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ISOLATION AND CHARACTERISATION OF METRIBUZIN-RESISTANT *CHLAMYDOMONAS REINHARDII* CELLS

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Chlamydomonas reinhardii **cells were treated with 5-fluorodeoxyuridine and ethylmethanesulfonate to induce mutagenesis. The mutant cells were analyzed for resistance against metribuzin (4-amino-6-(*t*-butyl)-3-methylthio-1,2,4-triazine-5-one). Clones with normal growth were isolated and the mutant cells further characterized. The photosynthetic rates of the mutant cells were about 20% lower than those of wild-type cells. The mutant cells were not only resistant against metribuzin (pI_{50} lowered from 6.65 to 3.41) but also against bromacil, atrazine, phenisopham and tolerant against 3-(3,4-dichlorophenyl)-1,1-dimethylurea. However, the mutant was more susceptible to phenolic electron-transport inhibitors like bromonitrothymol, ioxynil and i-dinoseb. 2,4-Dinitrophenyl-2′-iodo-3′-methyl-4′-nitro-6′-isopropyl phenyl ether inhibited the wild-type thylakoids more than the mutant. The analysis of the electron transport with artificial electron donors and acceptors showed that only Photosystem II was affected by the mutation and not Photosystem I. Binding experiments with isolated thylakoids of resistant and susceptible cells using [^{14}C]metribuzin and [^{3}H]-i-dinoseb revealed that metribuzin did not bind specifically to the thylakoids of the mutant cells, but that i-dinoseb did bind to the thylakoids of the mutant, and even better than to the thylakoids of the wild-type cells. Fluorescence studies confirmed these results.**

Introduction

The investigations of herbicidal resistance in higher plants have mainly concentrated on weed species [1–8]. The major components of the electron-transport system which react with herbicide molecules are the shielding protein of PS II, or synonymously called the B-protein [9,10]. A recent model proposes multiple binding sites for different herbicides on the B-protein [11,12].

It was of special interest to study how different classes of herbicides, including the -NH-C(=O)- group, and phenolic compounds, affected the binding properties of the B-protein. Photoaffinity labels of the phenolic type bind to a 42000 and an 18000 Da protein of the PS II complex [13–15]. Furthermore, PS II particles depleted of the B-protein were still sensitive to phenolic electron-transport inhibitors [16].

Our present study is an analysis of the inhibitory effects of different classes of herbicides on the electron transport in *Chlamydomonas reinhardii*.

Abbreviations: metribuzin, 4-amino-6-(*t*-butyl)-4-methylthio-1,2,4-triazine-5-one; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; i-dinoseb, 2,4-dinitro-6-isobutylphenol; ioxynil, 3,5-diiodo-4-hydroxybenzonitrile; BNT, 2-bromo-4-nitrothymol; DNP-INT, 2,4-dinitrophenyl-2′-iodo-3′-methyl-4′-nitro-6′-isopropyl phenyl ether; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; WT, wild-type; MZ-1, metribuzin-resistant cells; R/S, I_{50} (resistant cells)/I_{50} (susceptible cells); Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Chl, chlorophyll; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

The binding studies with radioactively labelled herbicides proved to be a very useful tool [17] for investigating their interaction with thylakoids of susceptible and resistant cells.

Materials and Methods

Strains and culture conditions. C. reinhardii WT strain 2137 mt + was selected for mutagenesis. This strain was isolated by Spreitzer and Mets [18] and maintains full photosynthetic competence under dark conditions. The cells were grown in Tris-minimal medium of Surzycki [19] supplemented with 18 mM sodium acetate.

Mutagenesis and selection. Cells were grown in Tris-minimal medium with 18 mM acetate in light (8000 lx). The cell suspension was diluted to $3.3 \cdot 10^5$ cells/ml and 1.2 mM 5-fluorodeoxyuridine was added. After a growing period of 5 days in light the suspension reached a cell content of $1.3 \cdot 10^7$ cells/ml in the stationary phase. These cells were treated with 0.1 M ethylmethanesulfonate to induce mutants. After an incubation period of 2 h the cells were thoroughly washed and resuspended in the culture medium. The survival rate after mutagenesis was approx. 16%. The cells were transferred to plates (culture medium with 2% agar) supplemented with 10^{-4} M metribuzin. Colonies which appeared after 4 days were selected and replated. Mutants were recovered with a frequency of about 1 per 10^3 cells.

