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Hormonal Activity in Detached Lettuce Leaves as Affected by Leaf Water Content[1]

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NEHEMIA AHARONI, AMOS BLUMENFELD, AND AMOS E. RICHMOND[2]
Volcani Center, Bet Dagan, Israel

ABSTRACT

The interrelationship between water deficiency and hormonal make-up in plants was investigated in detached leaves of romaine lettuce (*Lactuca sativa* L. cv. 'Hazera Yellow'). Water stress was imposed by desiccating the leaves for several hours in light or darkness at different air temperatures and relative humidity. In the course of desiccation, a rise in abscisic acid content and a decline in gibberellin and cytokinin activity were observed by gas-liquid chromatography, by both the barley endosperm bioassay and radioimmunoassay and by the soybean callus bioassay. Gibberellin activity began to decline in the stressed leaves before the rise in abscisic acid, the rate of this decline being positively correlated with the rate of increase in leaf water saturation deficit. Recovery from water stress was effected by immersing the leaf petioles in water while exposing the blades to high relative humidity. This resulted in a decrease in leaf water saturation deficit, a reduction in abscisic acid content, and an increase in gibberellin and cytokinin activity.

Application of abscisic acid to the leaves caused partial stomatal closure in turgid lettuce leaves, whereas treatment with gibberellic acid and kinetin of such leaves had no effect on the stomatal aperture. In desiccating leaves, however, gibberellic acid and kinetin treatment considerably retarded stomatal closure, thus enhancing the increase in leaf water saturation deficit. These results suggest that the effect of desiccation in changing leaf hormonal make-up, *i.e.* a rapid increase in abscisic acid and a decrease in both cytokinin and gibberellin activity, is related to a mechanism designed to curtail water loss under conditions inducing water deficiency.

In leaves, water stress causes a rapid increase in ABA content (15, 31) and a sharp decrease in cytokinin activity (18). ABA is known to induce stomatal closure with resulting decreased transpiration in many types of leaves (9, 16, 19, 23, 27, 28). Furthermore, a very close relationship between leaf ABA content and the extent of stomatal opening was found in leaves of plants exposed to a cycle of mineral deprivation or salination and subsequent recovery (7). Kinetin was reported to enhance transpiration in leaves (22, 24, 25, 27, 29), and partially to overcome the ABA effect of reducing transpiration in both attached (28) and detached (27) leaves. It was thus suggested that these hormonal changes were probably conducive to the maintenance of a balanced water economy in the intact plant, effecting increased water intake by the root system (12) and reducing water loss from transpiration in the leaves (15, 23, 28).

There is as yet no firm evidence for the involvement of other hormones in the course of plant adaptation to water stress (17). Treatment with GA_3 increased transpiration in excised barley leaves (22) but the hormone was ineffective when applied to excised oat leaves (24). Reid *et al.* (30) reported a marked reduction in the movement of GAs from the root to the shoot in flooded tomato roots where water stress occurred owing to reduced permeability of roots to water (21). This indicates that modification of gibberellin activity in the leaf could be a phenomenon characterizing water stress.

The present work deals with an attempt to elucidate the relationship between water content and hormonal make-up of the leaf.

MATERIALS AND METHODS

Plant Material. All of the experiments were performed with fully expanded, mature leaves of romaine lettuce (*Lactuca sativa* L. cv. 'Hazera Yellow'), grown under field conditions during the period January to May. To overcome possible extreme effects of prevailing field conditions on leaf water status and to reduce the effects of wound caused by detachment of the leaves from the stems, the leaf petioles were immersed in water and placed in a humid chamber (100% relative humidity, 25 C, 100 lux) for a period of 18-24 hr immediately after detachment. This treatment will be referred to as "preconditioning."

