflet".

Experimental procedure. On the evening before an experiment was performed, a leaf was detached under water. The leaf was left to hydrate overnight by standing it in a beaker of water in a darkened cabinet (relative humidity close to 100%, at 25° C). After hydration, the ψ_{leaf} was always $\geqq -2$ bar (1 bar = 0.1 MPa). The fresh weight of the leaf which corresponded to a ψ_{leaf} of -2 bar was taken as the leaf's original fresh weight, and will hereafter be referred to as such.

The measurements of ψ_{leaf} were made with a pressure chamber as described in Pierce and Raschke (1980a). In some experiments measurements of ψ_{leaf} were also made by the dew-point method, as also described in Pierce and Raschke (1980a).

The experiments were of three general types: (1) Accumulation of ABA and its metabolites in leaves of *P. vulgaris* was studied as a *function of leaf water deficit.* Individual leaves were removed from their water supply and allowed to wilt slowly. Sections were cut from these leaves as they lost water. Concomitantly, ψ_{leaf} and fresh weight were monitored on the remaining leaf for plotting a curve relating $-\psi_{leaf}^{-1}$ versus fresh weight (see Talbot et al. 1975). The measurements of fresh weight were corrected for the sections of the leaf that had been excised during the dehydration. The reciprocal bulk leaf π could be read from the linear portion of the curve or its extrapolation to the ordinate. By this procedure it was possible to know the ψ_{leaf}, π, and turgor values of a series of samples cut from a leaf. The samples, each having a different ψ_{leaf}, were then maintained at the same water content for 8.5 h from the time that the leaf had reached zero turgor. By that time the samples should all have reached close to a new steady-state level of ABA for their respective ψ_{leaf} values. At the end of the experiment the samples were frozen in liquid nitrogen and lyophilized before extraction. The method for experiments of this type has been described in more detail in Pierce and Raschke (1980a).

(2) Abscisic-acid synthesis and metabolism were studied as a *function of time during and upon recovery from water stress.* Individual, detached leaves were removed from their water supply and allowed to transpire until they reached 82% of their original fresh weight, the degree of water deficit which produced the greated accumulation of ABA in the first type of experiment. They were then wrapped in foil and maintained at constant weight for up to about 8 h from the time the leaf lost turgor. Part of the leaf was removed from foil after usually 5 h, and fully rehydrated by floating on water in a covered dish for up to 0.5 h. This part of the leaf was kept further in the rehydrated condition. Sections of wilted and rehydrated parts of the leaf were frozen at various times for subsequent analysis of the levels of ABA and its metabolites.

(3) Metabolism of ABA was studied as a *function of degree of rehydration.* Curves for $-\psi_{leaf}^{-1}$ versus fresh weight were determined for leaves until they reached 82% of their original fresh weight. These curves were used to determine by what percentage the fresh weight of a wilted leaf section would have to be increased in order to re-establish a desired degree of turgor. After allowing a wilted leaf to accumulate ABA for a period of about 5 h, leaf sections were cut. These sections were frozen immediately, left wilted for 3 h longer, or else rehydrated to varying degrees and kept for a further 3 h in the rehydrated state. Leaf sections were rehydrated by floating them on water in a covered dish until they reached the desired, pre-determined weight.

Analysis for ABA and its metabolites. Each lyophilized sample was homogenized at room temperature in 15 ml methanol containing 1% (v/v) glacial acetic acid and 10 mg l^{-1} of the antioxidant 2,6-di-*tert*-butyl-4-methylphenol (Milborrow and Mallaby 1975). The methanol extract was separated from the debris by vacuum filtration, and the debris was re-extracted by shaking overnight in another 15 ml of methanol. The second methanol fraction was combined with the first. Tritiated (±)-ABA, [^{3}H]PA, [^{3}H]DPA, and conjugated [^{3}H]ABA (250 Bq each) were added to the methanol extract for monitoring recovery. Hereafter, (±)-[^{3}H]ABA is referred to simply as [^{3}H]ABA. Five ml H_2O were added to the methanol extract, and the methanol was evaporated under reduced pressure. Material insoluble in H_2O was removed by filtration through a Millipore (Bedford, Mass., USA) AP prefilter.

The filtered aqueous solution was loaded on a SEP-PAK C_{18} cartridge (Waters Associates, Milford, Mass., USA) and followed by a wash with 5 ml 1% aqueous acetic acid. This method of extraction and preliminary purification was highly efficient. A single extraction of the tissue with methanol and subsequent purification of the treated extract on a SEP-PAK cartridge recovered more than 95% of the radioactivity added through the application of a known amount of [^{3}H]ABA to leaf sections just prior to freezing. Ethanol and 1% acetic acid were combined in a ratio 40:60 (v/v), and ABA, PA, DPA, and conjugated ABA were all removed from the cartridge with 7 ml of this solvent mixture. A sample was evaporated to a smaller volume under reduced pressure, filtered through a 0.45-μm Millipore filter, and evaporated to dryness under a stream of N_2.

