

Mutations in the D₁ Subunit of Photosystem II and Resistance to the Phenol Type Herbicide Ioxynil in *Synechocystis* PCC 6714 and 6803

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Several herbicides block the photosystem II electron transfer because they compete with Q_B, the second stable electron acceptor of photosystem II for binding to the D₁ subunit. We have previously isolated a mutant of *Synechocystis* 6714 in which Asn is replaced by Thr at position 266 of D₁ (G. Ajlani, I. Meyer, C. Vernotte, and Astier, FEBS Lett. **246**, 207–210 (1989)) and presenting resistance to ioxynil but not to DCMU. In this report we present selection, from this mutant, of a double mutant with an additional substitution at position 264 (Ser by Ala). The sensitivity of this mutant toward several herbicides is given and compared to those of the mutants having only one substitution at 266 and one substitution at 264. It was also compared to a mutant of *Chlamydomonas* having the same substitutions. This allows us to discuss the interaction of various herbicides with the D₁ protein and to compare the herbicide binding niches of *Chlamydomonas* and *Synechocystis*.

Introduction

Cyanobacteria, like green algae and higher plants, perform oxygenic photosynthesis involving two photosystems. Electrons are transferred from water to plastoquinones by photosystem II (PS II). Different classes of herbicides block the electron transfer between the primary (Q_A) and the secondary (Q_B) electron acceptor of PS II [1]. The herbicides and the Q_B quinone bind in the same region of the D₁ protein which is part of the PS II core complex [2]. Several herbicide resistant mutants of cyanobacteria and of green algae and several herbicide tolerant biotypes of higher plants have been characterized. They have various amino acid substitutions in the Q_B binding niche of D₁. These results have allowed prediction of the folding of this polypeptide and specially of the Q_B binding niche [3] and see [4] for a review.

In *Synechocystis* 6714, the correlation of the amino acid substitutions in D₁ with the herbicide cross resistances and with the functional properties in electron flow through PS II [5, 6] has refined the understanding of the structure and function of the PS II. Recently we have selected *Synechocystis* mutants resistant to ioxynil [7]. Ioxynil, a phenol-type herbicide, was shown to inhibit both the donor

and the acceptor side of PS II but at different concentrations [8]. Two mutants, IoxIA and IoxIIA were characterized. In these mutants only the sensitivity of the acceptor side was modified, the resistance to ioxynil was increased 10-fold and 3-fold in IoxIA and IoxIIA respectively. The two mutants remained completely sensitive to other herbicides as DCMU and atrazine. Mutations were found to map within the *psbA* 1 gene [7, 9]. The same codon 266 was modified in IoxIA and IoxIIA, Asn codon AAC in wild type was substituted by Thr codon ACC and by Asp codon GAC, respectively.

The effect of the two different amino acid substitutions at position 266 on the ioxynil resistance phenotype has allowed us to construct molecular models and to calculate the hydrogen bonding energies between the hydroxyl group and the respective amino acid at this position [9]. The results of this modeling underline the relation which appears between the hydrogen bond capability of each amino acid chain and the effect of the substitution at position 266 on resistance to ioxynil.

However it was impossible to control that these mutations transfer the ioxynil resistance phenotype to the wild type strain, as has been shown for other mutants [5], for lack of adequate selective pressure, inhibition by ioxynil depending upon medium pH which is poorly controlled on plates.

Furthermore a double mutant 264 (Ser-Ala) and 266 (Asn-Thr) obtained by site specific mutagenesis

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sis of *psbA* gene coding for D₁ in *Chlamydomonas* does not present a significant resistance to ioxynil [10].

For these two reasons, we decided to accumulate new mutations on our ioxynil resistant mutant IoxIA. The phenotype of this mutant (10-fold resistance to ioxynil, no cross resistance to DCMU) permits selection of new mutants on DCMU. The association of another resistance should allow us to have good selective pressure and obtain transformants, and the analysis of the transformant phenotypes will be used to confirm the role of the 266 substitution in the ioxynil resistance.

Results

Selection of mutants and herbicide cross-resistance

During the log growth-phase, 5 μ M DCMU was added to a suspension of IoxIA cells (500 ml at 8×10^7 cells per ml). The suspension bleached during the first days, then it regreened after about 3 weeks. The suspension was diluted in fresh medium containing the same concentration of DCMU and was plated on agar medium containing DCMU. Forty isolated colonies were streaked on either DCMU (5×10^{-6} M) or atrazine (8×10^{-5} M) or ioxynil (5×10^{-4} M). All colonies seemed to present comparable phenotype, *i.e.* resistance to the three inhibitors.

