Isolation and Characterization of Herbicide Resistant Mutants in the Cyanobacterium *Synechococcus* R2

Joseph Hirschberg, Nir Ohad, Iris Pecker, and Ana Rahat

Department of Genetics, The Hebrew University of Jerusalem, Jerusalem, 91904, Israel

Z. Naturforsch. **42c,** 758–761 (1987); received January 7, 1987

Herbicide Resistance, *psbA* Gene, Photosystem II, Cyanobacteria, Mutants

A variety of mutants which are resistant to triazine – and urea – classes of herbicides have been isolated in the cyanobacterium *Synechococcus* R2.

All the mutants that have been analyzed, show some cross-resistance to different herbicides suggesting that these herbicides share a common binding site in photosystem II.

Three *psbA* genes have been identified in *Synechococcus* R2. The *psbA*-copy I gene was cloned from three mutants and used in DNA-mediated genetic transformation. It was found that in all three mutants this gene could transfer the mutation for herbicide resistance indicating that this gene codes for the herbicide resistant protein.

Introduction

A large number of commercial herbicides inhibit photosynthesis by blocking electron transport at the second stable electron acceptor of photosystem II (PS II) [1, 2]. Their mode of action involves binding to a thylakoid membrane polypeptide of 32000 dalton identified as the apoprotein of Q_B which is part of the PS II complex ([3, 4] for recent review see [5]). The Q_B protein (also called D1) is encoded by the chloroplast gene – *psbA* [6, 7]. Atrazine resistance in higher plants was found to result from a point mutation in *psbA,* which changes the amino acid residue at position 264 of the Q_B protein from serine to glycine [8–11]. This alteration reduces the affinity of the Q_B protein to triazine herbicides [2]. Molecular analysis of herbicide resistant mutants in the green alga *Chlamydomonas reinhardtii* revealed that mutations at other locations in the *psbA* gene can confer herbicide resistance [12].

Abbreviations: DCPIP, 2,6-dichlorophenolindophenol; PS II, photosystem II; Q_B, secondary quinone acceptor of photosystem II; kb, kilobase pairs; kDa, kilodalton; w.t., wild type. Herbicides: atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; bromacil, 3-*sec*-butyl-5-bromo-6-methyluracil; diuron (DCMU), 3-(3,4-dichlorophenyl)-1, i-dimethylurea; ethidimuron, N-[5-(ethylsulfonyl)-1,3,4-thiadiazol-2-yl]-N,N-dimethylurea; metamitron, 4-amino-3-methyl-6-phenyl-1,2,4-triazin-5(4H)-one; metribuzin, 4-amino-6-*tert*-butyl-3-(methylthio)-*as*-triazin-5(4H)-one; tebuthiuron, N-[5-(1,1-dimethylethyl)-1,3,4-thiadiazole-2-yl]-N,N′-dimethylurea; terbutryn, 2-(*tert*-butylamino)-4-ethylamino-6-methylthio-*s*-triazine.

Reprint requests to J. Hirschberg.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/87/0600–0758 $ 01.30/0

Cyanobacteria offer an excellent model system for studying herbicide resistance at the molecular level. PS II structure and function in these organisms are similar to higher plants [13] and their PS II dependent electron transport is inhibited by herbicides [14, 15]. Molecular analysis of a diuron-resistant mutant of *Synechococcus* R2 has revealed a point mutation in *psbA* at the same site as in higher plants [16]. Yet, their prokaryotic genetic nature enables isolation of a large number of mutants and allows for sophisticated genetic manipulations such as DNA-mediated genetic transformation.

We describe here the isolation and characterization of herbicide resistant mutants in *Synechococcus* R2.

Materials and Methods

Strain and growth conditions

The strain *Synechococcus* R2 (*Anacystis nidulans* R2) [17] was kindly given by Dr. J. G. K. Williams.

Cells of *Synechococcus* R2 were grown in BG11 medium [18] at 35 °C in 2000–3000 lux of "warm white" fluorescent light as described in ref. [19].

Methods for isolation of cyanobacterial DNA and DNA-mediated transformation of *Synechococcus* R2 were according to Williams and Szalay [19].

Mutagenesis

Cells from 200 ml of logarithmic suspension culture (10^8 cells/ml) of *Synechococcus* R2 were harvested, washed twice with 200 ml of sterile distilled water and resuspended in 5 ml of 0.03 M phosphate buffer, pH 7.0. 180 µl of ethylmethanesulfonate

(EMS) (Sigma) were added and the suspension was incubated at 35 °C for 1 h in the light. The cells were then washed in 50 ml of a sterile solution of 5% sodium thiosulfate, resuspended in 50 ml BG11 medium and allowed to grow for 24 h before selection.

