Photoautotrophic *Chenopodium rubrum* Cell Suspension Cultures Resistant against Photosynthesis-Inhibiting Herbicides
I. Selection and Characterization

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For establishing metribuzin-resistant, photoautotrophic *Chenopodium rubrum* cell cultures plated cells, callus cultures or suspension cultures were subjected to selection procedures. The most effective procedure was the stepwise increase in the concentration of the herbicide from 0.01 μM to 10 μM in suspension cultures, which resulted in the isolation of eight different metribuzin-resistant photoautotrophic cell lines. Conjugation metabolism or a decrease in the uptake and translocation of the selective agent were not responsible for resistance, which was stable in the absence of the inhibitor over numerous growth cycles. Measurements of the photosynthetic electron transport, analyses of fluorescence induction kinetics and determination of the binding properties of ^{14}C-labelled metribuzin to isolated thylakoids indicated that resistance of the cell lines is based on an alteration in the photosystem II herbicide-binding protein (D 1 protein). RFLP analysis of the *psb*A gene of the eight resistant cell lines demonstrated that none of them possess an amino acid exchange in position 264 of the D 1 protein leading to altered herbicide-binding properties.

Introduction

Plant cell cultures are applied both for basic research as well as for biotechnology and plant breeding. In contrast to heterotrophic cells all photosynthesis reactions can only be examined in photoautotrophic cells. Therefore green cell cultures provide a useful system for physiological and biochemical studies with those herbicides, which either interfere with the differentiation of plastids, inhibit electron transport or cause membrane destruction by initiating photooxidations [1].

Selection of cells resistant to a photosynthesis-inhibiting herbicide could be achieved with *Nicotiana tabacum* cells growing photomixotrophically. The visual screening for cell aggregates remaining green in the presence of the triazines atrazine and terbutryne led to triazine-resistant, photosynthetically active cell cultures [2]. Under strictly photoautotrophic conditions, Rey *et al.* [3] were able to select atrazine-resistant *Nicotiana tabacum* mutants using mesophyll-derived protoplasts subjected to mutagenesis and plating on selective medium.

We used the potential of the photoautotrophic cell culture of *Chenopodium rubrum* for the selection of different herbicide-resistant cell lines. These photoautotrophic cells of *Chenopodium rubrum* possess well developed chloroplasts, express high photosynthetic activity [4] and provide a suitable material for the isolation of mutants resistant to the herbicide metribuzin.

Metribuzin is a potent photosystem II inhibitor applied for the control of broadleaf and grass weeds in potatoes, tomatoes, soybeans, and other tolerant crops. Like the better known herbicides atrazine and diuron, metribuzin inhibits the electron transfer at the reducing side of photosystem II by replacing the secondary electron acceptor, a plastoquinone, from its binding site at the D 1 protein. This protein is also called Q_B protein, herbicide-binding protein, or according to its size, 32 kDa polypeptide [5].

At the chloroplast level, resistance to photosystem II herbicides can be attributed to an alteration

Abbreviations: metribuzin, 4-amino-6-(*tert*-butyl)-3-methylthio-*as*-triazine-5(4 H)-one; atrazine, 2-chloro-4-(ethylamino)-6-(*iso*-propylamino)-*s*-triazine; diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-*sec*-butylphenol; fw, fresh weight; PPQ, phenyl-*p*-benzoquinone; Q_A, Q_B, primary and secondary quinone acceptors of photosystem II, respectively; R, metribuzin-resistant; S, metribuzin-sensitive; F_o, F_j, F_p, low, intermediate and peak chlorophyll-fluorescence level, respectively; RFLP, restriction fragment length polymorphism; MS, Murashige and Skoog.

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of the herbicide-binding site of the D 1 protein resulting from a point mutation in the chloroplast *psb*A gene, which leads to a specific loss of herbicide-binding capacity. A substitution at position 264 (ser to gly or ser to thr) was found in all triazine-resistant higher plants tested so far [2, 6], whereas in the green algae *Chlamydomonas reinhardtii* additional mutations at the positions 219, 251 and 275 occur [7, 8]. Resistance to one herbicide, for example metribuzin, also led to cross resistance to other photosystem II inhibitors like atrazine and diuron. Differential cross resistance towards various herbicides has been described for higher plants and for algal mutants [9–11].