Biochemical analysis. The photosynthetic rate of CO_2 fixation was measured with wild-type and mutant cells. The phototrophically grown cells were collected in the logarithmic growth phase and washed with 50 mM Tris-sulfate buffer, pH 7.2, containing 10 mM $MgCl_2$. The cells were resuspended in the same buffer to yield 1 mg Chl/ml cell suspension. The assay mixture contained cells equivalent to 0.1 mg Chl, 10 mM Tris-sulfate buffer, pH 7.2, 10 mM $MgCl_2$ and 10 mM sodium bicarbonate ($1.5 \cdot 10^7$ Bq/mmol). The cell suspension was kept in darkness for 5 min and the reaction was started in the light (10^5 lx) by the addition of bicarbonate and terminated by acidification. The incorporated ^{14}C atoms were determined by liquid scintillation counting.

The photosynthetic oxygen evolution was recorded in an oxygraph cell (equivalent to 0.05–0.1 mg Chl, Rank Brothers, Cambridge, U.K.). The cell suspension was added to 1.5 ml of 50 mM Tris-sulfate, pH 7.2, containing 10 mM $NaHCO_3$ and 10 mM $MgCl_2$.

Isolation of thylakoids. Cells were grown in 10-l carboys under mixotrophic conditions (5% CO_2 and 18 mM acetate) and harvested in the logarithmic growth phase. The cells were washed and resuspended in 50 mM Hepes, pH 7.5, containing 50 mM KCl, 2.5 mM $MgCl_2$ and 0.4 M sorbitol and broken in a Ribi cell fractionator (Sorvall) at 5000 lb/inch^2. The cell debris were collected at $1000 \times g$ (90 s) and the chloroplasts were sedimented at $8000 \times g$ (5 min).

Membrane protein analysis. The thylakoids were dissolved in SDS and the proteins were separated by acrylamide gel electrophoresis according to the procedure of Chua [20].

Determination of PS II activity ($H_2O \rightarrow Fe(CN)_6^{3-}$). The reaction assay mixture contained thylakoids in 1.5 ml (0.075 mg Chl) and the isolation medium of the thylakoids was supplemented with 1 mM ferricyanide. The oxygen evolution was recorded polarographically in light (10^5 lx).

Fluorescence analysis. The fluorescence induction curves were recorded with an instrument described by Bauer and Wijnands [21]. The cell suspensions were adjusted to 0.013 mg Chl/ml in 50 mM Tris-sulfate, pH 7.5, 10 mM $NaHCO_3$ and 10 mM $MgCl_2$. The suspension was illuminated for 1 s (exciting light: 632.8 nm, $6.6 \cdot 10^4$ erg $\cdot$ cm$^{-2} \cdot$ s^{-1}) and the resulting fluorescence emission was monitored. The dark periods of 10 min between additions and excitations were kept constant for all measurements.

Binding studies with [^{14}C]metribuzin and [^3H]dinoseb. Thylakoids were isolated from wild-type cells and from MZ-1 and purified by centrifugation in a vertical rotor (Sorvall SS90) on a sucrose gradient (35–60%, 20 min, 10000 rpm).

The photosynthetic activities of the thylakoid were determined in the assay system with H_2O as electron donor and ferricyanide as electron acceptor and yielded rates of about 200 μequiv./mg Chl per h. The incubation conditions for the binding experiments were as follows: in 2 ml of 20 mM Tricine-NaOH, pH 8.0, containing 20 mM $MgCl_2$ and thylakoids equivalent to 0.1 mg Chl ^{14}C-labelled metribuzin ($1.26 \cdot 10^6$ Bq/mg) and ^3H-

labelled dinoseb ($1.8 \cdot 10^7$ Bq/μmol) were added in the concentration range of 0.05–5 mM, incubated for 5 min at room temperature and centrifuged at $23000 \times g$ for 10 min. The residual radioactivity in the pellet and in the supernatant was analyzed.

Results

Isolation and characterization of the mutant strain

The treatment of wild-type cells with 5-fluorodeoxyuridine favors a decrease in the number of chloroplast DNA molecules [22]: the growth of cells at concentrations of 5-fluorodeoxyuridine inhibiting the thymidylate synthetase causes a reduction of the number of DNA molecules per chloroplast. The cells were cultivated in a medium with 1.2 mM 5-fluorodeoxyuridine until they reached the stationary phase (40-fold increase in cell number).

