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Control of Pyrophosphate:D-Fructose-6-Phosphate 1-Phosphotransferase Activity in the Cotyledons of *Citrullus lanatus*[1]

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ABSTRACT

After initiation of radicle elongation, the pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase (PFP) activity sharply increases in the cotyledons of *Citrullus lanatus*. Removal of the radicle early during incubation prevents the increase in PFP activity in the cotyledons evident in the control. Removal of the radicle at any stage after germination results in a decrease in PFP activity in the cotyledons. Application of kinetin (0.5 micromolar) or 2-chlorophosphonic acid (0.1 micromolar) to isolated cotyledons replaces the effect of the radicle. Gibberellic acid (0.09 micromolar GA_3) also partially mimics the presence of the radicle. Anaerobic conditions, as well as cycloheximide application (0.18 micromolar) to intact embryos or to kinetin and ethrel treated isolated cotyledons prevent the increase in PFP activity evident in the control.

PFP,[2] EC 2.7.1.90 is a cytosolic enzyme (2, 3, 9, 13, 24, 32) that reversibly interconverts fru-6-P/fru-1,6-P_2 and PPi/Pi. Since the discovery of this enzyme in plants (16) several papers on the kinetic properties have been published (6, 7, 23, 32, 33). From these results it is evident that fru-2,6-P_2 is probably the most important regulator of the enzyme. Whether PFP functions primarily as a glycolytic or gluconeogenic enzyme *in vivo* is still unclear.

It has been proposed that high PFP activity might be indicative of tissues which primarily breaks down sucrose (2, 3). In these tissues PFP might be functioning in the gluconeogenic direction for the synthesis of PPi required for sucrose degradation via sucrose synthase and UDP-glucose pyrophosphorylase (2, 3, 9, 22). Changes in PFP activity in the *Arum spadix* and germinating bean seeds are in agreement with such a role for PFP (2, 13). However, in germinating *Ricinus communis* and *Citrullus lanatus* seeds PFP activity also sharply increases in tissues in which a net sucrose synthesis occurs (12, 24), *i.e.* cotyledons.

Little is known about the control of PFP levels in plant tissues. In *C. lanatus* increased PFP activity is not due to activation of existing PFP protein but is a direct result of increased levels of the PFP protein (12). In the same investigation it was found that a light treatment which inhibited germination also prevented the increase in PFP activity, but this was not due to a direct effect of light on the activity of PFP. This led us to propose that the elongating radicle might play an important role in controlling PFP activity in the cotyledons of *C. lanatus*. It is well documented that the radicle plays an important role in plastid and mitochondrial development in the cotyledons of the Cucurbitaceae (1, 18, 19, 30, 34) but little is known regarding the role of the radicle in controlling the levels of cytosolic enzymes.

Here we report that the radicle plays an important role in the control of the level of PFP activity in the cotyledons. In addition, it was found that kinetin and ethrel can mimic the effect of the radicle. In all cases increased levels of PFP activity appear to be dependent on aerobic protein synthesis.

MATERIALS AND METHODS

Materials

Seeds of *Citrullus lanatus* (Thunb.) Matsumura and Nakai were obtained from plants growing in the wild, isolated, and stored as previously described (10). Embryos were isolated by removal of the dark lignified testa as described (10). Auxiliary enzymes, cofactors, substrates, kinetin, and GA_3 were obtained from Boehringer Mannheim and the Sigma Chemical Company. Ethrel was obtained from Agricura Ltd. N_2 was obtained from Afrox (SA). All other chemicals employed were of analytical grade.

Methods

Germination and Removal of the Radicles

Germination tests were conducted at 27°C in the dark. Isolated embryos were incubated on Whatman No. 1 filter paper moistened with 4 mL distilled water or test solution in 500 mL Schott Duran glass bottles capped with cotton wool. When the effect of anaerobic conditions and growth substances was tested the isolated embryos were incubated on Whatman No. 1 filter paper wetted with 4 mL test solution in 600 mL vaccine capped conical flasks. To obtain anaerobic conditions, two hypodermic needles were inserted through the vaccine cap. High purity N_2 was circulated through the flasks at a flow rate of 500 mL·min^{-1} for 30 min. The needles

