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A Possible Role for Abscisic Acid in Regulation of Photosynthetic and Photorespiratory Carbon Metabolism in Barley Leaves[1]

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ABSTRACT

The influence of abscisic acid (ABA) on carbon metabolism, rate of photorespiration, and the activity of the photorespiratory enzymes ribulose bisphosphate oxygenase and glycolate oxidase in 7-day-old barley seedlings (*Hordeum vulgare* L. var. Alfa) was investigated. Plants treated with ABA had enhanced incorporation of labeled carbon from $^{14}CO_2$ into glycolic acid, glycine, and serine, while ^{14}C incorporation into 3-phosphoglyceric acid and sugarphosphate esters was depressed. Parallel with this effect, treated plants showed a rise in activity of RuBP oxygenase and glycolic acid oxidase. The rate of photorespiration was increased twofold by ABA treatment at 10^{-6} molar while the CO_2-compensation point increased 46% and stomatal resistance increased more than twofold over control plants.

Intense research has been conducted in recent years to clarify the regulatory role of phytohormones on the photosynthetic process. ABA has been a primary object of study, since up to 90% of its total content in mesophyll cells is located within the chloroplast (6). Although the site of ABA biosynthesis has not been sufficiently documented (5, 14, 15), it has been indisputably demonstrated that ABA influences electron transport (1) involved in CO_2 fixation and reduction (17) and in carbohydrate and nitrogen metabolism (7, 22). ABA also inhibits formation of the membrane system of the plastids (8) and Chl biosynthesis (2). Compared with the control, leaves of ABA-treated plants have a lower Chl content, lower CO_2 fixation rate, and lower activity of RuBPCase[2] in barley and PEPCase in maize (17–19).

Two possibilities exist to explain the mechanism of action by ABA on photosynthesis: (a) an indirect effect mediated by stomatal closure causing a reduction in CO_2 supply (3); or (b) direct effect on the photosynthetic machinery, although this mechanism has not been clarified (20). According to the second hypothesis, ABA brings about changes in the adenylate system in the chloroplasts and reduces the energizing of the chloroplast membranes. The reduced capacity of the carboxylase reaction, RuBPCase, has not yet been explained. The assumption is that the RuBP regeneration capacity of the photosynthetic apparatus is more strongly affected by ABA treatments than is the carboxylation capacity. The possibility of changes in the kinetic properties of RuBP carboxylase-oxygenase have not been ruled out (20).

The aim of this paper was to investigate the influence of ABA on photosynthetic and photorespiratory carbon metabolism and the activity of the basic photorespiratory enzymes RuBP oxygenase and glycolate oxidase in barley. An attempt has been made in these investigations to clarify certain aspects of the hormonal regulation of photosynthesis and photorespiration.

MATERIALS AND METHODS

Plant Material. Seeds of barley (*Hordeum vulgare L. var. Alfa*) were germinated for 3 d in two layers of moist filter paper in moist vermiculite at 25°C in the dark. Seeds were then transferred in Petri dishes (9 cm diameter) containing 40 ml distilled H_2O or equal amounts of water solutions from the required ABA concentrations (10^{-6} M to 10^{-4} M ABA). The solutions were changed every 24 h. ABA treatment was for 7 d. During the experimental period, seedlings grew in a growth chamber under white fluorescent lamps (35 W m^{-2}) with 12 h light and dark periods. Day/night temperatures were 25/20°C; RH was about 50%.

