Regulation of Mitochondrial Function and Biogenesis in Cucumber (Cucumis sativus L.) Cotyledons during Early Seedling Growth

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ABSTRACT

The aim of this work was to characterize the respiratory metabolism of the green cotyledons of cucumber (Cucumis sativus L.) during early seedling growth and to investigate how this is integrated with changes in mitochondrial biogenesis and function. In light-grown cotyledons, lipid mobilization extended from germination to 6 days postimbibition, reaching a maximum at 3 to 4 days postimbibition. The rate of dark oxygen uptake reached a maximum at 2 days postimbibition in dark-grown and 3 days postimbibition in light-grown cotyledons. Development of photosynthetic capacity occurred from 4 to 7 days postimbibition. In dark-grown cotyledons, lipid mobilization extended beyond 7 days postimbibition, and there was no greening or acquisition of photosynthetic competence. Measurements of mitochondrial function indicated that the respiratory capacity of the tissue changed such that during lipid mobilization there was a much greater capacity for the operation of the noncarnitine cycle of the citric acid cycle (succinate to oxaloacetate), whereas during the development of photosynthetic function, the activity of the remainder of the cycle (oxaloacetate to succinate) was induced. Comparison of the maximum capacities for mitochondrial substrate oxidations in vitro with the rates of in vivo substrate oxidations, predicted from the rate of lipid breakdown, indicated that mitochondria in this tissue operate at or below state 4 rates, suggesting limitation by both availability of ADP and substrate.

The aim of the work presented in this paper was to characterize the respiratory metabolism of the cotyledons of cucumber (Cucumis sativus L.), during the immediate postgerminative phase in development, and to investigate how this is integrated with mitochondrial biogenesis. Plant mitochondria play a role in a number of distinct metabolic pathways, including lipid mobilization (14), photosynthesis (9), and the generation of intermediates necessary for biosynthesis (for example, acetate; 25). In addition, both photosynthetic and nonphotosynthetic tissues, mitochondrial oxidative phosphorylation is a major source of ATP (11, 13, 20). These pathways have been well characterized (see, for example, ref. 10), but little is known about their regulation, particularly in terms of the interactions between mitochondria and the rest of cellular metabolism.

The cotyledons of cucumber provide an amenable system for the study of the interactions between mitochondria and other metabolic processes. During the immediate postgerminative period, the cotyledons pass through two metabolic phases: during the initial stages, the primary activity is the mobilization of stored lipid; this is then followed by lipid mobilization and greening of the cotyledons to produce a fully photosynthetic tissue (3). It is expected that specific mitochondrial functions are required during the lipid mobilization phase, the photosynthetic phase (photorespiration), and the transition between the two.

In this study, the dark respiration rate of the cotyledons, the properties of isolated cotyledon mitochondria, and the variation in mitochondrial enzyme activities were measured during the course of development. These parameters were then compared with known markers of the lipid mobilization and photosynthetic phases of development.

MATERIALS AND METHODS

Plant Material

Seeds of cucumber (Cucumis sativus L. var Masterpiece) (Wm. K. McNair, Edinburgh, UK) were imbibed in tap water in the dark at 4°C for 16 h. The seeds were sown onto wadding (Robinson and Sons Ltd., Chesterfield, UK), thoroughly soaked in distilled water, and germinated at 28°C on a 12 h light/dark cycle with a 5°C night temperature depression in a Fisons model 600G3/THTL growth chamber. The RH around the seedlings was maintained at a high level using a transparent plastic propagator. The light intensity was 200 μmol quanta m⁻² s⁻¹. For dark-grown material, the seed trays were wrapped in tinfoil and placed in a black plastic bag within the same growth chamber as the light-grown material. Cotyledons were harvested onto ice within 1 h of the start of the light period and used immediately.
Enzyme Assays

Twenty cotyledons (or 2 g leaf tissue) were ground, first in a pestle and mortar, then in a Teflon-in-glass homogenizer in 8 mL extraction buffer (50 mM triethanolamine-HCl, pH 7.0, 2 mM MgCl₂, 1 mM EDTA, 2% [w/v] PVP-40), and the final volume measured. This extract was used immediately for enzyme assays.