Further purification was achieved with a high-performance liquid chromatography system (Model SP8000; Spectra-Physics, Santa Clara, Cal., USA) using a procedure modified from the one developed by Zeevaart (1980). The two solvents used for the reverse-phase column were re-distilled 95% ethanol (A) and 1% (v/v) aqueous acetic acid (B). A sample was dissolved in 0.5 ml of 10% A: 90% B and injected on a 0.5 ml sample loop connected

to a guard column filled with ODS pellicular packing (Whatman, Clifton, NJ., USA) followed by an analytical column packed with 10 µm Spherisorb ODS (Spectra-Physics). The sample was eluted with a linear gradient of 10–30% A in B in 1 h following 10 min at initial conditions. Solvent flow rate was 1 ml min^{-1} at 7 MPa.

The collection times for ABA, PA, DPA, and conjugated ABA were based on retention times of standards, which were detected in the eluant by absorption of light at 254 nm. Abscisic acid (racemic mixture) was purchased from Calbiochem, La Jolla, Cal., USA. Phaseic acid and DPA were a gift from T.D. Sharkey (see Sharkey and Raschke 1980). The conjugated ABA (β-D-glucopyranoside ester of (±)-ABA) was a gift from J.A.D. Zeevaart (see Zeevaart 1980). The retention time of alkaline-hydrolyzable conjugated ABA in extracts of *P. vulgaris* leaves was originally determined from trials in which fractions throughout the gradient were subjected to mild alkaline hydrolysis and re-run, collecting at the retention time for ABA. Subsequently it was found that the retention time originally determined for alkaline-hydrolyzable conjugated ABA of *P. vulgaris* corresponded to the retention time of synthetic glucosyl ester of (±)-ABA and to the retention time of a major radioactive metabolite when [^{3}H]ABA was supplied to leaves of *P. vulgaris*.

Conjugated ABA which was collected from the reverse-phase column was hydrolyzed at pH 13 (adjusted with 5 N NaOH) at 60° C for 30 min (Milborrow and Mallaby 1975). After hydrolysis, the pH was adjusted to 2.5 with phosphoric acid, and ABA was extracted from the solution by partitioning three times with an equal volume of ethyl acetate. The ABA in ethyl acetate was further purified using the same procedure as for the free-ABA fraction collected from the reverse phase column.

The fractions containing ABA, PA or DPA were taken to dryness under a stream of N_2 and methylated with diazomethane in ethyl acetate. The metyhlated fractions were filtered through a 0.5-µm Millipore FHLP filter and taken to dryness. A sample was dissolved in 0.5 ml of a mixture of ethyl acetate and hexane and injected on a 0.5-ml sample loop connected to a guard column filled with PAC pellicular packing (Whatman) followed by a µ-Bondapak-NH_2 analytical column (Waters Associates). A sample was injected and eluted with solvent of constant composition. A mixture of 40% ethyl acetate in hexane was used for ABA; 50% ethyl acetate in hexane was used for PA; 70% ethyl acetate in hexane was used for DPA. Solvent flow rate was 1 ml min^{-1} at 2 MPa.

The extracts of the individual leaf sections contained amounts of ABA and its metabolites too small to be analyzed quantiatively by absorption of light at 254 nm. Quantitative analysis was performed with aliquots of the samples by gas-liquid chromatography with a 3% SE-30 column and using an electron capture detector as described in Pierce and Raschke (1980a). The concentrations of standards for calibration were determined by ultraviolet spectrophotometry in methanol using published extinction coefficients (Milborrow and Robinson 1973; Harrison and Walton 1975).

Overall recovery was estimated by determining the radioactivity in the samples using a Packard (Downer's Grove, Ill., USA) model 3255 Tri-Carb Liquid Scintillation Spectrometer. Recovery of ABA from the original methanol extract was generally between 60% and 70%; recovery of the metabolites of ABA was generally between 40% and 60%. The [^{3}H]PA, [^{3}H]DPA, and conjugated [^{3}H]ABA were extracted and purified from *P. vulgaris* leaves which had been fed [^{3}H]ABA through the transpiration stream and incubated for 24 h (see Harrison and Walton 1975). The specific activities of the radioactive metabolites varied from one preparation to another. In no case did the amount of radioactive metabolite, which was added to a sample for monitoring recovery, equal more than 10% of the endogenous metabolite, and in most cases it was less than 1%. When warranted, the results were corrected for the quantity of radioactive material that was added to the samples. All results were corrected for degree of recovery and expressed as pmol mg^{-1} dry weight.

Results

Accumulation of ABA and its metabolites as a function of leaf water deficit. Leaf samples were prepared according to the first type of experiment outlined above in the Material and Methods section. The result of the experiment, shown in Fig. 1, illustrates what we had found before for ABA (Pierce and Raschke 1980a): Eighty percent or more of the maximum increase in ABA content, that leaves developed above the unstressed level, occurred at a turgor of less than 1 bar (=0.1 MPa). Samples having a high ψ_{leaf} (>-5 bar) contained nearly equal amounts of PA, conjugated ABA, and free ABA. Unstressed leaf sections contained about three times as much DPA as ABA. The DPA content was not measurably affected by decreasing ψ_{leaf} (<-5 bar) within the time of the experiment (≦10 h). Samples with a low ψ_{leaf} contained twice as much conjugated ABA as samples with a high ψ_{leaf}. In contrast, with loss of leaf turgor, PA content increased in parallel with increasing ABA content to more than 10 times the basal level. The sum of the contents of ABA and PA more accurately reflects how much ABA was synthesized during the incubation period as a result of water deficit than does the ABA content alone. A repetition of the experiment produced similar results, although the extent of the accumulation of PA in the wilted samples was somewhat less than in the experiment shown in Fig. 1. We conclude that the continuation of metabolism of

