Photoautotrophic Chenopodium rubrum Cell Suspension Cultures Resistant against Photosynthesis-Inhibiting Herbicides

II. Physiological and Biochemical Properties

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Dedicated to Professor Rolf Wiermann on the occasion of his 60th birthday
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Eight photoautotrophic cell cultures of Chenopodium rubrum, which are resistant against the photosystem II inhibitor metribuzin, were characterized for their growth parameters, chlorophyll content and photosynthetic capacity. Herbicide resistance of the eight lines results from different mutations in the D1 protein of photosystem II, which is the target for different photosystem II inhibitors. In the presence of 10^{-5} M metribuzin the eight lines showed substantial growth reduction depending on the degree of resistance, and this effect is explained by a reduced electron transport in photosystem II. The impaired photosynthetic capacity of the green cells in the presence of high metribuzin concentrations, leads to compensation effects similar to shade accommodation of plants. Adaptation includes an increase of the chlorophyll content, a decrease of the chlorophyll a/b ratios as well as an increase of thylakoid stacking and cell number per unit fresh weight. In the absence of the herbicide photosynthetic electron transport is not impaired, as indicated by measurements of electron transfer rates in photosystem II and flash-induced reduction kinetics of P-700$. In summary the alterations of the D1 protein of the eight cell lines do not result in a reduced electron transport in photosystem II.

Introduction

Stepwise adaptation of a photoautotrophic cell suspension culture of Chenopodium rubrum to increasing metribuzin concentrations resulted in the selection of eight different herbicide-resistant lines, which are able to grow with a 100- to 1000-fold higher mbz concentration than the wild type cells (Thiemann and Barz, 1994).

Metribuzin is known to interrupt the photosynthetic electron transport between the primary and secondary electron acceptor of photosystem II by displacing the quinone Q$_B$ from its binding niche at the D1 protein (also known as herbicide-binding or Q$_B$-binding protein). Binding studies performed with [14C]mbz and isolated thylakoids from resistant cells showed that a low specific binding of the herbicide only occurred in case of the variants 5 and 6, whereas all other variants had lost the ability of specific mbz-binding (Thiemann and Barz, 1994). Such data and our sequence analyses (Schwenger-Erger et al., 1993) of the psbA gene coding for the D1 protein corroborated that herbicide resistance of the photoautotrophic cell cultures is due to an altered D1 protein.

All PSII herbicide-resistant higher plants known so far show a mutation at position ser264 either to threonine or to glycine. According to Wildner et al. (1989) such mutations impair the electron transport at this site, because of a slower exchange rate with the plastoquinone pool of the membrane. In comparison with the wild type plants herbicide-resistant plants generally show lower fitness, reduced biomass and decreased pro-
ductivity (Hobbs, 1987; Ireland et al., 1988; Ric-roch et al., 1987; Stowe and Holt, 1988).

Numerous triazine-resistant organisms with reduced photosynthetic capacity evolve compensa-
tory mechanisms resembling the effects of low-
light adaptation. The plastids from such resistant
plants show an increase in the degree of thylakoid
stacking and also in the amount of light-harvesting
chlorophyll a/b protein complexes. Furthermore,
changes in temperature sensitivity, membrane
lipid composition and starch accumulation were
reported (Ducruet et al., 1990; Pillai and St. John,
1981; Vaughn and Duke, 1984). These effects can
also be observed by application of sublethal con-
centrations of an inhibitor of photosynthesis.
Under such conditions an increase in chlorophyll
content ("greening effect") occurs in the treated
plants (Koenig, 1987), which is thought to be
caused by an increase in the number of chloro-
plasts per cell (Anderson, 1986).

In order to study the relationship between mbz
resistance and modifications of the photosynthetic
apparatus, we have now compared the relevant
aspects of the wild type and the resistant cell cul-
ture lines of Chenopodium rubrum. It should be
demonstrated whether the new double and triple
mutations detected within the psbA gene at the
positions 219, 220, 229, 251, 266, 270 and 272
(Schwenger-Erger et al., 1993) also cause an im-
pairment of electron transport. In addition the
effects of high doses of metribuzin although still
sublethal for the resistant strains were investigated
with regard to possible induction of low-light
adaptation phenomena.

