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Biosynthesis of Stress Ethylene Induced by Water Deficit[1]

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ABSTRACT

Wheat leaves normally produced very little ethylene, but following a water deficit stress which caused a loss of 9% initial fresh weight, ethylene production increased more than 30-fold within 4 hours and declined rapidly thereafter. The changes in ethylene production were paralleled by an increase and subsequent decrease in 1-aminocyclopropanecarboxylic acid (ACC) content. The level of *S*-adenosylmethionine was unaffected, suggesting that the conversion of *S*-adenosylmethionine to ACC is a key reaction in the production of water stress-induced ethylene. This view was further supported by the observation that application of ACC to nonstressed leaf tissue caused a 70-fold increase in ethylene production, while aminoethoxyvinylglycine, a known inhibitor of the conversion of *S*-adenosylmethionine to ACC, inhibited ACC accumulation as well as the surge in ethylene production if the inhibitor was applied prior to the stress treatment. Cycloheximide, an inhibitor of protein synthesis, effectively blocked both ethylene production and ACC formation, suggesting that water stress induces *de novo* synthesis of ACC synthase, which is the rate-controlling enzyme in the pathway of ethylene biosynthesis.

Subjecting plants or detached leaves to water deficit stress results in a surge in ethylene production (4, 8, 16, 18). In avocado fruits it has been shown (3) that an accelerated loss of fresh weight due to water deficit hastened the peak of ethylene production and thus resulted in earlier fruit ripening. Increased ethylene production by tissues which normally produce very little ethylene has been reported to occur as a response to the stresses represented by drought, mechanical wounding, and radiation (4, 10, 11, 13, 16, 19, 24). As ethylene is a plant hormone known to be involved in many processes, including growth, development, ripening, and senescence (1); this stress-induced ethylene may assist the plant to cope with stress conditions. It has been suggested that under drought conditions ethylene production would cause leaf abscission, and thereby reduce water loss (16).

Adams and Yang (2) have studied ethylene biosynthesis in apple tissue and established the following biosynthetic pathway: methionine → SAM[3] → ACC → ethylene. The validity of this pathway has since been confirmed in other systems (20), including auxin-induced ethylene production (23) and stress-induced ethylene production (6, 24). The present study was conducted to determine which step in the ethylene biosynthetic pathway is responsible for the surge of ethylene production caused by water deficit stress.

MATERIALS AND METHODS

Plant Material. Wheat (*Triticum aestivum* L. cv. Anza) seeds were soaked for 3 h in distilled H_2O and sown in vermiculite. Seedlings were grown in a growth chamber under a 14-h photoperiod with a light intensity of 80 μE m^{-2} s^{-1} provided by a mixture of cool-white and Grolux tubes at 22 C and a RH of 70%. Four h after the beginning of the ninth light period, primary leaves (10–12 cm long) were excised above the coleoptile and trimmed to 8 cm.

Water Stress. The technique used was described by Wright (18). Batches of excised leaves (1 g) were allowed to wilt while spread in Petri dishes at room temperature (20–22 C) and 55% RH. When the leaves reached the desired degree of water stress, as indicated by loss of fresh weight (it took about 30 min to reach 9% weight loss and 10–15 min to reach 2% weight loss), they were inserted into 12-ml glass test tubes (15 × 90 mm) which were then closed with serum caps for the desired period. Nonstressed leaves used as controls were inserted in test tubes containing several drops of water to maintain high RH. The sealed tubes were kept in the growth chamber at 22 C under light. Gas samples (1 ml) were taken periodically from each tube for ethylene analysis. The tubes were flushed with ethylene-free air every 2 h. After the final sample was taken, the leaves were reweighed to determine loss of fresh weight during the sampling period. The total water loss, expressed as per cent of initial fresh weight, was used as an expression of the degree of water stress.

Feeding Technique. Leaves were placed in small beakers with the cut surfaces dipped in aqueous solutions of the precursor or inhibitor at the concentrations indicated. The beakers were placed for 3 to 4 h in a growth chamber under light as described above and the leaves were then subjected to water stress as described.

Chemicals. SAM was obtained from Boehringer, and AVG was a gift from J. P. Scannel (Hoffman-LaRoche). ACC was purchased from Calbiochem, CHI from Sigma, and SP-Sephadex from Pharmacia Fine Chemicals.