The conditions of the mutagenesis by ethylmethanesulfonate were selected so that a large number of cells (up to 16%) survived, from which a high percentage of mutants (20%) was available. The screening of the mutants for the herbicide metribuzin was carried out at a concentration of 10^{-4} M in the medium (both liquid culture and plates). Metribuzin-resistant clones which resembled the wild-type cells in size and color were selected, and designated as MZ-1.

The properties of the mutant MZ-1 were studied and compared to those of the wild-type cells; the results are summarized in Table I. The cells grew slower in photoautrotrophic medium but almost at the same rate under mixotrophic conditions with acetate as additional carbon source. The chloroplyll content as well as the ratio of Chlo a/b of both strains (Table I) were similar.

The photosynthetic rates were measured as light-dependent $^{14}CO_2$ fixation and as CO_2-dependent oxygen evolution in light. Both methods revealed that the mutant cells had a 20–25% diminished photosynthetic rate compared to the wild-type cells (Table I).

The analysis of the thylakoid membrane proteins, dissolved by sodium dodecyl sulfate and separated by gel electrophoresis, could answer the question as to whether there was a difference in the protein pattern. Thylakoids were isolated as described in Materials and Methods and the protein separation was carried out following the procedure of Chua [20]. No significant differences (especially in the molecular weight range of 28000–35000) could be observed between the two gel traces (data not shown). This result was not surprising, since ethylmethanesulfonate causes mainly point mutations [23], and therefore, the changes in the amino acid composition of the proteins are not sufficient to affect the mobility in SDS gels.

Specificity of the herbicide resistance

The resistance against metribuzin of MZ-1 was tested for specifity, i.e., whether the cells were also resistant against other herbicides. Intact cells or isolated thylakoids were incubated in the dark for 5 min with the inhibitors and oxygen evolution was measured. The following inhibitors of electron transport were selected: DCMU (urea), atrazine (triazine), bromacil (uracil), phenisopham (carbamate). These compounds bind to the B-protein of the thylakoids and block the electron transport on the reducing site of PS II (see Ref. 11 for a review). Inhibitors which block the electron transport at other sites, different from the B-protein, were also included in the test series: *o*-

TABLE I

COMPARISON OF METRIBUZIN RESISTANT (MZ-1) AND SUSCEPTIBLE (WT) CELLS

	MZ-1	WT	$\frac{\text{MZ-1} \times 100}{\text{WT}}$
Chl (mg/10^8 cells)	0.39	0.43	91
Chl a/b	2.0	2.0	
CO_2 fixation (μmol/mg Chl per h)	106	138	76
O_2 evolution (μmol/mg Chl per h)	101	128	79

TABLE II

EFFECT OF ELECTRON-TRANSPORT INHIBITORS ON METRIBUZIN SUSCEPTIBLE (WT) AND RESISTANT (MZ-1) CELLS

Reaction mixtures contained in 1.5 ml: 50 mM Tris-sulfate buffer, pH 7.5, supplemented with 10 mM $MgCl_2$, 10 mM $NaHCO_3$ and cells equivalent to about 75 μg Chl. The cells were kept in the presence of the electron-transport inhibitors for 5 min in the dark and then illuminated with white light (10^5 lx) at 25°C.

Inhibitor	pI_{50}		*R/S*
	WT	MZ-1	
Metribuzin	6.65	3.41	1740
Bromacil	5.74	3.80	106
Atrazine	6.11	4.41	50
Phenisopham	6.09	4.48	40
DCMU	6.33	5.17	14.5
i-Dinoseb	5.70	5.90	0.7
Bromonitrothymol	6.49	7.12	0.2

phenanthroline and DNP-INT. Phenolic compounds which inhibit PS II: bromonitrothymol, ioxynil and picric acid were of special interest, since these compounds probably do not bind to the B-protein [14].

The results of these experiments are summarized in Table II. It can be seen that MZ-1 was resistant not only to metribuzin but also to bromacil, atrazine and phenisopham, and tolerant to DCMU. The degree of the susceptibility varied in a wide range between 1740- to 14-fold. Contrary to the '-NH-C(=O)-' class of herbicides [24,25] the phenolic compounds, dinoseb and bromonitrothymol, inhibited the MZ-1 cells even more strongly than the wild-type cells.