Materials and Methods

Growth parameters

Photoautotrophic cell suspension cultures of
Chenopodium rubrum were grown as described in
Thiemann and Barz (1994). For analyses of growth
parameters a new culture cycle was started by
transfering 2 g fresh weight of 14 days old cells
into 40 ml of fresh MS medium (Murashige and
Skoog, 1962). The herbicide was applied as a filter-
sterilized methanolic solution at a concentration
of $5 \times 10^{-6}$ M. In intervals of two days fresh
weight, dry weight, cell number and chlorophyll content
of the cell cultures were determined. The measure-
ments of chlorophyll a and b and also of total
chlorophyll content were performed as described
by Ziegler and Egle (Ziegler and Egle, 1965),
the cell number was determined after disintegra-
tion of cell aggregates by incubation with a 10%
chrome(VI)oxide solution for 10 min at 70°C
(Thiemann, 1990).

Measurement of photosynthetic oxygen production
and electron transport

Cells were harvested at the beginning of the
stationary growth phase and transfered into fresh
medium with an inoculum of 50 mg/ml. After an
adaptation period of 30 min under normal growth
conditions, metribuzin was applied as a methanolic
solution using that concentration, which was usu-
ally used for cell cultivation. The final concen-
tration of the solvent did not exceed 0.2% of the
total reaction volume. After further incubation for
15 min the photosynthetic oxygen production was
measured using a Clark electrode (Hansatech,
Fa. Bachofer, Reutlingen, Germany). Cells were
illuminated with white light of an intensity of
1050 $\mu$E $\text{m}^{-2}$ $\text{s}^{-1}$ after a short preincubation
in the dark.

For measurements of the photosynthetic electron
transport in photosystem I and II, isolated
thylakoids were used. The isolation of thylakoids
was performed according to Nelson et al. (1970).
After the application of the herbicide the reaction
mixture was preincubated in the dark for a short
time and subsequently the oxygen production
(PS II) or uptake (PS I, linear electron transport)
were measured in white light (light intensity
1050 $\mu$E $\text{m}^{-2}$ $\text{s}^{-1}$) using a Clark electrode (Han-
satech, Fa. Bachofer, Reutlingen, Germany). The
reaction mixture for measurement of PS I con-
tained standard buffer (20 mM tricine-NaOH,
pH 7.6, 20 mM KCl, 3 mM MgCl$_2$·6H$_2$O), 0.1 mM
methylviologen, 0.5 mM NaN$_3$, 0.1 mM TMPD,
2.5 mM Na-ascorbate, 15 $\mu$M DCMU and 1.5 $\mu$M
gramicidin D in a total volume of 1 ml. The reac-
tion mixture contained thylakoid membranes
equivalent to 15 $\mu$g chlorophyll.

The reaction mixture for linear electron trans-
port analysis contained standard buffer (see above),
0.1 mM methylviologen, 0.5 mM NaN$_3$, 1.5 $\mu$M
gramicidin D in a total volume of 1 ml. Thy-
lakoids equivalent to 30 $\mu$g chlorophyll were
added.
The measurements of electron transport of PS II were performed as described before (Thiemann and Barz, 1994).

**Measurements of reduction kinetics of P-700**

The absorption change of P-700 was monitored at 703 nm after short flash induction according to Haehnel and Trebst (1982). The equipment was kindly provided by Prof. Dr. W. Haehnel (Institute of Biochemistry and Biotechnology of Plants, University of Münster, Germany), the measurements and calculations were performed as described by Ratajczak (1987).

To estimate the relative amount of photosystem II, thylakoids of 14 days old mutant cells and wild type cells of *Chenopodium rubrum* were isolated according to Nelson *et al.* (1970). The reaction mixture contained 0.2 mM methylviologen and 1.5 μM gramicidin D in a total volume of 3 ml. Each of three reaction assays prepared from wild type and mutant thylakoids, respectively, was used for the determination of 100 single flash-induced reduction kinetics. The amount of P-700 was determined after the application of 0.1% (v/v) Triton X-100, 1 mM Na-ascorbate and 0.1 mM DCPIP to the reaction mixture.

**Results and Discussion**

**Growth characteristics**

Among the photosynthesis-inhibiting herbicides, triazinones, when applied in sublethal concentrations, are known for their “greening effect” and for their stimulating activity on cell division, nitrate uptake and subsequently increasing activity of nitrate reductase, amino acid content and soluble protein (Koenig, 1987; Fedtke, 1979). These effects – also known as “Hormesis effect” – only occur in a narrow concentration range, whereas with increasing concentrations they rapidly become phytotoxic (Fedtke, 1982).