Hormone Treatments. Kinetin (Calbiochem) was dissolved in H_2O for 20 min at 120 C in an autoclave. GA_3 (Sigma) and (±)-ABA (Hoffman-La Roche) were dissolved in ethanol and then diluted with water, an identical amount of ethanol being added to all comparable controls. The detached leaves were immersed in the hormone solutions for 30 min immediately after harvesting; subsequently the leaves were petiole-fed with the desired hormone throughout preconditioning.

Water Stress and Recovery Treatments. The detached leaves were placed horizontally on a table and exposed to room atmosphere for up to 6 hr. Details of air temperature and relative humidity are presented together with the results. To increase water loss, the leaves were illuminated by Cool White fluorescent lamps (2,500 or 5,000 lux) and exposed to forced air produced by an electric fan. A more moderate water loss was achieved by leaving the leaves in the laboratory in darkness, without forced air. After such inducement of water deficiency, the leaves were transferred to a ventilated humid chamber (100% relative humidity, 25 C, 100 lux) for recovery, the petioles being freshly trimmed at the cut ends and immersed in water.

Determination of Water Status in Leaves. The water status of the leaf blades was estimated in leaf discs by determining the WSD[3] (6) according to the following equation:

$$\text{WSD} = \frac{\text{SW} - \text{FW}}{\text{SW} - \text{DW}} \times 100$$

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[2] Dept. of Biology and the Institute for Desert Research at Sede Boqer, Ben-Gurion University of the Negev, Beer Sheva, Israel.

[3] Abbreviations: WSD: water saturation deficit; Me-ABA: methyl ester ABA; R: stomatal diffusion resistance.

where FW is the fresh weight of the leaf discs (24 mm diameter); SW is the weight of water-saturated leaf discs that were floated abaxially for 20 hr on distilled H_2O at 4 C while illuminated by a single incandescent bulb (200 lux); and DW is the dry weight of the leaf discs after dehydration for 24 hr in an oven at 85C. WSD was determined in four to eight replicates, each consisting of two discs excised from the distal third part of two half-blades.

Stomatal Diffusion Resistance. Resistance (R) was measured on the adaxial side of the leaf by a diffusion porometer (20). The R for zero time (before desiccation) was determined after the leaves had been exposed to light (5,000 lux) for 30 min, placed abaxially on moist filter paper, and covered with 0.01-mm-thick polyethylene sheeting to maintain high relative humidity.

Extraction and Separation of Endogenous Hormones. After removal of the petioles and midribs, the leaf blades were frozen in liquid N_2 and thereafter freeze-dried, ground, and stored desiccated in darkness at −18 C until being analyzed.

Weighed samples of the ground leaf tissue were homogenized for 5 min with 80% methanol (60 ml/g dry wt) in a Sorvall Omni-Mixer. The homogenate was shaken for 24 hr at 2 C and subsequently filtered through a Büchner funnel. The residue was shaken again with methanol for 1 hr and then filtered. The filtrates were combined and the organic solvent was evaporated under vacuum with a rotary evaporator (bath temperature 36–38 C). The hormones in the extract were separated by fractionation in a manner essentially similar to that described by Barendse *et al.* (5). GA and ABA activity were determined in the ethyl acetate fraction (pH 2.5); and cytokinin activity was determined in the acidic aqueous fraction (pH 2.5). The aqueous fraction was also subjected to acidic hydrolysis (5) and the GAs were transferred to ethyl acetate after the pH was adjusted to 2.5.

The fraction in which hormone activity was to be tested was evaporated under vacuum, dissolved in 100% methanol, loaded on strips of chromatography paper (Whatman No. 3, 3 cm wide), and separated by ascending chromatography for 20 cm with isopropyl alcohol-28% ammonia-water (10:1:1, v/v) being used as developer. After drying, the strips were cut into 10 equal sections for determining hormone activity in the various assays.