Measurement of PS II-dependent electron transport

Cells from 500 ml of logarithmic suspension culture were harvested, washed once in 200 ml of distilled water and resuspended in 5 ml of 50 mM MES buffer, pH 6.5, at 4 °C. Cells were broken by sonication (3 times for 15 seconds each) and centrifuged for 10 min at $2500 \times g$. The cell-free supernatant containing the membranes was used for electron transport measurements. The rate of photochemical reduction of DCPIP (the electron acceptor), using H_2O as electron donor, was measured in Aminco-Chance dual wavelength spectrophotometer as described in ref. [20].

DNA methods

Methods for restriction endonuclease digestion, gel electrophoresis, Southern hybridization and molecular cloning were according to Maniatis *et al.* [21]. The plasmid pBR328 was used as a vector for cloning in *E. coli* strain HB101.

Results and Discussion

Herbicide-resistant mutants of *Synechococcus* R2 were selected on plates with solid BG11 medium containing the herbicides atrazine (5 μM) or diuron (5 μM), following mutagenic treatment with ethylamethane sulfonate (EMS). Herbicide-resistant colonies, which appeared following selection of mutagenized cells, were isolated and grown on BG11 medium containing the appropriate herbicide. The frequency of mutations that give rise to atrazine resistance following mutagenesis is approximately $1{:}10^8$.

An additional round of mutagenesis and selection has been carried out in the cells of one of the herbicide resistant mutants – Dil. This time the selection was done at a higher concentration (20 μM) of the herbicide atrazine or diuron. Highly resistant mutants which have been isolated – D5, Di22 and Di33, are probably a result of two mutational events.

The degree of resistance of the wild type (w.t.) strain and 5 mutants to various herbicides has been examined by plating 0.2 ml of cell suspension (5×10^3 cells/ml) on solid BG11 media containing increasing concentrations of the herbicides. The results shown in Table I indicate that each of the mutants shows some cross-resistance to all other PS II-herbicides, however they differ in their degree of resistance. Using this methodology we have so far analyzed over 20 different herbicide-resistant mutants.

In order to identify the *psbA* genes of *Synechococcus* R2, Southern hybridization analysis of cyanobacterial DNA has been carried out using the *psbA* gene from *Amaranthus hybridus* as a heterologous probe.

As shown in Fig. 1A, under low stringency conditions of hybridization and washing, two fragments are detected in each restriction cleavage, indicating that at least two genes in *Synechococcus* R2 are homologous to the higher plant *psbA*. The *psbA* gene which is located in the 2.7 kb *Hind*III-*Bam*HI

Table I. Resistance of w.t. and four mutants to various herbicides. The highest concentration (in μM) in which cell growth on solid BG11 medium is not arrested is given for each herbicide (n.d. = not detected).

Herbicide	Mutants	Wild type	Di1	D5	D6	Di22	Di33
s-Triazines	atrazine	< 2.0	5.0	25	25	< 2.0	15
	terbutryne	< 2.0	5.0	15	15	< 2.0	2.5
as-Triazines	metribuzin	< 2.5	25	200 <	40	20	35
	metamitron	25	200 <	200 <	100	100	200
Dimethylureas	diuron	< 2.0	12	7	n.d.	50 <	50 <
	tebuthiuron	15	250 <	50	25	50	100 <
	ethidimuron	10	150	100 <	50 <	100	250 <
	bromacil	< 2.0	50 <	5.0	20	10	50 <

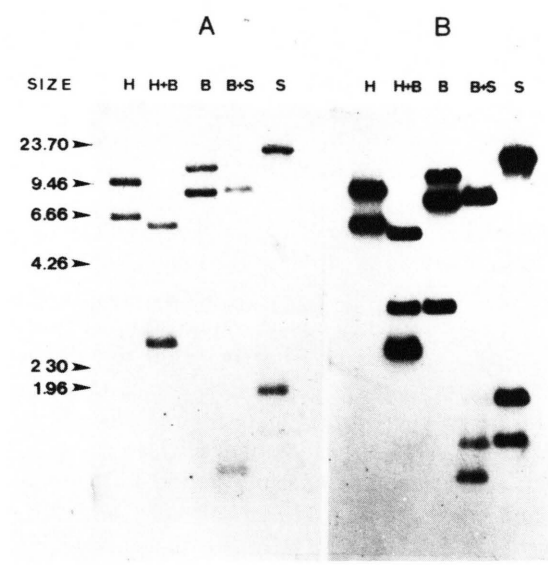

Fig. 1. Identification of the *psbA* genes of *Synechococcus* R2. A Southern blot of cyanobacterial DNA digested with the restriction enzymes *Hind*III (H), *Hind*III plus *Bam*HI (H + B), *Bam*HI (B), *Bam*HI plus *Sal*I (B + S) and *Sal*I [5], was hybridized with ^{32}P-labelled, *Hind*III-*Xba*I internal fragment of *psbA* from *Amaranthus hybridus* [8] (A) or with the 2.7 kb insert of the plasmid pAN27R2 containing *psbA*-copy II of *Synechococcus* R2 (B). Size is in kb.

