Volume 329, number 1,2, 43–46 FEBS 12856 August 1993


# Metribuzin resistance in photoautotrophic *Chenopodium rubrum* cell cultures

## Characterization of double and triple mutations in the psbA gene

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Received 24 May 1993; revised version received 21 June 1993

Sequence analyses of eight metribuzin-resistant mutants of photoautotrophic *Chenopodium rubrum* cell cultures revealed new mutations in the psbA gene coding for the 32 kDa herbicide binding protein. Mutants were found to possess either two or three changes in the amino acid sequence of the D1-protein between positions 219 and 272.

Herbicide resistance; Herbicide; psbA gene; D1 protein; Photosystem II; *Chenopodium rubrum*

## 1. INTRODUCTION

The $Q_B$ binding protein (or D1 protein or, according to its size, 32 kDa protein) is one of the most studied plant proteins [1]. It is part of the reaction center of photosystem II (PSII) [2], provides the $Q_B$ binding site for the secondary electron acceptor, $Q_B$, and is the target for a number of herbicides blocking electron transport between the primary and secondary plastoquinone electron acceptors [3]. Inhibition by herbicides through interaction with the binding niche prevents $Q_B$ binding to the $Q_B$-protein. The $Q_B$-protein is encoded by the psbA gene, which is located on the chloroplast genome [4] and is highly conserved in plants, algae and cyanobacteria. The psbA gene sequences from several organisms have been determined (see e.g. [4–9]). Quite frequently, resistance of photosynthetic organisms to PSII herbicides, like triazinones, triazines and DCMU, results from mutations in the psbA gene, leading to an amino acid exchange in the $Q_B$ binding protein. In higher plants a mutation affecting amino acid 264 (Ser) leads to resistance against triazine herbicides [5,6,9]. In algae mutations in position 219, 251, 255, 256, and 275, are also known, which confer resistance to several classes of herbicides [7,10,11]. All these mutations are located on helices IV and V and their connecting loop, and thus they are exactly on that part of the protein, which is directly involved in $Q_B$ and herbicide binding [12].

In the Münster Institute eight cell lines of *Chenopodium rubrum* highly resistant to metribuzin were selected and well characterized for their photosynthetic capacity and growth performance [13]. Further investigations on [$^{14}$C]metribuzin metabolism and [$^{14}$C]metribuzin binding studies with isolated thylakoids led to the assumption [13] that the resistance results from an altered D1-protein. Furthermore RFLP analyses had shown that none of the resistant cell lines carried a mutation in Ser-264, which is the sole mutation site in the $Q_B$-protein so far detected in herbicide-resistant higher plants.

We have now carried out partial DNA sequence analyses of the essential part of the psbA gene of the *Chenopodium rubrum* cells and thus revealed amino acid exchanges in the region between 219 and 272 of the $Q_B$-protein. Furthermore we determined factors of cross-resistance against several classes of herbicides to obtain information about the significance of the revealed mutation sites for herbicide resistance.

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*Abbreviations:* atrazine, 2-chloro-4-(ethylamino)-6-(iso-propylamino)-*s*-triazine; bromacil, 5-bromo-3-(sec-butyl)-6-methyl-uracil ; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; dinoseb, 2,4-dinitro-6-sec-butylphenol; kDa, kilodalton; metribuzin, 4-amino-6-(tert-butyl)-3-methylthio-as-triazine-5(4H)-one; PCR, polymerase chain reaction; phenmedipham, methyl *m*-hydroxy-carbanilate *m*-methylcarbanilate; PSII, photosystem II; $Q_A$ and $Q_B$, primary and secondary quinone acceptors; propanil, *N*-(3,4-dichlorophenyl)propionamide; RFLP, restriction fragment length polymorphism.

## 2. MATERIALS AND METHODS

### 2.1. *Cell material*

Eight photoautotrophic metribuzin-resistant cell suspension cultures were selected from a metribuzin-sensitive wild-type of *Chenopodium rubrum* via 'multiple step selection'. The selection procedure was

started with a herbicide concentration of $10^{-8}$ M metribuzin, which was increased in 0.05 $\mu$M steps twice, followed by 0.01 $\mu$M steps, as soon as the cells showed sufficient growth at one particular concentration. Finally six of the resistant cell lines were able to grow at a herbicide concentration of $10^{-5}$ M metribuzin (lines 1–4, 7 and 8), whereas two variants (lines 5 and 6) could only tolerate metribuzin concentrations of $2 \cdot 10^{-6}$ M [13].