Gibberellin Determination. The barley endosperm bioassay developed by Coombe *et al.* (8) as modified by Goldschmidt and Monselise (13), was used. Barley seeds (cv. 'Omer') were dehusked by being soaked in 60% H_2SO_4 solution for 3 hr and subsequently imbibed in sterilized water at 10 C for 20 to 22 hr. Two embryoless seeds were each placed in a bioassay vial, containing 1.2 ml sterile water and a section of the chromatogram strip from the 0.4 to 0.7 R_F zone (where GA activity was observed) or its eluate. The vials were rotated horizontally at 2 rpm for 30 hr at 30 C. Incubation was stopped by removing the vials to −18 C, at which temperature they were kept until analysis. The amount of reducing sugar in the incubation liquid was tested with Sumner reagent and determined spectrophotometrically at 550 nm. For relative comparison of activity, the response to GA_3 solutions at various concentrations, ranging from 10^{-9} M to 10^{-6} M, was also measured. The GA content of each R_F was calculated from a log dose response curve which was fitted for each bioassay. This quantitative estimation enabled comparison of the changes taking place during leaf desiccation.

To nullify the effect of potential inhibitory factors in the extracts, ethyl acetate fractions were irradiated with UV light at 254 nm (2) at a height of 2 cm for 3 hr. Preliminary experiments showed that under such conditions, all of the ABA, as measured by GLC, was destroyed. As a result of UV irradiation, gibberellin activity increased without any change in its chromatographic location, which remained constant in the 0.4 to 0.7 R_F zone (1).

A radioimmunoassay, as described by Fuchs and Gertman (10), was used to verify the results of the barley endosperm bioassay. Since this assay is not sensitive to ABA (10), it enables detection of GAs even when they are not separated from inhibitors present in the chromatogram strips.

Abscisic Acid Determination. ABA was eluted twice with methanol from the 0.55 to 0.85 R_F zone of the chromatogram. This zone corresponded to (±)-ABA which was co-chromatographed and identified by UV light absorption. The combined eluates were subsequently evaporated under vacuum and the extracted ABA was then methylated with diazomethane. The Me-ABA was dissolved in 1 ml ethyl acetate and a 1-μl aliquot, equivalent to 1.5 mg dry wt of leaf tissue, was injected into a Packard gas chromatograph equipped with an electron capture detector with radioactive tritium foil. Separation was carried out on glass columns (1,800 × 3 mm) packed with 1.5% QF 1 on Gas-chrom Q, 60 to 80 mesh. Temperatures of the injection port, column, and detector were 210 C, 190 C, and 195 C, respectively, and the N_2 flow was 50 ml min^{-1}. Under these conditions, the retention time for Me-ABA was 6.75 min. The amount of Me-ABA in the extract was calculated from the height of the peak relative to that obtained when 10 to 500 pg Me (±)-ABA was injected. A standard containing approximately the same amount of Me-ABA as in the sample was injected after every three samples. Each sample was injected at least twice.

Cytokinin Determination. Cytokinin activity was determined by the soybean callus bioassay (26), according to Gazit and Blumenfeld (11).

RESULTS

In order to observe the detailed changes in the hormonal make-up of detached lettuce leaves during water stress, experiments were conducted in which hormone levels were investigated at various intervals starting from the initiation of leaf desiccation. ABA content increased while gibberellin activity decreased in the course of desiccation (Fig. 1). While the overall pattern of these hormonal changes was similar under different degrees of water stress, their extent differed. After 6 hr of desiccation under illumination of 2,500 lux at 25 C and 40 to 50% relative humidity which increased leaf WSD by 18.1%, ABA content increased 20-fold and gibberellin activity decreased to 10% of its initial level (Fig. 1A). The assay of chromatograms of the extracts taken before and after 6 hr of desiccation is also depicted in Figure 2, A and B. During a milder water stress of 6 hr performed in darkness, at 25 C and 60 to 70% relative humidity which caused a leaf WSD increase of