![Graphs showing growth characteristics](image_url)
Since the eight metribuzin-resistant cell lines were routinely cultivated with rather high concentrations of the herbicide, it should be measured whether these concentrations cause stimulating or inhibiting effects. These effects were thought to become evident during the various phases of a growth cycle and/or by alterations of cell division activity.

Growth characteristics (fresh and dry weight, cell number and chlorophyll content) of the eight mutant lines were determined over a time period of three weeks in comparison to the wild type cells.

As an example the results obtained for line 7 are shown in Fig. 1. It is evident that the cells grow in a linear mode even in the presence of the herbicide. In comparison to the wild type cells the mutant line shows a 2 days longer lag phase with regard to the fresh weight, enters the linear phase at day 6 of the culture cycle and this growth phase ends four days later.

Furthermore, the herbicide-resistant line shows a higher chlorophyll content in comparison to the wild type (Fig. 1), which can be explained by the high number of cells per gram fresh weight.

The other six mutant lines of *Chenopodium rubrum* possess very similar growth characteristics (data not shown in detail). Therefore, it was assumed that metribuzin does not influence the general growth performance because of this high degree of similarity. Nevertheless, some changes in growth parameters could be detected and these were further analyzed.

**Terminal estimation of growth parameters**

At the end of the selection procedure six of the eight resistant cell lines constantly grew in the presence of $10^{-5}$ M metribuzin, whereas lines 5 and 6 were cultivated with $10^{-6}$ M metribuzin only.

In parallel experiments with wild type cells and all mutant lines growth parameters were determined 14 days after onset of a growth cycle with the media enriched with $10^{-5}$ M or $10^{-6}$ M metribuzin, respectively. The characterization of the growth behaviour was designed to reveal strain specific variations from the original wild type cells (Table I).

The values for the fresh weight indicate that the eight resistant lines grow slower than the wild type cells. The reduction of the growth rate can directly be correlated with the degree of resistance, exhibited by the various resistant lines (Thiemann and Barz, 1994). Thus the highly resistant lines 1 and 8 suffered the smallest growth reduction. However, the growth inhibitory effect of metribuzin may also depend on other factors which remain to be elucidated.

Further metabolic effects of metribuzin exerted even on the highly resistant strains are documented by the values obtained for cell number and chlorophyll content. In comparison to the wild type cells most of the resistant strains show a significant increase in chlorophyll content either per gram fresh weight or per $10^6$ cells. This phenomenon of “stronger greening” could also be visually detected by simply comparing the various suspension cultures during growth in the two-tier culture vessels. One reason for the high chlorophyll content appears to be the higher cell number per gram fresh weight. Only lines 1 and 8 possess a chlorophyll content similar to the wild type cells especially when based on cell number (Table I).

The data presented in Table I corroborate that metribuzin notably reduces the growth capacity of the resistant cell cultures although the degree of reduction appears to be low in view of the very potent inhibitor action of metribuzin on the wild type cells. The applied concentrations can therefore be considered as a sublethal dose which cause an increase in cell number and chlorophyll content.

The alterations shown in Table I clearly documented the so-called “greening effect” (Koenig, 1987) shown by sensitive plants, and this effect results from a higher chlorophyll content in the presence of low photosynthesis-inhibiting herbicides. Furthermore, a higher cell division activity (compare Table I), an increased protein content and a more pronounced accumulation of soluble amino acids have been reported. The latter fact could be correlated with a stimulation of the *in vitro* activity of nitrate reductase paralleled by an increase in total nitrogen concentration (Fedtke, 1982). These last mentioned responses have not yet been measured in photoautotrophic cell cultures of *Chenopodium rubrum*.

**Photosynthetic capacity**

The differential effects of metribuzin on the growth rates of the resistant strains (Table I) and
their different mutations in the D1 protein (Schwenger-Erger et al., 1993) required determinations of a putative impairment of photosynthesis or electron transport. Therefore, the photosynthetic oxygen production of all cell lines was determined in the presence and absence of the inhibitor.