Metribuzin has been chosen as a selective agent mainly for selection procedures with seedlings [12] but also with the green algae *Chlamydomonas reinhardtii.*

Our work was designed to clarify, whether and which kind of mutants could be selected using photoautotrophic cell cultures of the higher plant *Chenopodium rubrum* and the herbicide metribuzin as selective agent.

Materials and Methods

Growth conditions of strains

Photoautotrophic cell suspension cultures of *Chenopodium rubrum* were cultured in double-tier Erlenmeyer flasks as described by Hüsemann and Barz [13]. They were agitated on a gyratory shaker (120 rpm) at 25 °C under continuous light (110 $\mu E \cdot m^{-2} \cdot s^{-2}$). The cells were subcultured every 14 days by transferring approx. 2 g fresh weight to fresh MS medium [14].

Selection

Metribuzin was applied as a sterile filtered methanol solution (0.25%, v/v) at every subculture cycle. The multiple step selection procedure was started with a herbicide concentration of 0.01 μM. As soon as the cells showed sufficient growth, the concentration was increased in 0.05 μM steps twice, followed by 0.01 μM steps.

Characterization of resistant cell cultures

Growth

The relative growth of the selected cell lines at the various herbicide concentrations was estimated by measuring the increase in fresh weight over a culture period of 14 days. This value was set in relation to the increase of fresh weight of a control (100%), which was cultivated under identical growth conditions after application of the solvent methanol (0.25%, v/v).

Determination of cross resistance

The herbicides atrazine, diuron, and dinoseb were dissolved in methanol and applied as sterile filtered solutions (0.25%, v/v) to the resistant cell variants and the wild type cells of *Chenopodium rubrum.* The effects of different concentrations were estimated after a time period of eight days by using the microtest system according to Thiemann *et al.* [15].

Preparation of thylakoids

Thylakoid membranes of photoautotrophic cell cultures of *Chenopodium rubrum* were isolated by the procedure of Nelson *et al.* [16]. After suspending 5 g cells of a culture from early stationary phase in 20 ml of medium 1 (see Nelson *et al.* [16]) the cells were disrupted with a Yeda press.

Electron transfer activity

For the measurements of electron transport activity in photosystem II fresh thylakoid preparations were used. Oxygen production was monitored with a clark type electrode (Hansatech, Fa. Bachofer, Reutlingen, F.R.G.) in a thermostated vessel at 20 °C and 30 μg/ml chlorophyll. The chlorophyll content was estimated by the method of Arnon [17]. The 1 ml reaction solution contained standard buffer (20 mM Tricine-NaOH, pH 7.6, 20 mM KCl, 3 mM $MgCl_2 \cdot 6\,H_2O$), the artificial electron acceptor PPQ (0.3 mM), and Gramicidin D (1.5 μM) as uncoupling agent. After a short preincubation in the dark, the thylakoids were illuminated with 1050 $\mu E \cdot m^{-2} \cdot s^{-1}$ and the oxygen production was monitored under continuous stirring.

Fluorescence measurements

For the determination of chlorophyll *a* fluorescence induction kinetics the chamber of the Hansatech oxygenemeter was filled with cells of different strains of herbicide-resistant *Chenopodium rubrum* cell cultures to a final chlorophyll content of

10 μg · ml^{-1}. The oxygenemeter had been equipped with a light source (36 LEDs, 60 W · m^{-2}, λ = 660 nm Hansatech, Reutlingen, F.R.G.). The chlorophyll fluorescence was collected by a fibre optic and transferred to a fluorescence detector with a 740 nm interference filter. The output light was sent to a transient recorder for measuring rapid kinetics.

After the application of metribuzin over a concentration range from 0.1 to 100 μM, the cells were kept in darkness for 5 min. For the estimation of the minimal fluorescence level the cells were given a light pulse of 100 ms and the level of F_o from the initial phases of the fluorescence induction curves (60 ms) was routinely determined according to Hipkins and Baker [18]. After further dark adaptation, the cells were illuminated for 1000 ms and the kinetics of the chlorophyll induction (600 ms) were recorded and analyzed.