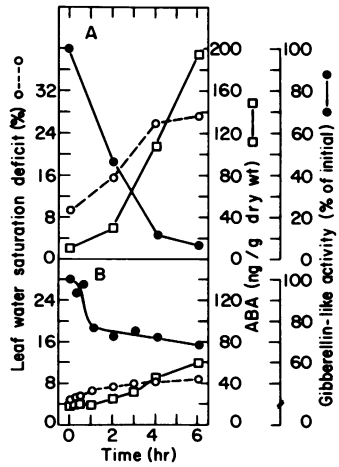

FIG. 1. Effect of the duration of desiccation on WSD, ABA content, and gibberellin-like activity in detached leaves. The leaves were exposed to dry air after 24 hr of preconditioning. A: desiccation under illumination (2,500 lux), 40 to 50% relative humidity, 25 C + forced air; B: desiccation in darkness, 60 to 70% relative humidity, 25 C. GA activity was determined by the barley endosperm bioassay in the acidic ethyl acetate fraction. GA content at zero time was 7.8 and 7.2 ng GA_3 equiv. g^{-1} dry wt in A and B, respectively.

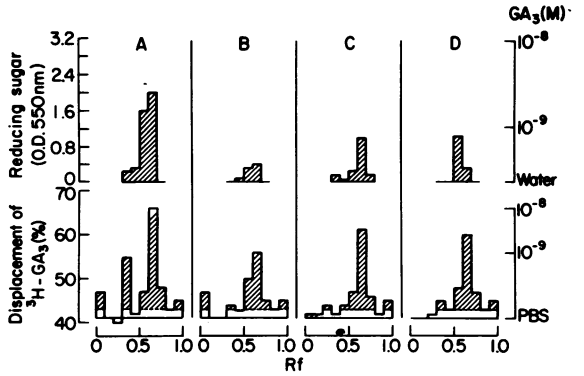

FIG. 2. Gibberellin-like activity in desiccating and recovered leaves. The activity was determined in the acidic ethyl acetate fraction. Upper row: measurements by the barley endosperm bioassay; chromatograms were loaded with 200 mg dry wt. Lower row: measurements by radioimmunoassay; chromatograms were loaded with 300 mg dry wt. Levels of GA in the radioimmunoassay are expressed as percentage of ^{3}H-GA_3 displaced from the immunoadsorbent column following its loading with extracts, or, for comparison, with unlabeled GA_3. Samples were dissolved in phosphate-buffered saline (PBS). Horizontal broken lines in the histograms in the lower row represent percentage of ^{3}H-GA_3 displacement double that of the SE of the PBS. A: 24-hr preconditioning (100% relative humidity, 25 C, 100 lux); B: 24-hr preconditioning as in A + 6-hr desiccation (40–50% relative humidity, 25 C + forced air, 2,500 lux); C: 24-hr preconditioning as in A + 6-hr desiccation as in B + 42-hr recovery as in A; D: 72-hr preconditioning as in A. Leaf WSD in A, B, C, and D were 9.2, 27.3, 5.1, and 6.8% respectively.

only 4%, (Fig. 1B), ABA rose only 4-fold and gibberellin activity was reduced to only 80% of its initial level. The purpose of the milder stress was to mitigate the changes in hormone levels, thereby permitting improved distinction between the patterns of hormonal modification. Under such moderate water loss, the first pronounced modificaton was the decline in gibberellin activity. This could not be observed under more severe conditions of water deficiency, since the first examination was conducted too late to establish the initial change in hormone levels.