Standard methods were used for the assay of ICL³ (7), HPR (21), fumarase (12), and ICDH (18).

Recovery experiments were performed as follows. For each extract, a duplicate extract was prepared using extraction buffer to which had been added a known amount of the enzyme to be assayed (commercial preparations from Sigma Chemical Company, Poole, UK). An activity approximately equivalent to the activity expected in the extract was added. The difference in activity between the two extracts gives a measure of the losses of activity during extraction. The recoveries obtained were: ICL, 91 to 102%; HPR, 91 to 99%; fumarase, 91 to 100%; ICDH, 89 to 97%.

Isolation of Intact Mitochondria

Mitochondria were isolated using a method modified from Day et al. (8). Cotyledons (10–50 g) were harvested onto ice, counted, washed with cold distilled water, and chilled for 30 min at 0°C. All subsequent operations were carried out at 4°C or below. The cotyledons were homogenized in 3 volumes of grinding buffer (0.3 M sucrose, 10 mM KH₂PO₄, 2 mM EDTA, 2 mM MgCl₂, 2 mM glycine, 1% [w/v] PVP-40, 1% [w/v] BSA, 30 mM iso-ascorbic acid, 25 mM pyrophosphate buffer, pH 7.6) with four 1 to 2 s bursts of a Polytron homogenizer at setting 7, fitted with a PTA-36 probe. The homogenate was filtered through 4 layers of muslin and the filtrate centrifuged at 1,000g for 5 min. The supernatant was collected and centrifuged at 12,000g for 20 min. The resulting pellet was resuspended in wash buffer (0.3 M sucrose, 1 mM glycine, 0.1% [w/v] BSA, 10 mM Tes buffer, pH 7.2) using a Teflon-in-glass homogenizer, and this suspension was centrifuged at 1,000g for 5 min. The supernatant was collected and centrifuged at 12,000g for 20 min. The pellet was resuspended in wash buffer and layered onto 38% (v/v) Percoll (Pharmacia), 1 mM glycine, 0.1% (w/v) BSA, 10 mM KH₂PO₄ (pH 7.5) containing a linear gradient of 0 to 10% (w/v) PVP-40. After centrifugation at 40,000g for 45 min, the mitochondrial band was removed, diluted in 5 to 10 volumes of wash buffer, and the mitochondria collected by centrifugation at 12,000g for 15 min. The pellet was resuspended in wash buffer (without glycine) and centrifuged at 12,000g for 15 min. The pellet was resuspended in wash buffer (without glycine) and used immediately.

Mitochondrial yield was measured by assaying both the initial homogenate and the mitochondrial fraction for fumarase as described above. Yields of 10 ± 2% were routinely obtained from 3- to 7-d-old light- and dark-grown cotyledons. Rates were expressed on a per cotyledon basis, after correcting for mitochondrial yield.

Mitochondrial outer membrane intactness was determined according to Neuburger (17). The results presented were obtained with mitochondrial preparations whose outer membrane intactness was 90% or greater. Mitochondrial preparation from cotyledons at 0 to 2 d postimbibition had an outer membrane intactness of less than 65%, and consequently were not used.

Oxygen Uptake and Evolution

Oxygen uptake and evolution by pairs of cotyledons due to dark respiration and photosynthesis were measured polarographically using a leaf disc electrode (Hansatech, Kings Lynn, UK) according to Walker (23). For photosynthetic measurements, cotyledons were illuminated with red light of an equivalent photon flux density to that of the growth conditions (200 μmol quanta m⁻² s⁻¹).