fragment (now designated "copy II") was cloned in the plasmid vector pBR328. The recombinant plasmid was designated pAN27R2. When DNA of the insert of pAN27R2 was used as a probe in hybridization to the same Southern blot shown in Fig. 1A, three rather than two homologous fragments were identified (Fig. 1B). These results establish the existence of three *psbA* sequences in the *Synechococcus* R2 genome. While a high sequence homology exists between all three copies, two of them have a greater sequence homology to the higher plant *psbA* gene. This result is in agreement with previous reports of a multi gene family of *psbA* in different cyanobacteria [16, 20, 21].

The 3.5 kb *Bam*HI fragment, containing the third *psbA* gene (now called – "copy I") has been cloned in the plasmid pBR328 and the recombinant plasmid was designated pAN35R2. The same gene was also cloned from the mutants Di1, D5 and Di22 in the plasmids pAN35Di1, pAN35D5 and pAN35Di22, respectively.

Transformation experiments with DNA of these plasmids showed that *psbA*-copy I from each of the three mutants, had the ability to transform w.t. *Synechococcus* R2 to herbicide resistance. A possible molecular mechanism by which such a stable transformation could have occurred is described schematically in Fig. 2. As shown by Williams and Szalay [19] homologous recombination between foreign DNA and the chromosome of *Synechococcus* R2 is very frequent and thus can result in a reciprocal exchange of homologous sequences.

The herbicide-resistant transformants were assayed for their degree of cross-resistance to the various herbicides and were found to have acquired the complete phenotype of the original mutants.

These results demonstrate that PS II type of herbicide resistance in cyanobacteria is encoded by the gene *psbA* as has been shown in higher plants and algae. It is also evident that in all three mutants, *psbA*-copy I conferred the resistance suggesting that this copy is encoding the 32 kDa herbicide-binding protein.

In order to further characterize the herbicide-resistant mutants, PS II-dependent electron transport was measured in isolated photosynthetic membranes of w.t. and of the transformant mutants Di1, D5 and Di22. These transformants are completely isogenic with the w.t. strain except for the *psbA*-copy I gene.

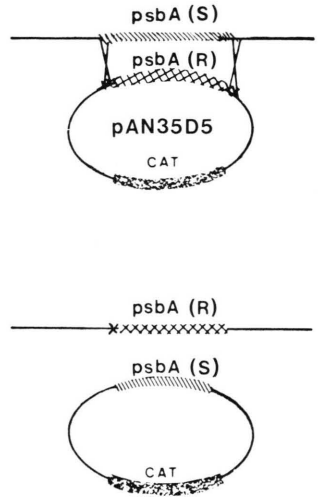

Fig. 2. A model for a possible recombination event between the circular plasmid pAN35D5, containing the herbicide-resistant *psbA*-copy I from mutant D5, and the bacterial chromosome (straight line). *psbA* (S) and *psbA* (R) are the "susceptible" and "resistant" genes, respectively. CAT is the chloramphenicol acetyl transferase gene. A double crossover event results in a reciprocal exchange of homologous sequences.

Table II. I_{50} concentrations (in M) of herbicides in w.t. and mutants Di1, D5, and Di22 (Hill reaction, water–DCPIP).

Herbicide	w.t.	Di1	D5	Di22
Atrazine	1.7×10^{-7}	3.0×10^{-6}	6×10^{-5}	4.5×10^{-7}
Terbutryne	1.5×10^{-8}	2.5×10^{-7}	1×10^{-5}	2.3×10^{-8}
Metribuzin	2.0×10^{-7}	1×10^{-3}	4×10^{-4}	3.5×10^{-5}
Metamitron	1.5×10^{-5}	5×10^{-4}	5×10^{-5}	6.0×10^{-4}
Diuron	1.7×10^{-8}	2.5×10^{-6}	5×10^{-6}	4.5×10^{-5}
Teburthiuron	4.0×10^{-6}	1×10^{-5}	1×10^{-5}	2.5×10^{-5}
Ethidimuron	1.5×10^{-6}	5×10^{-5}	3×10^{-5}	2.5×10^{-5}
Bromacil	1.5×10^{-7}	4.5×10^{-5}	5×10^{-6}	5.5×10^{-6}

Photosynthetic electron transport rates were measured using H_2O as the electron donor and DCPIP as the electron acceptor in the presence of different concentration of various herbicides. The control values (without herbicide) were considered to be the rate of 100%. The concentration of herbicide that inhibits 50% of the rate of DCPIP reduction (I_{50}) has been determined for each of the herbicides in the w.t. and in the mutant strains (Table II). Since the data in Table II were measured *in vitro* in isolated thylakoid membranes, they reflect the changes in binding affinities of the Q_B protein from the different strains to various herbicides. Mutant Di1 is 18 times more resistant to atrazine and 150 times more resistant to diuron than the w.t. strain. Mutant D5 is very highly resistant to both atrazine (350-fold) and diuron (300-fold) and Di22 is extremely resistant to diuron (2600-fold) but shows very low resistance to atrazine (less than 3-fold).