For further exploration of the relationship between leaf water content and hormone activity, the stressed leaves were allowed to recover in a humid chamber for 42 hr at 25 C and 100% relative humidity. During recovery the leaves absorbed water through petioles which led to a decline in leaf WSD. The results obtained by the barley endosperm bioassay were verified by performing a radioimmunoassay of the same chromatographed extracts: this yielded similar results (Fig. 2). After desiccation for 6 hr the GAs declined (Fig. 2, A and B), returning to the nonstressed level following recovery (Fig. 2, C and D). The GA activity was located in the 0.4 to 0.7 R_F zone in the two assays. In the radioimmunoassay, an additional zone of GA-like activity was detected in R_F 0.3 to 0.4, which disappeared in water-stressed leaves and did not reappear upon recovery. In all experiments, performed either by bioassay or radioimmunoassay, the level of hydrolyzable ("bound") GAs analyzed in the acidic aqueous fraction was extremely low in both stressed and recovered leaves. The level of cytokinin activity in the acidic aqueous fraction of the same extracts, already low before initiation of the water stress (Fig. 3A), was undetectable after 2 hr of stress (Fig. 3B). After recovery, cytokinin activity rose to a level equal to, or higher than that found in comparable, nonstressed leaves (Fig. 3, C and D). Initially, ABA content in the turgid leaves was 11.5 ng·g^{-1} dry wt, rising after 6 hr of desiccation to 196.6 ng·g^{-1} dry wt. After 42 hr of recovery, the ABA content of the stressed leaves decreased to 15.1 ng·g^{-1} dry wt, comparing well with the relevant control which contained 16.6 ng·g^{-1} dry wt.

To examine hormonal effect on the water status of the leaf under conditions of water deprivation, detached leaves were treated before desiccation with various concentrations of GA_3, kinetin, and ABA, and the WSD was measured after 4 hr of desiccation (Fig. 4). Treatments with increasing concentrations of GA_3 and kinetin caused a continuous rise in WSD, except in the 10 μl/l kinetin treatment, which was apparently supraoptimal in its effect on leaf WSD. ABA treatment reduced the WSD in stressed leaves, the magnitude of the effect being proportional to the concentration of the hormone.

When a known concentration of a hormone was applied and the WSD was measured during desiccation, the different hor-

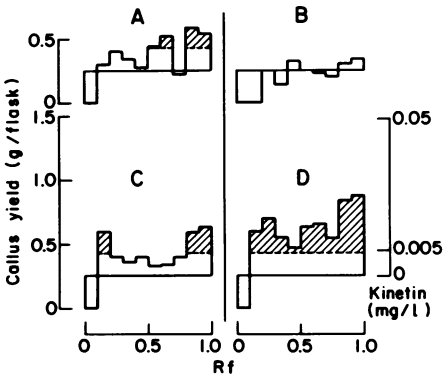

FIG. 3. Cytokinin activity in desiccating and recovered leaves. Activity was determined by the soybean callus bioassay in the acidic aqueous fraction; chromatograms were loaded with 300 mg dry wt. Horizontal broken line in each histogram represents callus yield double that of the SE of the control. A: 24-hr preconditioning (100% relative humidity, 25 C, 100 lux); B: 24-hr preconditioning as in A + 2-hr desiccation (40–50% relative humidity, 25 C + forced air, 2,500 lux); C: 72-hr preconditioning as in A; D: 24-hr preconditioning as in A + 6-hr desiccation as in B + 42-hr recovery as in A.

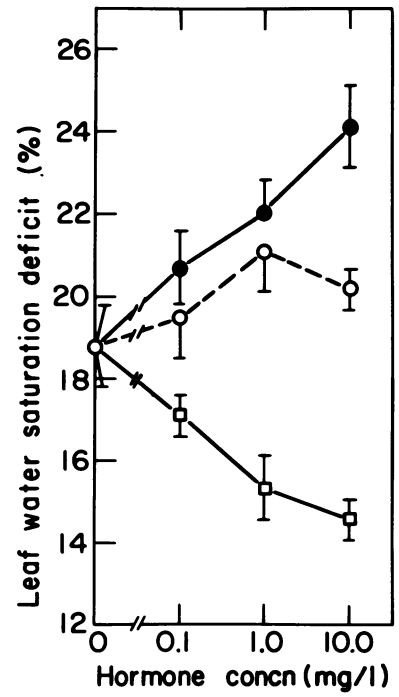

FIG. 4. Effect of pretreatment with various concentrations of GA_3 (●), kinetin (○), and ABA (□) on the WSD of detached leaves desiccated for a 4-hr period. Desiccation was performed under light (2,500 lux), with forced air at 50 to 55% relative humidity and 28 C. WSD of the control leaves before desiccation was 7.3%. Vertical lines represent the SE of the four replicates of each treatment.

