Effects of Light and 3-(3,4-Dichlorophenyl)-1,1-Dimethylurea on Levels of ATP in *Lemna paucicostata* 6746 and a Photosynthetic Mutant with Abnormal Flowering Responses

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**ABSTRACT**

The effects of light, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), and ammonium on pool sizes of ATP were studied in *Lemna paucicostata* 6746 (wild type) and a photosynthetic mutant (strain 1073) with abnormal flowering responses. Wild-type fronds were capable of endogenous and phenazine methosulfate-catalyzed cyclic photophosphorylation. The endogenous cyclic photophosphorylation was inhibited by DCMU. The mutant fronds showed little endogenous but appreciable rates of phenazine methosulfate-catalyzed cyclic photophosphorylation. Treatment with DCMU during prolonged exposure to light did not result in elevated levels of ATP. Ammonium ion in the medium did not inhibit light-induced increases in pool sizes of ATP. It is concluded that the previously reported effects on flowering of DCMU, the photosynthetic mutation or ammonium ion, were not due to altered pool sizes of ATP.

Modification of flowering responses by altering photosynthetic activity has been described for a number of species (4, 13). Kandeler reported that long-day flowering of the duckweed *Lemna gibba* G3 was enhanced by DCMU or adenylylates (10), and inhibited by ammonium ion (11). On the basis of such results, it was suggested that promotion of flowering by DCMU might be due to increased levels of ATP resulting from cyclic photophosphorylation, and inhibition of flowering by ammonium ion to an uncoupling of phosphorylation (9, 13).

Promotion of flowering by DCMU and adenylylates has also been observed in *Lemna paucicostata* strain 6746—a photosynthetic mutant, strain 1073, flowered in the absence of DCMU (16–18). Under certain conditions, flowering of both strains was inhibited by ammonium ion (6, 16, 18).

Studies on chloroplast suspensions (20) showed that the photosynthetic electron transport chain of the mutant was blocked between PQ2 and Cyt f, but was capable of PMS-catalyzed PSI cyclic phosphorylation, results consistent with the ATP hypothesis described above.

The purpose of these experiments was to test the hypothesis in vivo. We have studied the effects of light, DCMU, and ammonium on the pool sizes of ATP in intact plants of wild type and the mutant.

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2 This clone was formerly designated as *Lemna paucicostata* (see ref. 12).

3 Abbreviations: PQ: plastoquinone; PMS: phenazine methosulfate.

**MATERIALS AND METHODS**

**Growth Media.** Stock cultures of *L. paucicostata* 6746 (wild type) and mutant strain 1073 were grown on half-strength Hutner’s medium (7) with 1% sucrose under continuous cool-white fluorescent light of 20 ft-c at 25°C. As indicated in the appropriate sections, experimental cultures were grown either on the same medium, on tenth-strength Hutner’s or on the ammonium-free version (tenth-strength KNO3 medium) in which the NH4NO3 (0.25 mM) was replaced by an equimolar amount of KNO3 (6). All media contained 1% (w/v) sucrose.

**O2 Measurements.** A Clark-type electrode was used to measure O2 concentration. The reaction mixture contained 5 ml half-strength Hutner’s medium plus 1% sucrose at 25°C. A nylon mesh screen prevented fronds from coming in contact with the probe membrane.

**Preparation and Illumination.** Chl was determined according to the method of Arnon (1). Under the growth conditions used, typical values for wild type and mutant fronds were 1.3 μg Chl/mg fresh weight and 0.7 μg Chl/mg fresh weight, respectively. Samples of fronds to be illuminated were blotted for 10 s with filter paper, weighed, and placed in 3 ml of medium in a 12-ml graduated conical centrifuge tube. The tube's interior bore a narrow conical shoulder which contained between 15 and 25 mg of fronds, were incubated in the dark for 2 h to establish dark levels of ATP. Those cultures being maintained in nitrogen were flushed with water-saturated nitrogen at a rate of 2 liter/h during the dark pretreatment and the illumination periods. For illumination periods of 1 min or less, most of the medium was removed, leaving the fronds adhering to the side of the tube. For long term exposures, the medium was removed at the termination of illumination; replicate cultures were used for weight and Chl determinations. There were three or four replicates for each treatment.

Illumination with white light for short term exposures was from a 300-W tungsten filament projector bulb filtered through 5 cm of water. For red and far red illumination, the light was filtered through Corning filters 4-97 + 2-73 or 7-59 + 2-73, respectively (3). For red, the peak wavelength was about 610 nm, the cut-off wavelengths were 560 and 680 nm, and the intensity at the surface of the plants was 93 μW cm−2. At this intensity of red light, the rate of O2 evolution in wild type fronds was 10.9 μmol/mg Chl-h. The far red light had a low wavelength cut-off at 680 nm; the incident intensity between 680 and 730 nm was 118 μW cm−2. This intensity of far red light did not cause any detectable O2 evolution in wild type fronds.