Binding of [^{14}C]metribuzin by thylakoids

Thylakoids were prepared as described above and suspended in standard buffer to a final chlorophyll concentration of 50 μg · ml^{-1}. The radioactively labelled metribuzin (specific radioactivity: 769 mBq · mmol^{-1}) was added in methanolic solution over a concentration range of 0.1 – 2 nmol · ml^{-1}: The total reaction volume was 1 ml. Samples were thoroughly mixed, incubated for 5 min and centrifuged at 12,000 × *g* for 10 min. The clear supernatant was carefully removed and assayed for radioactivity. The determination of the binding parameters like binding constant K_B, number of binding sites x_t, and number of chlorophyll molecules per bound inhibitor z were calculated according to Tischer and Strotmann [19] from the double reciprocal plots obtained for the binding curves. The binding parameters could be estimated from the specific binding.

RFLP analysis

DNA of wild type and the eight different metribuzin-resistant cell lines was isolated and prepared for restriction following the procedures of Taylor and Powell and Thomzik and Hain [20, 21]. The restriction of the DNA with *Mae* I was performed according to Bettini *et al.* [22] and blotting of DNA according to Southern [23]. As hybridization probes a subcloned *Pst* I/*Eco* RV fragment from the *psb*A gene of *Solanum nigrum,* supplied by Dr. P. H. Schreier was synthesized by nick translation. For more details see Thomzik and Hain [21].

Results and Discussion

Selection techniques

Initial attempts to select herbicide-resistant cells were performed with photomixotrophic *Chenopodium rubrum* suspension cultures. By regular plating single cells on metribuzin-containing medium, several clones were obtained, which finally grew on herbicide concentrations of 10^{-4} M. However, these clones, despite of a high chlorophyll content, failed to show any photosynthetic activity. Thus, it has to be assumed that these cell lines returned to heterotrophic growth and that photomixotrophic cells are not an useful system for the selection of resistant lines.

Single step selection procedures in which cell suspensions, plated cells or callus cultures, respectively, were incubated with 10^{-5} M metribuzin also did not allow the isolation of resistant lines. Finally, plated photomixotrophic cells in feeder layer systems were found to be no suitable alternative. In all cases, the large portion of dying cells obviously suppressed the growth of any resistant cells.

Multiple step selection

Wild type photoautotrophic cells of *Chenopodium rubrum* were exposed to an initial metribuzin concentration of 10^{-8} M. At this concentration, the majority of cells survived although they only showed a very low rate of cell division. Using this approach with initial minimal inoculum being applied single mutant cells with unmodified cell division capacity are able to enrich, whereas the propagation of the wild type cells was inhibited. As soon as such suspensions showed visible growth the level of the herbicide was stepwise increased.

Application of mutagenic compounds like 5-fluorodesoxyuridine or UV irradiation according to Wurtz *et al.* [24] did not lead to an acceleration of the selection process. Compared to non-mutagenized cells the treated cultures should have shown a faster growth if the mutagenesis had caused an increase in mutant number [25]. Nevertheless, the metribuzin-resistant cell line 3 traces back to wild type cells irradiated with UV, while cell line 8 was treated with 5-fluorodesoxyuridine for three cell

cycles before an UV irradiation was performed. All other cell variants are descendents from untreated wild type cells.

After a time period of about 2 years eight cell lines were received. Six of them were able to grow at a herbicide concentration of 10^{-5} M, whereas two of them (L 5 and L 6) did not tolerate metribuzin concentrations higher than 10^{-6} M.

The possibility that a mixture of different mutants within one suspension flask of photoautotrophic *Chenopodium rubrum* cells may have been selected does not seem to be very likely because of the very long duration of the selection procedure. Furthermore, after two years of subcultivation, different mutants which show identical growth characteristics are assumed not to occur within one suspension [25].

Characterization of cell lines

Uptake, translocation, and metabolism of metribuzin

To find out whether the resistance mechanism of the selected strains of *Chenopodium rubrum* was based on a decrease in uptake of the herbicide or on its degradation or conjugation to less toxic metabolites, [^{14}C]metribuzin was applied to the cells. Analysis of uptake and metabolism of the radioactive product in the cell culture medium, cells, and cell walls, indicated that the herbicide was taken up by the wild type and all mutant cells in a similar amount [26, 27]. Since no metabolites could be detected by chromatographic analyses of cell extracts, a significant degree of detoxification of metribuzin can be excluded as the cause of resistance.