2.2. *Growth conditions of the strains*

The eight resistant strains and the wild-type cells were grown under photoautotrophic conditions [14] in a mineral salt medium according to Murashige and Skoog without vitamins and hormones [15]. The mutants received in the medium metribuzin in different concentrations: $10^{-5}$ M metribuzin for lines 1–4, 7 and 8, and $2 \cdot 10^{-6}$ M metribuzin for lines 5 and 6. For isolation of DNA/RNA the cell material was harvested after a culture period of 14 days by suction of cells and shock freezing of the cell material in liquid nitrogen.

2.3. *R/S- and $pI_{50}$ values*

Cross-resistance against different herbicides was determined by measuring the inhibition of uncoupled electron transport from water to phenyl-*p*-benzoquinone in thylakoid membranes isolated from wild-type and mutant cell cultures of *Chenopodium rubrum* after a culture period of 14 days using increasing concentrations of the herbicides. The determination of the $pI_{50}$ ($-\log I_{50}$) values and R/S ($I_{50}$ resistant/$I_{50}$ wild-type) values involved estimation of the concentration required for 50% inhibition ($I_{50}$) [16].

2.4. *DNA/RNA isolation*

Cellular DNA and RNA were isolated simultaneously following a modified protocol of the cetyl-trimethyl-ammonium bromide (CTAB) nucleic acid precipitation method of Taylor and Powell [17], as described by Naber et al. [18]. This method is based on precipitation of nucleic acids in high salt concentration in the presence of CTAB.

2.5. *Amplification of a fragment of the psbA gene*

For amplification of the DNA fragment of interest in the psbA gene the PCR technique was used. Two oligonucleotides were used as primers which were synthesized on an Applied Biosystems model 381 A DNA synthesizer. The first primer (1) had a length of 23 nucleotides (5′-CCA(G) TTT AA(G)G TTG AAA GCC ATA GT) and the second (primer 2) one a length of 20 nucleotides (5′-GTA(T) GCT GGT GTA TTC GGT(C) GG). Primer 1 hybridizes to bases 874–896 of the RNA-like strand, and primer 2 to bases 604–623 of the coding strand. Using these oligonucleotide primers, a fragment of 293 bases was amplified. This fragment codes for amino acids 209–291 of the $Q_B$-protein. The PCR reactions were passed through 40 cycles with temperatures of 50°C for primer annealing (1 min), 70°C for primer elongation (3 min) and 90°C for denaturation (1 min) in a water bath thermal cycler.

2.6. *Cloning and transformation*

After amplification, the PCR fragment was purified by centrifugation through a Centricon-30 microconcentrator, treated with Klenow DNA polymerase to obtain blunt ends, ligated into the *Eco*RV restriction site of the Bluescript SK$^-$ vector (Stratagene) and used for transformation of *E. coli* TG1. Transformants were selected for plasmid-encoded ampicillin resistance. Plasmid DNA was isolated following the method of Birnboim and Doly [19].

2.7. *DNA sequencing*

Sequencing of the plasmid DNA was carried out with (5′-P)-labeled oligonucleotides, T3 and T7, according to Zagursky et al. [20].

## 3. RESULTS AND DISCUSSION

In view of the presently known mutations in the psbA gene conferring herbicide resistance [21], a DNA fragment representing amino acids 209–291 was selected for the investigations. This fragment of wild-type and mutant cells of *Chenopodium rubrum* was amplified by the PCR technique, cloned and then fully sequenced. Table I shows the detected mutation sites and the affected amino acid residues in the relevant region of the $Q_B$-protein of the eight resistant cell lines of *Chenopodium rubrum*. The data are based on sequencing 2 clones of each line. Some aspects of the data are especially noteworthy. First it is conspicuous that all cell lines carry the same mutation at position 219 (Val → Ile), which suggests that this is a typical feature of our cell cultures selected for resistance against metribuzin and that this amino acid exchange is decisive for the expression of triazinone resistance. Furthermore none of the mutants carries a mutation at Ser-264 of the D1-protein, which up to now has been the only mutation site in the D1-protein detected in herbicide-resistant higher plants [22–24]. Six of the eight cell lines possess an amino acid exchange at the Ala-251, either for Val (lines 1, 2 and 8) or for Thr (lines 4, 6 and 7). Finally, none of the mutants carries a single mutation only, but in every case two or even three amino acid exchanges in the D1-protein were present. This suggests that a single mutation at position 219 is not sufficient for the expression of the selected metribuzin resistance.

