Light-Dependent Sucrose Synthesis in Cell-Free (Reconstituted) Lysates of Evacuolated Mesophyll Protoplasts

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Oat mesophyll protoplasts were evacuolated by centrifugation on a Percoll gradient and used as starting material for establishing a functional cell-free system. For this purpose evacuolated protoplasts were osmotically lysed. From the resulting homogenate a cytosolic fraction was obtained by silicon oil filtration. This fraction was used to dilute preparations of lysed evacuolated protoplasts in order to reduce the number of organelles per volume cytosol. The latter was necessary to increase light-dependent oxygen evolution of the cell-free system. The resulting kind of "reconstituted" system showed light-dependent sucrose formation (about 100 nmol (mg Chl)$^{-1} \cdot h^{-1}$) with bicarbonate as the only substrate. As this property depends on a functional interaction of chloroplast and cytosolic reactions, this cell-free system appeared to perform essential steps of partitioning of CO_2 between starch and sucrose. Addition of about 12 μM fructose 2,6-bisphosphate, an inhibitor of fructose 1,6-bisphosphatase and an activator of the PPi-dependent fructose 6-phosphate phosphotransferase, caused sucrose degradation in the light. Thus, this cell-free system allows both the study of cytosolic enzyme activities under quasi *in vivo* conditions and the manipulation of cellular reaction sequences by plasma membrane-impermeable compounds.

Introduction

Many biochemical pathways of cells depend on a coordinated interaction of cytosolic and organellar reaction sequences. For example, sucrose synthesis occurs in the cytosol of photosynthesizing cells [1], but it largely depends on the export of carbon from the chloroplasts which, in turn, is determined by the cytosolic level of inorganic phosphate. In addition, sucrose formation is controlled by the levels of activity of sucrose-P synthetase, fructose 1,6-bisphosphatase, and PFP [2–4]. The latter two enzymes have been shown to be regulated in a multiple way by the interaction of several metabolites, such as fructose 1,6-bisphosphate, fructose 6-phosphate, triose phosphates, phosphoglyceric acid, Pi, pyrophosphate, AMP, and fructose 2,6-bisphosphate (for a recent review see [4]).

Our knowledge about the regulation of carbon partitioning between cytosolic sucrose and chloroplast-located starch largely stems from the determination of metabolite pools in intact leaves or leaf discs [5–8] or from *in vitro* assays with tissue extracts or purified enzymes [*e.g.* 7, 9–11]. Both approaches only give limited insight into events taking place in the intact system because manipulation is either restricted to changes in the environmental conditions (tissue) or takes place in an artifical medium (buffer instead of the complex cytosol: *in vitro* experiments).

Protoplasts constitute some intermediary stage of metabolic analysis. Methods are available which, *e.g.,* allow for the determination of compartmented pool sizes of metabolites and their kinetics. However, owing to the rather limited permeability of the plasma membrane towards charged metabolites, manipulation of metabolic

Abbreviations: EP, evacuolated protoplasts; EPL, osmotically lysed EP; PEPC, phospho-enol pyruvate carboxylase; PFP, pyrophosphate-dependent fructose 6-phosphate phosphotransferase; P_i, inorganic phosphate; VP, vacuolated [control] protoplasts.

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pathways by the addition of effectors is also very limited with this experimental system.

With the reconstitution of broken chloroplasts it was demonstrated that transport-limiting membranes must not necessarily cause an obstacle [*e.g.* 12]. Such an approach, however, is not possible with ordinary leaf mesophyll protoplasts. These contain vacuoles with constituents which are detrimental to a proper cytosolic functioning. During protoplast homogenation the vacuolar membrane will also be disrupted and thus mixing of cytosolic and vacuolar fractions cannot be avoided.

We therefore investigated a possible reconstitution of evacuolated mesophyll protoplasts. We found that these cells are photosynthetically competent and constitute an ideal system for the determination of cytosolic metabolite pools [13, 14]. In this paper we demonstrate that lysates of evacuolated oat mesophyll protoplasts can be used to obtain an only slightly diluted cytosolic fraction. Protoplast lysates supplied with this fraction showed light-dependent synthesis of sucrose with bicarbonate as the only substrate. Furthermore, sucrose formation could be manipulated by the addition of fructose 2,6-bisphosphate.