mone effects became apparent after 1 to 2 hr (Fig. 5). Consequently, we examined the effects of the various hormones on the stomatal apertures as indicated by R values (Fig. 6). We observed that before the water stress was initiated (after a 30-min exposure of the turgid leaves to light, 5,000 lux, at high relative humidity, see zero time in Fig. 6), R values for ABA-treated leaves were higher than for the control, kinetin and GA_3-treated leaves. Stomatal closure induced by the stress treatment (5,000 lux, 35-40% RH) began after 30 min in all treatments but was much higher in the untreated leaves. There was no significant difference in the WSD of hormone-treated leaves prior to desiccation (average of 4.1% WSD). After 120 min of stress, WSD values were 16.9 ± 0.8% for GA_3; 17.1 ± 0.7% for kinetin, 14.7 ± 0.4% for control, and 12.5 ± 0.5% for ABA. WSD for GA_3 and kinetin leaves corresponded with their R values. WSD

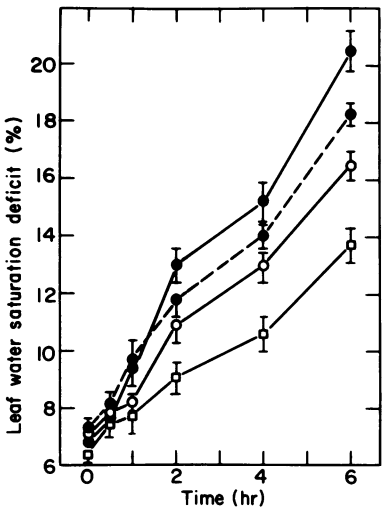

FIG. 5. Effect of GA_3, kinetin, and ABA on the WSD of detached leaves in the course of desiccation. Leaves were pretreated with 10 mg/l GA_3 (●——●), 5 mg/l kinetin (●- - -●), 10 mg/l ABA (□——□), or water (○——○) and desiccated under light (2,500 lux), at 27 C and 50 to 60% relative humidity. Vertical lines represent the SE of the eight replicates of each treatment.

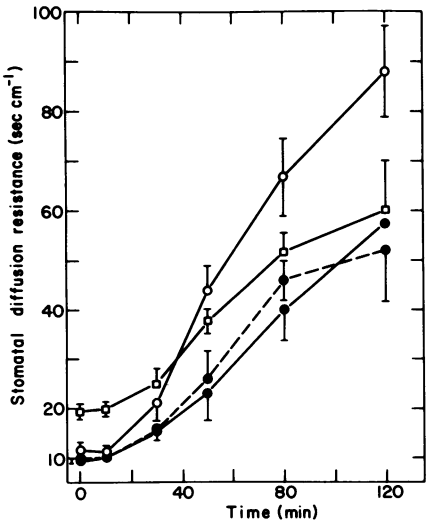

FIG. 6. Effect of GA_3, kinetin, and ABA on stomatal diffusion resistance in detached leaves during desiccation. Leaves were pretreated with 10 mg/l GA_3 (●——●), 5 mg/l kinetin (●- - -●), 10 mg/l ABA (□——□), or water (○——○). Desiccation was performed under light (5,000 lux) at 35 to 40% relative humidity and 30 C. Vertical lines represent the SE of four replicates (different leaves) of each treatment.

for ABA-treated leaves was lower than that of the control, apparently because of lower water losses during the first 30 min of desiccation. In our experimental conditions, pretreatments with kinetin or GA_3 had no significant effect on stomatal opening in turgid leaves that were exposed to light for 30 min under relative humidity. However, the effect of these hormones in retarding the rate of stomatal closure induced by water loss became clearly evident in leaves exposed to desiccation.

