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Ethylene-Induced Increase in Fructose-2,6-Bisphosphate in Plant Storage Tissues[1]

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ABSTRACT

Treatment of carrot roots with ethylene led to: (a) a doubling of the fructose-2,6-bisphosphate content; (b) a general increase in the concentration of glycolytic intermediates; and (c) an increase in the extractable activity of fructose-6-phosphate,2-kinase, the enzyme synthesizing fructose-2,6-bisphosphate from fructose-6-phosphate and adenosine triphosphate.

Fructose-2,6-bisphosphate is a regulatory metabolite that makes an important contribution to the regulation of carbohydrate metabolism in different types of eukaryotic organisms. In mammalian and yeast cells, Fru-2,6-P_2[4] promotes glycolysis and restricts gluconeogenesis by respectively activating phosphofructokinase and inhibiting fructose-1,6-bisphosphatase (5, 15) Through a similar inhibition of fructose-1,6-bisphosphatase, Fru-2,6-P_2 functions in higher plants in regulating sucrose synthesis, a process which is analogous to gluconeogenesis, in leaves (3, 13), and in germinating castor bean seedlings (6). The contribution of Fru-2,6-P_2 to regulation of respiratory metabolism in plants is, however, not clear. To gain information on this point, we have exposed plant storage tissues to ethylene—a hormone known to increase respiration (9). We report that Fru-2,6-P_2 increases when such tissues are treated with ethylene and that this is accompanied by an increase in the activity of the enzyme synthesizing Fru-2,6-P_2—Fru-6-P,2K.

MATERIALS AND METHODS

Plants. Freshly topped carrots (10–13 cm long, 1.5–2.5 cm maximum diameter) or potatoes (4–6 cm cross-section) purchased from a market, were pretreated in water-saturated air for at least 30 h before introducing 50 μl/L ethylene for 19 to 22 h. Each sample was made from a single carrot or potato. Control experiments were with matched carrots and potatoes incubated for 50 h in the absence of added ethylene. The flow rate was 0.5 L min^{-1} per carrots, and 1.0 L min^{-1} per four potatoes. The air was water-saturated.

Extraction for Fru-2,6-P_2 and Metabolites. Within 5 sec of cutting the storage tissue, a cross-sectional sample (0.6–0.9 g fresh weight) was frozen in liquid N_2. Samples were cut so that the width was 2 mm or less. The samples were deproteinized using a chloroform-methanol mixture at sub-zero temperatures as described in Stitt *et al.* (10) for carrot, and as modified in Stitt and Heldt (12) for potato. The extracts were stored at −85°C. Fru-2,6-P_2 was measured by the PFP bioassay as in Stitt and Heldt (12) and Glu-6-P, Fru-6-P, DHAP, and PGA were assayed spectrophotometrically in a dual wavelength spectrophotometer as in Stitt *et al.* (10). In experiments where Fru-6-P,2K was assayed (see below), the activation of PFP by the zero-time sample (compared to PFP activity in the absence of sample) was also used to provide an estimate of the Fru-2,6-P_2 present in the same sample of carrot tissue. There was no significant hydrolysis of Fru-2,6-P_2 during the time required to prepare the samples under these conditions.

Extraction of Fru-6-P,2K. Within 60 s of slicing, a cross-sectional sample (0.3–0.5 g fresh weight) was homogenized with glass powder (200–400 μm mesh) at 4°C in a pestle and mortar with 1500 μl of a solution containing 50 mM Tricine-KOH (pH 7.7), 5 mM $MgCl_2$, 2 mM EDTA, 14 mM 2-mercaptoethanol, 20 mM diethyldithiocarbamate, 2 mM benzamidine HCl, 2 mM ε-amino-*n*-caproic acid, 0.5 mM phenylmethylsulfonylfluoride, 1.5% (w/v) insoluble PVP, 10% (v/v) glycerol. The homogenate was centrifuged, 2 min, and aliquots of the supernatant fraction were assayed immediately for Fru-6-P,2K. Protein was measured as in Cséke and Buchanan (2).

Assay of Fru-6-P,2K. Fru-6-P,2K was assayed as in Stitt *et al.* (14), using 20 μl aliquots of extract in a final volume of 100 μl containing 100 mM Tris-HCl (pH 7.3), 5 mM $MgCl_2$, 1 mM EDTA, 2 mM ATP, 5 mM Fru-6-P, 5 mM Pi, and with no or 1 mM PGA. At 0 (two samples), 400, and 800 s, 20 μl aliquots were transferred into 30 μl 0.25 M KOH and stored on ice (5–15 min) before assaying for Fru-2,6-P_2 using the PFP bioassay. One of the two samples taken at zero-time received 1 pmol Fru-2,6-P_2, and acted as an internal standard. For practical details see Stitt *et al.* (14).