The uncoupled electron-transport rate of the thylakoids of the wild-type cells and of the MZ-1 cells varied between 160 and 320 μequiv./mg Chl per h. The thylakoids were incubated for 5 min with the inhibitors in the dark and then assayed for oxygen evolution. The results of these experiments are summarized in Table III. As already observed in the experiments with intact cells the inhibitors of the -NH-C(=O)-group had a similar effect on the thylakoids. The effect of the phenolic compounds could also be repeated with the isolated thylakoids: MZ-1 cells were more susceptible to ioxynil, i-dinoseb, bromonitrothymol and picric acid than the wild-type thylakoids. Ioxynil, picric acid and DNP-INT could not be tested in intact cells due to their impermeability. DNP-INT was similar to the DCMU-type inhibitors but not to the phenolics. An interesting observation was the greater tolerance of MZ-1 against *o*-phenanthroline, which could be related to the observed effect with DNP-INT.

Similar results were obtained when using diphenylcarbazide as the electron donor and dichlorophenolindophenol as the electron acceptor (data not shown).

TABLE III

EFFECT OF ELECTRON-TRANSPORT INHIBITORS ON THYLAKOIDS OF METRIBUZIN-SUSCEPTIBLE AND -RESISTANT CELLS

The reaction mixture contained in 1.5 ml: 50 mM Hepes buffer, pH 7.5, supplemented with 0.4 M sorbitol, 50 mM KCl, 2.5 mM $MgCl_2$ and 1 mM ferricyanide and thylakoids (about 75 μg Chl). The incubation with the inhibitors occured in darkness for 5 min at 25°C.

Inhibitor	pI_{50}		*R/S*
	WT	MZ-1	
Metribuzin	7.02	4.38	437
Atrazine	6.45	5.08	23
Bromacil	6.71	5.02	49
Phenisopham	6.68	5.07	39
DCMU	6.72	5.70	9.5
DNP-INT	7.15	6.13	10
Ioxynil	5.60	6.20	0.3
i-Dinoseb	6.64	7.15	0.3
Bromonitrothymol	6.76	7.21	0.35
Picric acid	4.35	5.76	0.38

Binding studies with [^{14}C]metribuzin and [^{3}H]dinoseb

The binding of herbicides to the B-protein could be studied with radioactively labelled substances. This method [17] was used to determine the equilibrium constant for binding. The distribution of the labelled inhibitor between the supernatant and the thylakoid pellet reflects the binding constant and the number of binding sites at the thylakoids.

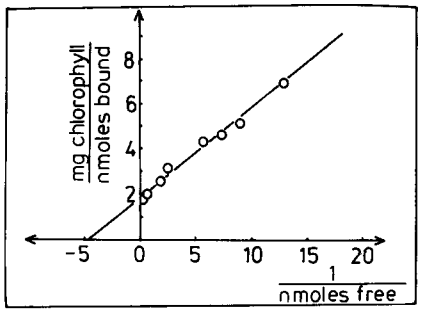

Fig. 1. Binding studies with [^{14}C]metribuzin and thylakoids isolated from metribuzin-resistant (MZ-1) and -susceptible (WT) cells. The binding experiments were carried out with 100 μg Chl in 2 ml and varying concentrations of the inhibitor from 0.1 to 10 μmol. Double-reciprocal plot for the specific binding of [^{14}C]metribuzin to the wild-type cells (O——O). The resistant cells did not show any specific binding and therefore, the data were omitted.

Specifically bound and unspecifically attached inhibitor molecules can thus be differentiated.

Metribuzin was bound to the wild-type cell thylakoids with a binding constant of $2 \cdot 10^{-8}$ M and 526 Chl molecules/bound metribuzin, i.e., one specifically bound inhibitor molecule per electron-transport chain (Fig. 1).

The lack of a biphasic binding curve indicated that the resistant mutant showed no specific binding at all and these results were in perfect agreement with the electron-transport studies.