Materials and Methods

Plant culture and protoplast preparation

Seeds of *Avena sativa* L. (*cv.* Arnold) were germinated in moist peat and grown in a green house at about 80% rel. humidity and 22 to 26 °C. If necessary, daylight was supplemented with light from HQLS lamps (Osram, about 75 W m^{-2}). Leaves were harvested from 7- to 10-day-old plants (about 12 cm long).

Mesophyll protoplasts were isolated and purified as described by Hampp and Ziegler [15], except that the lower epidermis was peeled off and a solution of Mes-NaOH (pH 6.0) containing 0.4 M sorbitol, 1 mM $CaCl_2$ and 20% (v/v) Percoll was used instead of the 0.5 M sucrose layer. These protoplasts were evacuolated on a Percoll gradient [16] essentially as described recently [14] and kept on ice for at least 1 h before carrying out the following steps.

Isolation of a cytosolic fraction

The efficiency of lysis of evacuolated protoplasts was compared for four different approaches: mechanically (by shearing forces), chemically (addition of DEAE-dextran or digitonin), osmotically, and *via* sonification. Osmotical lysis appeared to be the least detrimental approach. For the latter evacuolated protoplasts were washed trice with unbuffered 0.6 M sorbitol (pH 7 to 8) and counted on a hemocytometer (Fuchs-Rosenthal). The final pellet (200 × *g*, 90 s) was dried as far as possible by carefully succing off the supernatant. Lysis was then induced by a slow addition (30 to 60 s) of about trice the protoplast volume of doubly distilled water (10^6 evacuolated protoplasts correspond to about 5.6 µl). This procedure resulted in complete protoplast rupture without affecting chloroplast integrity (assayed *via* ferricyanide reduction [17]). Fractionation of the protoplast homogenate was by silicon oil filtration [18]. Best results were obtained by filtration through AR 130 (see Results; mixture of the oil types AR 20 and AR 200; Wacker Chemie, Burghausen, F.R.G.). Microfuge tubes (400 µl, Beckman) were filled with 100 µl silicon oil on top of which 20 µl of lysed evacuolated protoplasts (about 10^6) were added. After centrifugation (10000 × *g*, 10 min, 4 °C; Hettich Mikrorapid/A, Tuttlingen, F.R.G.) the clear supernatant was collected. Aliquots were either used directly for assays and the determination of marker enzyme activities [19, 20], or were frozen in liquid nitrogen and kept at −80 °C.

Reconstitution of protoplasts and incubation

Incubation of reconstituted EP was in a miniaturized, self-constructed Clark-type oxygen electrode. The thermostated cuvette (inner diameter 2 mm) had a variable volume of between 5 and 50 µl. Stirring of the sample was from above with a motor-driven nylon loop. For studying photosynthetic activities protoplast lysis was in 1 mM $NaHCO_3$ (pH 7.5). In order to decrease the viscosity of the lysate and to increase its volume for better illumination efficiency each protoplast lysate was diluted with one volume of a separately isolated cytosolic fraction (see above). Illumination of the samples was with cold light (about 2000 $\mu E \cdot m^{-2} \cdot s^{-1}$; KL 1500, Schott). In a typical experiment about 2×10^6 protoplasts (45 µl) were incubated under continuous stirring (200 rpm) at 20 °C. After a preincubation period of 5 min in the dark illumination was started and 2- to 5-µl-ali-

quots were removed at given time intervals (see Results). These were injected into acid or base and used for the determination of metabolites.

Determination of metabolites

Adenine nucleotides were determined from neutralized $HClO_4$ extracts by luminometry [21], sucrose according to Outlaw and Tarczynski [22] from boiled (5 min) KOH (0.2 M) extracts. The amount of total adenine nucleotides (ATP + ADP + AMP: AdN) was always assayed in aliquots taken from the protoplast suspension after lysis and referred to cell number. As we could not detect any change in the total amount during the incubation of lysates, this measure was always taken in parallel as an internal reference in order to determine the loss of cells during the withdrawal of supernatant (step before lysis). Fructose 2,6-bisphosphate was standardized as described [14].

Results and Discussion

Lysis of evacuolated protoplasts. Properties of the cytosolic fraction

In order to increase the volume of protoplast lysates (= decrease of number of chloroplasts per volume) without an unacceptable dilution of the cytosolic metabolite pools or enzyme concentrations fractions enriched in cytosol had to be prepared. This was achieved by silicon oil filtration of lysed protoplasts. We tested a range of different silicon oils. From these the most efficient types are shown in Table I. The retention of the cytosolic marker PEPC was best with AR 130. Fumarase activity (mitochondria) was absent, while aldolase (more than 95% of total activity chloroplast associated) was present only to a limited extent.