$^{14}CO_2$ Fixation and Analysis of ^{14}C Products. Photosynthetic rates were measured using leaf slices by the method of Rathnam and Chollet (21). With the use of a sharp razor blade, 1 g of leaf blade tissue was cut perpendicular to the veins into 1-mm slices. Slices were incubated in 5 ml buffer in a 25 ml Erlenmeyer flask at 25°C for 5 min at 120 W m^{-2} light intensity. The buffer contained: 0.33 M sorbitol, 0.05 M Hepes-NaOH, 0.002 M KNO_3, 0.002 M EDTA, 0.001 M $MnCl_2$, 0.001 M $MgCl_2$, 0.0005 M K_2HPO_4, 0.02 M NaCl, and 0.2 M Na-isoascorbate, pH 7.6. At the end of the preincubation period, 20 mM $NaHCO_3$ containing 40 μCi $NaH^{14}CO_3$ (14.3 μCi/μM) was added to each sample. They were allowed to fix $^{14}CO_2$ for 10 min. The reaction was killed by adding boiling 80% ethanol. Tissues were subsequently extracted eight times with boiling ethanol of the same concentration. Combined extracts were brought to dryness *in vacuo* at 40°C and were dissolved in 10 ml distilled H_2O. An aliquot was measured into 5 ml of scintillation fluid for radioactivity assay using a Packard Tri-Carb liquid scintillation counter.

The radioactive products of photosynthesis were analyzed by taking aliquot samples of the water-soluble mixture (usually 100 μl) and subjecting them to a combination of two-dimensional paper chromatography and autoradiography. The solvent system used was 98% ethanol: 1 M ammonium acetate: 0.1 M EDTA (75:30:1, v/v) for the first dimension, and *n*-butanol:propionic acid: H_2O (10:5:7, v/v) for the second dimension. The chromatograms were exposed to X-ray films (Fuji Photo Film). Radioactive areas from chromatograms were located and radioactivity

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[2] Abbreviations: RuBP, ribulose bisphosphate carboxylase; RuBP, ribulose bisphosphate; PEPCase, phosphoenolpyruvate carboxylase; 3-PGA, 3-P-glyceric acid; R_1, photorespiration; R_d, mitochondrial respiration.

in each compound was determined by elution with 10% ethanol, followed by scintillation counting. Counting efficiency was 93%. Percent recovery of radioactivity from chromatograms ranged from 85 to 95%. Results were expressed as a percent of the total $^{14}CO_2$ fixation.

Enzyme Assays. The second leaf tissues of 7-d-old seedlings were harvested. Leaf tissue without the major veins was ground in a mortar on ice at a ratio of 1 g fresh weight to 5 ml cold extraction medium containing the above buffer for CO_2 fixation. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000*g* for 15 min. All enzyme determinations were made immediately after extraction.

Activity of RuBP oxygenase was measured polarographically from the O_2 uptake in a standard reaction mixture containing in a final volume of 1 ml: 50 mM Tris buffer, pH 9.3, 10 mM $MgCl_2$, 5 mM DTT, 0.3 mM RuBP, and 100 μl enzyme extract (0.30–0.35 mg protein in each sample).

Activity of glycolate oxidase was measured by the method of Kolesnikov (9). The method is based on the quantitative assay of glyoxylic acid by the modified Fosse reaction. For enzyme extraction, 25 mg of leaves were ground in a prechilled mortar with purified sea sand and 20 ml of 1/15 M K/Na phosphate buffer, pH 8.0. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20,000*g* for 15 min. To 5 ml of extracts was added 0.5 ml of 0.1 M Na-glycolate (H_2O for the controls). Reaction time was 10 min at 25°C. At the end of the reaction, extracts were precipitated with (TCA final concentration 3%) and developed a color reaction with 0.3% phenylhydrazine hydrochloride and 1.5% $K_3Fe(CN)_6$. The amount of glyoxylic acid was assayed spectrophotometrically at 530 nm (Specol 10, GDR).

Rate of photorespiration was determined by the method of Catsky and Ticha (4), where photorespiration is estimated as the increase in net CO_2 exchange rate with ambient CO_2 between 20% O_2 and 1% O_2. A closed system was used which included an IR gas analyzer (Infralyt 4, GDR) and a paramagnetic O_2 analyzer (Perrmolyt 2, GDR). The plants investigated were placed in a thermostatic leaf chamber irradiated with light intensity 200 W m^{-2} by a projection apparatus Profil (Poland). The leaf temperature was maintained at 25°C.