Oxygen uptake by isolated mitochondria was measured polarographically using a liquid phase oxygen electrode (Hansatech) at 25°C in 1 to 2 mL standard reaction medium (0.3 M sucrose, 10 mM KH₂PO₄, 5 mM MgCl₂, 0.1% [w/v] BSA, 10 mM Tes buffer, pH 7.0) containing mitochondria (0.2–1.0 mg protein). Respiration was initiated by the addition of 10 mM substrate followed by 100 nmol ADP. The following additions were also made: 0.25 mM ATP during succinate oxidation, 10 mM glutamate during malate oxidation, and 0.1 mM thiamine pyrophosphate during pyruvate and 2-oxoglutarate oxidation. The rate of substrate oxidation was unaffected by the addition of CoA. Mitochondria were passed through one state 3/4 transition before measurement of the state 3 and following measurement of state 4 rates.

Other Assays

The protein content of mitochondrial preparations was determined, following precipitation with TCA, using the method of Bradford (4), with BSA as standard. Chl content was measured in cotyledon extracts in 80% (v/v) acetone according to Arnon (1). Total lipid content was determined using methanol:chloroform extracts (2:1, v/v) according to Radin (19).

RESULTS

Lipid Mobilization, Oxygen Uptake, and Photosynthesis

Figure 1 shows the variation in the lipid and Chl content, and the rates of lipid breakdown, Chl synthesis, photosynthesis, and respiration during the early seedling development of cucumber cotyledons. The net rates of lipid mobilization and Chl synthesis were calculated as the difference between lipid or Chl contents on successive days. These calculations were carried out on the data from individual replicates, resulting in a positive rate of lipid breakdown between days 6 and 7 light-grown, despite the mean lipid content increasing. The rate of lipid breakdown reached a maximum at 4 d postimbibition in the cotyledons of both light- and dark-grown seedlings, but in the latter the rates were lower and lipid mobilization was observed up to day 7 (Fig. 1A). The rate of oxygen uptake in the dark reached its maximum value at 2 d postimbibition in dark-grown cotyledons, and 3 d postimbibition in light-grown cotyledons (Fig. 1C). The de-
cline following the maximum rate of oxygen uptake was more rapid in light-grown than in dark-grown cotyledons. Both the capacity to carry out photosynthesis and the presence of Chl could be detected, in cotyledons from light-grown seedlings only, from 3 d postimbibition onwards (Fig. 1, B and D). The Chl content and photosynthetic rate reached their maximum values at day 7 (data not shown), whereas the maximum rate of Chl synthesis occurred between days 5 and 6 (Fig. 1B). The developmental variation in the activities of the marker enzymes ICL and HPR is shown in Figure 2, A and B. In light-grown material, ICL activity appeared soon after imbibition, reached a maximum at 4 d postimbibition, and declined to near zero by 7 d postimbibition (Fig. 2A). A similar pattern was observed in cotyledons from dark-grown seedlings, except that there was still appreciable ICL activity at 7 d postimbibition (Fig. 2A). The activity of this enzyme therefore appears to be temporally regulated and modulated by light.

HPR activity increases in light-grown cotyledons from 3 d postimbibition onwards, but remains low in dark-grown cotyledons (Fig. 2B). Therefore, HPR is induced in a light-dependent manner, and the development of this enzyme parallels the increase in photosynthetic rate (Fig. 1D).

Mitochondrial Enzyme Activities

The developmental variation in the activity of fumarase and ICDH is shown in Figure 2, C and D. Between 3 and 7 d postimbibition, there was a fivefold increase in fumarase activity in light-grown cotyledons and a fourfold increase in dark-grown cotyledons (Fig. 2C). ICDH activity increased in light-grown cotyledons after day 5, but did not change in dark-grown cotyledons (Fig. 2D). In cotyledons from both light- and dark-grown seedlings, the activity of fumarase was significantly greater than that of ICDH (Fig. 2, C and D), in contrast with the situation in the fully expanded first leaf, where the activities of these enzymes were similar, being 68 ± 9 and 69 ± 4 nmol min⁻¹ g⁻¹ fresh weight, respectively.