Such double and triple mutants have never been described for higher plants. It is also surprising that lines 4 and 7, and lines 1 and 8 show identical mutation sites in the sequenced gene fragment yet their growth behaviour is quite different [13].

Table I

Mutation sites of the psbA gene of eight metribuzin-resistant cell lines of *Chenopodium rubrum* (L1–L8) in the region coding for amino acids 209–291 of the $Q_B$-protein in comparison to the wild-type (wt)

| Line | Codon | | | | | | |
|---|---|---|---|---|---|---|---|
| | 219 | 220 | 229 | 251 | 266 | 270 | 272 |
| wt | GTA | ACT | GAA | GCT | AAC | TCT | CAC |
| | Val | Thr | Glu | Ala | Asn | Ser | His |
| L1 | ATA | | | GTT | | | |
| | Ile | | | Val | | | |
| L2 | ATA | | | GTT | ACC | | |
| | Ile | | | Val | Thr | | |
| L3 | ATA | | GGA | | | TTT | |
| | Ile | | Gly | | | Phe | |
| L4 | ATA | | | ACT | | | |
| | Ile | | | Thr | | | |
| L5 | ATA | GCT | | | | TAT | |
| | Ile | Ala | | | | Tyr | |
| L6 | ATA | | | ACT | | | CGC |
| | Ile | | | Thr | | | Arg |
| L7 | ATA | | | ACT | | | |
| | Ile | | | Thr | | | |
| L8 | ATA | | | GTT | | | |
| | Ile | | | Val | | | |

To evaluate the mutations and their significance for herbicide resistance, cross-resistance of the various cell lines against different herbicide was measured. Table II shows the R/S- and $pI_{50}$ values, determined by measuring the inhibition of the uncoupled electron transport from water to phenyl-*p*-benzoquinone in isolated thylakoid membranes. The results obtained demonstrate different cross-resistance patterns for each of the resistant cell variants. The most favourable combination of mutation sites for metribuzin-resistance in our cell cultures is an amino acid exchange of Val-219 for Ile, and Ala-251 for Val (lines 1 and 8). The cell variants carrying these mutations also show a comparatively high cross-resistance to bromonitrothymol and bromacil. The additional mutation at position 266 of the D1-protein of line 2 seems to have a negative effect on the metribuzin-resistance but provides a moderate phenmedipham resistance and intensifies the bromacil cross-resistance. The triple mutant is remarkable, because all three substitutions have been reported as single mutations in *Chlamydomonas* mutants [7,10,25], and all of them, when occurring individually, confer moderate resistance to phenol-type inhibitors like dinoseb or ioxynil. As shown in Table II, line 2 shows the highest resistance to dinoseb. Line 4, which shows the amino acid exchanges Val-219 for Ile and Ala-251 for Thr, also shows high metribuzin resistance and cross-resistance to bromacil, propanil, and diuron. In contrast line 7 possesses a comparatively low metribuzin resistance, an inferior diuron and atrazine cross-resistance, and hardly any resistance to propanil and bromacil, although this line carries the same mutations in the examined part of the psbA gene as line 4. Line 6 carries an additional amino acid exchange at the position 272 (His for Arg). This substitution is surprising, since it is believed that the central iron ion in the reaction center is coordinated by four conserved histidines supplied by the D1- and D2-proteins, one such being His-272 of the D1-protein [12]. Our data indicate that, due to the structural similarities between His and Arg, the latter may at least partially fulfill this complexing function. The additional mutation seems to have a negative effect on metribuzin resistance, which is only expressed to a low extent, indicating a contribution of Arg-272 to metribuzin binding. Furthermore line 6 shows a reduced atrazine resistance and hardly any diuron- and bromacil resistance, whereas the bromonitrothymol resistance is slightly increased. Finally the cell variants without a mutation at position 251 (lines 3 and 5) show a higher sensitivity to bromonitrothymol. In algae the mutation (Val → Ile) at position 219 was found by selection for resistance against DCMU and atrazine, and this mutation site also provided cross-resistance against dinoseb and ioxynil [26]. The mutation at position 251 (Ala → Val) was found by selecting algae for resistance against metribuzin, and the mutants showed cross-resistance to atrazine, ioxynil and dinoseb [26]. All these data on single-site mutations cannot easily be transferred to the mutants shown in Table I because these cells all carry more than one mutation, and at present it appears difficult to analyse the interaction between the various point mutations. Furthermore, the microenvironment of the D1-protein may be different in algae in comparison to higher plants and this difference may effect resistance characteristics. Therefore, it will be necessary to sequence the complete psbA gene of *Chenopodium rubrum* especially of lines 1 and 8, as well as 4 and 7, because the presently known mutation sites in the sequenced gene fragment are identical while the established differences (Table II) [15] suggest that further mutations in other parts of the psbA gene, or in other proteins of the photosynthetic apparatus entirely, still exist. Investigations to determine such questions are in progress.