The binding with [^{3}H]dinoseb, a phenolic compound, revealed that both types of thylakoids could specifically bind this inhibitor (Fig. 2). The unspecific and the specific binding of dinoseb to the MZ-1 thylakoids was significantly higher as compared with the wild-type thylakoids. Therefore, the binding constant was smaller for the mutant ($2.5 \cdot 10^{-8}$) than for the wild type ($2.7 \cdot 10^{-7}$). The number of binding sites in both samples was equivalent to 833 Chl molecules per dinoseb molecule, i.e., two electron-transport chains per inhibitor. These figures are in sound agreement with recently published data [25].

Fluorescence analysis

Fluorescence induction curves can be useful to study the redox state of the acceptor of PS II, the quencher Q [26]. Since the electron transition from

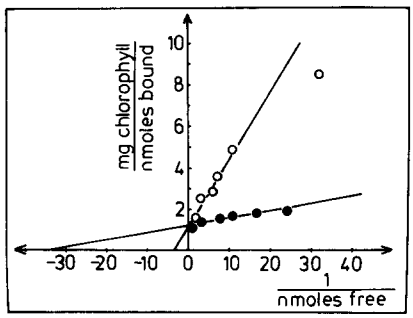

Fig. 2. Binding studies with [^{3}H]dinoseb and thylakoids isolated from metribuzin-resistant (MZ-1) and -susceptible (WT) cells. The binding experiments were carried out with 100 μg Chl in 2 ml and varying concentrations of the inhibitor from 0.1 to 10 μmol. Double-reciprocal plot for the specific binding of [^{3}H]dinoseb to the WT and MZ-1 thylakoids. The open symbols represent the data obtained with the thylakoids from the WT cells and the closed symbols those of the MZ-1 thylakoids.

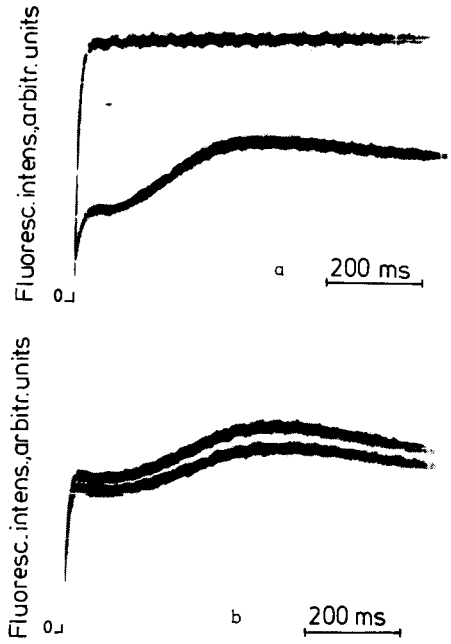

Fig. 3. (a) Chlorophyll fluorescence transient changes of WT cells. The cells (13 μg Chl/ml) were incubated for 10 min in the dark with or without $5 \cdot 10^{-6}$ M metribuzin. The cells were illuminated for 1 s (632 nm, $6.6 \cdot 10^{4}$ erg/cm^2 per s). The upper curve shows the experiment in the presence of $5 \cdot 10^{-6}$ M metribuzin, the lower curve shows the control experiment. (b) Chlorophyll fluorescence transient changes of MZ-1 cells. The conditions were the same as described in a, except that the metribuzin concentration was raised to 10^{-5} M (upper curve).

TABLE IV

FLUORESCENCE INDUCTION ANALYSIS OF METRIBUZIN-SUSCEPTIBLE AND -RESISTANT CELLS

Inhibitor	$\Delta F_I/\Delta F_M$	
	WT	MZ-1
Control	0.51	0.71
Metribuzin ($5 \cdot 10^{-6}$ M)	1.0	0.70
DCMU ($3 \cdot 10^{-7}$ M)	1.0	0.78
Bromonitrothymol ($5 \cdot 10^{-8}$ M)	0.89	0.98

Q to B is blocked by DCMU, the binding of an inhibitor molecule to the B-protein has a direct impact on the chlorophyll fluorescence.

Cell cultures (0.013 mg Chl/ml) were incubated in the dark with 10 mM bicarbonate and after a short period of light (1 s) the fluorescence emission was measured. Typical fluorescence induction curves for the metribuzin-resistant and susceptible cells are presented in Fig. 3a and b. To both cell samples, either $5 \cdot 10^{-6}$ M (WT cells) or 10^{-5} M (MZ-1 cells) metribuzin was added, they were incubated in the dark for 10 min, and subsequently the fluorescence emission was recorded. The results of these experiments with inhibitors are summarized in Table IV.