Long-term effects

To analyze for the long-term effects of herbicides, wild type cells and the eight different metribuzin-resistant cell lines were incubated with increasing concentrations of the photosynthesis inhibitors metribuzin, atrazine, diuron and dinoseb and cultured for 14 days under normal growth conditions. The increase of fresh and dry weight was estimated and set in relation to the growth of cells treated with the solvent methanol only.

The concentration I_{50}, which inhibited cell growth by 50%, the pI_{50} (-log I_{50}) and the resistance (R/S) ratios (I_{50} resistant/I_{50} wild type) are presented in Table I. It is evident that lines 1, 4, and 8 show the highest degree of metribuzin resistance followed by the strains 2, 3, and 7, which possess a moderate degree of resistance. The cell variants 5 and 6 growing with only 10^{-6} M metribuzin belong to a group of low resistance only.

Furthermore cell line 6 shows similar degrees of resistance to metribuzin and diuron, whereas these cells possess a slightly higher sensitivity to atrazine. However cell lines 2 and 5 seem to be more sensitive to this inhibitor at the cellular level. Variant 1 and to a lower degree variant 7 are able to grow with low doses of atrazine. Diuron as a member of the urea-herbicides is tolerated by cells of line 4 and 7 and in low concentrations also by the other cell variants.

Dinoseb as a phenolic inhibitor of photosynthesis, for which uncoupling properties have been reported, showed pronounced toxic effects to each of the examined cell cultures of *Chenopodium rubrum* during the growth period of 14 days even at the low concentration of 10^{-7} M. Therefore, cross-resistance at the cellular level could not be determined.

The results obtained for photosynthetic oxygen production in relation to increasing herbicide concentrations (data not shown) revealed identical degrees of resistance to metribuzin as shown in Table I. The cross resistance patterns for the selected cell lines towards the other photosystem II inhibitors were also similar.

For the interpretation of these values determined for inhibition of growth and photosynthetic activity long-term effects of the herbicides like inhibition of RNA- and protein synthesis must be con-

Table I. Effect of metribuzin, atrazine, diuron and dinoseb on growth of metribuzin-resistant (lines 1 – 8 and wild type (wt)) cell suspension cultures of *Chenopodium rubrum* cultivated in two-tier flasks for 14 days documented by pI_{50} values (-log I_{50}) and resistance ratios (I_{50} resistant/I_{50} wild type).

Line	Metribuzin		Atrazine		Diuron		Dinoseb	
	pI_{50}	R/S	pI_{50}	R/S	pI_{50}	R/S	pI_{50}	R/S
wt	6.82	1	6.80	1.0	7.15	1.0	6.49	1.0
L 1	4.06	587	5.80	10.0	7.08	1.2	6.52	0.9
L 2	5.05	60	7.00	0.6	7.03	1.3	6.34	1.4
L 3	5.04	60	6.35	2.8	6.44	5.1	6.24	1.8
L 4	4.74	120	6.66	1.4	6.21	8.7	6.31	1.5
L 5	5.59	17	6.89	0.8	6.74	2.6	6.57	0.8
L 6	6.21	4	6.92	0.8	6.48	4.7	6.32	1.5
L 7	5.05	60	6.11	4.9	6.08	12.0	6.36	1.4
L 8	4.22	400	6.46	2.2	6.29	7.3	6.26	1.7

sidered. Therefore, short-term effects of the different herbicides also had to be measured in order to calculate the actual degree of resistance more precisely.

Short-term effects

Since the eight metribuzin-resistant cell lines of *Chenopodium rubrum* were able to tolerate other herbicides, it could be expected that the resistance was associated with the herbicide binding protein. Thus, resistance should also be detectable at the level of broken chloroplasts; these assays were expected to show a rather unimpaired electron transport after metribuzin application.

Furthermore, secondary effects caused by the herbicides at the cellular or plastid level should not occur and thus disturb the determination of resistance.

The uncoupled electron transfer in photosystem II from H_2O to PPQ was measured using thylakoids isolated from the resistant and wild type cells according to Nelson *et al.* [16]. Table II shows the I_{50}, pI_{50} and R/S values obtained for various classes of herbicides. Five variants (lines 1, 2, 4, 5 and 8) showed a drastic increase in their R/S values under these assay conditions with thylakoids (compare Table I), whereas two cell lines (lines 3 and 7) and also the wild type were now more sensitive to metribuzin.