Oxidation of Respiratory Substrates by Cotyledon Mitochondria

The variation in the state 3 rates of succinate, malate, pyruvate, and glycine oxidation during early seedling growth are shown in Figure 3. These data are expressed on a per cotyledon basis. Because the cell number in the cotyledons does not change during development (3), this is equivalent to expressing the data on a per cell basis. In light-grown cotyledons, there was a fourfold, and in dark-grown cotyledons a threefold, increase in the rate of succinate oxidation between 3 and 7 d postimbibition (Fig. 3A). The rates of oxidation of malate, pyruvate, and glycine showed light-dependent increases during development (Fig. 3B). The malate oxidation rate increased from day 3 to day 7; glycine oxidation increased.

![Figure 1](image1.png)

**Figure 1.** Developmental changes in the lipid content (A), Chl content (B), dark oxygen uptake rate (C), and photosynthetic rate (D) of cotyledons from light- and dark-grown cucumber seedlings. Lipid breakdown and Chl synthesis rates (histograms) were calculated from the difference between lipid or Chl content on consecutive days. Calculations were carried out on the raw data before any statistical treatment. Values are the mean of three (A and B) or five (C) separate determinations; bars represent the se. Photosynthetic rate (D) is based on a single representative experiment. Open symbols, light-grown seedlings; closed symbols, dark-grown seedlings.
Figure 2. Developmental changes in the catalytic activity of ICL (A), HPR (B), fumarase (C), and ICDH (D) in the cotyledons of light- and dark-grown cucumber seedlings. Values are the mean of three separate determinations; bars represent the se. Open symbols, light-grown seedlings; closed symbols, dark-grown seedlings.

Figure 3. Developmental changes in state 3 rate of oxidation of succinate (A) and malate, pyruvate, and glycine (B) by mitochondria isolated from the cotyledons of light- and dark-grown cucumber seedlings. Data are expressed on a per cotyledon basis, and are corrected for mitochondrial yield. Values are the mean of three separate determinations; bars represent the se. Open symbols, light-grown seedlings; closed symbols, dark-grown seedlings.
between day 3 and day 5; and, in contrast, pyruvate oxidation began to increase at day 5 (Fig. 3B). It should be emphasized that under the conditions used (10 mM glutamate and pH 7.0), both malate dehydrogenase and NAD-malic enzyme contributed to malate oxidation. The rate of externally supplied NADH oxidation increased during development in cotyledons from both light- and dark-grown seedlings (Table I), although the magnitude of the increase was greater in light-grown cotyledons. The state 4 (ADP-limited) rates of succinate and NADH oxidation are also shown in Table I. There is also an increase in the capacity of the tissue for oxidation at state 4 rates.

The rates of oxygen uptake with the range of substrates, expressed in terms of mitochondrial protein, are shown in Table II. The only oxidation rate that showed significant developmental variation when expressed on this basis was that of glycine, which is oxidized only by mitochondria from 7-d-old light-grown cotyledons. This indicates that the changes observed in the respiratory capacity of the tissue are primarily due to a net synthesis of mitochondrial protein.

### Discussion

The measurement of lipid content and ICL activity, and Chl content, in vivo photosynthetic rate, and HPR activity, established the developmental pattern of expression of parameters associated with either lipid mobilization or photosynthesis. Any variation in dark respiration rate or respiratory capacity of the tissue during development, therefore, could be correlated with the metabolic phases of development. Following germination, lipid mobilization was initiated irrespective of the light environment, although growth in complete darkness reduced the rate of lipid breakdown and extended the lipid mobilization phase (Figs. 1A and 2A). Photosynthetic development was dependent on light (Figs. 1, B and C, and 2B). Similar phenomena have been previously described in the immediate postgerminative development of cucumber (3, 22) and soybean cotyledons (2).