Determination of Ethylene. Gas samples (1 ml) withdrawn from the tubes to be tested were injected into a gas chromatograph equipped with an alumina column and flame ionization detector.

Determination of ACC. The tissue was homogenized in 70% ethanol. After centrifugation, the ethanol was evaporated under vacuum at 40 C. The residue was dissolved in 1 ml water, and an aliquot was used for assay of ACC according to the method of Lizada and Yang (12). The sample to be assayed was placed in a 10-ml test tube in an ice bath, 1 μmol $HgCl_2$ was added, and the volume was brought to 0.4 ml with water. The tube was then sealed with a rubber serum cap. Approximately 100 μl cold mixture of 5% NaOCl and saturated NaOH (2:1, v/v) was injected into the tube. The reaction mixture was kept in ice for 5 to 10 min, the tubes were shaken vigorously, and the ethylene liberated was assayed by GC. The efficiency of the conversion of ACC to ethylene by this method in the presence of the ethanol extract of

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[3] Abbreviations: SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine[2-amino-4-(2'-aminoethoxy)-*trans*-3-butenoic acid]; CHI, cycloheximide.

the tissue was determined by adding a known amount of authentic ACC as an internal standard. The yield was usually between 66 and 70%. The amount of ACC was calculated from the quantity of ethylene liberated and the conversion efficiency.

Determination of SAM. SAM was isolated according to the method of Glazer and Peale (9). Batches of wheat leaves (3 g) were homogenized and extracted with 15 ml cold 10% TCA (w/v). After centrifugation at 10,000*g* for 10 min at 4 C, a known amount of [^{14}C]SAM was added as an internal standard used to monitor recovery during purification. The supernatant was extracted three times with water-saturated ether to remove the TCA. One ml 0.15 M HCl was added to the sample and it was passed through a column (1 × 5 cm) of SP-Sephadex C-25 equilibrated with 10 mM HCl. The column was washed with 0.15 M HCl until the effluent showed no absorption at 257 nm. SAM was then eluted with 15 ml 0.5 M HCl and the eluate was collected in 2-ml fractions which were used for determination of total SAM. Profiles of radioactivity in the various fractions were found to correspond to adsorptivity at 257 nm. To verify the presence of SAM, the adsorption spectrum of the eluate was compared with that of authentic SAM. The concentration of SAM was determined spectrophotometrically, assuming a molar adsorptivity of 15,000 at 257 nm. Recovery, based on the radioactivity of SAM, ranged from 65 to 72%. All experiments were repeated three times with two or three replicates for each treatment. The data presented are results obtained from a representative experiment.

RESULTS AND DISCUSSION

Wheat leaves which were excised 4 h after the beginning of the ninth light period and kept in a humid atmosphere produced ethylene at a low rate ranging from 0.3 to 0.6 $nl \cdot g^{-1} \cdot h^{-1}$ for 24 h after excision (Fig. 1). If these leaves were subjected to a water deficit stress, increased ethylene production was observed. Stress ethylene production was small but apparent when water loss reached 2% of the initial fresh weight. This production increased progressively to a maximum rate when the water loss reached 9%, but then declined as the water loss continued (Fig. 2). The maximum rate of ethylene production for leaves which had lost 9% of their initial fresh weight was about 37 $nl \cdot g^{-1} \cdot h^{-1}$ 4 h after the end of the stress period (Fig. 1). Thereafter, ethylene production declined, falling to 8 $nl \cdot g^{-1} \cdot h^{-1}$ after 12 h and to the control level after 24 h (Fig. 1). Similar results have been reported by Wright (18) with wheat leaves and by others with various tissues (4, 8, 11, 16).