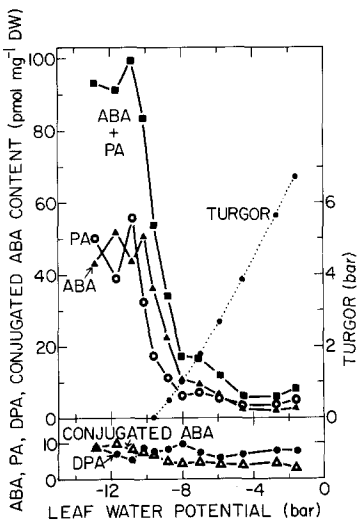

Fig. 1. The relationship between turgor (····) and ψ_{leaf}, and the effect of ψ_{leaf} on the content of ABA, PA, DPA, and conjugated ABA in samples from a single, detached leaf of *Phaseolus vulgaris*

ABA, especially to PA, during water stress is certainly a factor in limiting the levels of ABA in stressed bean leaves.

Identification of metabolites of ABA in bean leaves during recovery from water stress. In detached leaves, changes in the pool sizes of an endogenous compound and its metabolites can be used to calculate rates of metabolism. In order to obtain a good estimate of the rate of synthesis of ABA, the pool sizes of ABA and of all of its important metabolites must be determined. Although the identity of metabolites of ABA is known for fresh and for wilted leaves of *P. vulgaris* (Harrison and Walton 1975; Zeevaart and Milborrow 1976), we felt it was necessary to determine the pattern of metabolism also for leaves recovering from water stress, when the rate of disappearance of ABA is especially high. Leaves were prepared according to the second type of experiment outlined in Material and Methods. In this particular experiment, leaf sections were rehydrated in water containing [^{3}H]ABA. The tissue was analyzed for radioactive compounds 3 h later. Other samples were prepared by applying a known amount of [^{3}H]ABA directly to rehydrated leaf sections and allowing no time for metabolism. Radioactive material was extracted from the tissue and transferred to SEP-PAK C_{18} cartridges (see Material and Methods). Three fractions were prepared from the cartridges: (1) the 1% acetic-acid solution from which the sample was loaded onto the cartridge; (2) the fraction eluted from the cartridge with 40% ethanol in 1% acetic acid; and (3) the fraction eluted with 95% ethanol. Whether a leaf sample was given time to metabolize the [^{3}H]ABA or not, most of the radioactivity recovered from the SEP-PAC cartridge was contained in the 40% ethanol fraction, an average of 96% in 8 samples that were rehydrated in the presence of [^{3}H]ABA.

The radioactive compounds in the 40% ethanol fraction were separated by high-performance liquid chromatography on a reverse-phase analytical column. The distribution of radioactivity in the elution profile is shown for several samples in Fig. 2. When [^{3}H]ABA was added to samples just before freezing them, most of the radioactivity co-chromatographed with ABA (Fig. 2b), an average of 83% of the total radioactivity, in two trials. The remaining radioactivity appeared in several small peaks as well as in a low level of radioactivity spread over the whole profile; presumably this residual radioactivity resulted from non-metabolic breakdown of the [^{3}H]ABA.

When 3 h were allowed for the metabolism of [^{3}H]ABA (Fig. 2a, c), major peaks of radioactivity appeared which co-chromatographed with PA, conjugated ABA, and residual free ABA. Very little, if

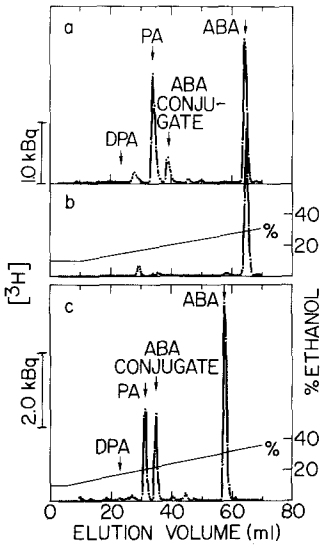

Fig. 2a–c. High-performance liquid chromatography elution profiles of radioactivity from leaf sections of *Phaseolus vulgaris*. The retention times of standards are indicated by arrows. For **b**, rehydrated leaf tissue was frozen immediately after a drop of [^{3}H]ABA solution was added to it. Profiles **a** and **c** show two examples of leaf tissue which had been wilted for 5 h, was rehydrated in water containing [^{3}H]ABA and allowed to metabolize the [^{3}H]ABA for 3 h. During rehydration the amount of ABA absorbed by the tissue was 1–2 pmol mg^{-1} DW. Profiles **a** and **b** were obtained by conditions as described in Material and Methods; **c** is from samples which were eluted with a slightly steeper gradient of solvent composition

any, radioactivity above background was associated with DPA. If DPA had been labeled to a measurable degree, we would see it in the elution profile because the fractions which eluted at the retention time for DPA were collected and concentrated, and actually found to contain DPA. The conversion of PA to DPA appears to be a relatively slow process. Harrison and Walton (1975) reported that, in wilted bean leaves, at 4 h after feeding [^{14}C]ABA, DPA contained one-sixth as much radioactivity as did PA.

