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Biochemistry

Herbicide resistance in *Chlamydomonas reinhardtii* results from a mutation in the chloroplast gene for the 32-kilodalton protein of photosystem II

(*psb*A/3-(3,4-dichlorophenyl)-1,1-dimethylurea/triazine herbicides/photosynthesis)

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ABSTRACT **We report the isolation and characterization of a uniparental mutant of *Chlamydomonas reinhardtii* that is resistant to 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) and 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine (atrazine). Such herbicides inhibit photosynthesis by preventing transfer of electrons in photosystem II from the primary stable electron acceptor Q to the secondary stable electron acceptor complex B, which is thought to contain a protein of 32 kDa and a bound quinone. It has been proposed that herbicide binding to the 32-kDa protein alters the B complex so that electron transfer from Q is prohibited. Both whole and broken-cell preparations of the mutant alga show a resistance to the effects of herbicide on electron transfer from Q to B, as measured by fluorescence-induction kinetics. In the absence of herbicide, mutant cells exhibit a slower rate of Q to B electron transfer than do wild-type cells. The 32-kDa protein from wild-type cells, but not mutant cells, binds azido[^{14}C]atrazine at 0.1 μM. We have isolated *psb*A, the chloroplast gene for the 32-kDa protein, from both wild-type and herbicide-resistant algae and sequenced the coding regions of the gene that are contained in five exons. The only difference between the exon nucleotide sequences of the wild-type and mutant *psb*A is a single T-A to G-C transversion. This mutation results in a predicted amino acid change of serine in the wild-type protein to alanine in the mutant. We suggest that this alteration in the 32-kDa protein is the molecular basis for herbicide resistance in the *C. reinhardtii* mutant.**

An increased understanding of herbicide susceptibility and resistance has obvious practical applications for crop production but may also reveal much about the basic photosynthetic apparatus in the chloroplasts of green organisms. Photosynthetic electron transport takes place in the chloroplast thylakoid membranes, mediated by the membrane-bound protein complexes of photosystem II (PSII) and photosystem I (PSI). Herbicides of the *s*-triazine or urea class, such as atrazine (2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine) or DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], disrupt electron flow at the reducing side of PSII, thus inhibiting photosynthesis (1, 2). Both algae and higher plants have developed resistance to these herbicides (3–5). The molecular basis for this resistance has been under intense investigation in which genetic, biochemical, and biophysical lines of evidence are now merging.

The green alga *Chlamydomonas reinhardtii* is particularly well-suited for a joint biochemical and genetic analysis of herbicide resistance. It has a single large chloroplast, undergoes a sexual zygotic stage in which the chloroplasts from each of two gametes fuse, and is the only organism in which chloroplast gene recombination has been observed (6). Both genetic (7, 8) and physical (9) maps of the chloroplast genome have been constructed and can be correlated by analysis of specific mutants. Primary‡ DCMU-resistant mutants have been isolated from *C. reinhardtii* (3, 4). These mutants exhibit a uniparental mode of inheritance, suggesting that a protein encoded in the chloroplast genome is central to the herbicide mode of action. Herbicide resistance in higher plants is also maternally inherited (10). These genetic data are consistent with the observation that a chloroplast-encoded thylakoid membrane polypeptide of 32 kDa (11) specifically binds the triazine analog 2-azido-4-ethylamino-6-isopropylamino-*s*-triazine (azidoatrazine) (12).

The 32-kDa protein has been extensively characterized in algae and higher plants (13–16). It has recently been postulated that this protein is part of the protein–plastoquinone complex B (17), which acts as the secondary stable electron acceptor of PSII (18). There is no direct proof that the quinone itself binds to the 32-kDa protein. The quinone may bind to another membrane polypeptide or several proteins of the PSII complex may interact to create a quinone binding site. One model proposed for the action of herbicides such as atrazine or DCMU suggests that these compounds bind with high affinity to the secondary electron acceptor site in PSII (19), altering this site such that the transfer of electrons from the primary electron acceptor Q is inhibited (5). Herbicide binding, presumably to the-32 kDa protein, may inhibit oxidation of Q^- by preventing binding of the quinone cofactor of the B complex (20). According to this model, herbicide resistance could be conferred to cells with a 32-kDa protein altered in such a way that it no longer binds herbicide at levels that interfere significantly with quinone binding.

We report here the isolation and characterization of a DCMU- and atrazine-resistant uniparental mutant of the green alga *C. reinhardtii*. We have compared the wild-type and mutant strains with respect to herbicide binding and fluorescence induction in the presence or absence of DCMU and atrazine. In addition, we have identified and sequenced the *C. reinhardtii* chloroplast gene for the 32-kDa polypeptide. Comparison of the deduced amino acid sequence for the protein from wild-type and herbicide-resistant cells shows a direct correlation between herbicide resistance and an altered 32-kDa protein.