*Acknowledgements:* This work was supported by Bundesministerium für Forschung und Technologie, Bonn, and Fonds der Chemischen Industrie.

Table II

R/S ($I_{50}$ resistant/$I_{50}$ wild-type) values and $pI_{50}$ ($-\log I_{50}$) values, determined by measuring the inhibition of uncoupled electron transport from water to phenyl-*p*-benzoquinone in thylakoid membranes isolated from wild-type (wt) and mutant cell lines of *Chenopodium rubrum*

| Line | Metribuzin | | Atrazine | | Bromonitro-thymol | | Bromacil | | Dinoseb | | Diuron | | Phenmedi-pham | | Propanil | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $pI_{50}$ | R/S | $pI_{50}$ | R/S | $pI_{50}$ | R/S | $pI_{50}$ | R/S | $pI_{50}$ | R/S | $pI_{50}$ | R/S | $pI_{50}$ | R/S | $pI_{50}$ | R/S |
| wt | 6.9 | 1 | 7.2 | 1 | 7.0 | 1 | 7.0 | 1 | 5.3 | 1 | 6.8 | 1 | 7.5 | 1 | 6.2 | 1 |
| L1 | 3.2 | 5,012 | 6.2 | 8 | 5.5 | 25 | 5.7 | 20 | 4.5 | 6 | 6.4 | 3 | 7.3 | 1.5 | 4.9 | 15 |
| L2 | 4.5 | 251 | 6.2 | 8 | 6.2 | 5 | 4.9 | 120 | 4.3 | 10 | 6.4 | 3 | 6.5 | 9 | 5.9 | 2 |
| L3 | 5.5 | 25 | 6.0 | 14 | 7.1 | 0.6 | 6.3 | 5 | 5.2 | 1.1 | 6.4 | 3 | 7.0 | 3 | 5.9 | 2 |
| L4 | 4.0 | 794 | 6.0 | 14 | 6.2 | 5 | 5.6 | 25 | 4.5 | 6 | 5.7 | 13 | 7.0 | 3 | 4.3 | 71 |
| L5 | 5.5 | 25 | 6.2 | 8 | 7.2 | 0.6 | 6.6 | 2 | 5.4 | 0.7 | 6.5 | 2 | 7.2 | 2 | 5.1 | 13 |
| L6 | 6.1 | 6 | 6.4 | 6 | 5.9 | 11 | 6.5 | 3 | 4.4 | 8 | 6.3 | 3 | 7.1 | 2 | 4.3 | 71 |
| L7 | 5.5 | 25 | 6.6 | 4 | 7.2 | 0.6 | 6.8 | 2 | 4.7 | 4 | 5.9 | 8 | 7.2 | 2 | 5.9 | 2 |
| L8 | 3.8 | 1,259 | 6.2 | 9 | 5.6 | 25 | 5.8 | 15 | 4.4 | 8 | 6.5 | 2 | 7.3 | 1.5 | 5.0 | 14 |

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