For long-term illumination, the conditions were similar to those used in previously reported flowering experiments: cool-white fluorescent light (350 ft-c) at about 25°C. At the end of the period of illumination, cold ethanol (−70°C) was rapidly added to the samples which were then stored at −20°C for at least 16 h to maximize the ATP recovered.
ATP Extraction. The samples were lyophilized, extracted with 6.0 mm glycine (pH 9.5) at 100 °C for 15 min, and then rapidly brought to room temperature. Tests showed that this procedure completely extracted ATP without significant loss.

ATP Assay. Standard or unknown samples of ATP (0.1 ml) were rapidly injected into a tube containing 50 μl of firefly tail extract (pH 7.6) (Sigma Chem. Co.). The resulting luminescence was detected by a fluorometer, the signal was amplified by an external amplifier, and displayed on a storage oscilloscope. The initial peak luminescence was directly proportional to the concentration of ATP.

RESULTS

Dark Levels of ATP. Before any period of irradiation, cultures of the wild type and mutant were incubated in darkness for 2 h to establish dark levels of ATP. These levels are indicated by the zero time values in Figure 1. On a fresh weight basis, levels of ATP of the mutant in air were markedly lower than those of wild type. In nitrogen, mutant and wild type fronds had similar levels of ATP.

Effects of Short Exposures to Light. During a 1-min illumination of wild type in air, ATP increased during the first 10 s and then decreased to levels slightly above dark values (Fig. 1). Similar results have been reported for pea leaves (15).

In nitrogen, similar initial increases in ATP occurred. In contrast to the results in air, the level of ATP did not decrease but continued to rise at a reduced rate during the final 30 s of illumination.

The pool sizes of ATP in the mutant, in air or nitrogen, were unaffected by light. Since photophosphorylation in nitrogen is usually considered to be cyclic (5), these results suggest that the mutant was defective in both cyclic and noncyclic in vivo photophosphorylation. In order to conclude that the mutant was defective in cyclic photophosphorylation, it was necessary to demonstrate unequivocally that cyclic photophosphorylation was occurring in wild type. It was possible, e.g. that in nitrogen, fermentation produced sufficient CO₂ to permit noncyclic electron flow. To rule out this possibility, light-induced increases in ATP levels were determined using a far red source that did not cause any detectable O₂ evolution. A 1-min irradiation with this far red light increased levels of ATP in wild type (ΔATP = 24.3 nmol/mg Chl) but not in mutant cultures. Further, 1 μM DCMU almost completely prevented this effect of far red light on wild type (ΔATP = 3.1 nmol/mg Chl).

Endogenous versus PMS-catalyzed Photophosphorylation. In vitro studies (20) had shown that wild type and mutant chloroplast suspensions were capable of appreciable rates of DCMU-resistant cyclic photophosphorylation. The next experiment was designed to resolve the apparent discrepancies between these in vitro results and the in vivo results described in the previous sections. The effect of light on levels of ATP was measured in intact fronds treated with PMS, the artificial cofactor that had been used in the in vitro experiments with chloroplast suspensions (20).

Table I shows that wild type and mutant fronds had comparable increases in ATP levels when illuminated in the presence of PMS. This photophosphorylation was DCMU-resistant as it was in the case of chloroplast suspensions, whereas endogenous photophosphorylation (i.e. in the absence of PMS) in wild type was sensitive to DCMU.

Prolonged Exposure to Light. Since the studies on flowering involved long-term illumination, it was of interest to determine the effects of such treatments on pool sizes of ATP. Wild type cultures given 8 h of light showed marked increases in levels of ATP (Table II). This effect of light was partially prevented by DCMU. Levels of ATP in the mutant were unaltered by light. Thus, even with prolonged exposures to light, ATP pool sizes were not increased in the mutant; and ATP pool sizes in light-grown wild type fronds treated with DCMU were not higher than in untreated light-grown controls.

Previous experiments had also shown that under certain conditions ammonium ion inhibited flowering of wild type and mutant cultures (6, 16, 18). The next experiment tested the possibility that this inhibition resulted from lowered levels of ATP due to an uncoupling effect. Cultures were incubated on tenth-strength KNO₃ medium or on ammonium-containing tenth-strength Hutner's medium, both with 1% sucrose. The latter medium was inhibitory to flowering in short and long days.

The results in Table III show that with KNO₃ medium, 8 h of light had little or no effect on levels of ATP in either the mutant or wild type. With ammonium-containing medium, light caused marked increases in ATP levels in wild type but not in mutant cultures.

DISCUSSION

Dark Levels of ATP. Anaerobic conditions or growth on dilute medium reduced dark levels of ATP in wild type more so than in the mutant (Tables II and III). Our tentative interpretation is that the mutant has a defective respiration, a possibility supported by the observation that on a fresh weight basis, the rate of O₂ consumption of the mutant is about half that of wild type. Further, mutant chloroplasts lack an iron-sulfur protein (14) which has properties similar to those of a protein found in mitochondria (19). It would be of interest to determine whether the mitochondria of the mutant also have a defective electron transport chain, and whether such a defect is related to an abnormal iron-sulfur protein.