The extent of cross-resistance to atrazine and diuron appears to be similar if the effects of the herbicides at the whole cell level are compared to those found with the thylakoids (Table II). In addition the high-resistant cell variants 1 and 8 show a slight cross-resistance to diuron and atrazine, respectively.

In case of dinoseb several differences on the cellular and thylakoid level were found. Using thylakoids the strains 2, 4, and 8 were less sensitive than the wild type because they showed only a small decrease in the electron transport activity even in the presence of high dinoseb concentrations.

In general it can be pointed out that none of the eight metribuzin-resistant cell variants show a higher sensitivity to the phenolic inhibitor dinoseb.

The occurrence of cross-resistance and the results obtained by measuring the photosynthetic electron flow led to the assumption that the eight metribuzin-resistant strains of *Chenopodium rubrum* possess an altered D 1 protein. For further support of this hypothesis experiments were performed which should reveal the binding properties of metribuzin to thylakoids.

Chlorophyll-fluorescence

The determination of chlorophyll-fluorescence offers a sensitive method for the rapid and non-destructive screening of photosynthetic performance of green cells. Even very low abnormalities in the photosynthetic electron transport activity cause changes in the chlorophyll a-fluorescence induction kinetics. Fluorometric methods also provide an appropriate tool for the analysis of herbicide tolerance and resistance in plant cells, because the fluorescence induction kinetics of susceptible and resistant biotypes are different [9, 28]. In cells susceptible to a herbicide rapid Q_A-reoxidation by Q_B is prevented and therefore the maximum fluorescence level F_{max} is rapidly reached after set on of

Table II. Effect of metribuzin, atrazine, diuron and dinoseb on the Hill reaction activity measured with chloroplast thylakoid membranes of metribuzin-resistant (lines 1–8) and sensitive (wt) cell suspension cultures of *Chenopodium rubrum* showed by pI_{50}-values and resistance ratios.

Line	Metribuzin		Atrazine		Diuron		Dinoseb	
	pI_{50}	R/S	pI_{50}	R/S	pI_{50}	R/S	pI_{50}	R/S
wt	6.82	1	6.51	1.0	6.85	1.0	5.35	1.0
L1	2.48	22000	5.23	19.0	6.07	6.1	4.74	4.0
L2	4.64	153	6.30	1.6	6.30	3.6	4.07	18.9
L3	5.37	29	6.12	2.5	6.46	2.5	5.00	2.2
L4	4.07	566	6.31	1.6	5.62	17.0	4.25	12.4
L5	5.36	29	6.21	1.9	6.35	3.2	4.77	3.8
L6	6.07	6	6.55	0.9	6.33	3.4	4.72	4.2
L7	5.25	37	5.89	4.2	5.77	12.1	5.09	1.8
L8	2.74	12000	5.22	19.2	5.96	7.9	4.29	11.0

illumination. The fluorescence quenching, which occurs during intact electron transport, does not occur any more and the level of fluorescence remains high. In herbicide-resistant plants the rise of variable fluorescence to the maximal level only occurs in the presence of high herbicide concentrations.

Fig. 1 shows typical patterns of fluorescence induction for the wild type cells and the resistant variants 1, 2, and 5 observed in the absence and presence of metribuzin.

The fluorescence induction kinetics exhibited by the wild type cells demonstrate that even the low inhibitor concentration of 10^{-7} M is sufficient to impair the electron transfer in photosystem II. This is evident from rise of the fluorescence to a higher level. For the low-resistant cells of the variants 5 and 6 (curves not shown) this rise of fluorescence requires a higher inhibitor concentration of 10^{-6} M. The total inhibition of electron transport can be reached by metribuzin when applied at 10^{-5} M. The maximum of fluorescence emitted from wild type cells is also caused by this concentration. Cells showing moderate levels of resistance such as variant 2, as well as lines 3 and 7 (curves not shown) did not reach maximum inhibition with 10^{-5} M, their electron transport was not completely inhibited unless a concentration of 10^{-4} M was used. In case of variant 1, as well as lines 4 and 8 (data not shown) the inhibitor dose had to be increased to 10^{-3} M in order to cause maximal fluorescence emission. A concentration of 10^{-5} M sufficient for the complete inhibition of electron transport in photosystem II in the wild type cells did not reveal any effect on these last mentioned high metribuzin-resistant cell lines.