Cells of mutant and wild type strains of *Chenopodium rubrum* were harvested at the early stationary growth phase, inoculated at 50 mg fresh weight per ml MS medium and applied either with the solvent (0.2%, v/v) or with the methanolic metribuzin stock solution (0.2%, v/v). The net rate of photosynthetic oxygen production was measured for the eight resistant lines using those metribuzin concentrations, which were usually taken for cultivation. For comparison the specific oxygen production of wild type cells was measured in the presence and absence of $10^{-6}$ M metribuzin.

The data shown in Table II reveal that all resistant lines except line 5 show a loss in photosynthetic capacity after incubation with high metribuzin concentrations. However, in comparison to the wild type cells this decrease is very low. In the absence of the inhibitor, the rates of photosynthetic oxygen production of the resistant lines except line 1 are very similar to those obtained for the wild type cells. These results correlate well with the growth rates of the resistant lines when measured in the absence of the herbicide (data not shown) because very similar values as shown for the wild type cells were now found.

In view of these data the repeatedly observed low photosynthetic capacity in some triazine-resistant plants is probably not caused by a low electron transfer rate in photosystem II. Ort and co-workers (1983) pointed out that a decreased rate of electron transfer from QA to QB is still much more rapid than the following steps of electron transport and thus not rate limiting. Other findings imply that low rates of CO2 reduction in resistant plants are possibly a consequence of poor efficiency in the use of separated charges at the photosystem II reaction center, an instability of the oxygen-evolving complex or a less efficient utilization of the pH gradient (Jursinic and Pearcy, 1988; Rashid and van Rensen, 1987). Furthermore, the detrimental effect of mutations leading to triazine resistance on plant growth may be the conse-

Table I. Growth parameters of metribuzin-resistant cell lines 1–8 of *Chenopodium rubrum* in comparison to the wild type cells (wt) after 14 days of cultivation with $10^{-5}$ M or $10^{-6}$ M (lines 5 and 6) metribuzin. The wild type cells were cultivated without the herbicide. The data represent the average of four independent growth experiments (deviation ±5%).

<table>
<thead>
<tr>
<th>Line</th>
<th>Fresh weight</th>
<th>Dry weight</th>
<th>Cell number</th>
<th>Chlorophyll</th>
<th>Chlorophyll</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>flask [g]</td>
<td>g fresh weight [mg]</td>
<td>g fresh weight $\times 10^{-6}$</td>
<td>[g]</td>
<td>$10^6$ cells</td>
</tr>
<tr>
<td>wt</td>
<td>10.1</td>
<td>35.1</td>
<td>23.6</td>
<td>471</td>
<td>19.9</td>
</tr>
<tr>
<td>L1</td>
<td>7.4</td>
<td>42.6</td>
<td>26.0</td>
<td>477</td>
<td>18.3</td>
</tr>
<tr>
<td>L2</td>
<td>2.8</td>
<td>54.3</td>
<td>29.2</td>
<td>1006</td>
<td>34.5</td>
</tr>
<tr>
<td>L3</td>
<td>3.1</td>
<td>54.5</td>
<td>35.2</td>
<td>1001</td>
<td>28.4</td>
</tr>
<tr>
<td>L4</td>
<td>3.5</td>
<td>60.3</td>
<td>23.3</td>
<td>709</td>
<td>30.4</td>
</tr>
<tr>
<td>L5</td>
<td>4.5</td>
<td>59.3</td>
<td>32.8</td>
<td>730</td>
<td>22.3</td>
</tr>
<tr>
<td>L6</td>
<td>3.8</td>
<td>49.9</td>
<td>21.6</td>
<td>524</td>
<td>24.3</td>
</tr>
<tr>
<td>L7</td>
<td>3.1</td>
<td>50.3</td>
<td>25.7</td>
<td>843</td>
<td>32.8</td>
</tr>
<tr>
<td>L8</td>
<td>6.5</td>
<td>45.5</td>
<td>36.1</td>
<td>738</td>
<td>20.5</td>
</tr>
</tbody>
</table>

Table II. Values for photosynthetic net-oxygen production determined in the absence of metribuzin with selected (lines 1–8) and wild type (wt) cells of *Chenopodium rubrum* in comparison to oxygen production rates measured after application of different metribuzin concentrations ($10^{-6}$ M; cells of wt and of lines 5 and 6 received $10^{-6}$ M only).