ATP Levels in the Light. In contrast to wild type, mutant fronds did not show increased pool sizes of ATP during exposures to light in either air or nitrogen, indicating that the mutant was defective in both cyclic and noncyclic photophosphorylation. Earlier work with chloroplast suspensions had shown that the mutant was blocked between PQ and Cytf, but was capable of PMS-catalyzed cyclic photophosphorylation (20). The present results indicate that mutant fronds are also capable of PMS-catalyzed photophospho-
Table I. Effects of PMS and DCMU on light-induced increase in ATP levels in wild type and mutant.

The irradiation was 1 min of white light (140 mm cm⁻²). Cultures were incubated in nitrogen in half-strength Hutner's medium with 1% sucrose.

<table>
<thead>
<tr>
<th>PMS (µM)</th>
<th>DCMU (µM)</th>
<th>ΔATP (nmoles/mg Chl)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>40.5</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>4.1</td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>35.4</td>
</tr>
</tbody>
</table>

Table II. Effects of DCMU on pool sizes of ATP in wild type and mutant during prolonged exposure to light.

Cultures, grown on half-strength Hutner's with 1% sucrose, were pretreated with 2 h of darkness and then given 8 h of either darkness or 350 ft·c cool white light at 25°C.

<table>
<thead>
<tr>
<th>Condition</th>
<th>DCMU (µM)</th>
<th>Conc of ATP nmoles/mg fr wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Wild type</td>
</tr>
<tr>
<td>Dark</td>
<td>0</td>
<td>105.2</td>
</tr>
<tr>
<td>Light</td>
<td>1</td>
<td>104.2</td>
</tr>
</tbody>
</table>

Table III. Effect of ammonium on pool sizes of ATP in wild type and mutant during prolonged exposure to light.

All cultures were given 2 h darkness followed by 8 h of either darkness or cool-white light (350 ft·c at 25°C). Ammonium-containing medium was tenth-strength Hutner's medium with 1% sucrose. Ammonium-free medium was the same medium with KNO₃ substituted for the NH₄NO₃.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Condition</th>
<th>Conc of ATP nmoles/mg fr wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>Mutant</td>
</tr>
<tr>
<td>Ammonium-free</td>
<td>Dark</td>
<td>50.1</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>57.7</td>
</tr>
<tr>
<td>Ammonium-containing</td>
<td>Dark</td>
<td>76.9</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>101.3</td>
</tr>
</tbody>
</table>

The results are consistent with the scheme in which the electron transport pathway for endogenous cyclic photophosphorylation, in contrast to the artificial pathway, involves PQ (22).

The endogenous cyclic photophosphorylation in wild type fronds was inhibited by DCMU, whereas the artificial PMS-catalyzed system in chloroplasts (20) or in intact fronds (Table I) was resistant to DCMU. Although such resistance is usually considered a criterion for cyclic photophosphorylation, there have been reports that endogenous cyclic flow is inhibited by DCMU (2, 5, 8). It has been suggested (2) that complete suppression of electron flow from PSII prevents appropriate redox states required for endogenous ferredoxin-catalyzed cyclic photophosphorylation.

Relationships to Flowering Responses. One of the possibilities suggested by Kandel (9-11) for the inhibitory effect of ammonium ion on duckweed flowering was decreased ATP levels caused by an uncoupling of photophosphorylation. This possibility seems to be ruled out by the finding (Table III) that ammonium ion in the medium increased rather than decreased pool sizes of ATP during illumination. Using a different approach, Tanaka and Takimoto (21) also concluded that ammonium inhibition of wild type flowering was not due to an uncoupling mechanism.

Earlier results also showed that under certain conditions, DCMU promoted flowering of wild type, whereas flowering of the photosynthetic mutant occurred in the absence of DCMU (16, 18). It has been hypothesized (10) that DCMU promotion of flowering resulted from increased levels of ATP caused by cyclic photophosphorylation in the absence of ATP-consuming CO₂ reduction reactions. The present results do not support this hypothesis. First, the mutant was defective in endogenous photophosphorylation, and even with prolonged illumination showed little or no change in pool size of ATP. Furthermore, endogenous cyclic photophosphorylation in wild type was not resistant to DCMU. During long term exposure to light, the presence of DCMU did not lead to higher levels of ATP (Table II). It appears that the flower-promotive effects of DCMU or of the photosynthetic defect cannot be explained on the basis of increased pool sizes of ATP. It remains to be determined whether these effects on flowering are due to altered levels of photosynthetic products other than ATP (13) or to some other phenomenon, such as abnormal redox states caused by a block in the electron transport chain.

LITERATURE CITED
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