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 [^{14}C]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 PS II herbicide-resistant higher plants known so far show a mutation at position ser 264 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-

Abbreviations: mbz, metribuzin, 4-amino-6(*tert*-butyl)-3-methylthio-*as*-triazine-5(4H)-one; atrazine, 2-chloro-4-(ethylamino)-6-(iso-propylamino)-*s*-triazine; diuron, DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; fw, fresh weight; LHC II, light-harvesting complex of photosystem II; PPQ, phenyl-*p*-benzoquinone; MV, methylviologen; TMPD red., 2,3,4,5-tetramethylphenylenediamine-reduced; DCPIP, dichlorophenol indophenol; Q_A, Q_B, primary and secondary electron acceptor of photosystem II; kDa, kilodalton; PS II, photosystem II; RFLP, restriction fragment length polymorphism; MS, Murashige and Skoog.

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ductivity (Hobbs, 1987; Ireland *et al.,* 1988; Ricroch *et al.,* 1987; Stowe and Holt, 1988).

Numerous triazine-resistant organisms with reduced photosynthetic capacity evolve compensatory 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 concentrations 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 chloroplasts 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 culture 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 impairment 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 5×10^{-6} M. In intervals of two days fresh weight, dry weight, cell number and chlorophyll content of the cell cultures were determined. The measurements 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 disintegration 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 usually used for cell cultivation. The final concentration 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 $\mu E\ xm^{-2}\ xs^{-1}$ 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 $\mu E\ xm^{-2}\ xs^{-1}$) using a Clark electrode (Hansatech, Fa. Bachofer, Reutlingen, Germany). The reaction mixture for measurement of PS I contained standard buffer (20 mM tricine-NaOH, pH 7.6, 20 mM KCl, 3 mM $MgCl_2\cdot6H_2O$), 0.1 mM methylviologen, 0.5 mM NaN_3, 0.1 mM TMPD, 2.5 mM Na-ascorbate, 15 μM DCMU and 1.5 μM gramicidin D in a total volume of 1 ml. The reaction mixture contained thylakoid membranes equivalent to 15 μg chlorophyll.

The reaction mixture for linear electron transport analysis contained standard buffer (see above), 0.1 mM methylviologen, 0.5 mM NaN_3, 1.5 μM gramicidin D in a total volume of 1 ml. Thylakoids equivalent to 30 μg 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).

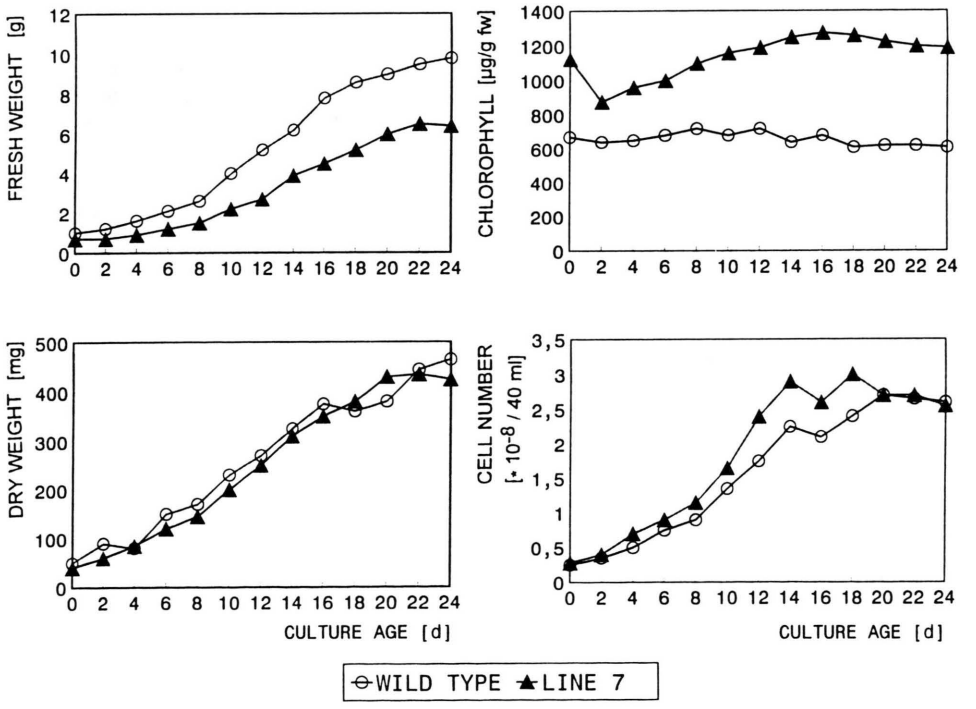

Fig. 1. Growth characteristics of the wild type cells without the herbicide and of the metribuzin-resistant line 7 of *Chenopodium rubrum*, cultivated with 5×10^{-6} M metribuzin over a time period of 22 days. The increase in fresh and dry weight per flask, in cell number and in chlorophyll content per gram fresh weight is depicted (standard deviation ± 10%).