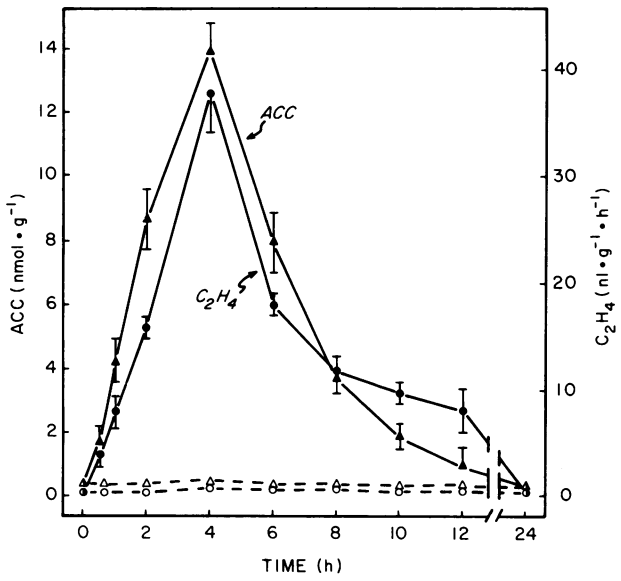

FIG. 1. Time course for the effect of water stress on ethylene production and ACC content in wheat leaves. Leaves were subjected to water stress conditions until they lost 9% their initial fresh weight (about 30 min at 22 C and 55% RH) and enclosed in sealed, 12-ml test tubes (1 g each). Nonstressed control leaves were enclosed in test tubes containing several drops of water to maintain high RH. Ethylene production and ACC content were determined periodically. The tubes were flushed with ethylene-free air every 2 h to prevent O_2 depletion and CO_2 accumulation. Vertical bars indicate ± SE limits. The values of ACC and ethylene were expressed per g of initial fresh weight.

The ACC content of nonstressed wheat leaves was very low ranging between 0.4 and 0.6 $nmol \cdot g^{-1}$ tissue during 24 h after the leaves were harvested (Fig. 1). In leaves stressed by water deficit, the ACC content first increased and subsequently declined as the stress was increased. These stress-induced changes in ACC content paralleled the changes in the rate of ethylene production (Fig. 1). The sharp rise in both ethylene production and ACC content became apparent when weight loss reached about 2% initial fresh weight, and continued to a peak value at a loss of about 9% initial weight (Fig. 2). The sharp reversal in the changes in ACC content and rate of ethylene production as the loss of weight exceeded 9 or 10% initial weight may reflect damage to the tissue.

When ACC was administered to nonstressed leaves, ethylene production was increased 70-fold (Table I). In this, as in other tissues (7, 20), ACC is probably the rate-limiting factor for ethylene production. The induction of ethylene production by water deficit stress, as by auxin treatment (22, 23) or by stress conditions such as waterlogging (6) and wounding (24), seems to cause an increase in ACC synthesis which ultimately results in increased ethylene production. This view was further supported by the analysis of the content of SAM and ACC. While water stress stimulated ethylene production and caused accumulation of ACC, it did not affect the level of SAM (Table II). These data are consistent with the view that the step converting SAM to ACC is rate-limiting and is accelerated by stress, whereas the step converting methionine to SAM is not rate-limiting, thereby the level of SAM can be maintained even when the conversion of SAM to ACC is accelerated. When AVG, a known inhibitor of ACC

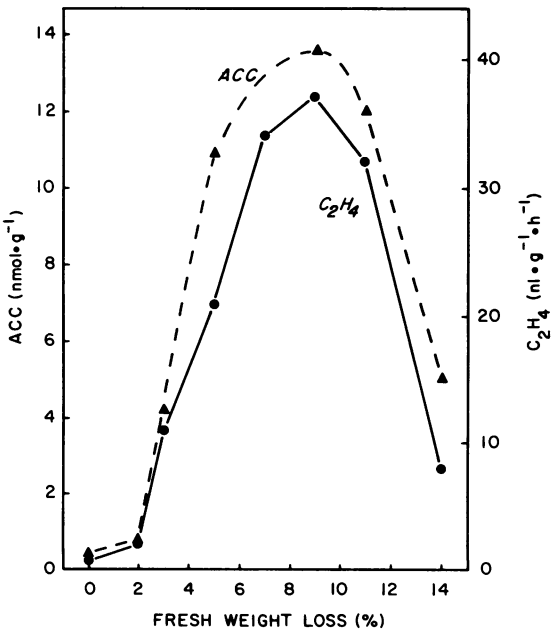

FIG. 2. Effect of the severity of water stress on ethylene production and ACC content in wheat leaves. Leaves were subjected to water stress conditions at 22 C and 55% RH for various lengths of time until they reached the indicated weight loss. Ethylene production and ACC content were determined 4 h after the end of the water stress treatment.