The peak of radioactivity which in all profiles eluted several minutes prior to the PA peak was probably an artefact of the extraction procedure. We are certain that it was not caused by an alkaline-labile conjugate of ABA, of PA, or of DPA since its retention time was not changed after the extract was subjected to conditions for mild alkaline hydrolysis.

We confirmed that the peak of radioactivity which co-chromatographed with synthetic glucosyl ester of (±)-ABA (designated "ABA conjugate" in Fig. 2) was indeed caused by a conjugate of ABA: after mild alkaline hydrolysis of this radioactive material, a second elution profile showed one major peak of radioactivity at the retention time for ABA.

When (±)-ABA is fed to tissue, conjugated ABA

contains an excess of the unnatural (−)-enantiomer (Milborrow 1970); the (−)-enantiomer is not easily metabolized to PA. Thus, the profiles of radioactive products in Fig. 2 overestimate (relative to PA) the extent to which conjugated ABA would be produced from endogenous ABA in bean leaves during 3 h after rehydration.

In the four trials conducted, the sum of the radioactivities which appeared in the eluates corresponding to DPA, PA, conjugated ABA, and free ABA accounted for an average of 86% of the total radioactivity in the elution profile. The remaining radioactivity can reasonably be attributed to non-metabolic breakdown of the [^{3}H]ABA or of its metabolites, based on the results presented above from leaf samples which were not given any time to metabolize the radioactive ABA. We conclude that changes in the endogenous pool sizes of PA, DPA, and alkaline-hydrolyzable conjugated ABA account for the bulk of metabolism of ABA in bean leaves during the first few hours of recovery from water stress, PA being by far the principal metabolite during this time.

Accumulation of ABA and its metabolites as a function of time during and upon recovery from water stress. Samples were prepared according to the second type of experiment described in Material and Methods. The time course of accumulation of ABA in the wilted leaf and of the disappearance of ABA after rehydration is plotted in Fig. 3. Two and one-half hours after turgor had been lost, ABA had increased to seven times its original value, and by 7.5 h the ABA content was leveling off at 16 times its original value. Rehydration required about 20 min, and by 40 min later the level of ABA had started to decline. After 4.5 h recovery, 84% of the ABA which had accumulated in 5 h of stress had been metabolized.

Figure 4 shows the time course of accumulation of PA and DPA in the same samples which were used for the determinations of ABA that are shown in Fig. 3. The level of DPA increased, but only slightly, during the course of the experiment. This is in agreement with the results presented in Figs. 1 and 2. Phaseic acid accumulated at a constant rate in water-stressed tissue after a lag period of less than 2.5 h. After 9.5 h, the wilted tissue had a level of PA which was 10 times the pre-stress level. While rehydration caused the level of ABA to fall rapidly, it caused a surge in the rate of synthesis of PA to as much as four times the rate that was found in the wilted tissue. In the face of a declining pool size of ABA, the conversion of ABA to PA was enhanced. Since DPA accumulates as an end product in bean leaves (Zeevaart and Milborrow 1976), and since the levels of DPA were higher rather than lower in the

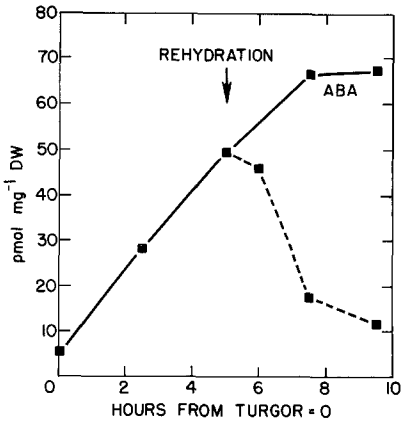

Fig. 3. Changes with time in content of ABA in sections of a single, detached leaf of *Phaseolus vulgaris* during water stress (—) and recovery (---)

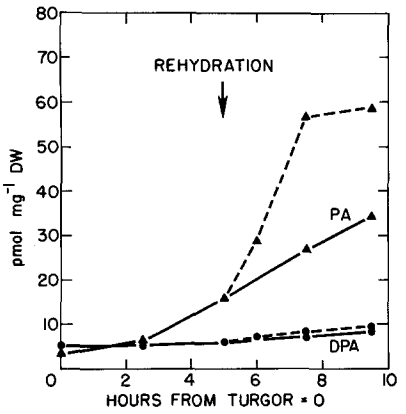

Fig. 4. Changes with time in content of PA (▲) and DPA (●) in the same bean leaf sections that were analyzed for ABA content (Fig. 3) during water stress (—) and recovery (---)

rehydrated tissue as compared to the wilted tissue, rehydration did not inhibit conversion of PA to DPA.