Stomatal resistance was determined by the method described by Laisk (11), using a thermocouple electropsychrometer at leaf temperature of 25 ± 0.2°C, CO_2 concentration of 360 μl/L, and light intensity of 200 W m^{-2}.

Protein was determined by the method of Lowry *et al.* (12), with BSA as standard.

Chemicals. ± ABA was purchased from Fluka AG, Chem Fabrik; all other chemicals were obtained from Sigma.

Table I. *Effect of ABA on the Distribution of $^{14}CO_2$ in the Photosynthetic Products in Barley Leaves*

Photosynthetic fixation of CO_2 was 34.10^{-6} dpm·g^{-1}·fresh weight for controls, ABA inhibited fixation by 42.0% at 10^{-6} M and by 37.2% at 10^{-5} M. Values are averages from analysis of three separate experiments ± SE (n = 3). Student's t test.

Compounds	Distribution of ^{14}C		
	Control	10^{-6} M ABA	10^{-5} M ABA
	% of total ^{14}C		
Sugar phosphate esters	4.8 ± 0.17	4.4 ± 0.24	4.0 ± 0.09*
Amino acids	36.4 ± 0.5	33.2 ± 0.5	34.0 ± 0.1
Organic acids	20.6 ± 0.5	23.0 ± 0.4	25.2 ± 0.3*
Sugars	36.4 ± 0.5	38.2 ± 0.1	36.2 ± 0.2
Unknown	2.6 ± 0.2	1.2 ± 0.1	0.2 ± 0.02

* $P < 0.05$.

Table II. *Effect of ABA on Distribution of $^{14}CO_2$ in the Main Photosynthetic Products in Barley Leaves*

Details are described in the text of Table I.

Compounds	Distribution of ^{14}C		
	Control	10^{-6} M ABA	10^{-5} M ABA
	% of total ^{14}C		
Sugar phosphate esters	4.8 ± 0.17	4.4 ± 0.24	4.0 ± 0.09*
3-PGA	3.4 ± 0.1	1.8 ± 0.1*	2.0 ± 0.1*
Glycine + serine	4.5 ± 0.2	8.7 ± 0.3*	7.5 ± 0.2*
Alanine	23.1 ± 0.5	19.1 ± 0.3*	21.2 ± 0.8
Glutamic acid	4.1 ± 0.2	3.3 ± 0.2	3.5 ± 0.3
Aspartic acid	2.7 ± 0.2	2.0 ± 0.2	1.8 ± 0.2
Phosphoenolpyruvate	0.01 (traces)	0.4 ± 0.1	0.2 ± 0.1
Malic acid	13.5 ± 0.3	15.6 ± 0.2*	16.8 ± 0.4*
Citric acid	4.2 ± 0.2	3.1 ± 0.2	4.0 ± 0.2
Glycolic acid	2.9 ± 0.1	3.9 ± 0.1*	4.2 ± 0.2*
Glucose + fructose	14.3 ± 0.3	18.7 ± 0.6*	15.6 ± 1.0
Sucrose	12.1 ± 0.3	15.0 ± 0.6	11.2 ± 0.7
Maltose	10.1 ± 0.5	4.4 ± 0.2*	9.8 ± 0.6

* $P < 0.05$.

Table III. *Effect of ABA on Activity of RuBP Oxygenase and Glycolate Oxidase in Barley*

Activity of the enzymes was determined as indicated in "Materials and Methods." Activity for RuBP oxygenase was 0.19 μmol O_2 mg^{-1} protein min^{-1} in the controls and for glycolate oxidase it was 0.13 mg glyoxylic acid mg^{-1} protein min^{-1}. Data are average of three experiments.