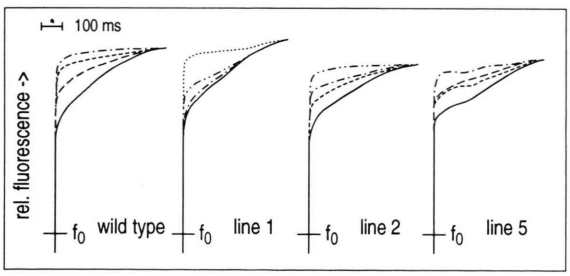

Fig. 1. Fluorescence induction kinetics obtained with wild type cells and metribuzin-resistant cell lines of *Chenopodium rubrum* (line 5: low resistance, line 2: moderate resistance and line 1: high resistance) after incubation of cells with different metribuzin concentrations in the dark.

The curves indicate:

with metribuzin:	without metribuzin:
10^{-7} M – – – – – –	————
10^{-6} M ----------	
10^{-5} M –·–·–·–·–	

[^{14}C]-metribuzin binding assay

Thylakoids from selected and wild type cells of *Chenopodium rubrum* were isolated and their capacity for binding [^{14}C]-metribuzin was assayed as described by Oettmeier *et al.* [29]. The binding of [^{14}C]-metribuzin to thylakoids is characterized by a small amount of unspecific binding *versus* a high extent of specific binding (Fig. 2). The thylakoids isolated from the wild type cells and to a lower extent those isolated from the strains 5 and 6 show a specific, high-affinity binding of the inhibitor as shown by the hyperbolic saturation kinetics. In contrast, the other six cell lines completely lost the ability of specific binding, because the data measured correspond to low-affinity binding of [^{14}C]-metribuzin to thylakoids. The linear relationship between the concentration of free (radioactivity in the supernatant) and bound metribuzin (radioactivity in thylakoids) indicates unspecific binding.

Double-reciprocal plots of the binding curves (Fig. 2) were used for the determination of the binding constant, K_B, pK_B ($-\log K_B$), the number of binding sites x_t, and the number of chlorophyll molecules per molecule of bound inhibitor [19]. These binding parameters, which can only be calculated for specific binding, are summarized in Table III. The data reveal that a metribuzin concentration of 0.027 μM is sufficient for the occupation of 50% specific inhibitor binding sites in thylakoids of the wild type cells. A 4.6- and even a 12.2-fold higher metribuzin concentration is necessary in case of thylakoids from variants 6 and 5, respectively. These results clearly indicate a lower affinity of the D 1 protein from the resistant strains 5 and 6 for metribuzin. Assuming the binding of one inhibitor molecule per molecule of D 1 protein a total number of 300 chlorophyll molecules per one electron carrier molecule can be calculated for the wild type cells of *Chenopodium rubrum.* In case of cell variants 5 and 6 the number of binding sites seems to have decreased. Under the assumption that there is no mixture of metribuzin-sensitive and -resistant chloroplasts within the cells of one strain the values for x_t

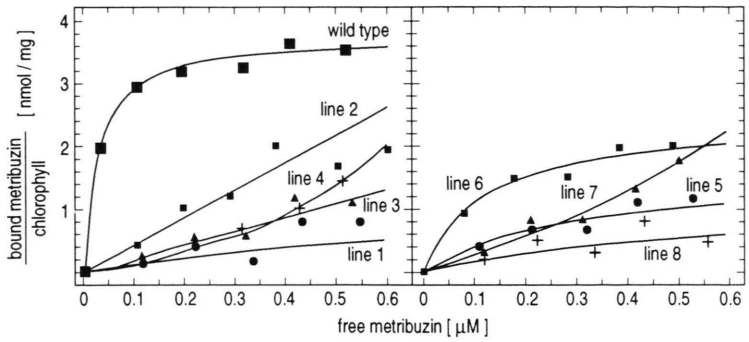

Fig. 2. [^{14}C]-metribuzin binding by thylakoids from wild type and metribuzin-resistant cell lines 1–8 of photoautotrophic *Chenopodium rubrum* suspension cultures.

should have been identical for resistant and wild type cells. One explanation for the observed deviation could be that chlorophyll was used as a reference. According to Anderson [30] an increase in the amount of light-harvesting complexes of PS II has been observed in inhibitor-resistant plant. This increased ratio of the number of chlorophyll molecules to the number of reaction centers and simultaneously to D 1 protein appears to indicate a lower number of binding sites per mg chlorophyll.

