

Short Communication

Increased Abscisic Acid Biosynthesis during Plant Dehydration Requires Transcription¹

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ABSTRACT

Excised pea plants were rapidly dehydrated to turgor pressures of 1.5 to 2.0 bars. After a 30 minute lag, abscisic acid (ABA) levels increased approximately 100-fold in the dehydrated plants. Pretreatment of plants with the transcription inhibitors actinomycin D or cordycepin or with an inhibitor of cytoplasmic protein synthesis prior to plant dehydration inhibited the synthesis of ABA. These results suggest that dehydration induced synthesis of ABA requires nuclear gene transcription.

Dehydration of plants has been shown to cause ABA levels to increase up to 50-fold (6). This increase in ABA is due to *de novo* biosynthesis (7, 13). A key factor which triggers ABA accumulation is a loss of cell turgor (10) or cell membrane perturbation which occurs as pressure potentials approach zero (1). Exactly how perturbation of the cell wall/plasmalemma is coupled to increased ABA biosynthesis is unknown. However, it has been recently reported that inhibitors of cytoplasmic translation can block dehydration induced increases in ABA (12). A model was proposed suggesting that enzymes required for ABA synthesis are encoded on nuclear DNA (11). In this paper we show that transcription is required for ABA induction in dehydrated plants, suggesting that a change in nuclear gene expression is required to produce elevated levels of ABA during plant water stress.

MATERIALS AND METHODS

Plant Growth, Dehydration, and Inhibitor Treatments. *Pisum sativum* (Progress No. 9) was grown for 10 d in a model E15 Conviron growth chamber with continuous illumination at 23°C. Well watered plants were excised with their stems submerged in water and were placed in 1 mM HEPES-KOH (pH 8.0) for pretreatment. Inhibitors were added to the 1 mM HEPES-KOH at the following concentrations; CHI² at 20 µg/ml, CAP at 100 µg/ml, actinomycin D at 25 µg/ml, and cordycepin at 25 µg/ml. The pretreatments were done with illumination at 23°C for 1 h. Plants were then removed from the pretreatment solution, weighed, and transferred to darkness where they were dehydrated under a stream of cool air for 15 min. Dehydration caused a 10 to 15% loss in fresh weight. Following dehydration the plants

were incubated in a dark, humid chamber. Five to 10 plants were used per assay.

Water Status Measurements. The water potential of pea leaves was measured with a thermocouple psychrometer using the isopiestic method (2). Osmotic potential measurements were done on cell sap which was expressed from pea leaves using a syringe. The osmotic potential of pea leaf contents was also measured after freezing pea leaves over Dry Ice and thawing. No correction was made for dilution of protoplast solution by cell-wall water after freezing and thawing since cell-wall volume was only 4% of the total cell volume (8). Turgor was calculated as the difference between the water potential and the osmotic potential. All tissue manipulations were carried out in a humid chamber, and one leaf from three different plants was bulked and used for each water status determination.

ABA Assays. Pea plants (5–10 plants, 1–5 gfw) to be analyzed for ABA content were extracted by grinding in acetone:acetic acid (99:1, v/v) and 100 mg/L 2,6-di-*tert*-butyl-4-methylphenol according to Zeevaert (13). All analytical work was done under dim light to prevent geometric isomerization of ABA. Approximately 20,000 cpm of (±)-[³H]ABA (39 Ci/mmol) were added to the initial ABA extract as an internal standard to allow correction for variation in ABA recovery. This corresponded to 0.6 ng ABA. The extract was then centrifuged at 10,000 *g* for 10 min, 4°C and the supernatant saved. The pellet was reextracted with the acetone mixture and the supernatants pooled and stored under N₂ at –20°C. The pellet was reextracted a third time with the acetone mixture by stirring for 12 h, under N₂ at 4°C. After extraction and centrifugation the supernatant from the third extraction was pooled with the first two acetone extracts. Five ml of 1 mM EDTA were added and the combined extract was then evaporated in a Rotovap (Brinkman Instruments) to remove acetone. The ABA extract was then partitioned 3 times against diethyl ether at pH 9 to 10 (ether phase discarded) and 3 times against diethyl ether at pH 3 (ether phase retained). The ether phase from the last extraction was evaporated to dryness and resuspended in 100% methanol. A portion of each sample was separated by HPLC on a 4.5 × 250 mm 5 µm C₁₈ reversed-phase column at a flow rate of 1 ml/min. ABA was eluted from the column using a 35 to 100% methanol (v/v) gradient which was developed over 30 min. Solvent A was 10% methanol (v/v) with the pH adjusted to pH 3 with acetic acid. Solvent B was 100% methanol. ABA fractions, assayed by following the elution profile of an authentic (±)-ABA standard and the [³H]ABA standard, were collected, evaporated to dryness, and resuspended in the mobile phase for the silica column. A portion of each sample was then separated by HPLC on a 4.5 × 250 mm 5 µm silica column (5) at a flow rate of 1 ml/min. The mobile phase for the silica column was chloroform: acetonitrile:acetic acid (85:14.85:0.15, v/v). Depending on the flow rate [³H]ABA eluted