<table>
<thead>
<tr>
<th>Line</th>
<th>Net-oxygen production [μmol/mg Chl x h]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without metribuzin</td>
</tr>
<tr>
<td>wt</td>
<td>38</td>
</tr>
<tr>
<td>L1</td>
<td>23</td>
</tr>
<tr>
<td>L2</td>
<td>37</td>
</tr>
<tr>
<td>L3</td>
<td>31</td>
</tr>
<tr>
<td>L4</td>
<td>31</td>
</tr>
<tr>
<td>L5</td>
<td>39</td>
</tr>
<tr>
<td>L6</td>
<td>41</td>
</tr>
<tr>
<td>L7</td>
<td>39</td>
</tr>
<tr>
<td>L8</td>
<td>41</td>
</tr>
</tbody>
</table>
quence of an impaired interaction between the plastid and the nuclear genome (Stowe and Holt, 1988; Bettini et al., 1987).

In essence our measurements of photosynthetic net rates in the absence of the inhibitor appear to indicate that the photosynthetic capacity of the resistant strains of Chenopodium rubrum is not impaired by the observed mutations (Schwenger-Erger et al., 1993).

**Chlorophyll a/b ratio**

Chlorophyll *a/b* ratios provide information on the size/number of the photosystem II antennae. An increase in the light-harvesting complex II can be detected by a decrease of the chlorophyll *a/b* ratio, because the amount of chlorophyll *b* within the antennae pigments is relatively higher in photosystem II than in photosystem I (Glazer and Melis, 1987). A lower chlorophyll *a/b* ratio and therefore an increase in the size/number of antennae of photosystem II indicates a secondary effect of adaptation to either low herbicide concentrations, resulting from a slightly reduced photosynthesis, or to a rate-limiting light intensity (Lemoine et al., 1985).

To evaluate the photosynthetic capacity under the influence of metribuzin, the chlorophyll *a/b* ratio of the eight resistant strains was examined. Cells of each mutant strain and the wild type cells of *Chenopodium rubrum* were cultivated in the presence and in the absence of metribuzin. The medium of lines 5 and 6 contained $10^{-6} \text{M}$ metribuzin whereas the other lines received $10^{-5} \text{M}$ of the herbicide. After 14 days of cultivation with metribuzin the resistant cells showed a lower chlorophyll *a/b* ratio than the wild type cells (Table III). The extent of decrease can easily be correlated with the degree of resistance (Thiemann and Barz, 1994), with the exception of lines 5 and 6, which were cultivated using the lower metribuzin concentrations. The higher the degree of resistance the lower the decrease in chlorophyll *a/b* ratios.

From this data it can be assumed that the resistant lines show some adaptation processes to compensate the effects of metribuzin. A slightly impaired electron transport may be the reason for these compensatory effects, which may result in an increase of photosystem II light-harvesting complexes.

Interestingly, all strains except the highly resistant cell line 1 show an increase of the chlorophyll *a/b* ratio after removal of the herbicide from the growth medium (Table III). Since a compensation of a lower electron transport rate by an increase of the antenna size/number of photosystem II is not required under such conditions, we assume that in the absence of metribuzin the electron transport is most likely not impaired. All results obtained so far indicate that the new mutations occurring in the *psbA* gene of the resistant strains of *Chenopodium rubrum* (Schwenger-Erger et al., 1993) do not cause a reduction of photosynthetic capacity.

**Electron transport in photosystem I and II**

In order to obtain further insight into the photosynthetic activity of the metribuzin-resistant lines, the rates of the uncoupled electron transport in photosystem II were measured. The determinations were performed with herbicide-free thylakoids isolated from metribuzin-resistant cells and wild type cells of *Chenopodium rubrum* and phenyl-β-benzoquinone was chosen as an artificial electron acceptor.

Since cell line-specific changes causing an impairment within photosystem I could also have occurred, the uncoupled electron transport from the artificial electron donor TMPD red. to methylviologen was measured. Furthermore, the linear electron transport from water to methylviologen was also recorded.