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[2] Abbreviations: PFP, pyrophosphate:D-fructose-6-phosphate 1-phosphotransferase (EC 2.1.7.90); PFK, ATP:fructose 6-phosphate phosphotransferase (EC 2.1.7.11); TPI, triose phosphate isomerase (EC 5.3.1.1); Fru-6-P, D-fructose-6-phosphate; Fru-1,6-P_2, D-fructose-1,6-bisphosphate; Fru-2,6-P_2, D-fructose-2,6-bisphosphate; Ethrel, 2-chlorophosphonic acid; Rubisco, ribulose bisphosphate carboxylase.

were removed after allowing the flask and atmospheric pressure to equilibrate. Hypoxic conditions were created by submerging the embryos in 50 mL distilled water in vaccine capped conical flasks. In all cases, 30 seeds per replicate and at least six replicates per treatment were used. The percentage germination as well as radicle length were recorded. A seed was considered germinated when the radicle started to elongate.

Extraction and Enzyme Assay

All procedures were carried out at 0 to 4°C. Crude extracts of cotyledons were prepared by homogenizing 30 cotyledons in 2 mL ice-cold extraction buffer. The extraction buffer contained 100 mM Tris-acetate (pH 8.00), 2 mM $MgCl_2$, 2 mM EDTA, 1 mM PMSF, 14 mM 2-mercaptoethanol, and 10% (v/v) glycerol. The homogenate was centrifuged at 27,000*g* for 15 min. The supernatant was removed and again centrifuged at 9,800*g* for 10 min to facilitate removal of the fat layer.

All assays were carried out at 25°C in a total volume of 1 mL. NADH oxidation was recorded at 340 nm with a Cary 219 UV/VIS spectrophotometer. PFP activity was measured by the production of Fru-1,6-P_2 as previously described (24). The standard assay medium contained 100 mM Tris-HCl (pH 8.00), 10 mM Fru-6-P, 1 mM $MgCl_2$, 0.1 mM NADH, 1 μM Fru-2,6-P_2, 1 IU aldolase, 10 IU TPI, and 1 IU glycerol-3-P dehydrogenase. The reaction was started by the addition of 1 mM PPi. The presence of possible activators or inhibitors were determined by preparing a series of extracts each containing at least two different tissues. The measured activity in these extracts was 95.3 ± 4.1% of that measured in the tissues extracted separately.

Gas Exchange Under Anaerobic Conditions

CO_2 evolution by cotyledons under anoxia was determined as previously described (11). To obtain a complete anaerobic atmosphere the 15 mL flasks containing the cotyledons, were flushed with pure N_2 for 5 min at a flow rate of 500 mL·min^{-1} prior to the measurement of gas exchange. CO_2 release was measured for 30 min after a 15 min equilibration period in the anoxic atmosphere.

Protein

Protein was measured by the method of Bradford (15) using bovine γ-globulin as a standard.

RESULTS

Incubation of *Citrullus lanatus* embryos at 27°C in the dark resulted in a final germination percentage of 98% (Fig. 1). Radicle protrusion commenced after only 2 h of incubation, and 55% germination was recorded after only 12 h of incubation. It is evident that the increase in PFP activity in the cotyledons of the intact embryos (Fig. 1) only commences well after the initiation of radicle elongation. The increase in PFP activity in the cotyledons is largely dependent on the presence of the radicle. Removal of the radicle at time zero

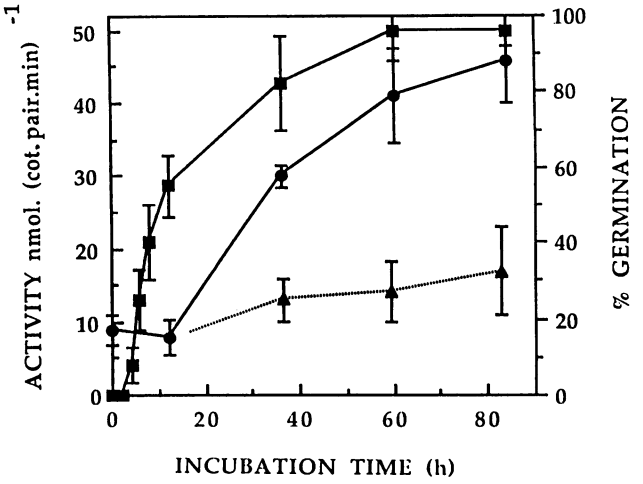

Figure 1. Percentage germination (■) and PFP activity of intact (●) and radicle excised (▲) *C. lanatus* embryos incubated for 84 h at 27°C in darkness. Error bars indicate standard deviation.