Conclusion

The ability to isolate a large number of different herbicide-resistant mutants makes cyanobacteria an attractive model system for studying the molecular mechanism of PS II-herbicide resistance.

All the herbicide-resistant mutants that have been analyzed show cross resistance to different PS II inhibitors indicating that these herbicides share the same binding site on the Q_B polypeptide. The ability to transform herbicide resistance to *Synechococcus* R2 by DNA of the gene *psbA* demonstrates, that, like in the case of higher plants and algae, this gene codes for herbicide resistance. The fact that only "copy I" of the *psbA* gene family codes for herbicide resistance in all three mutants suggests that this gene copy is the one that codes for the Q_B polypeptide.

Acknowledgement

This research was supported by a grant from N.C.R.D. Israel and G.S.F. München, Germany.

[1] A. Trebst and W. Draber, Adv. Pesticide Sci. **2,** 223–234 (1979).
[2] C. J. Arntzen, K. Pfister, and K. E. Steinback, in: Herbicide Resistance in Plants (H. M. LeBaron and J. Gressel, eds.), pp. 185–224, Wiley, New York 1982.
[3] W. Tischer and H. Strotmann, Biochim. Biophys. Acta **460,** 113–125 (1977).
[4] K. Pfister, K. E. Steinback, G. Gardner, and C. J. Arntzen, Proc. Natl. Acad. Sci. USA **78,** 981–985 (1981).
[5] G. Renger, Physiol. Vég. **24,** 509–521 (1986).
[6] L. Bogorad, S. O. Jolly, G. Link, L. McIntosh, C. Paulsen, Z. Schwartz, and A. Steinmetz, in: Biological Chemistry of Organelle Formation (T. Bucher, W. Sebald, and H. Weiss, eds.), pp. 87–96, Springer, Berlin 1980.
[7] G. Zurawski, H. J. Bohnert, P. R. Whitfeld, and W. Bottomley, Proc. Natl. Acad. Sci. USA **79,** 7699–7703 (1982).
[8] J. Hirschberg and L. McIntosh, Science **222,** 1346–1348 (1983).
[9] J. Hirschberg, A. Bleecker, D. J. Kyle, L. McIntosh, and C. J. Arntzen, Z. Naturforsch. **39c,** 412–420 (1984).
[10] P. Goloubinoff, M. Edelman, and R. B. Hallick, Nucl. Acid Res. **24,** 9489–9496 (1984).
[11] M. Schönfeld, T. Yaacoby, A. Ben-Yehuda, B. Rubin, and J. Hirschberg, Z. Naturforsch. **42c,** 779–782 (1987).
[12] J. M. Erickson, M. Rahire, L. Metz, and J.-D. Rochaix, Science **228,** 204–207 (1985).
[13] K. K. Ho and D. W. Krogmann, in: The Biology of the Cyanobacteria (N. G. Carr and B. A. Whitton, eds.), pp. 191–214, University of California Press 1982.
[14] C. Astier, C. Vernotte, M. Der-Vartanian, and M. Joset-Espardellier, Plant Cell. Physiol. **20,** 1501–1510 (1979).
[15] S. S. Golden and L. A. Sherman, Biochim. Biophys. Acta **764,** 239–246 (1984).
[16] S. S. Golden and R. Haselkorn, Science **229,** 1104–1107 (1985).
[17] S. V. Shestakov and N. T. Khyen, Mol. Gen. Genet. **107,** 372–375 (1970).
[18] R. Rippka, J. Deruelles, J. B. Waterbury, M. Hardman, and R. Y. Stanier, J. Gen. Microbiol. **111,** 1–61 (1979).
[19] J. G. K. Williams and A. A. Szalay, Gene **24,** 37–51 (1983).
[20] D. S. Cahen, S. Malkin, S. Shochat, and I. Ohad, Plant Physiol. **60,** 845–849 (1976).
[21] T. Maniatis, E. F. Fritsch, and J. Sambrook, Molecular Cloning – A Laboratory Manual, Cold Spring Harbor Laboratory, New York 1982.