This cytosolic fraction had a pH of 7.0 (S.D. ± 0.2, n = 10) which was surprisingly constant for all preparations. Addition of 1 mM $NaHCO_3$ resulted in an increase of 0.2 pH units which was about the same when the complete cell-free system was exposed to light (not shown). Thus, bicarbonate did not cause unphysiological changes in the assay pH.

Oxygen consumption in the dark and light-dependent oxygen evolution of the cell-free system

Changes in the oxygen content of suspensions of mesophyll protoplasts can easily be assayed with conventional Clark-type oxygen electrodes (*e.g.* [23]). Owing to the small volume of our cell-free system we had to construct a miniaturized electrode arrangement which allowed the use of volumes between 10 and 50 µl. In Table II rates of oxygen production or consumption are compared for vacuolated, evacuolated and "reconstituted" protoplasts. Clearly, manipulation of vacuolated protoplasts resulted in decreased rates. For EP this should be a consequence of the very tight packing of chloroplasts (compare [13]) which will cause a reduced efficiency of illumination within a single protoplast. With the cell-free system packing is even tighter (about 10 mg chlorophyll · ml^{-1}) and illumination efficiency thus still lower. The addition, *via* lysis, of $NaHCO_3$ (about 20 nmol · (10^6 protoplasts)$^{-1}$) was thus sufficient to sustain linear rates for up to 10 min. These could be extended by further addition of bicarbonate.

As the mutual shadowing of chloroplasts should not affect respiration, ratios of the rates of oxygen production in the light and consumption in the dark should be different with the respective cell system. The data given in Table II show that in-

Table I. Oil filtration of lysed evacuolated oat mesophyll protoplasts. Percentage of total marker enzyme activities retained in the supernatant after centrifugation (10,000 × g, 10 min, 4 °C) of lysed protoplast preparations through silicon oils of different physical properties.

Type of oil	Viscosity (cST 25 °C)	Spec. Density [kg/l]	Fumarase	PEPC	Aldolase
AR 120	120	1.02	0	63	19
AR 130	130	1.025	0	81	14
AR 140	140	1.03	0	36	14

Table II. Rates of respiratory consumption or light-dependent evolution of oxygen by vacuolated (VP), evacuolated (EP) and "reconstituted" (EPL) oat mesophyll protoplasts. Lysed protoplasts were diluted with the same volume of a cytosol fraction prepared in parallel. Lysis was with 1 mM $NaHCO_3$. Values are μmol·(mg Chl·h)$^{-1}$.

	Light	Dark	Light/dark
VP (n = 17)	20 to 114	8 to 33	2.2 to 6.0
EP (n = 20)	8 to 53	3 to 18	2.0 to 5.7
EPL (n = 11)	0.8 to 3	0.8 to 1.4	0.9 to 2.0

deed highest L/D ratios were found in control (vacuolated) protoplasts, while those determined for the cell-free system were considerably lower. This implies that dark respiration was less affected by reconstitution.

In general, there was a very high fluctuation of oxygen rates between individual protoplast preparations depending on season (Table II). This should largely be due to the growth conditions of our seedlings (greenhouse with supplemented natural daylight) and is also expressed by the individual data obtained during an experimental period of about nine months (Fig. 1). Clearly, highest rates of light-dependent oxygen evolution of vacuolated oat mesophyll protoplasts were obtained between April and August, and this was largely reflected by the rates measured after evacuolation (Fig. 1), or with the cell-free system (not shown). Reconstitution was thus only appropriate with reasonable photosynthetic activities of the respective protoplast preparation. Otherwise no light-dependent sucrose synthesis could be detected (see below).

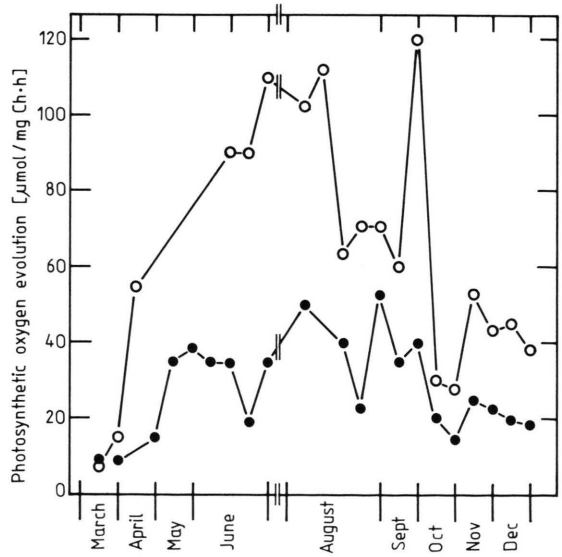

Fig. 1. Rates of photosynthetic oxygen evolution of vacuolated (○; control) and evacuolated (●) oat mesophyll protoplasts prepared between March and December.