Three of these colonies were chosen and were further tested for their stabilities by culture in the absence of herbicides for several generations. Then we tested their phenotypes more precisely, as described by Ajlani *et al.* [8]. We measured the chlorophyll variable fluorescence of the cells in the presence of various concentrations of herbicides. The I_{50} was determined as the concentration needed to block half of the variable fluorescence. The three strains selected presented a 700-fold resistance to DCMU, a 5-fold resistance to ioxynil, a 100-fold resistance to atrazine and a >1000-fold resistance to metribuzin. We chose one of these three clones to perform molecular analysis and we call it "ID".

Molecular analysis

In *Synechocystis* 6714, all prior mutations conferring herbicide resistance have been localized on the C-terminal moiety of *psbA*1 (copy 1 of the *psbA* gene), [6]. After digestion of the genomic

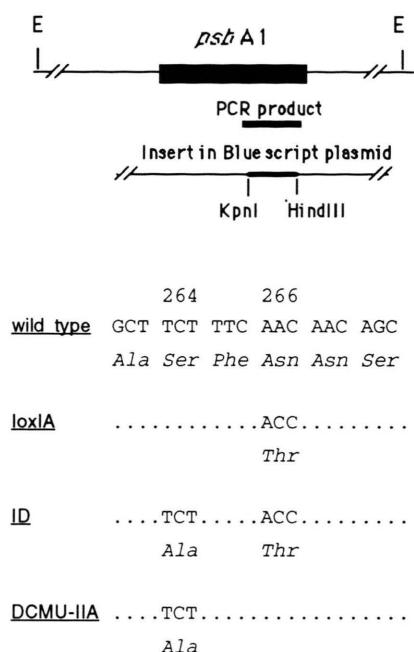


Fig. 1. Scheme of molecular analysis and various mutations in the *psbA* 1 gene of the *Synechocystis* mutants.

DNA by *Eco*RI, the 5 Kb fragments, containing the *psbA* 1, were isolated. Polymerase Chain Reaction (PCR) was used to amplify the C-terminal moiety of *psbA* 1. The PCR product was digested and inserted in a Bluescript plasmid to be cloned and sequenced. The original mutation at codon 266 was conserved and an additional mutation at codon 264 was found, TCT (Ser) was substituted by GCT (Ala). This substitution was already obtained in a single mutant called DCMU-IIA. In Fig. 1 we have schematized the cloning protocol and given the part of the sequence containing the mutations of the three mutants strains.

Transformants

The double mutant ID is highly resistant to DCMU. Therefore, we now have a good means of selecting transformants with ID DNA. *Synechocystis* 6803 wild type strain was transformed with the plasmid containing the cloned fragment of *psbA* 1 of the ID mutant (see Fig. 1). Transformants were selected on 5 μ M DCMU. Two transformants were isolated and their phenotypes analyzed and compared to that of the *Synechocystis* 6803 wild type. They are 700 \times resistant to DCMU

and 5 × to ioxynil. These transformants have acquired a phenotype similar to that of ID, the donor strain. As a control, a transformant obtained with DCMU-IIA DNA containing only the 264 substitution was analyzed. Like DCMU-IIA, this *Synechocystis* 6803 transformant is resistant to DCMU (× 700) and less resistant to ioxynil (× 0.8) than the wild type.

Discussion

Table I presents the relative resistances (R/S) of the double mutant ID toward various herbicides. R/S is the ratio of the inhibitor concentration blocking half of the PS II activity of the mutant to that of the wild type. For comparison, R/S of the single mutant 266 (Thr) and of the single mutant 264 (Ala) are also presented. Concerning ioxynil, the double mutant is resistant but less than the single mutant 266 (Thr), R/S being 5 instead of 10. It has been previously shown that the single mutant 264 (Ala) was slightly more sensitive than the wild type (R/S = 0.8). Therefore there is cooperativity of the two mutations for binding of ioxynil. Concerning others herbicides, DCMU, atrazine and metribuzin, the presence of the 266 substitution does not modify the resistances induced by the 264 substitution. The phenotype of the double mutant

266(Thr)-264(Ala) confirms the involvement of the residue 266 of the D₁ protein in binding the herbicide ioxynil.

These results obtained on *Synechocystis* 6714 or 6803 were compared to those obtained with *Chlamydomonas* mutants described in [10]. The authors have selected a double mutant (called A 264 T 266) having two mutations, one at 266 (Thr) and one at 264 (Ala), *i.e.* exactly the same substitutions as our double mutant ID. They compared its phenotype with that of a single mutant 264 (Ala) previously described. Unfortunately they did not have a single mutant 266 (Thr). In Table I we recall the R/S values they determined. Concerning resistance to ioxynil, the double mutant 266 (Thr)-264 (Ala) is slightly more resistant than the single mutant 264 (Ala) (× 0.8 instead of × 0.5). Then, the presence of the 266 substitution in *Chlamydomonas*-induced resistance to ioxynil as in *Synechocystis* but with a

	220	240
<i>Synecho</i>	TSSLVRETTE	VESQ NYGY KFG
<i>Chlamy</i>	TSSLIRETTE	NESAN EGYR FG

Fig. 2. Part of the amino acid sequence of D₁ corresponding to the region of the binding niche presenting differences (bold letters) in *Synechocystis* and *Chlamydomonas*.