Table I. *Effect of Different Treatments on Cotyledon PFP Activity as well as the Radicle Length of C. lanatus*

Treatment	Incubation Time	Radicle Length	PFP Activity
	h	*mm*	*nmol/(cot/pair/min)*$^{-1}$
Air	0	0.0 ± 0.0[a]	7.60 ± 3.7
	12	1.4 ± 0.2	6.93 ± 0.7
	36	3.3 ± 0.7	32.30 ± 1.6
	60	21.3 ± 5.5	42.17 ± 1.6
	84	36.5 ± 7.9	45.29 ± 4.6
Hypoxia	0	0.0 ± 0.0	7.60 ± 3.7
	12	ND[b]	3.40 ± 0.9
	36	ND	6.60 ± 2.4
	60	ND	6.60 ± 1.3
	84	0.9 ± 0.1	5.40 ± 1.8
Anoxia	0	0.0 ± 0.0	7.60 ± 3.7
	12	ND	6.93 ± 1.4
	36	ND	8.40 ± 2.1
	60	ND	7.53 ± 1.0
	84	0.8 ± 0.1	7.93 ± 2.8

[a] ± = standard deviation. [b] Not determined.

largely prevented the increase in PFP activity in the cotyledons evident in the control tissue (Fig. 1). The specific activity in the control tissue increases from 2.3 nmol.(mg protein·min)$^{-1}$ to 5.8 nmol.(mg protein·min)$^{-1}$. When the radicle is removed the final specific activity is 3.3 nmol.(mg protein·min)$^{-1}$.

In this study, hypoxia and total anoxia were found which resulted in germination, but with only very limited radicle elongation (Table I). The protrusion and limited radicle elongation under these conditions is not due to merely a physical extension of the radicle, resulting from inhibition, as we have previously shown that dormant seeds will fully imbibe without any radicle elongation (10). The PFP activity in cotyledons of the embryos exposed to these two treatments remained low throughout the incubation period (Table I). From the data it

appears that the increase in PFP activity, in the cotyledons is dependent on the continuous growth of the radicle.

Removal of the radicle after different incubation times clearly showed that the dependence of PFP activity in the cotyledons on the presence of the elongating radicle is maintained throughout the incubation period (Fig. 2). Furthermore, removal of the radicle at different stages of incubation not only prevented a further increase in PFP activity, but also resulted in a large decrease in activity (Fig. 2). It is evident that removal of the radicle at any stage of incubation, causes PFP activity to decrease in 24 h to almost the same level as in tissues where the radicle was removed at time zero (Figs. 1 and 2).

Exposure of intact embryos to anoxia at different stages of incubation had the same effect on cotyledon PFP activity as removal of the radicle (Fig. 3). It appears as if the PFP activity decreases to a basal level of approximately 8 to 15 nmol.(cotyledon·pair·min)$^{-1}$ (2–3 nmol.[mg protein·min]$^{-1}$) when the radicle is removed (Fig. 2) or during exposure to N_2 (Fig. 3). A similar decrease to this level of PFP activity was found during exposure to hypoxia (not shown). It is evident that exposure of the tissue to N_2, after 36 h of incubation, resulted in a rapid decrease in PFP activity. After only 4 h of incubation in N_2, the activity is already significantly lower than that of tissue in air. The kinetics of the decrease in PFP activity also indicate that a certain level of PFP activity remains in the tissue regardless of the type of treatment. In contrast to the decrease in PFP activity during exposure to N_2, a limited further increase in radicle length (40% of that of the control tissue) was found (results not shown).