Changes of metabolite pools in the reconstituted cell-free system

Adenylates

Compartmentational studies on adenylates in mesophyll cell protoplasts [19, 24, 25] showed characteristic fluctuations upon dark-light transition. In Table III the amounts of ATP and ADP in EPL within 150 s after the onset of illumination are presented. The data, best expressed by the ratio of ATP/ADP, showed a clear increase in ATP formation within 30 s of illumination which was almost the same as shown earlier for intact oat mesophyll protoplasts and fractions thereof [19, 24].

Sucrose

Changes during dark incubation

When protoplast preparations were transferred from storage (about 4 °C) to assay conditions (20 °C) sucrose levels decreased with time under continuous darkness (Fig. 2).

Degradation of sucrose was more pronounced with reconstituted protoplast lysates. As dark respiration is lower in the cell-free system this increased degradation of sucrose should not be due to increased rates of respiration but could indicate the establishment of a new equilibrium between cytosolic metabolite pools, possibly in response to the preceding dilution during lysate preparation.

Table III. Changes in the amounts of ADP and of ATP in reconstituted protoplast lysates during dark-light transition. Values (nmol (10^6 protoplasts)$^{-1}$) are from 8 independent experiments. Numbers in parentheses: SD.

Period of illumination (s)	0	30	60	150
ATP	7.4	10.4	9.6	8.8
	(4.8)	(4.7)	(4.5)	(3.1)
ADP	5.3	2.8	3.6	4.3
	(2.5)	(1.8)	(1.7)	(1.8)
ATP/ADP	1.4	3.7	2.7	2.0

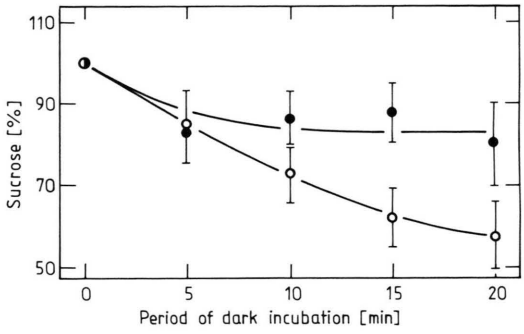

Fig. 2. Sucrose degradation in the dark in preparations of evacuolated (●) and lysed protoplasts (○); 100% are equivalent to about 35 nmol sucrose $(10^6$ protoplasts$)^{-1}$.

Changes during illumination

Light-dependent sucrose synthesis depends on both chloroplasts and cytosol. Triosephosphates, synthesized in the chloroplast stroma and exported *via* the phosphate translocator of the inner chloroplast membrane [26], form the substrate for cytosolic sucrose formation. With bicarbonate as the only added substrate this pathway should thus be an indicator for the functionality of our cell-free system as far as carbon partitioning is concerned. In Fig. 3 sucrose levels in cytosol-enriched protoplast lysates are shown during an initial period of illumination. Upon illumination there was a switch from degradation to formation of sucrose. Net synthesis of sucrose generally occurred from 10 min of illumination onwards.

The absolut sucrose content at the beginning of the experiment varied considerably with each preparation and was between 10 and 90 nmol·$(10^6$ protoplasts$)^{-1}$ during an experimental period of several months (compare Fig. 1). On average the dark level (100%) immediately before the illumination was started was about 30 nmol·$(10^6$ protoplasts$)^{-1}$. The average increase in sucrose between 10 and 15 min (Fig. 3) converts thus into a rate of about 100 nmol sucrose formed (mg Chl)$^{-1}\cdot$h^{-1} (1 mg of chlorophyll equals about 5×10^6 protoplasts). This is considerably lower compared to rates published in the literature (between below 1 and up to 10 µmol (mg Chl)$^{-1}\cdot$h^{-1}; calculated from drawings which show the initial period of illumination of soybean or pea leaves [6]; spinach leaf discs [27]; mesophyll protoplasts from wheat [28], or determined for intact vacuolated or evacuolated oat mesophyll protoplasts (about 1.5 µmol (mg Chl)$^{-1}\cdot$h^{-1}; unpublished data).