The rate of oxygen uptake by cotyledons in the dark consists of two components; that due to β-oxidation of fatty acids, specifically the reaction catalyzed by acetyl-CoA oxidase, and a mitochondrial component, dependent on the availability of substrates such as succinate, NADH, malate, and ADP. The mitochondrial rate of succinate and NADH oxidation required to support the measured rate of lipid breakdown can be predicted. For example, on day 1 in light-grown cotyledons, the rate of lipid breakdown, if the lipid is assumed to have been tripalmitin glycerol, would have been 11.49 nmol acetate units min⁻¹ cotyledon⁻¹. For each acetate unit, the β-oxidation cycle consumes 0.5 units of oxygen, so that the peroxisomal oxygen uptake would be 5.74 nmol min⁻¹ cotyledon⁻¹. For every 2 acetate units, 1 unit of succinate is produced, so that the succinate synthesis rate is 5.74 nmol succinate min⁻¹ cotyledon⁻¹. Because oxidation of 1 unit of succinate results in the uptake of 0.5 unit oxygen, the oxygen uptake due to succinate is 2.87 nmol min⁻¹ cotyledon⁻¹. For each acetate unit, the β-oxidation cycle produces 1 NADH, so that the NADH production rate is 11.49 nmol NADH min⁻¹ cotyledon⁻¹. Sucrose synthesis requires 1 unit NADH for every 2 units of acetate that are converted to hexose phosphate, so that the net NADH synthesis rate is 5.74 nmol min⁻¹ cotyledon⁻¹. Because the oxidation of 1 unit of NADH consumes 0.5 unit of oxygen, the oxygen uptake due to NADH is 2.87 nmol min⁻¹ cotyledon⁻¹. Similar calculations were carried out for the other time points and the results are summarized in Table III. This table also summarizes the relationship between the predicted rates of substrate oxidation and state 3 rates measured in vitro. The following points are emphasized. First, between 2 and 3 d following imbibition, the tissue oxygen uptake rate predicted from the rate of lipid 

### Table I. Oxidation of Succinate and NADH by Cucumber Cotyledon Mitochondria

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Light-grown</th>
<th>Dark-grown</th>
</tr>
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<tbody>
<tr>
<td>Succinate</td>
<td>275 ± 67</td>
<td>250 ± 43</td>
</tr>
<tr>
<td>Malate</td>
<td>210 ± 53</td>
<td>185 ± 48</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>16 ± 2</td>
<td>9.2 ± 0.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
<td>86 ± 27</td>
</tr>
<tr>
<td>NADH</td>
<td>212 ± 41</td>
<td>187 ± 32</td>
</tr>
</tbody>
</table>

* DPI = days postimbibion.
breakdown was lower than the measured in vivo oxygen uptake rate, suggesting that other substrates were respired at this stage. Second, the rate of oxygen uptake allowed for the mitochondrial oxidation of both succinate and NADH generated by fatty acid breakdown, except at 4 d postimbibition in cotyledons from light-grown seedlings and at 6 d postimbibition in dark-grown seedlings. This indicates that cytosolic NADH oxidation was not linked to oxygen uptake, but is oxidized cytosolically. This may reflect an increased cytosolic demand for NADH due to active biosynthesis. Third, at 7 d postimbibition the rate of oxygen uptake, which at this stage was largely mitochondrial due to the low rates of lipid breakdown (Fig. 1A), was greater in light-grown than in dark-grown cotyledons, suggesting a greater rate of dark respiration in photosynthetic tissue (Fig. 1C). Fourth, mitochondria from this tissue operated at no more than 42% of the maximum state 3 rates of substrate oxidation, at least during the period examined. For succinate oxidation, mitochondria in vivo were estimated to operate at or below rates equivalent to state 4 in vitro (Table III). This was also true for NADH oxidation, except in 4-d-old light-grown seedlings (Table III). This suggests that cucumber cotyledon mitochondria were limited in vivo by both ADP and substrate availability. It should be noted that this analysis only applies to the nonphotosynthetic stages of development and photosynthetic cotyledons in the dark. When photosynthetic cotyledons are illuminated, oxidation of glycine generated by photorespiration may result in much higher mitochondrial O2 uptake rates. This may explain the "excess" mitochondrial capacity that appears unused for lipid breakdown.