To determine the glycolytic capacity of cotyledons after different stages of incubation in air and/or N_2, isolated cotyledons were rapidly exposed to an anaerobic environment and measurements were made of CO_2 evolution under anoxia. It is evident that the amount of CO_2 that can be released

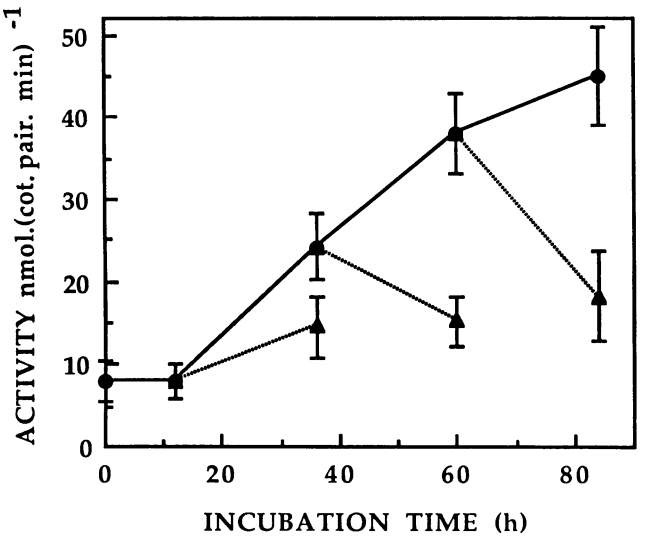

Figure 2. Changes in PFP activity in the cotyledons of intact *C. lanatus* embryos (●) or in cotyledons where the radicle was removed 24 h before enzyme extraction (▲). Error bars indicate standard deviation.

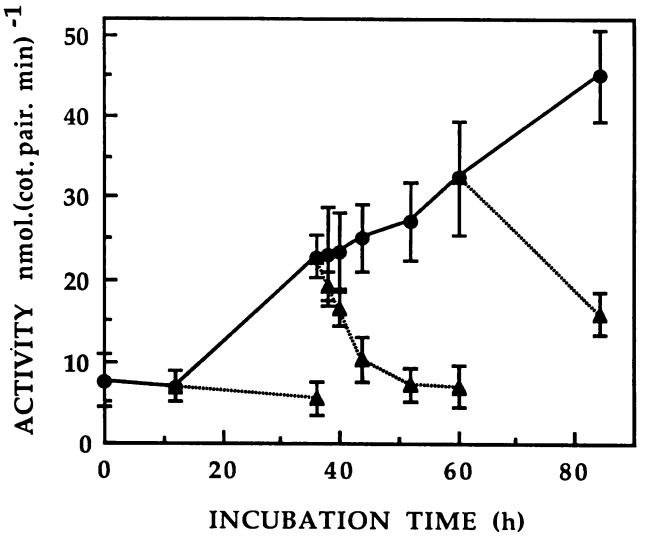

Figure 3. Changes in PFP activity in cotyledons of intact *C. lanatus* embryos (●) and in the cotyledons of intact embryos exposed to anoxia for 24 h prior to enzyme extraction (▲). Error bars indicate standard deviation.

Table II. *Rate of CO_2 release by C. lanatus Cotyledons Under Total Anoxia at Different Stages of Incubation*

Treatment	Rate of CO_2 Evolution
	nmol/(cot/pair/min)$^{-1}$
36 h in air	8.16
12 h in air followed by 24 h in N_2	2.50
60 h in air	14.58
36 h in air followed by 24 h in N_2	4.23
84 h in air	15.35
60 h in air followed by 24 h in N_2	8.00

under anoxia increases over the first 60 h of incubation, whereafter, it levels off (Table II). Exposure of isolated cotyledons to N_2 for 24 h, after 12, 36, or 60 h of incubation in air, not only prevented a further increase in CO_2 release capacity, but resulted in a decrease (Table II).

Treatment of cotyledons, excised at time zero, with kinetin, ethrel, or Ga_3 stimulated PFP activity in the cotyledons (Fig. 4, A, B, and C). The optimum kinetin, GA_3, and ethrel concentration for the stimulation of PFP activity in the excised cotyledons were 1.0, 0.9, and 0.1 μM, respectively (Fig. 4). Treatment of the excised cotyledons with the optimum kinetin and ethrel concentrations resulted in PFP activities similar to those of the cotyledons of intact embryos after 84 h of incubation (Table III). The PFP activity in the presence of GA_3 was significantly higher than that of the excised cotyledons. Continuous exposure of the excised cotyledons to N_2 during the treatment with kinetin and ethrel largely prevented the increase in PFP activity evident in the tissue incubated in air (Table III). It is also evident that the PFP activity in the excised cotyledons after treatment with anoxia was significantly lower than that of the excised cotyledons in air.