The values obtained for pK_B (Table III) and pI_{50} (Tables I and II) also should be identical if the chlorophyll content is extrapolated to zero within the I_{50} determination [19]. The discrepancy of the values obtained for pK_B and I_{50} can be satisfactorily explained by regarding the difference in chlorophyll content used for the two estimation processes.

RFLP analysis

Six of the eight metribuzin-resistant cell lines of *Chenopodium rubrum* had lost the ability for specific metribuzin binding, indicating that the herbicide-binding protein of these strains was altered.

Table III. Binding constants K_B, pK_B (-log K_B) values, number of binding sites K_t and number of chlorophyll molecules per bound molecule metribuzin z in thylakoids isolated from susceptible (wt) and metribuzin-resistant *Chenopodium rubrum* cell lines 1 to 8.

Line	K_B [µM]	pK_B	x_t [nmol · mg · chl^{-1}]	z
wt	0.027	7.57	3.7	300
Line 6	0.125	6.90	2.4	463
Line 5	0.329	6.48	1.6	694
Line 1–4, 7 and 8	no specific binding			

Since all higher plants detected so far with an altered D 1 protein possess a point mutation at the position 264 (ser) replaced by glycine or threonine [2, 31, 32], the resistant cell variants of *Chenopodium rubrum* were also thought to show a nucleotide exchange at the corresponding position of the *psb*A gene. Therefore, a RFLP analysis was performed comparing MAEI-generated restriction fragments of wild type with mutant DNA using a subcloned *Pst* I/*Eco* RV fragment from *Solanum nigrum* as a probe [21].

The specific site of nucleotide exchange was checked for an alteration by the method of Bettini *et al.* [22]. As a surprising result it was found that the eight herbicide-resistant strains of *Chenopodium rubrum* do not possess an altered nucleotide triplet at the site of the *psb*A gene, coding for amino acid 264 in the D 1 protein.

Since all mutations leading to triazine-resistant plants, algae or photosynthetically active bacteria, respectively are located in a distinct region of the *psb*A gene, coding for helix IV, the interhelical loop and a part of helix V [33], other amino acids than position 264 should be checked in our eight resistant strains of *Chenopodium rubrum.* A comparison of R/S values and cross resistance pattern obtained for triazine-resistant green and blue-green algae [6] with our data possibly points at an amino acid exchange at position 251. This mutation has also been detected in metribuzin-resistant green algae [7].

A different selection experiment with metribuzin and *Chlamydomonas reinhardtii* cells [8], revealed a mutation of the amino acid position 275. The amino acids within this region (helix V and facing the reaction center) are supposedly not directly involved in herbicide binding. The *Chlamydomonas* mutant cells show some similarities with the low-

resistant strains 5 and 6 of *Chenopodium rubrum* (metribuzin R/S ratio: 20; a slight cross resistance against diuron and dinoseb).

Mutations at position 275 as reported for *Chlamydomonas reinhardtii* do not impair the electron transfer from Q_A to Q_B as reported for mutations at position 264 [8, 34]. In this context the selected cells of *Chenopodium rubrum* much more resemble mutant green algae, with alterations in the D 1 protein at other sites than 264. Thus, measurements of chlorophyll *a* fluorescence emitted from wild type and resistant cells of *Chenopodium rubrum* clearly showed that the electron transfer of photosystem II is not altered in these mutant strains. The initial rise of fluorescence after illumination and the ratio $(F_p - F_i)/(F_p - F_o)$ were identical for the selected and wild type cells of *Chenopodium rubrum.* This observation contrasts with reports by Bettini *et al.* [22] or Erickson *et al.* [34] who compaired wild type and mutant cells of *Chenopodium album* and *Chlamydomonas reinhardtii* respectively.

The data presented in this report and other findings concerning the ratios of resistance against metribuzin, cross-resistance pattern, physiological and morphological differences between the lines indicate that the eight cell variants are distinct mutants [35]. Results of sequence analyses of the *psb*A gene [36] supported this assumption because the eight strains of *Chenopodium rubrum* carry several new point mutations, which have so far never been found in higher plants. Therefore, the different mutant strains of *Chenopodium rubrum* represent valuable systems for the evaluation of the topography of the D 1 protein and for gaining further insight into the binding mechanisms of plastoquinone and herbicide molecules.

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