RESULTS

Change in Fru-2,6-P_2. Whole carrots were treated for 20 h either in air or in air supplemented with 50 μl/L ethylene, in seven separate experiments carried out during a period of 6 weeks, using immature carrots (4 per treatment). The Fru-2,6-P_2 content (mean ± SE of 28 carrots) increased from 140 ± 12 pmol·g^{-1} fresh weight in the absence of ethylene to 250 ± 21 pmol·g^{-1} fresh weight after exposure to ethylene. A similar increase was observed with mature carrots, from 80 ± 29 to 170

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[4] Abbreviations: Fru-2,6-P_2, fructose-2,6-bisphosphate; DHAP, dihydroxyacetonephosphate; Fru-6-P,2K, fructose-6-phosphate,2-kinase; Fru-2,6-P_2ase, fructose-2,6-bisphosphatase; PFP, pyrophosphate:fructose-6-phosphate-1-phosphotransferase; PGA, 3-phosphoglycerate; Fru-6-P, fructose-6-phosphate; Glu-6-P, glucose-6-phosphate.

± 40 pmol·g^{-1} fresh weight where the number of carrots tested (n) was 4, and with white potato tubers, from 30 ± 3 to 60 ± 5 pmol·g^{-1} fresh weight, n = 4. In related experiments, ethylene also led to an increase of Fru-2,6-P_2 in young carrots when exposure took place in the presence of 100% O_2 (from 101 ± 10 to 210 ± 19 pmol·g^{-1} fresh weight n = 12).

Changes in Glycolytic Metabolites. To relate the ethylene-induced changes in Fru-2,6-P_2 to other metabolites, we determined the effect of ethylene on intermediates of glycolysis which are known to regulate Fru-6-P,2K (2, 6, 8, 11, 14). In the experiments summarized in Table I, Fru-2,6-P_2 and glycolytic intermediates were measured in the same samples of carrot roots or potato tubers. Consistent with an enhanced mobilization of carbohydrates, we observed in both cases a small but general increase in metabolite levels after exposure to ethylene. The percentage increases produced by ethylene, expressed respectively for carrots and potatoes, were: Fru-2,6-P_2, 108 and 100%; Glu-6-P, 34 and 19%; Fru-6-P, 7 and 16%; DHAP, 124 and 200%; and PGA, 32 and 13%.

Change in Fru-6-P,2K. To determine whether there was a correlation between Fru-2,6-P_2 levels and extractable Fru-6-P,2K activity, we tested the activity of the enzyme in extracts rapidly prepared from control and ethylene-treated carrot roots (Table II). In these experiments, less than 5 min elapsed between the time at which a sample was removed from the exposure chamber and the time at which the Fru-6-P,2K assay was initiated. By minimizing preparation time, we considered that any alterations in enzyme activity following exposure to ethylene would be best preserved. Fru-6-P,2K was assayed in both the absence (control) and presence of PGA, which is an inhibitor of Fru-6-P,2K from carrot and other plant tissues (2, 6, 8, 11, 14). For comparative purposes, the Fru-2,6-P_2 content was estimated in the sample used to measure Fru-6-P,2K activity.

Considering first the measurements in the absence of PGA (in which assay conditions were arranged to give about 80% of the V_{max} activity), the results demonstrated that there was a small increase in Fru-6-P,2K activity in carrot tissues that had been exposed to ethylene (Table II). This increase was somewhat larger when Fru-6-P,2K activity was measured in the presence of 1 mM PGA. With PGA, the activity of Fru-6-P,2K was, in three separate experiments, 94, 51, and 40% higher in carrots that had been exposed to ethylene (*versus* respective values of 29, 15, and 32% for assays carried out in the absence of PGA). Parallel to these increases in Fru-6-P,2K activity were increases in Fru-2,6-P_2 content of 130, 63, and 110% (Table II). These results are related to protein content, but similar increases were found when activity was related to fresh weight.

DISCUSSION

In this communication, we present evidence that ethylene, a known stimulator of respiration in plants, brings about a significant increase in Fru-2,6-P_2 content of nonphotosynthetic storage tissues (carrot roots, potato tubers). This increase was found whether Fru-2,6-P_2 content was related to fresh weight or to protein content. Although higher in ethylene-treated tissues, the Fru-2,6-P_2 content of individual carrots was found to vary, even within single lots. It seems likely that this variation may reflect the prehistory of each individual carrot and result from isolated treatments, such as wounding during handling and storage, which could result in up to a 10-fold increase in Fru-2,6-P_2 (A Balogh, JH Wong, BB Buchanan, unpublished data; M Stitt, unpublished data). A similar increase in Fru-2,6-P_2 has also been found in response to ethylene production in naturally ripening avocado (AB Bennet, unpublished data).