ABA	RuBP oxygenase	Glycolate oxidase
M	*activity, % of controls*	
H_2O/controls	100	100
10^{-6}	101.5 ± 6.1	116.6 ± 2.8
10^{-5}	115.2 ± 8.6	123.0 ± 5.4
10^{-4}	140.2 ± 11.6	128.8 ± 11.8

RESULTS

ABA inhibited photosynthesis by 42% at 10^{-6} M and 37.2% at 10^{-5}M. An analysis of the products of photosynthesis in the controls and ABA-treated plants revealed no essential changes in the proportional amount of CO_2 incorporated into the amino acids and sugar fractions. (Tables I and II). There was only a slight inhibition of the incorporation of labeled carbon into the sugar phosphate fractions and of the same degree stimulation into the organic acid fraction (Table I).

Significant differences were observed among the individual compounds (Table II). ABA-treated and control plants showed greatest differences in ^{14}C incorporation into 3-PGA, glycine, serine, glycolic acid, and certain amino acids and sugars. The amounts of labeled glycine and serine are nearly twice as high as those of the controls. There is an almost 40% increase in the labeling of glycolic acid. At the same time this hormone sharply reduced the relative incorporation of carbon into 3-PGA.

The absence of differences in the labeling of aspartic acid, malic acid, and PEP (and also generally in the proportion organic acids) did not support the hypothesis of Sankhla and Huber (23) on the role of ABA as a regulator of the balance between the C_3 and C_4 pathways of photosynthesis.

The results of the present investigation provide grounds for the prediction that ABA acts on certain units of the carbon photorespiratory metabolism. This hypothesis made it necessary to determine the intensity of photorespiration, stomatal resistance, the CO_2 compensation point, and the activity of the photorespiratory enzymes-RuBP oxygenase and glycolate oxi-