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² Abbreviations: gfw, grams fresh weight; CHI, cycloheximide, CAP, chloramphenicol.

from the silica column between 12 and 20 min. The ABA peak was collected, evaporated to dryness, and a portion counted in a scintillation counter to ascertain recovery of [³H]ABA. The amount of ABA eluted from the silica column was determined by preparing a standard curve using an ABA standard (Sigma Chemical Co.) while monitoring *A* at 269 nm. Results of ABA quantifications are based on ng ABA/gfw of tissue where fresh weight is measured following the excision and pretreatment in Hepes solution for 1 h.

GC was used to verify that the designated ABA peak eluting from the HPLC silica column did not contain comigrating impurities. Standard grade (±)-ABA was separated on the silica column, the ABA peak was collected, evaporated to dryness, and methylated with 1 ml of freshly prepared diazomethane. A plant sample from the ABA induction experiments was treated similarly. Both solutions were evaporated to dryness, resuspended in hexane, and analyzed using a Varian 3400 gas chromatograph equipped with electron capture detection.

All solvents were HPLC grade. [³H]ABA was purchased from New England Nuclear. Diazomethane was prepared using the mini-diazald preparation kit from Aldrich Chemical Co. All other chemicals were from Sigma Chemical Co.

RESULTS AND DISCUSSION

ABA Assays. An ABA assay was developed which is based on the following: partitioning properties of ABA at high and low pH (3). The separation of ABA by HPLC on a C18 reversed phase

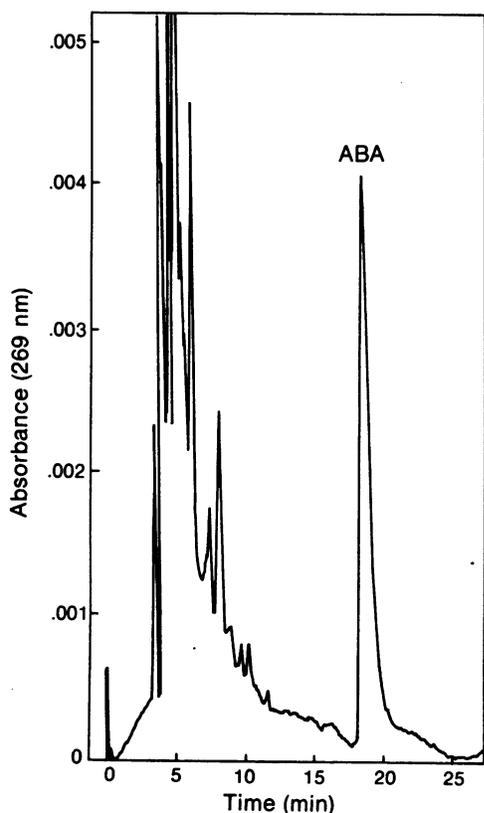


FIG. 1. HPLC chromatogram of the final step in the quantitation of ABA. The sample shown was extracted from pea leaves (0.16 gfw) which had been dehydrated for 15 min and incubated for 4 h. ABA fractions were separated by HPLC on a C₁₈ column, then evaporated, resuspended in the silica column mobile phase, and separated by HPLC on a 4.5 × 250 mm silica column. Flow rate was 0.85 ml per min. The peak of ABA (310 ng) indicated in the figure (elution 19.4 min) was identified using a [³H]ABA standard.