Cycloheximide (0.18 μM) prevented the increase in PFP

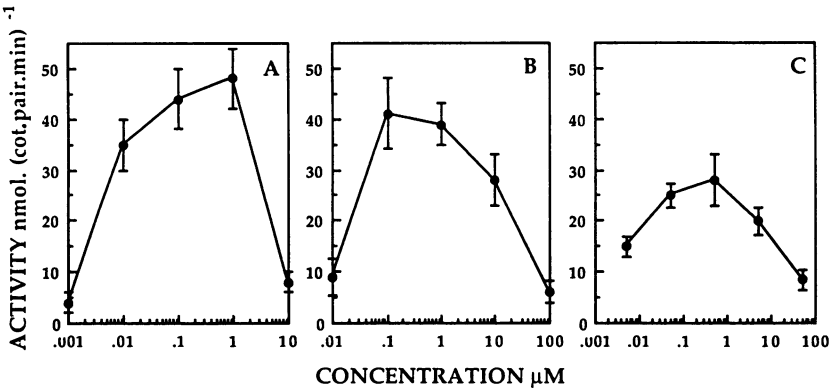

Figure 4. Effect of different kinetin (A), ethrel (B), and GA_3 (C) concentrations on PFP activity of isolated cotyledons of *C. lanatus* embryos incubated for 84 h at 27°C in darkness. Error bars indicate standard deviation.

activity in the cotyledons of intact embryos as well as in the isolated cotyledons treated with kinetin or ethrel (Table III).

DISCUSSION

It has been suggested that increased PFP activity might be indicative of tissues where sucrose breakdown occurs (2, 3, 9, 22). Although this seems to hold for certain tissues (2, 3) it was recently found that PFP activity also markedly increases in the gluconeogenic cotyledonary tissue of *Citrullus lanatus* (12). At present, nothing is known about possible factors controlling PFP levels in plant tissues. Following radicle protrusion PFP activity increases sharply in *C. lanatus* seeds as a result of an increase in the PFP protein concentration (12). Although the total protein increases during the first 84 h of incubation the specific activity of PFP more than doubles, indicating that there is a specific increase in PFP expression.

As is the case with Rubisco levels (14), our results clearly show that the radicle plays a major role in controlling the levels of PFP activity in the cotyledons of *C. lanatus*. Removal of the radicle after different incubation times showed that the effect of the radicle in controlling Rubisco synthesis rapidly diminishes following radicle protrusion (14). In contrast, removal of the radicle at any stage after radicle protrusion resulted in a large decrease in PFP activity in the cotyledons. This decrease is also evident in the specific activity of PFP. To our knowledge this is the first report showing that PFP activity in a specific tissue can be controlled by another distant tissue.

During postgerminative growth the radicle apparently plays an important role in mobilization of reserve material such as proteins in peas and lipids in marrows, cucumbers and other lipid rich seeds (for review, see 20). Removal of the radicle of the Cucurbitaceae, not only inhibits lipid mobilization, but also affects plastid and mitochondrial development in general. The effect of the radicle on mobilization of reserves, and levels of certain enzymes could be linked to a hormonal signal derived from the elongating radicle (18, 19, 21, 31, 34) or to the radicle acting as a sink and thereby preventing feedback inhibition by certain metabolites (18). Especially kinetin and GA_3 have been implicated as the hormones derived from the radicle (8, 27, 28, 31).

Kinetin, ethrel, and GA_3 (to a lesser extent) stimulate PFP activity in isolated cotyledons, and thereby, apparently mimic the effect of the radicle. The effect of these compounds is completely alleviated by the simultaneous application of cycloheximide, indicating that their effect is most probably due to a stimulation of synthesis of PFP. It is well documented that kinetin stimulates the activity of a large number of

Table III. *Effect of Kinetin, Ethrel, and Cycloheximide on PFP Activity in the Cotyledons of C. lanatus Embryos after 84 h Incubation at 27°C in Darkness*

In all cases the treatments were applied for the complete 84 h incubation period. With the exception of the intact control, all other treatments were done on excised cotyledons.