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

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%).

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

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 coworkers (1983) pointed out that a decreased rate of electron transfer from Q_{A^-} to Q_B is still much more rapid than the following steps of electron transport and thus not rate limiting. Other findings imply that low rates of CO_2 reduction in resistant plants are possibly a consequence of poor efficiency in the use of separated charges at the photosystem II reaction center, an unstability 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 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 μM; cells of wt and of lines 5 and 6 received 1 μM only).

Line	Net-oxygen production [μmol/mg Chl × h]	
	Without metribuzin	With metribuzin
wt	38	13
L1	23	21
L2	37	32
L3	31	21
L4	31	28
L5	39	39
L6	41	38
L7	39	30
L8	41	35

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.*, 1986).

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} M metribuzin whereas the other lines received 10^{-5} 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-*p*-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} M, lines 5 and 6 10^{-6} M) or without metribuzin. The data represent the average of 4 parallel assays.

Line	Cultivation	
	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} M to lines 5 and 6 and 10^{-5} 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 IV. Rates of uncoupled electron transfer in photosystem I (2,3,4,5-tetramethylphenylenediamine-red. → methylviologen) photosystem II (H_2O → phenyl-*p*-benzoquinone) and photosystem I + II (H_2O → 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 (10 µM or 1 µM 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.

Line	Without metribuzin		With metribuzin	
	PS I O_2 uptake µmol/mg Chl×h	PS II O_2 production µmol/mg Chl×h	PS II O_2 production µmol/mg Chl×h	PS I + PS II O_2 uptake µmol/mg Chl×h
wt	146	59	0	(47)
L1	200	77	72	38
L2	169	85	52	21
L3	132	61	19	19
L4	194	76	56	33
L5	208	41	34	42
L6	156	83	38	40
L7	153	72	26	29
L8	213	88	85	40

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-*

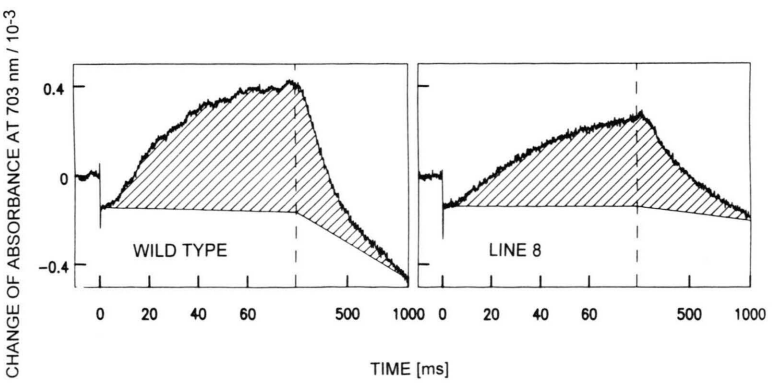

Fig. 2. Flash light-induced reduction kinetics of P-700$^+$ during continuous illumination with far-red light, shown for thylakoids of wild type and metribuzin-resistant line 8. The areas below the positive transients are indicated. The vertical dashed lines indicate a change from linear to logarithmic scale.

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 $P\text{-}700^+$ reduction. The data in columns 1, 4 and 5 were obtained by flash light-induced reduction kinetics of $P\text{-}700^+$ 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.

Column Line	1 Area rel.	2 [P-700] rel.	3 Chlorophyll / P-700 [μg]	4 Half-life time [ms]	5 Lag phase [ms]
wt	1.000	1.000	1061	19.2	6.11 ± 0.22
L1	0.804	1.061	1000	16.1	5.05 ± 0.30
L2	0.637	0.541	1963	16.4	3.50 ± 0.33
L3	0.838	0.730	1454	15.8	5.89 ± 0.46
L4	1.543	1.520	698	17.1	3.01 ± 0.13
L5	0.949	1.196	887	20.8	5.57 ± 0.54
L6	1.157	1.142	929	16.6	4.33 ± 0.36
L7	0.642	0.892	1189	16.9	6.18 ± 0.16
L8	0.859	0.858	1236	20.3	4.61 ± 0.22

podium rubrum. 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 siza/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 $P\text{-}700^+$ 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

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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