DISCUSSION

Desiccating detached lettuce leaves showed a rapid decline in gibberellin and cytokinin activity, and a rise in ABA content. These changes in hormonal activity were proportional to the intensity and duration of the stress. The desiccation-induced decline in GA activity could not have been an artifact of the bioassay, since the ABA in the extracts was destroyed by UV irradiation. Furthermore, the results stemming from changes in GA activity were corroborated by a radioimmunoassay which preliminary studies had shown to be independent of the effects of extractable inhibitors from lettuce leaves.

Gibberellin and cytokinin activity which became barely assayable after 6-hr desiccation, reverted to control level after 42 hr of recovery. Itai and Vaadia (18), who first reported on decreased cytokinin activity after water stress and its increase following recovery, suggested that these changes resulted from inactivation of the hormone and subsequent reversal of this inactivation. Our data support these findings without having any bearing on the detailed mechanism involved in the modifications in cytokinin activity induced by water stress. The decline in gibberellin activity was closely related to the rise in leaf WSD, and preceded the start of the rise in ABA (Fig. 1). Also, the GA decrease in the desiccating leaves leveled off long before the rise in ABA, which continued to increase well after the increase in leaf WSD had reached equilibrium. Thus, under conditions of moderate desiccation, the sharp decline in GA activity took place for only the first hr (Fig. 1B), whereas under severe desiccation it progressed for 4 hr (Fig. 1A).

During leaf desiccation, gibberellin and cytokinin activity declined, rising again upon leaf recovery, whereas ABA in similar conditions increased rapidly and then declined to prestress levels. These findings support the thesis that in detached lettuce leaves, the levels of these hormones and leaf water status are related.

While exogenous GA_3 and kinetin did not affect light-induced stomatal opening in turgid, nonstressed leaves, these hormones considerably retarded stomatal closure during water stress, thereby accelerating the wilting process (Fig. 6). The marked hormonal effects on stomatal aperture and on the extent of transpiration in water-deficient leaves effected an unusual relationship between R values and leaf WSD. As indicated in Figure 6, the R value of the ABA-treated leaves which were fed with ABA for 24 hr before the experiment began is already significantly greater at zero time. This is in fact expected, owing to the effect of the hormone on stomatal closure, and the reason for the WSD of the desiccating leaves being lowest in those treated with ABA. Thus, the effect of ABA on R in the course of leaf desiccation simply reflects the relative effect of this hormone, as compared with that of the others, on the maintenance of cell water during desiccation. With regard to the over-all effect on the stomatal aperture then, the initial effect of ABA on prevention of rapid water loss finally resulted in more limited stomatal closure than observed in nontreated leaves. In the latter, accelerated water loss followed by rapid stomatal closure and increased R occurred soon after the leaves were exposed to desiccation. Also understandable was the discrepancy between WSD and R in desiccating leaves treated with GA and kinetin. In contrast to the pronounced stomatal closure caused by ABA, kinetin and GA treatments retarded closure. This led to the

fastest water loss and the highest WSD (Fig. 5) which, rather than effecting the highest R due to loss of turgor, brought about the lowest R because of stomatal reaction to the specific influence of the hormone on extending the stomatal aperture.

This paper presents further evidence for the involvement of the hormonal system in regulating plant water balance during water deprivation, (3, 4). On the one hand, leaf hormone levels markedly change when cell water decreases. On the other hand, the hormonal make-up of the cells in itself modifies the extent of water loss from the leaves, clearly bearing upon cell water and thus upon the metabolism (3, 4) and senescence (14) of the leaf.

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