During lipid mobilization, the capacity for the flow of carbon through the decarboxylating side of the citric acid cycle was restricted, but during chloroplast biogenesis and the associated development of photosynthetic function, the capacity existed for operation of the complete citric acid cycle (Figs. 2, C and D, and 3). The restriction of citric acid cycle decarboxylation is consistent with evidence from tissue slices of castor bean endosperm (6). In this tissue, the activity of enzymes and oxidation rate of substrates on the nondecarboxylating side of the citric acid cycle were higher than those on the decarboxylating side (7, 15). An exception to this was the oxidation rate of malate. This was low due to the accumulation of oxaloacetate, which is exported at very low rates from castor bean endosperm mitochondria (15). The equilibrium position of the malate dehydrogenase reaction does not favor oxaloacetate production (9). In our experiments, the oxidation of malate (Fig. 3B) was measured in the presence of glutamate to remove oxaloacetate by transamination. Very low rates of pyruvate oxidation were observed by cucumber cotyledon mitochondria (Table II). Pyruvate was not metabolized via the citric acid cycle in castor bean endosperm (16), and this appeared at least in part to be due to a low capacity for mitochondrial pyruvate oxidation (15). Cucumber cotyledon mitochondria show higher activities of the external NADH dehydrogenase than those from castor bean endosperm (Table I) (15). This would enable a greater proportion of the NADH generated during lipid breakdown to be oxidized by the mitochondria without the need for a reducing equivalent transport mechanism, such as that proposed by Mettler and Beevers (14).

During early seedling growth of soybeans, similar developmental changes have been described in the dark respiration rate of the cotyledons (2). In this species, the variation is brought about by an increase in mitochondrial capacity for
the oxidation of a range of substrates (2). In contrast, in cucumber cotyledons there is no significant change in the mitochondrial capacity (Table II). For example, in light-grown cotyledons, the rate of succinate oxidation by isolated mitochondria changes only from 275 to 250 nmol min⁻¹ (mg protein)⁻¹ between day 3 and day 7 (Table II), whereas the capacity of the tissue to oxidize this substrate increases from 16 to 49 nmol min⁻¹ cotyledon⁻¹ (Fig. 3A). There was, therefore, a net synthesis of mitochondrial protein, resulting in an increased respiratory capacity of the tissue (Fig. 3). In addition, these species also differ in that the difference between the capacity for the oxidation of substrates from the nondecarboxylating and the carboxylating sides of the citric acid cycle is less marked in soybean (5) than in cucumber (Fig. 2). This may reflect the importance of lipid, rather than protein, as respiratory substrate in cucumber.

Mitochondria from soybean cotyledons exhibited their maximum rates of citric acid cycle substrate oxidation when the Chl content of the cotyledons was highest (2). Similarly, the maximum respiratory capacity of cucumber cotyledons also correlated with the greening and photosynthetic phases. These data complement the growing body of evidence that suggests that operation of the citric acid cycle and oxidative phosphorylation is important in illuminated photosynthetic tissues (11, 13, 20).

The mitochondrial parameters described show two distinct types of developmental variation. The first resembles the expression of ICL, in that it occurred irrespective of the light environment, but the magnitude was dependent on illumination (Fig. 2A). This behavior was exhibited by fumarase activity and succinate oxidation, which are both involved in lipid mobilization (Figs. 2C and 3A). The second resembles HPR, in that changes were absolutely dependent on illumination (Fig. 2B), suggesting that these steps (ICDH activity and malate, pyruvate, and glycine oxidation; Figs. 2D and 3B) were required during photosynthesis or the development of photosynthetic function.

A major question concerning the respiratory properties of plant tissues is the rate at which mitochondria operate in vivo, and how this corresponds to the state 3 and state 4 conditions that are generally used for studies on mitochondria in vivo. This study provides evidence that mitochondria in cucumber cotyledons operate at rates well below the available state 3 capacity. Thus, even in a tissue where there is rapid mobilization of substrate, mitochondrial rates are near state 4. Mitochondrial oxidation potentially may be limited by not only the availability of ADP but also the rate of supply of oxidizable substrate. This may be the general case for many plant tissues (24), so that in vitro studies of mitochondrial respiration, under state 3 conditions, may not reflect the actual properties and behavior of the mitochondria in vivo.

**LITERATURE CITED**