Table I. Relative resistance (R/S) of mutants of *Synechocystis* and of *Chlamydomonas* to various herbicides. For *Synechocystis* 6714, the *pI*₅₀ values were determined in whole cells as the inhibitor concentration which blocks half of the variable fluorescence. R/S is the ratio of the *pI*₅₀ of the mutant to that of the wild type.

Mutations	266 Asn-Thr	264 Ser-Ala	266 Asn-Thr 264 Ser-Ala
<i>Synechocystis</i> 6714 ^a	Iox I A	DCMU II A	ID
Ioxynil	10	0.8	5
DCMU	0.8	700	700
Atrazine	1	100	100
Metribuzin	6	1000	>1000
<i>Chlamydomonas</i> <i>reinhardtii</i> ^b		MZ 1	A 264 T 266
Ioxynil		0.5	0.8
DCMU		200	80
Atrazine		160	40
Metribuzin		5000	1580

^a Ref. [5, 8] and this work.

^b Ref. [10].

much lower amplitude. In contrast, the effect of the additional substitution at 266 to the 264 mutant on the resistance to DCMU and atrazine is different in *Chlamydomonas* as there is a 3- to 4-fold decrease in resistance of the double mutant compared to the single 264 mutant.

These differences are probably due to difference in the structure of the herbicide-binding niche of *Chlamydomonas* and *Synechocystis*. In Fig. 2 are presented the amino acid sequences of D₁ of *Chlamydomonas* and *Synechocystis*, from amino acid 220 to 240. Five residues, at positions 224, 230, 233, 235, and 238, are different. Only two, 224 and 238, can be considered to be homologous. This part of the sequence corresponds to a loop between transmembrane helices IV and V. In a recent model, it was proposed that this amino acid sequence might be involved in a contact site between the two reaction center polypeptides D₁ and D₂ [11]. It is remarkable that in this part of the sequence, many amino acid residues differ between the two species [12]. This might be the cause of the differences observed in the phenotypes of equivalent mutants in *Synechocystis* and *Chlamydomonas*. Replacement of these amino acids by directed mutagenesis might be a way of answering this question.

Materials and Methods

Cyanobacterial strains

Synechocystis PCC 6714 and PCC 6803 were grown photoautotrophically. The mineral medium was that described in [13] with twice the concentration of nitrate. For the solid medium, 1.5% agar autoclaved separately was added. Standard growth was achieved by incubation in a Gallenkamp rotatory shaker at 34 °C in a CO₂-enriched atmosphere, under 70 µE · m⁻² · s⁻¹.

Measurements of herbicide resistance

The inhibition of photosystem II electron transport between Q_A and Q_B by herbicides was meas-

ured by the changes in chlorophyll fluorescence as in [8]. It is known that chlorophyll fluorescence yield is controlled by the redox state of Q_A. In conditions where the photosystem I is preferentially excited, there are very few PS II centers in Q_A⁻ state and fluorescence yield is low. Addition of herbicide-blocking electron transfer between Q_A and Q_B produced an increase of the fluorescence quasi proportional to the number of PS II centers blocked in the Q_A⁻ state by the herbicide. The apparatus was previously described in [14]. Cell suspensions contained 1 µg chlorophyll per ml.

DNA preparation, polymerase chain reaction and sequencing

Genomic DNA from *Synechocystis* 6714 was isolated from exponentially growing cultures as in [5]. DNA fragments (4.5 to 5.5 Kb) containing only the copy 1 of the *psbA* gene were obtained by digestion with *Eco*RI. Polymerase Chain Reaction (PCR) was performed with DNA amplification reagent kit (Gene Amp) from Perkin Elmer Cetus. Two primers were used: one corresponding to nucleotides 441 to 458 and one corresponding to nucleotides 1048 to 1072 with an additional *Hind*III site on the 5' end. The PCR product was digested by *Kpn*I and *Hind*III and the 531 bp fragment was inserted in Bluescript plasmid. Sequencing was done by dideoxy chain termination according to [15], using a Sequenase kit from USB. Oligonucleotide primers were synthesized on a Milligen 7500 DNA synthesizer.

Transformation of Synechocystis 6803 wild type

0.5 ml at 2 × 10⁸ cells per ml of wild type *Synechocystis* 6803 growing exponentially were mixed in 2 ml top agarose and plated on mineral medium. About 3 µg of cloned DNA (in 10 µl) were dotted onto this lawn of recipient cells. After 16 h incubation in light at 34 °C, selective herbicide was added by the underlying technique.

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