The analysis of the data in Table IV shows that the control with MZ-1 cells already has a higher $\Delta F_I/\Delta F_M$ value than the wild-type cells. Furthermore, the addition of metribuzin raises the intermediate fluorescence to the maximal level in the experiment with wild-type cells but the same procedure with the MZ-1 cells causes no significant change of the fluorescence.

Similar results were obtained in the experiments with DCMU as inhibitor. However, the addition of the phenolic compound bromonitrothymol has the opposite effect: the fluorescence level ratio is close to 1 for MZ-1 but lower for the wild-type cells.

Discussion

A classical approach for depicting sequences of components of the electron-transport chain was introduced by Levine [27] with mutants of *C. reinhardii* cells.

Mutants of *C. reinhardii* which showed tolerance against DCMU and against simazine (2-chloro-4,6-bisethylamino-*s*-triazine [28,29] were isolated. The I_{50} value of the mutant was equivalent to $1.7 \cdot 10^{-6}$ M for DCMU and $1.9 \cdot 10^{-6}$ M for simazine, the corresponding R/S values (I_{50} resistant cells/I_{50} susceptible cells) were 10.6 for DCMU, and 2.5 for simazine [29].

The tolerance against DCMU was similar to that observed with the mutant MZ-1, where an R/S value of 9.5 was calculated. Furthermore, the general growth characteristics were similarly unaffected by the mutation. However, the data of the fluorescence analysis with the MZ-1 mutant indicated that the electron transfer from Q to plastoquinone seems to be slower compared to the wild-type cells. Atrazine-resistant plants of *Chenopodium album* [5] and of *Amaranthus retroflexus* [8] showed similar fluorescence kinetic curves. The intermediate fluorescence (F_I) was much higher in the experiments with thylakoids of resistant plants than with those of the susceptible plants [5,8].

The interaction of the electron-transport inhibitors like metribuzin, atrazine and DCMU with the binding protein in the thylakoid membrane and the resulting inhibition of the electron-transport chain are still not elucidated. The shield protein or B-protein is assumed to be the binding site for this type of herbicide (see Ref. 11 for a recent review). Its tryptic digestion causes the exposure of the electron acceptor of PS II and the interruption of the electron flow to the plastoquinone pool [30,31]. By the use of the photoaffinity label, azidoatrazine, a single protein of 32000 Da could be labelled [32], and it was concluded that the herbicide-binding site is the apoprotein of B [10,12]. A comparison of chloroplasts from triazine-resistant and triazine-susceptible *Amaranthus* plants showed that only the B-protein of the latter type could be covalently modified [10].

From the results of the binding studies and from the competetive displacement experiments, the conclusion can be drawn that the B-protein can bind diverse herbicides with common properties (like the -NH-C(=O)-group) at different binding domains at the protein [11,12]. The results of this investigation are in agreement with the proposed model with respect to the inhibitors metribuzin,

bromacil, DCMU and phenisopham. The mutant was resistant to these inhibitors to different degrees (Tables II and III).

The resistance of the MZ-1 mutant against metribuzin could not be extended to the class of phenolic electron-transport inhibitors. In contrast, these substances showed even lower R/S values, i.e., the mutant was more susceptible. Our data support the results recently obtained with the photoaffinity label 2-azido-4-nitro-6-[2′,3′-^{3}H]isobutylphenol: the predominantly labelled thylakoid protein was in the range of 42000 Da and not in the 32000 Da B-protein [14] range. The photoaffinity labelling of thylakoids from atrazine-resistant *A. retroflexus* yielded almost identical labelling patterns [15]. It appears that the phenolic inhibitor compounds bind to the reaction center of PS II; whether the binding occurs at the acceptor site Q and in competition to Q is still a matter for speculation. The increased sensitivity of the resistant MZ-1 thylakoids indicates that the mutation of the B-protein facilitates the penetration to or reaction of these phenolic inhibitors with the PS II reaction center (Tables II and III). This assumption is supported by the observation that dinoseb, ioxynil, bromonitrothymol and related compounds increased their inhibitory activity after a mild trypsin digestion of thylakoids [33,34] when the B-protein is preferentially attacked by trypsin [35].

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