During stress and during recovery the formation of conjugated ABA occurred to a much smaller extent than did the formation of PA, and rehydration did not accelerate the conjugation of ABA. Unstressed bean leaves were found to contain about 3.8 pmol of conjugated ABA mg^{-1} dry weight. In the time-course experiment illustrated in Figs. 3 and 4, the content of conjugated ABA increased to 11 pmol mg^{-1} dry weight in the sample which was kept wilted for 9.5 h and to 5 pmol mg^{-1} dry weight in the sample which had been wilted but then was allowed to rehydrate for 4.5 h. In another experiment, the corresponding wilted sample also contained more than twice as much conjugated ABA as the rehydrated sample did.

Rates of synthesis and metabolism of ABA were estimated from the data of the time-course experi-

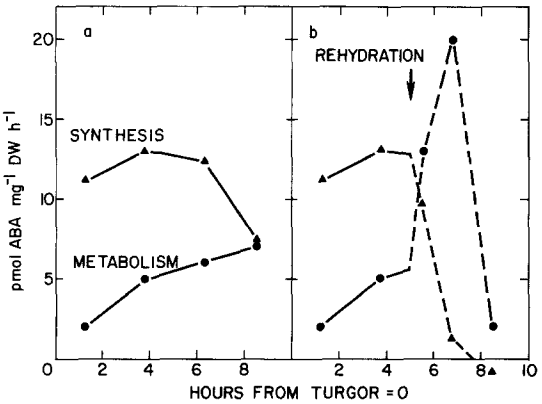

Fig. 5a, b. Effect of wilting (—) and rehydration (---) on the average rates of synthesis (▲) and metabolism (●) of ABA during intervals of time after loss of turgor in samples from a single, detached leaf of *Phaseolus vulgaris*. **a** Continued wilting; **b** rehydration after 5 h. The data points are placed at the midpoints of the intervals. Each data point is the sum of differences between pairs of determinations of three or four different compounds and is not more than an estimate

ments. This was possible because detached leaves were used; translocation of ABA or its metabolites from the leaf did not contribute to the changes that were observed. The rate of synthesis of ABA equals the rate of change in the level of ABA plus the rates of change in the levels of its metabolites. The rate of metabolism of ABA equals the sum of the rates of change in the levels of just the metabolites of ABA. The maximum rate of synthesis of ABA averaged 15 pmol mg^{-1} dry weight h^{-1} in five experiments, with a range from 6 to 23 pmol mg^{-1} dry weight h^{-1}. The calculations for the results shown in Figs. 3 and 4 are presented in Fig. 5. After wilting occurred, the rate of synthesis of ABA jumped to a maximum that averaged 13 pmol mg^{-1} dry weight h^{-1} between 2.5 and 5 h. Thereafter, the rate of synthesis of ABA subsided to an average of 7 pmol mg^{-1} dry weight h^{-1}. In wilted tissue the rate of metabolism of ABA climbed steadily until it matched the rate of synthesis between 7.5 and 9.5 h.

Upon rehydration of the leaf tissue, the synthesis of ABA did not cease abruptly. The pool size of the metabolites of ABA increased by 13 pmol mg^{-1} dry weight, and the level of ABA fell by 3 pmol mg^{-1} dry weight during the first hour after rehydration, which meant that ABA was being synthesized at an average rate of 10 pmol mg^{-1} dry weight h^{-1}, down from the maximum rate found just prior to rehydration. Synthesis of ABA fell rapidly after that to zero within about 3 h after rehydration.

If the loss of stimulation of ABA synthesis had been the only effect of rehydration, ABA would have disappeared at a rate of 6 or 7 pmol mg^{-1} dry weight h^{-1}, which was the average rate of metabolism of ABA in wilted tissue. However, after 1 h of recovery, ABA disappeared at a rate of more than 19 pmol mg^{-1} dry weight h^{-1}, which was possible only if rehydration actually enhanced the metabolic removal of ABA. Later, as synthesis of ABA slowed down and conversion to PA proceeded rapidly, the supply of ABA was depleted and turnover of ABA slackened in the last period of recovery.

Table 1. Effect of wilting and rehydration on the average rate of accumulation of PA in samples from individual, detached leaves of *Phaseolus vulgaris*. Leaves were wilted for approx. 5 h, after which part of each leaf was rehydrated. The change in level of PA that occurred during the subsequent approx. 3 h was determined for both the rehydrated tissue and tissue which was kept wilted. Coefficient of conversion: pmol ABA converted to PA h^{-1} $pmol^{-1}$ ABA present

Expt.	Treatment	Average rate of change in PA during period following approx. 5 h of wilting	
		pmol PA mg^{-1} DW h^{-1}	Coefficient of conversion (h^{-1})
1	Continued wilting	1.6	0.05
	5.5 h wilted	7.8	0.51
	+2.5 h rehydrated		
2	Continued wilting	4.3	0.07
	5 h wilted	16.3	0.49
	+2.5 h rehydrated		
3	Continued wilting	9.3	0.13
	5.75 h wilted	20.9	0.42
	+3.25 h rehydrated		
4	Continued wilting	10.4	0.29
	5 h wilted	9.7	0.36
	+3 h rehydrated		
5	Continued wilting	9.4	0.27
	5 h wilted	10.6	0.42
	+3 h rehydrated		