The increase of Fru-2,6-P_2 in response to ethylene treatment provides new evidence that this regulatory metabolite is involved in promoting carbohydrate mobilization and breakdown in plants. A similar correlation between increased Fru-2,6-P_2 and carbohydrate breakdown is found (a) during wound-induced respiration in Jerusalem artichoke (16) and carrot or potato storage tissues (A Balogh, JH Wong, and BB Buchanan, unpublished data; M Stitt, unpublished data); (b) after making tissues anaerobic (7); (c) after supplying endogenous sugars to leaves (10) or roots (M. Stitt, unpublished data); and (d) during malate accumulation as guard cell protoplasts swell (4). It remains to be seen how Fru-2,6-P_2 brings about this stimulation. As Fru-2,6-P_2 does not increase the linear rate of phosphofructokinase activity in plants (1), one site of action could lie in its activation of PFP, either the forward or reverse reaction. Depending on the direction in which PFP operates *in vivo*, increasing Fru-2,6-P_2 could enhance the glycolytic direction, thereby increasing the use of PPi for phosphorylation of Fru-6-P, or, alternatively, Fru-2,6-P_2 could stimulate the reverse reaction, thereby increasing the pool of PPi needed for sucrose mobilization via sucrose synthase and UDP-glucose phosphorylase.

The question arises as to the basis of the ethylene-induced increase in Fru-2,6-P_2, *i.e.* what is responsible for altering the activities of the enzymes catalyzing the synthesis and degradation of Fru-2,6-P_2. Current evidence suggests that plants resemble animal systems in having both Fru-6-P,2K and Fru-2,6-P_2ase activities on a single bifunctional protein (3). Hence the amount of Fru-2,6-P_2 depends on the relative extent to which each of these activities functions. In carbohydrate exporting tissues (sources) like leaves (3, 8, 11) and germinating bean endosperm (6), the extent of function seems to be fixed, at least in part, by the level of metabolites. The recent finding that the Fru-6-P,2K preparations from different carbohydrate importing tissues (sinks) (14), including carrot roots, show properties similar to the enzyme from sources opens the possibility that Fru-2,6-P_2 content of plant sink tissues could also be controlled by metabolite levels. However, the increase of Fru-2,6-P_2 following ethyl-

Table I. *Changes of Fru-2,6-P_2 and Respiratory Metabolites Following Exposure of Storage Tissues to Ethylene*

Whole carrot roots, or potato tubers, were treated in air, or in air containing 50 μl/L ethylene, and then quenched, extracted and analyzed as in "Materials and Methods." The results are the mean ± SE of three separate experiments with 4 carrots for each treatment (n = 12), and of one experiment with 4 potatoes for each treatment (n = 4).

Tissue	Ethylene Added	Content				
		Fru-2,6-P_2	Glu-6-P	Fru-6-P	DHAP	PGA
	μl/L	pmol·g^{-1} fresh wt		nmol·g^{-1} fresh wt		
Carrot	0	120 ± 9	111 ± 6	40 ± 3	2.5 ± 0.4	28 ± 3
Carrot	50	250 ± 13	149 ± 16	43 ± 4	5.6 ± 0.6	37 ± 2
Potato	0	30 ± 3	47 ± 3	17 ± 2	0.6 ± 0.1	23 ± 1
Potato	50	60 ± 5	56 ± 6	20 ± 2	1.8 ± 0.4	26 ± 2

Table II. *Changes of Fru-2,6-P_2 Content and Fru-6-P,2K Activity Following Exposure of Carrot Storage Tissue to Ethylene*

Whole carrots were treated in air, or in air containing 50 μl/L ethylene and extract rapidly prepared and assayed as in "Materials and Methods." The assay in the absence of PGA yielded an activity which was about 80% of the V_{max} of Fru-6-P,2K. Fru-2,6-P_2 content of the carrots was estimated during the Fru-6-P,2K assay as in "Materials and Methods." Three experiments with separate batches of carrots are shown, the number of carrots used per experiment being shown in the footnote, and results are as mean ± SE.

Ethylene Added	Fru-2,6-P_2	Fru-6-P,2-Kinase Activity	
		Control	1 mM PGA
μl/L	*pmol·mg^{-1} prot*	*nmol·mg^{-1} prot·h^{-1}*	
0	100 ± 28	2.4 ± 0.4	0.48 ± 0.13
50	230 ± 46	3.1 ± 0.2	0.93 ± 0.08
0	80 ± 20	2.0 ± 0.4	0.70 ± 0.19
50	130 ± 18	2.3 ± 0.3	1.06 ± 0.21
0	80 ± 11	1.9 ± 0.3	0.65 ± 0.18
50	170 ± 13	2.5 ± 0.3	0.92 ± 0.09

[a] The number of carrots used for each of the three experiments were 4, 4, and 5, respectively.

ene treatment could not be accounted for by concordant changes in regulatory metabolites, *i.e.* a strong activator of Fru-6-P,2K (Fru-6-P) rose, but to a lesser degree than did inhibitors of the enzyme (PGA and DHAP). While uncertainty about the subcellular compartmentation of these metabolites (as well as changes in Pi, another activator) means that a degree of interpretative caution is in order, the results suggest that regulation by metabolites is not primarily responsible for the increased Fru-2,6-P_2 levels following exposure to ethylene but that additional mechanisms may be required.

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