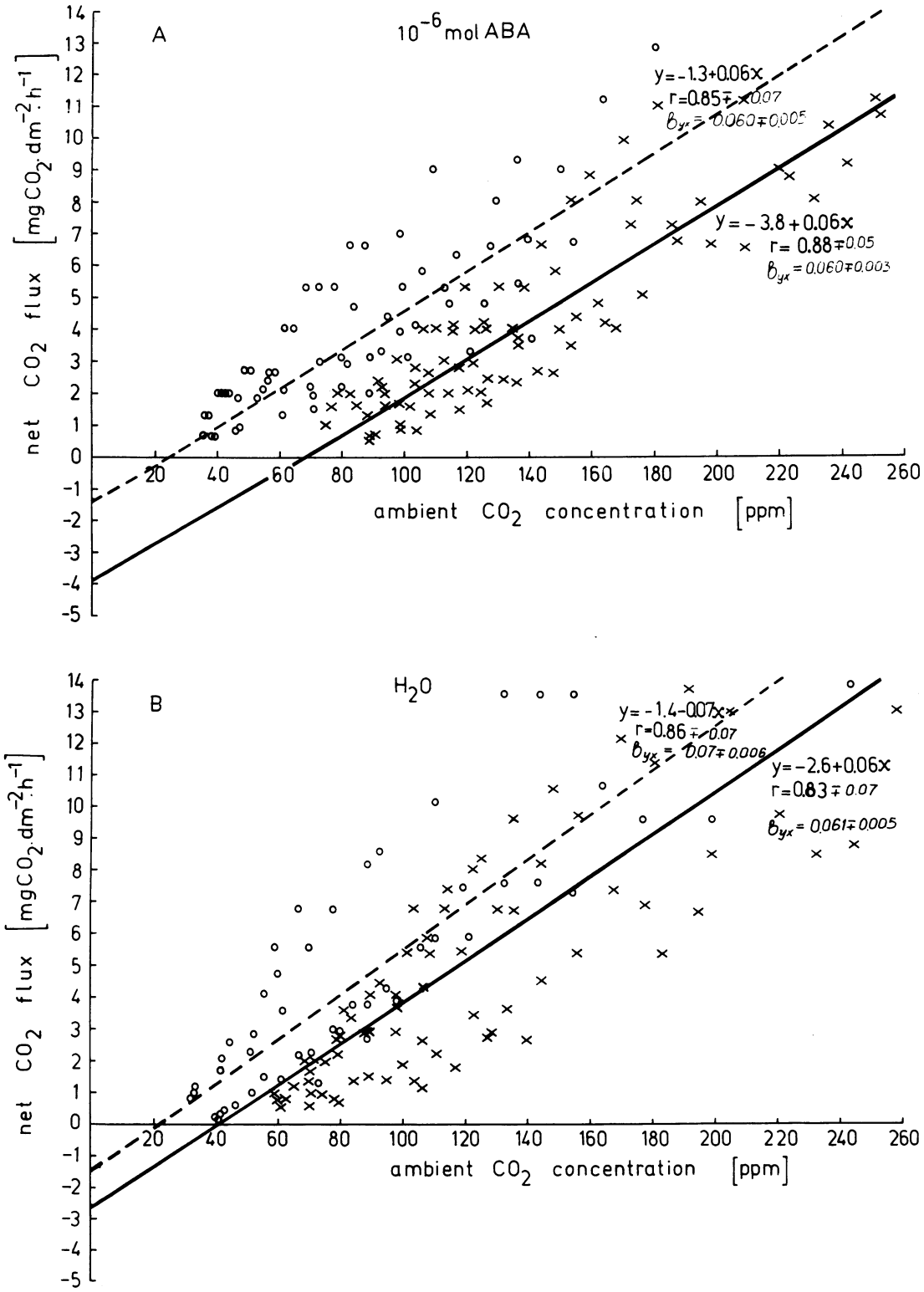

FIG. 1. Responses of the net CO_2 flux to ambient CO_2 concentration in barley seedlings grown at 10^{-6} mol ABA (A) and at H_2O (B). Experimental data are marked with "O" for 1% O_2 and with "×" for 20% O_2. The dashed line represents regression line for 1% O_2, and the solid line—regression line for 20% O_2.

dase.

RuBP oxygenase and glycolate oxidase activities were greater in plants treated with ABA (Table III). The activity depends on the ABA concentration. The greatest effect was found with 10^{-4}M ABA; 10^{-6}M causes almost no change in the activity of RuBP oxygenase, but its positive effect on the activity of glycolate oxidase is preserved. The effect of ABA on the activity of photorespiratory enzymes is consistent with the rate of photorespiration (Fig. 1), and about the value of stomatal resistance (Table IV). Figure 1 shows the dependence of the net CO_2 exchange rate on the ambient concentration of the CO_2 in the controls (B) and in the plants cultivated on 10^{-6}M ABA (A),

Table IV. *Effect of ABA on the Stomatal Resistance in Barley*

ABA	Stomatal Resistance
M	$s \cdot cm^{-1}$
H_2O (control)	7.3 ± 0.8
10^{-6}	11.7 ± 1.0
10^{-5}	16.1 ± 1.2*

* $P < 0.05$.

Table V. *Effect of ABA on the Rate of Photorespiration on the Mitochondrial Respiration and on the CO_2 Compensation Point*

Experimental conditions as described under "Materials and Methods."

Treatment	Photo-respiration	Mitochondrial Respiration	Compensation Point	
			1% O_2	20% O_2
	$mg\ CO_2 \cdot dm^{-2} \cdot h^{-1}$		$\mu l\ CO_2/l$	
H_2O (control)	1.2	1.4	20	43
10^{-6} M ABA	2.5	1.3	22	63

recorded at 20% O_2 and 1% O_2 for determining the photorespiration rate. The regression lines of the type $Y = a + bx$ have been determined. The respiration rates are determined by extrapolating the dependence of the net CO_2 photosynthetic rate to zero CO_2 concentration (Fig. 1). Since for the regression lines $Y_{x=0} = a$, then at 1% O_2 $a = R_d$, and at 20% O_2 $a = R_d + R_l$, where R_d is the rate of mitochondrial respiration and R_l is the rate of photorespiration (Table V). In an analogous manner at $Y = 0$ estimates CO_2 compensation point. At 10^{-6}M ABA there are no changes in mitochondrial respiration, while photorespiration increases twofold. The value of the CO_2 compensation point increases at about 50% (Table V) in 20% O_2.