The rate of accumulation of PA during the period of rehydration was compared with the rate in tissue that was left wilted. Results of five experiments are shown in Table 1. Individual leaves varied considerably in their response. In three experiments, rehydration caused increased production of PA; in two experiments no large change occurred (Table 1, left column). In spite of the declining size of the precursor pool, accumulation of PA in rehydrated bean leaves was equal to or greater than it was in wilted tissue. An acceleration of the conversion of ABA to PA becomes strikingly apparent if the rates of PA formation are related to the sizes of the ABA pools and coefficients of conversion are obtained (Table 1, right column). The same data also indicate that the relative rates of ABA conversion to PA in *rehydrated tissue*

were similar in all experiments (between 0.4 and 0.5 pmol ABA converted $pmol^{-1}$ ABA present h^{-1}). What appears to have varied from one experiment to the next is the rate of conversion of ABA to PA in the *wilted tissue*. The cause of this variation is not known at present.

Conversion of ABA to PA as a function of degree of rehydration. In leaf samples differing in degree of water deficit, synthesis of ABA was stimulated by loss of turgor (Fig. 1 and Pierce and Raschke 1980a). Is regulation of the metabolism of ABA to PA also related to turgor? The following experiments were designed to test whether enhanced conversion of ABA to PA correlates with reestablishment of turgor. The experimental procedure was of the third type described in Material and Methods. In general, leaves were wilted and left to accumulate ABA for 5 h. Then leaf sections were rehydrated to varying degrees and 3 h later examined for their production of PA.

A graph of $-\psi_{leaf}^{-1}$ versus percent of original fresh weight, which was determined during dehydration of a leaf, was used as a calibration curve for calculating turgor when sections from the same leaf were subsequently rehydrated to different percentages of their original fresh weights. It was important to determine whether the original relation between ψ_{leaf} and fresh weight could be used to calculate the turgor of the rehydrated samples. Figure 6 shows the results of one kind of test. A bean leaf was dehydrated, yielding one set of ψ_{leaf} and fresh-weight values. When the same leaf was rehydrated and then dehydrated a second time, the second set of ψ_{leaf} and fresh-weight values yielded a curve which closely matched the original relationship. The two curves fell within about 0.25 bar of each other.

In another kind of test (Fig. 7) a relation between ψ_{leaf} and fresh weight was determined using a pressure bomb, then the leaf was cut into sections, and these were rehydrated to varying degrees. The ψ_{leaf} values of the rehydrated samples were determined by the dew-point method. Once again the agreement was good between the two sets of measurements, but Fig. 7 shows that ψ_{leaf} values of individual pieces of leaf after rehydration, as determined by the dew-point method, *may* deviate by as much as 1 bar from ψ_{leaf} values as originally determined by the pressure-bomb method for the entire leaf.

Bean leaf samples were prepared for analysis of the content of ABA and its metabolites. Tissue was frozen to assess the contents after 5 h of wilting. A number of leaf sections were rehydrated to different extents and kept for another 3 h, after which they were also frozen. The results of this experiment are shown in Fig. 8. The changes in ABA, PA, and conjugated ABA from the levels after 5 h of wilting, that occurred during the 3 h recovery period, were plotted versus the ψ_{leaf} which a sample had during the recovery period. The relation between ψ_{leaf} and turgor for these samples is also indicated. The sample having the lowest ψ_{leaf} (−10.9 bar) and no turgor had not been rehydrated at all. It can be compared to the samples in the time-course experiments (Figs. 3, 4) which were kept wilted throughout the experiment.

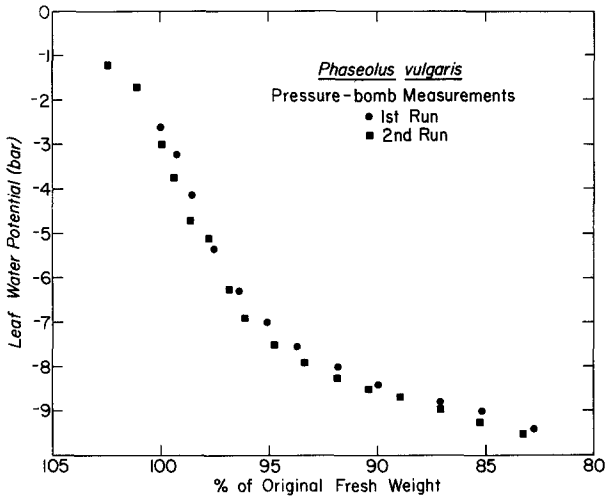

Fig. 6. A comparison of duplicate determinations on the same bean leaf of the relationship between ψ_{leaf} and fresh weight. After the first set of measurements, the leaf was floated on water until it had rehydrated