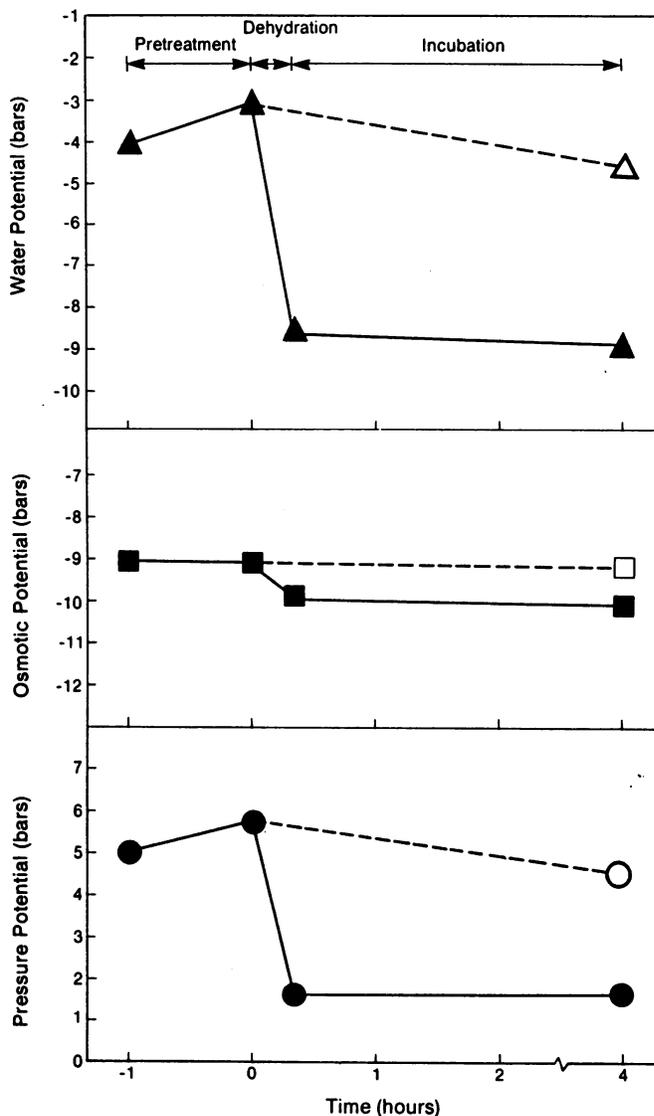


FIG. 2. Water status of excised pea plants after various treatments. Measurements of water potentials, osmotic potentials and calculated pressure potentials were obtained on leaves taken from intact plants, after 1 h pretreatment in light at 23°C in 1 mM Hepes-KOH, after pretreatment plus a 15 min dehydration period (23°C, dark) and after a 4 h incubation period (23°C, dark) following dehydration (solid symbols). The water status of excised plants which were pretreated and then incubated for 4 h in the dark at 23°C without dehydration was also determined (open symbols). Errors for each assay point are within the symbols. Data for one experiment are shown. Two additional experiments gave similar results although each set of plants varied in initial water potential by as much as 2.5 bars. The water status points after 15 min dark dehydration were calculated for the experiment shown based on three experiments which showed a 0.2 to 0.7 bar decrease in water potential during the 4 h incubation. The data shown correspond to experiment 1 of Table I.

column (13), and separation of ABA by HPLC on a silica column (5). Each of these steps has been used previously in ABA purification procedures. The particular combination used here gives 60 to 85% recovery of ABA and has a detection limit of 1 ng ABA. This procedure allows for sample recovery and is relatively simple and fast. Following the ether partitioning step, approximately 2 h per sample are required for quantification with little sample handling and minimal potential for losses. In addition

Table I. ABA Content of Pea Plants after Various Treatments

Pretreatment	Dehydrated	Incubated	ABA Content ^a			
			Exp 1 ^b	Exp 2 ^c	Exp 3 ^c	Exp 4 ^b
			% of dehydrated control			
1. None	No	No	4			
2. Hepes	No	No	2	2	7	2
3. Hepes	No	4 h	12			
4. Hepes	Yes	4 h	100	100	100	100
5. CHI	Yes	4 h		32	33	
6. CAP	Yes	4 h		95	100	
7. Actinomycin D	Yes	4 h			40	<2
8. Cordycepin	Yes	4 h			53	<2

^a ABA concentration in dehydrated controls (line 4) was 600 to 800 ng/gfw in 8 d old plants and 1000 to 3000 ng/gfw in 10 d old plants. ^b Ten d old plants; experiment 1, 1 h pretreatment; experiment 4, 2 h pretreatment. ^c Eight d old plants; 1 h pretreatment.

the reproducibility for the purification procedure (two triplicate samples) was $\pm 10\%$. An example of the separation of ABA at the final step of purification is shown in Figure 1 (*i.e.* separation on the silica column). To determine if the ABA peak from the HPLC silica column was contaminated by other compounds, an ABA standard and ABA extracted from pea leaves were run on the silica column, the ABA peaks were collected, methylated, and analyzed by GC. This analysis showed that no compounds comigrated with ABA at the HPLC silica column quantification step (results not shown). It has previously been shown that ABA can be separated from *t*-ABA by GC (4). Scintillation analysis of eluted peaks showed that 100% of [³H]ABA injected on the HPLC silica column was accounted for in one ABA peak. Analysis of the ABA peak by gas chromatography did not show the presence of *t*-ABA.