Treatment	PFP Activity	
	nmol/(cot/pair/min)$^{-1}$	*nmol/(mg protein·min)*$^{-1}$
Control (intact)	45.29 ± 7.19[a]	5.8 ± 0.8
Control (excised)	8.67 ± 1.32	3.3 ± 0.9
Cycloheximide 0.18 μM	9.26 ± 3.08	ND[b]
Kinetin 0.5 μM	47.83 ± 7.40	6.9 ± 1.2
Ethrel 0.1 μM	37.23 ± 6.59	4.8 ± 0.6
Ga_3 0.09 μM	26.86 ± 4.02	ND
Kinetin + anoxia	7.67 ± 4.94	ND
Ethrel + anoxia	5.13 ± 1.93	ND
Kinetin + cycloheximide	7.05 ± 3.11	ND
Ethrel + cycloheximide	4.13 ± 1.21	ND
Anoxia	3.00 ± 1.98	0.5 ± 0.01

[a] ± = standard deviation. [b] Not determined.

enzymes, probably by increasing synthesis and/or inhibiting breakdown (20, 21, 25, 26, 31). Ethylene apparently can stimulate the activity of some glycolytic enzymes (4, 5, 17, 35). In cereals GA_3 causes a large increase in the activity of several enzymes involved in reserve mobilization. The role and effect of GA_3 in the mobilization of reserves in the storage organs of dicots, however, is less evident (for review, ref. 8).

The present report, however, is the first to indicate that kinetin, ethrel, and GA_3 have an effect on PFP activity in plant tissue. Whether this is a direct effect of these substances on PFP, or an indirect effect through an alteration in carbohydrate metabolism, is currently under investigation.

Apart from the dependence of PFP on the radicle during early seedling growth, it is also evident that the synthesis of PFP in *C. lanatus* is an aerobic process. Anaerobic stress has been shown to repress normal protein synthesis and induce the synthesis of a characteristic set of proteins in cereal roots. Some of these anaerobic proteins have been characterized as glycolytic and fermentative enzymes (for review, see 29). Our results most probably indicate that PFP in *C. lanatus* cotyledons is not a typical anoxic protein, because activity sharply decreases during anoxia. Very little lactic acid is produced by *C. lanatus* cotyledons under anaerobic conditions while ethanol production increases (FC Botha, unpublished results). We assume that the CO_2 evolution under anoxia largely reflects the glycolytic capacity of the tissue. The apparent glycolytic capacity, between 36 and 84 h of incubation varies from 4.1 to 7.7 nmol.(cotyledon·pair min)$^{-1}$. Exposure of the tissue for 24 h to N_2, after 36 or 60 h in air, resulted in a 80 and 53% decrease in PFP activity, respectively. During the same treatment, however, the apparent glycolytic capacity decreased with 48 and 44% to 2.1 and 4.0 nmol.(cotyledon·pair min)$^{-1}$, respectively. It appears therefore, that regardless of the level of PFK activity, the total PFP activity, by itself, during all stages of incubation, is sufficient to catalyze the apparent maximum glycolytic flux present in the cotyledons.

The *in vivo* concentrations of Pi and Fru-2,6P_2, however, could result in activities substantially lower than that of the maximum *in vitro* activity which was measured at saturating Fru-2,6P_2 concentrations.

At 60 h of incubation the maximum gluconeogenic flux is approximately 20 nmol hexose produced.(cotyledon·pair min)$^{-1}$ (FC Botha, unpublished results). At this stage of incubation, the PFP activity of 32 nmol.(cotyledon·pair min)$^{-1}$ is also adequate to sustain the gluconeogenic flux.

The rapid drop in PFP activity following exposure to N_2 or removal of the radicle could be the result of an arrest in synthesis and/or an increase in inactivation or breakdown. In this regard it is important to note that no indication was found that activation or inactivation was responsible for the observed changes in PFP activity in *C. lanatus*. The similar effect of cycloheximide to radicle removal or N_2 treatment on PFP activity, however, suggests that the effect is probably due to an inhibition of PFP synthesis. Furthermore, the rapid decrease in the PFP activity during exposure to hypoxia, anoxia or after radicle removal clearly shows that PFP breakdown continues under these conditions.

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