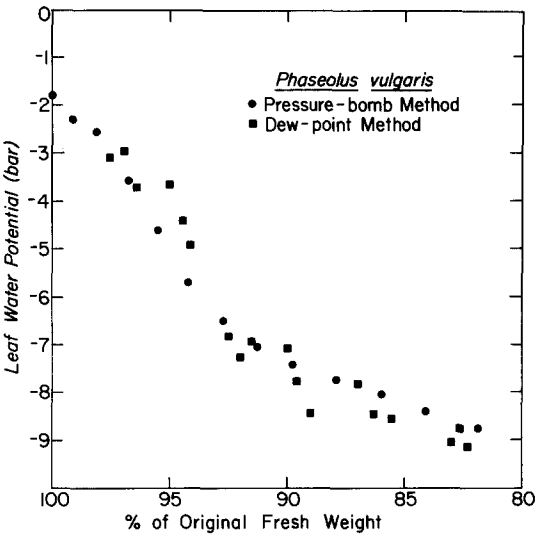

Fig. 7. Measurements of ψ_{leaf}, which were determined with a pressure bomb, versus percent of the leaf's original fresh weight compared to measurements of ψ_{leaf} on sections of the same bean, which were made by the dew-point method, versus the percent to which those sections were rehydrated

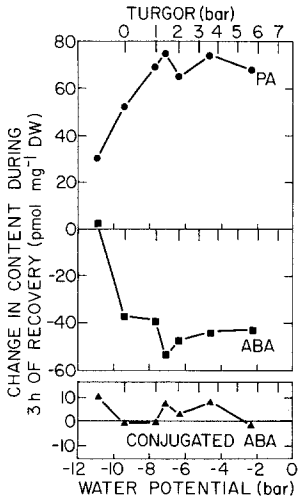

Fig. 8. The effect of degree of rehydration on the change in content of ABA, PA, and conjugated ABA in sections of a single leaf of *Phaseolus vulgaris* during 3-h period of recovery from water stress. Degree of rehydration is indicated by (i) ψ_{leaf} and (ii) turgor which the leaf sections had during the period of recovery

The sample having the highest ψ_{leaf} (-2.3 bar) and the highest turgor (5.6 bar) had been rehydrated to 100% of its original fresh weight and can be compared to the samples which were rehydrated in the time-course experiments.

Rehydration triggered the disappearance of ABA and increased the production of PA (Fig. 8). Samples having turgor accumulated more than twice as much PA during the recovery period than did the sample having the lowest ψ_{leaf}. In all samples, the magnitude of the decrease in ABA was less than the rise in PA, which indicates that synthesis of ABA must have continued during at least part of the recovery period. These results are consistent with those presented in Figs. 3 and 4.

Increased synthesis of PA and disappearance of ABA were not linear functions of the degree of rehydration. Re-establishment of the slightest turgor was sufficient to elicit maximum enhancement of the conversion of ABA to PA. We can not tell whether the response anticipated re-establishment of turgor in the leaf or not. The position of zero turgor on the ψ_{leaf} scale in Fig. 8 could be incorrect by about ± 1 bar (refer to Fig. 7). Re-establishment of turgor did not increase the production of conjugated ABA (Fig. 8).

Discussion

Synthesis and metabolism of ABA after loss and gain of turgor. Our results demonstrate that leaf turgor affects both the metabolic system which catalyzes the synthesis of ABA, and that which catalyzes the conversion of ABA to PA. The systems respond to changes in turgor in opposite ways. Synthesis of ABA is more rapid in wilted than in turgid leaves; metabolism of ABA to PA is more rapid in leaves that have regained turgor than in wilted leaves. The results are a net accumulation of ABA in wilted tissue, and a decline of the ABA content to a low level in tissue to which turgor has returned.

Stimulation of ABA synthesis after loss of turgor. The conclusion that turgor loss causes an increase in the rate of synthesis of ABA is based on the elimination of other possible causes of ABA accumulation, namely (1) inhibition of the metabolism of ABA to PA, (2) release of ABA from a conjugated form, or (3) inhibition of the formation of ABA conjugates. With respect to the first possibility, Harrison and Walton (1975) were the first to show that metabolism of ABA continued in leaves under water stress. The content of PA and DPA actually built up in wilted bean leaves. As shown by Harrison and Walton (1975) and confirmed by us (Figs. 1, 4), inhibition of the metabolism of ABA through the PA-DPA pathway was not responsible for the accumulation of ABA that had occurred. Hydrolysis of ABA conjugates could not have been the source of free ABA either. As demonstrated by Fig. 1, bean leaves which had not experienced water stress did not contain sufficient alkaline-labile conjugated ABA to yield the amounts of ABA which appeared in the wilted tissue (see Zeevaart 1980, and review by Wright 1978). Turning to the third possibility, we see from Fig. 1 that the level of conjugated ABA was slightly higher in wilted than in turgid leaves. Inhibition of the metabolism of ABA to conjugated ABA cannot have been the cause of the observed accumulation of ABA. As the pathways via PA and alkaline-labile conjugated ABA account for the bulk of the metabolism of ABA in bean leaves (e.g. Fig. 2), it follows that the principal cause of the accumulation of ABA in water-stressed bean leaves was the stimulation of the synthesis of ABA.

Simultaneously with the accumulation of ABA in wilted leaves occurred an accumulation of PA and, to a much smaller degree, of conjugated ABA. Since ABA and PA (and DPA) increased in parallel fashion with respect to changes in ψ_{leaf} (Fig. 1) and with respect to time (Figs. 3, 4), we can explain the accumulation of the metabolites of ABA in wilted tissue to be an effect of mass action of ABA.