| Table III. Chlorophyll *a/b* ratios of wild type (wt) and metribuzin-resistant cells (lines 1–8) of *Chenopodium rubrum* after cultivation for 14 days with ($10^{-5} \text{M}$, lines 5 and 6) or without metribuzin. Values represent the average of 4 parallel assays. |
|---|---|---|
| Line | With metribuzin Chlorophyll *a/b* | Without metribuzin Chlorophyll *a/b* |
| wt | – | 3.02 ± 0.25 |
| L1 | 2.51 ± 0.12 | 2.49 ± 0.08 |
| L2 | 2.25 ± 0.01 | 2.66 ± 0.25 |
| L3 | 2.37 ± 0.10 | 2.50 ± 0.11 |
| L4 | 2.66 ± 0.05 | 3.04 ± 0.21 |
| L5 | 2.52 ± 0.10 | 2.92 ± 0.12 |
| L6 | 2.58 ± 0.14 | 3.17 ± 0.02 |
| L7 | 2.37 ± 0.09 | 2.90 ± 0.16 |
| L8 | 2.56 ± 0.09 | 3.24 ± 0.07 |
The results (Table IV) obtained with the thylakoid preparations of the resistant and the wild type cells in the absence of metribuzin show that the resistant lines (with the exception of line 3 in PS I and line 5 in PS II, respectively) only exhibit slightly higher rates for electron transport in both photosystem I and II.

Taking into account that thylakoids represent a very sensitive system and that the D1 protein shows rapid turnover (Wettern, 1986) the absolute values of Table IV should be interpreted with caution. Nevertheless, a real decrease in electron transport can be excluded for the herbicide-resistant lines of *Chenopodium rubrum*. In contrast to this result a substantial change was found in triazine-resistant *Chenopodium album* plants. They exhibited only 44% of the electron transfer rate in photosystem II shown by the susceptible plants (Jansen *et al.*, 1986).

After application of metribuzin (10^{-6} \text{m} to lines 5 and 6 and 10^{-5} \text{m} to the other resistant strains) the resistant lines showed an impaired electron flow, which appears to be inversely proportional to their degree of resistance (Schwenger-Erger *et al.*, 1993). In view of the values recorded for linear electron transport it is understandable that the resistant lines when compared to the wild type cells exhibit reduced growth rates if they are cultivated with high inhibitor doses.

In general, our data lead to the assumption that the photosystem II inhibitor metribuzin exerts inhibitory effects even in case of highly resistant lines. Since the binding properties of thylakoids for metribuzin are altered (Thiemann and Barz, 1994), the effects might be unspecific. This phenomenon of unspecific binding could be detected in triazine-resistant *Amaranthus retroflexus* plants if they were treated with high inhibitor concentrations (Oettmeier *et al.*, 1982).

The partial decrease in electron transport is supposed to originate from other effects shown by the resistant lines in the presence of high metribuzin concentrations. Such additional factors may be compensatory effects, which are visible as an increase in chlorophyll content and antenna size/number of photosystem II. Furthermore, changes in membrane lipid composition and ultrastructural modifications in chloroplast structure (increased ratio of grana to stroma thylakoids) have been observed and may be of importance (Thiemann, 1990; Thiemann and Barz, 1994; Barz *et al.*, 1994; Schwenger-Erger and Barz, unpublished).

### Function and relative amount of photosystem II

The results presented so far indicate that changes in the amount of composition of light-harvesting complexes as well as in the number of reaction centers may have occurred in the mutant lines. To clarify such points, flashlight photometry offers a possibility to measure the amount and function of photosystems and this technique may

<table>
<thead>
<tr>
<th>Line</th>
<th>PS I O₂ uptake μmol/mg Chl×h</th>
<th>PS II O₂ production μmol/mg Chl×h</th>
<th>PS II O₂ production μmol/mg Chl×h</th>
<th>PS I + PS II O₂ uptake μmol/mg Chl×h</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>146</td>
<td>59</td>
<td>0</td>
<td>(47)</td>
</tr>
<tr>
<td>L1</td>
<td>200</td>
<td>77</td>
<td>72</td>
<td>38</td>
</tr>
<tr>
<td>L2</td>
<td>169</td>
<td>85</td>
<td>52</td>
<td>21</td>
</tr>
<tr>
<td>L3</td>
<td>132</td>
<td>61</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>L4</td>
<td>194</td>
<td>76</td>
<td>56</td>
<td>33</td>
</tr>
<tr>
<td>L5</td>
<td>208</td>
<td>41</td>
<td>34</td>
<td>42</td>
</tr>
<tr>
<td>L6</td>
<td>156</td>
<td>83</td>
<td>38</td>
<td>40</td>
</tr>
<tr>
<td>L7</td>
<td>153</td>
<td>72</td>
<td>26</td>
<td>29</td>
</tr>
<tr>
<td>L8</td>
<td>213</td>
<td>88</td>
<td>85</td>
<td>40</td>
</tr>
</tbody>
</table>