Table I. *Effect of CHI, AVG, and ACC on Ethylene Production and ACC Content in Water-Stressed Wheat Leaves*

Leaves were preincubated for 4 h with the cut ends dipped in H_2O or in solutions containing the precursor or inhibitor at the indicated concentration. At the end of the preincubation period, leaves were subjected to water stress conditions until they lost 9% their initial fresh weight (about 30 min at 22 C and 55% RH). Ethylene production and ACC content were determined 4 h after the end of the water stress treatment. Results presented are the mean ± SE of three replications.

Treatment	Ethylene	ACC
	$nl \cdot g^{-1} \cdot h^{-1}$	$nmol \cdot g^{-1}$
Nonstressed leaves		
H_2O	0.5 ± 0.08	0.4 ± 0.03
ACC, 0.5 mM	22.2 ± 1.7	126 ± 6
AVG, 0.05 mM	0.3 ± 0.04	0.5 ± 0.02
ACC + AVG	21.7 ± 1.9	106.0 ± 10
Water-stressed leaves		
H_2O	35.2 ± 2.3	12.8 ± 2.2
ACC, 0.5 mM	61.1 ± 5.0	126 ± 9
AVG, 0.05 mM	3.5 ± 0.2	1.2 ± 0.2
ACC + AVG	58.4 ± 4.7	115 ± 10
CHI, 0.5 mM	0.8	0.4

Table II. *Effect of Water Loss on Ethylene Production, ACC Content, and SAM Content in Wheat Leaf*

Leaves were subjected to stress conditions until 9% initial fresh weight was lost. Ethylene production, ACC content, and SAM content were determined 4 h after the water stress treatment. Results presented are the mean ± SE of three replications.

Treatment	Ethylene	ACC	SAM
	$nl \cdot g^{-1} \cdot h^{-1}$	$nmol \cdot g^{-1}$	
Non-water-stressed	0.6 ± 0.08	0.5 ± 0.03	38
Water-stressed	32.0 ± 2.5	17.0 ± 2.2	39

synthase, which catalyzes the conversion of SAM to ACC (2, 5), was applied to wheat leaves prior to water stress treatment, it inhibited both ACC accumulation and the surge of ethylene production by 90% (Table I). Since the accumulation of ACC in the tissue seems to be the direct cause for increased ethylene production, it is reasonable to assume that the decline in ethylene production after 4 h (Fig. 1) was due to the depletion of ACC below a critical level. However, the depletion of ACC could not be attributed entirely to its conversion to ethylene. ACC content of the tissue increased to 14 $nmol \cdot g^{-1}$ 4 h after the end of the stress treatment and rapidly decreased thereafter, declining to 1 $nmol \cdot g^{-1}$ at the end of 24-h incubation period. During the course of the accumulation and decline of ACC, however, less than 9 nmol ethylene were produced. These data suggest that ACC might be metabolized by some pathway other than ethylene production. The stimulatory influence of water deficit stress was not restricted to ACC synthesis. In addition to increased ACC synthesis, the capacity of the system to convert exogenous ACC to ethylene was approximately 3 times greater in water deficit-stressed leaves than in controls (Table I).

The formation of ACC from SAM is known to be catalyzed by ACC synthase (5, 21), and any increase in ACC must involve either activation or *de novo* synthesis of this enzyme. Our attempts to isolate and study the activity of ACC synthase from wheat leaves were not successful. If *de novo* synthesis of proteins is involved in water stress-induced ethylene production, it is expected that application of CHI, a known inhibitor of protein synthesis, would inhibit the stress-induced ACC accumulation and ethylene production. In tissue incubated with 0.5 mM CHI for 3 h prior to stressing treatment, both ACC synthesis and ethylene production were effectively blocked (Table I). The present results suggest that during the course of ethylene synthesis induced by water stress *de novo* synthesis of an enzyme, probably ACC synthase, is induced and is in turn responsible for the accumulation of ACC and the increase in ethylene production. The evidence indicates that the pathway of the biosynthesis of water stress-induced ethylene and its induction, are similar, if not identical, to those of stress ethylene induced by auxin (23) or mechanical wounding (24). The biochemical mechanism by which these different stimuli result in stimulation of the same biochemical step remains to be elucidated.

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