With confidence that determinations of changes in the levels of PA, DPA, and alkaline-labile conjugated ABA would account for the metabolism of ABA during a short-term wilt-recovery cycle in bean leaves (see Fig. 2, and Harrison and Walton 1975), we calculated rates of synthesis and metabolism of ABA. Seven hours after turgor was lost, accumulation of ABA

leveled off, then production and metabolic removal of ABA balanced each other. At this time, the rate of synthesis or metabolism was 7 pmol mg^{-1} dry weight h^{-1} (Fig. 5). This is about two times the rate Harrison and Walton (1975) determined for a steady-state level of ABA in wilted primary leaves of two-week-old bean plants (at 15% dry weight, 0.15 μg ABA g^{-1} fresh weight h^{-1} correspond to 3.8 pmol mg^{-1} dry weight h^{-1}). Harrison and Walton felt that twice the rate they calculated was closer to being accurate. Wilted *Xanthium* leaves appear to possess a capacity to synthesize and metabolize ABA at rates very similar to those of wilted bean leaves. From the data in Figs. 3 and 4 (time interval 5–12 h) of Zeevaart (1980) we estimate a rate of 8 pmol mg^{-1} dry weight h^{-1} for the steady state.

Stimulation of ABA metabolism after recovery of turgor. After the return of turgor to the tissue, conversion of ABA to PA accelerated (Figs. 4, 8, Table 1). Since this stimulation of the metabolism of ABA occurred at a time of declining ABA content (Figs. 3, 8), recovery of turgor must have caused an increase in the amount or the activity of the enzyme(s) involved in the conversion of ABA to PA; or, possibly, recovery of turgor increased the accessibility of critical substrates to the enzyme(s). A stimulation of the conversion of ABA to PA after rehydration was also discovered by Zeevaart (1980) in leaves of *Xanthium strumarium*. Previously, it was possible to consider that the concentration of ABA falls when plants are rewatered because of diminished capacity for synthesis in combination with, as Milborrow (1979) wrote, "a constant, large degradative capacity for ABA...". Our evidence indicates that the capacity of bean leaves to remove ABA by metabolism is not constant but increases when turgor returns to a wilted leaf (Table 1, Fig. 8). This acceleration becomes particularly evident when the rate of ABA conversion is expressed relative to the amount of ABA present in the tissue (yielding a coefficient of conversion; second column in Table 1). In rehydrated tissue, almost one half of the amount of ABA present was converted to PA h^{-1} ($0.44 \pm 0.06\ h^{-1}$, as compared to $0.16 \pm 0.11\ h^{-1}$ in wilted tissue). An increase in the rate of conversion appears also in Zeevaart's data on *Xanthium* leaves. Calculating from Figs. 3 and 4 of Zeevaart, during periods when PA was increasing linearly with time, the coefficient of conversion averaged $0.40\ h^{-1}$ for leaves rehydrated after four hours of wilting (time interval: 0–4 h after rehydration), and it averaged $0.12\ h^{-1}$ for wilted leaves (time interval: 4–12 h of wilting).

The stimulation of the metabolic removal of ABA after rehydration was restricted to the PA pathway. Conjugation of ABA continued at a low rate, if at all (Fig. 8). An early report by Hiron and Wright (1973) indicated some conversion of ABA to conjugated ABA during recovery of bean seedlings from water stress. But neither we, nor Dörffling et al. (1974), who tested pea seedlings, nor Zeevaart (1980), who analyzed *Xanthium* leaves, found evidence for the formation of conjugated ABA to be of importance during short-term stress and recovery from it. Prolonged or repeated stress may be a different matter.

Conclusion

For accumulation of ABA to be stimulated by *loss* of turgor and disappearance of ABA to be stimulated by *regain* of turgor in leaves (Figs. 1, 8, and Pierce and Raschke 1980a), indicates that control of the level of ABA is important in the stress physiology of plants. Plants have the ability to adapt to stress conditions by adjustment of their solute content (Turner and Jones 1980). Linkage of the ABA level to turgor automatically takes into account the plant's success in making an osmotic adjustment, when ABA is produced as a signal of water stress. Several responses which could ameliorate water stress in plants, for instance stomatal closure as well as enhanced ion transport and water permeability of roots (Glinka 1980), may involve ABA as a signal.

Accumulation of ABA sets in before turgor of the bulk of the leaf has become zero (Fig. 1); conversion of ABA to PA is accelerated at the slightest indication of a regain of turgor (Fig. 8). Anticipatory production or removal of ABA could be caused by deviations from bulk properties in the solute content or the cell-wall elasticity of individual cells in the tissue (see also Pierce and Raschke 1980a).

Changes in ABA levels are net changes (this paper; Zeevaart 1980). Conversion of ABA to PA continues when ABA is being synthesized in wilted tissue and, coupled with a slackening of ABA synthesis, leads to a steady state. Synthesis of ABA continues for about 1 h after turgor has been regained and conversion of ABA to PA has been accelerated. Thereafter, synthesis of ABA declines.

Of the questions arising from this work, what the turgor sensor is and how it causes changes in the opposing mechanisms of synthesis and metabolic removal of ABA appear particularly important and challenging.

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