Table IV. Rates of uncoupled electron transfer in photosystem I (2,3,4,5-tetramethylphenylenediamine-red. → methylviologen) photosystem II (H₂O → phenyl-p-benzoquinone) and photosystem I + II (H₂O → methylviologen) of thylakoids isolated from metribuzin-sensitive (wt) and -resistant cell variants (lines 1–8) of *Chenopodium rubrum* cell cultures. The electron transfer rates were estimated in the absence and presence of metribuzin (10μm or 1μm for lines 5 and 6); uncoupler was gramicidin D. The linear electron transport PS I + II) of the wild type cells was measured without metribuzin.
also provide data on electron transfer rates between $Q_B$ and the plastoquinone pool.

The measurements were performed with thylakoids isolated from the eight metribuzin-resistant lines and the wild type cells of *Chenopodium rubrum*. According to Haehnel and Trebst (1982) the relative amount of P-680 was estimated by recording the reduction kinetics of P-700$^*$. The measurements were performed using established procedures and equipment (Haehnel, 1984; Ratajczak, 1987).

The reduction kinetics of P-700$^*$ following a short light flash were measured in the thylakoids after oxidizing the electron carriers between the two photoreactions and most of the P-700 by far-red light. The subsequent time course of the P-700 absorption change induced by a short flash was monitored at 703 nm. This short flash leads to an oxidation of residual P-700 as indicated by the negative absorption change shown in Fig. 2. The positive amplitude after the flash is caused by reduction of P-700$^*$ with electrons from photosystem II. At a constant intensity of the far-red light the area below the positive transient shaded in Fig. 2 is proportional to the number of electrons produced by photosystem II and due to this relation also to the number of reaction centers. The relative values obtained by calculating the areas for resistant and wild type cells are presented in Table V (column 1).

The half-time of P-700 reduction can graphically be estimated (Haehnel, 1984). This reduction is caused by the electron transfer from the cytochrome $b_{6}/f$ complex via the rate-limiting oxidation of the plastoquinone pool. Low proton concentrations inside the thylakoids will increase this half-time of P-700$^*$ reduction, therefore this phenomenon was avoided by the addition of the uncoupler gramicidin D to the thylakoid assays. The data for the half-time measurements are also listed in Table V (column 4).

An initial lag phase of 3–6 ms in the reduction of P-700 can be detected by using a different time course for recording the change of extinction at 703 nm. Such a lag phase is due to all electron transfer steps occurring before the rate-limiting oxidation of plastoquinol. These earlier steps also enclose the electron transfer from $Q_A$ to $Q_B$ and this reaction might be of special interest with regard to the mutant cells. The relevant data are included in Table V (column 5).

Since an alteration in the number and composition of light-harvesting complexes might also have led to changes in the amplitude and consequently in the area below the positive transient (Fig. 2) the concentration of P-700 was also determined. Under the assumption that the reaction centers of both photosystems are present in approximately equal concentrations (Kaplan and Arntzen, 1982), a direct determination of the concentration of P-700 might also offer a means to obtain more data on the concentration of P-680.

The reaction mixtures for the aforementioned spectroscopic assays were completed with artificial electron donors for photosystem I and the detergent Triton X-100. The reduction of P-700 was induced by a short light flash of low intensity and carried on without additional far-red illumination. The concentration of P-700 is proportional to the extent of absorption change at 703 nm. The relevant concentrations were calculated for the eight resistant lines and for the wild type cells of *Cheno-

![Fig. 2. Flash light-induced reduction kinetics of P-700$^*$ during continuous illumination with far-red light, shown for thylakoids of wild type and metribuzin-resistant line 8. The areas below the positive transients are indicated. The vertical dashed lines indicate a change from linear to logarithmic scale.](image)
The values listed in Table V (column 2) show that the data for P-700 although different for the various lines are in fair agreement with those estimated for the relative amounts of P-680 (Table V, column 1). This is in line with the assumption by Kaplan and Arntzen (1982). It is also evident that remarkable increases (line 4) or decreases (lines 2, 3, 7 and 8) in reaction centers and/or light-harvesting complexes occurred.