ABA Induction by Plant Dehydration. Leaf water potential measurements during pretreatment, dehydration and incubation are shown in Figure 2. Excised plants could be pretreated in a 1 mM Hepes-KOH (pH 8.0) solution for 1 to 2 h without significant induction of ABA (Table I). Other than the dehydration, no adverse effects from the plant pretreatments were noted. Excised plants, pretreated for 1 h in 1 mM Hepes and subsequently dehydrated, synthesized large amounts of ABA during a 4 h incubation (Table I, line 4). Conversely, excised plants pretreated for 1 h in 1 mM Hepes but not dehydrated did not show appreciable changes in ABA with or without incubation (Table I, line 2, 3). During the preincubation, leaf water potential and pressure potential increased by approximately 1 bar (Fig. 2). If plants were placed in a dark humid chamber without a dehydration treatment, water and pressure potentials gradually decreased 1 to 2 bars over 4 h (Fig. 2, dotted line). Associated with the decrease in water and pressure potential was a 6-fold increase in tissue ABA content.

To determine the effect of leaf dehydration on ABA induction, plants were dehydrated for 15 min and subsequently transferred to a dark humid chamber for 4 h. Leaf water potentials and ABA levels were determined at selected points during dehydration and the subsequent incubation period (Fig. 2, Table I). Dehydration of plants for 15 min was found to lower the leaf water potential 4 to 6 bars and to cause a decrease in osmotic potential of 1 bar (Fig. 2, solid line). This corresponded to a decrease in pressure potential to a final level of 1.5 bars. Incubation of the dehydrated plants in dark humid chambers resulted in a slight decrease in water and pressure potential of less than 0.5 bar over a 4 h incubation (Fig. 2).

Pierce and Raschke (10) reported that decreasing a plant's pressure potential to 1 bar caused a 4- to 8-fold increase in ABA, whereas maximal ABA induction (50-fold) occurred at zero turgor. The onset and extent of ABA induction was somewhat

species specific (10). Under the present experimental conditions, no increase in ABA occurred for 30 min following dehydration. ABA levels then increased linearly to 1000 ng/gfw over a 4 h period (data not shown). This represents approximately a 100-fold induction of ABA, indicating that in peas a decrease in leaf pressure potential to 1.5 to 2.0 bars is sufficient to cause large increases in ABA. It is possible that under the conditions of rapid dehydration used here some leaf cells are actually at zero turgor and could promote a significant ABA induction. The present method detects only the average leaf water status.

Effect of Inhibitors on ABA Induction. We next tested the effect of various inhibitors on the dehydration induced increase in ABA. The water status of plants treated with inhibitors was not measured. However, all inhibitor treatments involved dehydrated plants and the pretreatment of plants with inhibitors did not alter water loss imposed in our experiment. Optimal conditions for treating plants with CHI and CAP were previously determined by following [³⁵S]methionine incorporation into chloroplast polypeptides (9). Initial experiments were done with 8 d old peas and using a 1 h pretreatment with inhibitors prior to dehydration (Table I). The results from this experiment showed that CHI (inhibitor of 80S ribosomes) inhibited ABA induction 60 to 70% whereas CAP (inhibitor of 70S ribosomes) did not alter ABA biosynthesis. Inhibition of dehydration-induced ABA biosynthesis by CHI but not CAP is consistent with the results of Quarrie and Lister (12) on wheat. In experiment 3 (Table I), 1 h pretreatment with actinomycin D or cordycepin (transcription inhibitors) inhibited ABA induction 50 to 60%. A second experiment was done with 10 d old plants using a 2 h inhibitor treatment (experiment 4, Table I). This experiment showed similar inhibition patterns but with actinomycin D and cordycepin inhibiting all dehydration-induced ABA accumulation. The inhibition we observe using actinomycin D and cordycepin indicates that transcription is required for dehydration-induced ABA biosynthesis. This conclusion assumes that the use of the inhibitors did not introduce other side effects which would interfere with ABA biosynthesis. Nevertheless, these results suggest that dehydration-induced changes in turgor are coupled to increased ABA biosynthesis through a change in nuclear gene transcription. We are now in the process of obtaining more direct evidence for this possibility by examining changes in polyA⁺RNA during the time course of ABA induction.

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