Effect of fructose 2,6-bisphosphate on sucrose synthesis

Fructose 2,6-bisphosphate affects carbon partitioning between starch and sucrose in that low levels favour sucrose synthesis while concentrations of 10 µM or more inhibit its formation [4, 14]. Addition of fructose 2,6-bisphosphate (final concentration about 12 µM) to our cell-free system in parallel to the onset of illumination resulted in an extended period of sucrose degradation (up to 15 min of illumination). This was significantly different from the response of controls (Fig. 3). According to accepted schemes of the interaction of fructose 2,6-bisphosphate with sucrose metabolism [4] this response can easily be explained. Increased levels of fructose 2,6-bisphosphate will inhibit FBPase and thus the gluconeogenic pathway in the cytosol which eventually will decrease sucrose synthesis. On the other hand, they will increase the activity of PFP which works in either direction (glycolysis and gluconeogenesis). In our system obviously the glycolytic path was favoured.

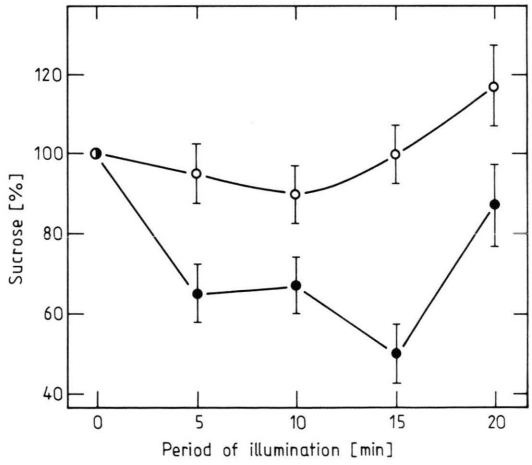

Fig. 3. Illumination-dependent changes of sucrose in a cell-free system prepared from evacuolated oat mesophyll protoplasts in the absence (○) or in the presence (●) of fructose 2,6-bisphosphate (final concentration: 12 µM). For better comparison levels of sucrose determined in each independent experiment (n = 10) at the end of a 5-min-dark incubation at 20 °C were set as 100%, which is 35.8 ± 25.4 nmol $(10^6$ evacuolated protoplasts$)^{-1}$.

On average, the cytosolic sucrose concentration in our cell-free system was only about 2 mM (30 nmol (10^6 protoplasts)$^{-1}$; 2×10^6 protoplasts per 45 µl total assay volume; cytosolic volume due to dilution about 80% of total, *i.e.* 36 µl) and thus considerably lower compared to evacuolated intact protoplasts. These contained about 15 mM sucrose (30 nmol (10^6 protoplasts)$^{-1}$; cytosol: 2 µl (10^6 protoplasts)$^{-1}$; [14]).

Periods of illumination longer than 15 min resulted in an increase of sucrose also in the fructose 2,6-bisphosphate-treated samples (Fig. 3). This change could be due to increased cytosolic levels of triose phosphates (exported from chloroplasts) which are known to support sucrose synthesis *via* the inhibition of fructose 2,6-bisphosphate formation (FBPase inhibition is suspended while PFP is no longer activated). Fructose 2,6-bisphosphate dephosphorylation is thus no longer counter-balanced and fructose 2,6-bisphosphate levels should decrease with time.

Conclusion

In this contribution we demonstrate that lysates of evacuolated oat mesophyll protoplasts, diluted with separately prepared cytosol, show light-dependent sucrose synthesis with bicarbonate as the only substrate. It thus appears to be functional as far as carbon partitioning into sucrose is concerned. Owing to the lack of transport-limiting plasma membranes, this system offers considerable potential for investigating, *e.g.*, the regulation of sucrose synthesis under quasi *in vivo* conditions. PFP, for example, is regulated in many ways *via* activation or product inhibition (*e.g.* [4, 11]). However, effective concentrations determined mainly *in vitro* show what happens with a purified enzyme in an artificial (buffer) environment. With our system manipulation of pathways by changing the concentrations of effectors or intermediates is possible at "nearly cytosolic" conditions.

Work is now under progress to determine cytosolic metabolite and effector concentrations connected to the synthesis or degradation of sucrose and their response towards the addition of substrates such as Pi, pyrophosphate and others.

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