Furthermore, the experimental determination of the P-700 concentrations enabled us to calculate the number of chlorophyll molecules per reaction center of photosystem I (Table V, column 3). The comparative data of Table V lead to interesting conclusions with regard to number and/or size of reaction centers, light-harvesting complexes and photosynthetic electron transport rates for the Chenopodium rubrum mutant lines. In general, compensatory effects are evident although different in extent for the various lines.

Pronounced variations in reaction centers/light-harvesting complexes of mutant strains (lines 2 and 3 versus lines 4 and 5) in comparison to wild type cells indicate that the concentrations of P-700 and P-680 may readily respond to mutations in the D1 protein of photosystem II. Furthermore, such variations are also reflected by massive changes in the number of chlorophyll molecules per reaction center (Table V, column 3). The highest increase in this specific chlorophyll content (lines 2 and 3) is assumed to result from a decrease in antenna size/number. These two lines are the only ones which carry mutations in the D1 protein at positions 229 and 266, respectively. Further work must show whether this assumption is correct.

Differences in half-time values for plastoquinol oxidation (Table V, column 4) are measurable but they are not considered to be significant (Ratajczak, 1987). Therefore, this oxidation reaction appears to proceed unimpaired in all cell lines.

The data on lag phase of P-700\(^*\) reduction (Table V, column 5) reveal that the events within photosystem II including the reduction of the plastoquinol pool are not slowed down by the various mutations. Some resistant lines even show a slightly reduced lag phase in comparison to the wild type. The significance of these observations remains to be evaluated by more intensive investigations.

All data taken together allow the conclusion that the eight metribuzin-resistant mutant lines do not show a mutation-induced impairment of photosynthetic electron transport. Only in the presence of high metribuzin concentrations slight inhibitory effect become evident. On the other hand, the various compensatory effects (i.e. increase in the size/number of light-harvesting complexes, changes in chlorophyll a/b-ratio, degree of

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**Table V.** Comparison of the mutant lines 1–8 and wild type cells of Chenopodium rubrum photoautotrophic cell cultures for photosynthetic reaction centers and rates of electron transfer reactions. Column 1: relative amounts of P-680 given as relative area; column 2: relative concentrations of P-700; column 3: number of chlorophyll molecules per P-700; column 4: half-times of plastoquinol reoxidation; column 5: durations of lag phase of P-700\(^*\) reduction. The data in columns 1, 4 and 5 were obtained by flash light-induced reduction kinetics of P-700\(^*\) with continuous illumination by far-red light. Values presented in columns 2 and 3 were measured by absorbance changes of P-700 after reduction with reduced dichlorophenol indophenol.

<table>
<thead>
<tr>
<th>Column</th>
<th>Line</th>
<th>Area rel.</th>
<th>[P-700] rel.</th>
<th>Chlorophyll P-700 [µg]</th>
<th>Half-life time [ms]</th>
<th>Lag phase [ms]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>1.000</td>
<td>1.000</td>
<td>1061</td>
<td>19.2</td>
<td>6.11 ± 0.22</td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>0.804</td>
<td>1.061</td>
<td>1000</td>
<td>16.1</td>
<td>5.05 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>L2</td>
<td>0.637</td>
<td>0.541</td>
<td>1963</td>
<td>16.4</td>
<td>3.50 ± 0.33</td>
<td></td>
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<tr>
<td>L3</td>
<td>0.838</td>
<td>0.730</td>
<td>1454</td>
<td>15.8</td>
<td>5.89 ± 0.46</td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>1.543</td>
<td>1.520</td>
<td>698</td>
<td>17.1</td>
<td>3.01 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>0.949</td>
<td>1.196</td>
<td>887</td>
<td>20.8</td>
<td>5.57 ± 0.54</td>
<td></td>
</tr>
<tr>
<td>L6</td>
<td>1.157</td>
<td>1.142</td>
<td>929</td>
<td>16.6</td>
<td>4.33 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>L7</td>
<td>0.642</td>
<td>0.892</td>
<td>1189</td>
<td>16.9</td>
<td>6.18 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>L8</td>
<td>0.859</td>
<td>0.858</td>
<td>1236</td>
<td>20.3</td>
<td>4.61 ± 0.22</td>
<td></td>
</tr>
</tbody>
</table>
thylakoid stacking) are well pronounced and the extent of these effects can well be correlated with the degree of resistance (Schwenger-Erger et al., 1993). The photoautotrophic mutant cell lines of Chenopodium rubrum are thus a highly versatile system for more detailed investigations on the adherent mechanisms.

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