DISCUSSION

ABA exerts an influence on the flow of carbon into the photosynthetic products of barley. Compared with the controls, the leaves of seedlings grow in the presence of ABA incorporated a lower proportion of radioactivity into the products of the Calvin cycle: sugar phosphate esters, 3-PGA, and also in aspartic and glutamic acids, while a larger proportion of ^{14}C was found in the products of the photorespiratory carbon metabolism: glycolic acid, glycine, and serine. These results are consistent with the inhibiting effect of the growth regulator on the rate of photosynthetic CO_2 fixation and on the activity of RuBPCase data established by us and other authors as well (17, 23). On the other hand with ABA treatment, the RuBP oxygenase and glycolate oxidase activities were increased, and more ^{14}C was incorporated into the products of photorespiratory carbon metabolism. ABA influences the flow of carbon in the photosynthetic process, which results in a change in the ratio between the reductive and oxidative actions of photosynthesis and photorespiration. The results provide no categorical explanation of the mechanism of ABA action.

The first possibility is an indirect effect mediated by the effect of ABA on stomatal closure: decrease in the partial pressure of CO_2 and change in the CO_2/O_2 ratio in the chloroplasts. Supporting the hypothesis about stomatal effects of ABA on photosynthesis are Mawson *et al.* (13), who report an absence of ABA effects on the rate of photosynthesis in cells isolated from *Phaseolus vulgaris*, *Nicotiana tabacum*, and *Lycopersicum esculentum*. The hypothesis of ABA action mediated by stomatal closure is valid only upon a sharp rise in ABA concentration, resulting from an application of exogenic concentration or a sudden stress (water or temperature). Two to three h are needed for the onset of partial restoration after a certain period during which the stomata are closed (20). Under conditions of continuous treatment of the plants with ABA, as in the case with our experiments, photosynthesis is only partially inhibited, showing an adjustment of the plants and reaching new steady state values.

Another mechanism may explain the action of ABA, *i.e.* regulation of the synthesis of some chloroplast proteins or on the kinetic characteristics of the RuBP carboxylase-oxygenase. This hypothesis is supported by our results indicating a rise in the CO_2 compensation point in ABA-treated plants. Raschke and Hedrich (20) made a similar observation with a number of plants of C_3 and C_4 types of photosynthesis.

Direct action of ABA on photosynthesis has been suggested. Bauer *et al* (1) reported that ABA induced an inhibition of CO_2 fixation in duckweed (*Lemna minor*) fronds, plants which have nonfunctional stomata. This effect was accompanied by reduced Hill reaction activity. The authors assume that ABA treatment acts directly on the photosynthetic apparatus, in addition to its action on the stomata.

It is well established that ABA induces strong inhibition of DNA and RNA synthesis (16, 24). More recent reports concern the inhibiting effect of ABA on the synthesis of chloroplastic rRNAs in pumpkin cotyledons (10). These results lead to the assumption that, as a consequence of the inhibition of the synthesis of the plastid rRNAs, there will also be inhibition of the synthesis of a number of chloroplast proteins, certain photosynthetic enzymes included.

The mechanism of ABA action on the carboxylating capacity of RuBPCase is not clear, but when ABA is increased there is inhibition of the enzyme activity. Nor should we rule out the possibility of a direct effect of ABA on the conformation properties of the allosteric enzyme RuBP carboxylase oxygenase which leads to changes in some of its kinetic characteristics.

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