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

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A variety of mutants which are resistant to triazine — and uracil — 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 Qb which is part of the PS II complex ([3, 4] for recent review see [5]). The Qb protein (also called D1) is encoded by the psbA gene. 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; Qb, secondary quinone acceptor of photosystem II; kb, kilobase pairs; kDa, kilodalton; w.t., wild type. Herbicides: atrazine, 2-chloro-4-ethylamino-6-iso-propylamino-s-triazine; bromacil, 3-sec-butyl-5-bromo-6-methyluracil; diuron (DCMU), 3-(3,4-dichlorophenyl)-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-dimethylthyl)-1,3,4-thiadiazole-2-yl]-N,N-dimethylurea; terbutryn, 2-(tert-butylamino)-4-ethylamino-6-methylthio-s-triazine.

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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 (107 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

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(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 × 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 H2O 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 ethylmethane 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 1:10^6.

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 HindIII-BamHI

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>Mutants</th>
<th>Wild type</th>
<th>Dil1</th>
<th>D5</th>
<th>D6</th>
<th>Di22</th>
<th>Di33</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-Triazines</td>
<td>atrazine</td>
<td>&lt; 2.0</td>
<td>5.0</td>
<td>25</td>
<td>25</td>
<td>&lt; 2.0</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>terbutryne</td>
<td>&lt; 2.0</td>
<td>5.0</td>
<td>15</td>
<td>15</td>
<td>&lt; 2.0</td>
<td>15</td>
</tr>
<tr>
<td>as-Triazines</td>
<td>metribuzin</td>
<td>&lt; 2.5</td>
<td>25</td>
<td>200</td>
<td>200</td>
<td>&lt; 40</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>metatmitron</td>
<td>25</td>
<td>200</td>
<td>&lt; 200</td>
<td>200</td>
<td>&lt; 100</td>
<td>200</td>
</tr>
<tr>
<td>Dimethylureas</td>
<td>diuron</td>
<td>&lt; 2.0</td>
<td>12</td>
<td>7</td>
<td>n.d.</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>tebuthiuron</td>
<td>15</td>
<td>1250</td>
<td>50</td>
<td>25</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>ethidimuron</td>
<td>10</td>
<td>150</td>
<td>100</td>
<td>100</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>bromacil</td>
<td>&lt; 2.0</td>
<td>5.0</td>
<td>20</td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
</tbody>
</table>
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.

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**Fig. 1.** Identification of the *psbA* genes of *Synechococcus* R2. A Southern blot of cyanobacterial DNA digested with the restriction enzymes *HindIII* (H), *HindIII* plus *BamHI* (H + B), *BamHI* (B), *BamHI* plus *SalI* (B + S) and *SalI* [5], was hybridized with 32P-labelled, *HindIII-XbaI* 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.

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**Fig. 2.** A model for a possible recombination event between th 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 \mu M) of herbicides in w.t. and mutants Di1, D5, and Di22 (Hill reaction, water–DCPIP).

<table>
<thead>
<tr>
<th>Herbicide</th>
<th>w.t.</th>
<th>Di1</th>
<th>D5</th>
<th>Di22</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atrazine</td>
<td>1.7 \times 10^{-7}</td>
<td>3.0 \times 10^{-6}</td>
<td>6 \times 10^{-5}</td>
<td>4.5 \times 10^{-7}</td>
</tr>
<tr>
<td>Terbutryn</td>
<td>1.5 \times 10^{-8}</td>
<td>2.5 \times 10^{-7}</td>
<td>1 \times 10^{-5}</td>
<td>2.3 \times 10^{-8}</td>
</tr>
<tr>
<td>Metribuzin</td>
<td>2.0 \times 10^{-7}</td>
<td>1 \times 10^{-6}</td>
<td>4 \times 10^{-5}</td>
<td>3.5 \times 10^{-6}</td>
</tr>
<tr>
<td>Metamitron</td>
<td>1.5 \times 10^{-5}</td>
<td>5 \times 10^{-5}</td>
<td>3 \times 10^{-5}</td>
<td>3.5 \times 10^{-5}</td>
</tr>
<tr>
<td>Diuron</td>
<td>1.7 \times 10^{-8}</td>
<td>2.5 \times 10^{-6}</td>
<td>5 \times 10^{-6}</td>
<td>4.5 \times 10^{-6}</td>
</tr>
<tr>
<td>Tebuthiuron</td>
<td>4.0 \times 10^{-8}</td>
<td>1 \times 10^{-7}</td>
<td>3 \times 10^{-4}</td>
<td>1 \times 10^{-5}</td>
</tr>
<tr>
<td>Ethidimuron</td>
<td>1.5 \times 10^{-6}</td>
<td>5 \times 10^{-6}</td>
<td>3 \times 10^{-5}</td>
<td>2.5 \times 10^{-5}</td>
</tr>
<tr>
<td>Bromacil</td>
<td>1.5 \times 10^{-7}</td>
<td>4.5 \times 10^{-5}</td>
<td>5 \times 10^{-6}</td>
<td>5.5 \times 10^{-6}</td>
</tr>
</tbody>
</table>

Photosynthetic electron transport rates were measured using H_{2}O as the electron donor and DCPIP as the electron acceptor in the presence of different concentrations 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.

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