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Abbreviations: atrazine, 2-chloro-4-ethylamino-6-isopropylamino-*s*-triazine; azidoatrazine, 2-azido-4-ethylamino-6-isopropylamino-*s*-triazine; PSI, photosystem I; PSII, photosystem II; B, a protein–quinone complex serving as secondary stable electron acceptor of PSII; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; kb, kilobase(s); bp, base pair(s); Q, plastoquinone serving as primary stable electron acceptor of PSII.

‡Primary herbicide resistance is described in ref. 3.

MATERIALS AND METHODS

Strains. *C. reinhardtii* wild-type strain 137c was used for mutagenesis. Algal strains were maintained in continuous dim light on solid Tris/acetate/phosphate (TAP) medium (21). *Escherichia coli* strains BHB2690 [N205 *recA*$^-$ (λ*imm*434 *cI*^{ts} *b2 red3 Dam15 Sam7*)/λ] and BHB2688 [N205 *recA*$^-$ (λ*imm*434 *cI*^{ts} *b2 red3 Eam15 Sam7*)/λ] were used to make λ packaging extracts (22). *E. coli* NM539 [*hsdR supF trpR met* (P2 *cox3*)] was obtained from N. Murray and used to select for recombinant λ phage (23). *E. coli* C600 (r_k^- m_k^- *suII*) was the host for propagation of λ phage and plasmids.

Mutagenesis and Selection. Cells of *C. reinhardtii* wild-type strain 137c were grown in liquid TAP medium and treated with 1 mM 5-fluorodeoxyuridine (FdUrd) as described (24). Enrichment was achieved by growing cells in liquid minimal medium (25) containing 1 μM DCMU. Cells were then plated on solid minimal medium containing 1 μM DCMU.

Genetic Analysis. Reciprocal crosses between DCMU-4 and 137c were performed. Twenty-five complete tetrads from each cross were tested for *in vivo* resistance to varying levels of DCMU and atrazine.

Fluorescence Induction. Fluorescence-induction kinetics (26) were carried out by using dark-adapted whole cells or broken-cell preparations (27).

Binding Studies. Cells at 2×10^6 cells per ml were concentrated by centrifugation and resuspended in TAP medium to a final density of 2.5×10^7 cells per ml (50 μg of chlorophyll per ml). Azido[^{14}C]atrazine (49.4 mCi/mmol; 1 Ci = 37 GBq) was added at a final concentration of 0.1 or 10 μM to 1.0-ml aliquots of the cell suspension in a small beaker. Cells were then irradiated with UV light from an unfiltered General Electric 4 W germicidal lamp placed 8 cm from the sample. After irradiation, 250-μl aliquots were removed and centrifuged and the cell pellet was resuspended in a buffer containing 2% $NaDodSO_4$, 30 mM sodium carbonate, 30 mM dithiothreitol, and 12% sucrose. These samples were heated at 100°C and electrophoresed on a 12–18% gradient polyacrylamide gel containing 8 M urea (28). Gels were analyzed for azido[^{14}C]atrazine by x-ray fluorography at −70°C using EN^{3}HANCE from New England Nuclear.

Cloning and Sequencing. *C. reinhardtii* chloroplast DNA (29), plasmid DNA (30), and λ DNA (31) were prepared as described. *psb*A, the gene for the 32-kDa protein, was isolated from DCMU-4 by cloning chloroplast DNA digested with *Bam*HI into the λ vector λEMBL 4 (32). Ligated DNA was packaged *in vitro* (22) and phage were plated on *E. coli* NM539, which prevents growth of the parental λ. Recombinant phage were screened by the method of Benton and Davis (33). Nick-translation, hybridization, and autoradiography were performed as described (34). Phage containing the DCMU-4 *psb*A were identified after hybridization with a ^{32}P-labeled fragment of the *C. reinhardtii* wild-type *psb*A contained on chloroplast *Eco*RI fragment R14 (9). The wild-type gene had been previously isolated (35) by heterologous hybridization with the cloned spinach *psb*A (36). The *C. reinhardtii psb*A is located entirely within the chloroplast inverted repeat (35) and hence present in two copies per genome. Digestion of chloroplast DNA with *Bam*HI allows for the unambiguous separation of these two copies, one of which is located on the restriction fragment Ba12 and the other on Ba14 (9). The mutant gene located on Ba12 was subcloned from λ into plasmid vectors as follows. The 6.0-kilobase (kb) *Eco*RI fragment (R14) was cloned into the *Eco*RI site of pBR328 (37). The adjacent 3.6-kb *Eco*RI–*Bam*HI fragment was cloned in pBR322 (38). DNA fragments labeled at the 5′ or 3′ end were sequenced by the method of Maxam and Gilbert (39).

Herbicides. Azido[^{14}C]atrazine was obtained from Pathfinders Laboratories (St. Louis), DCMU was from DuPont, and atrazine was from Ciba–Geigy. Unlabeled azidoatrazine was a gift from K. Pfister. Stock solutions of DCMU and atrazine were made in ethanol and methanol, respectively, and stored at −20°C. The concentration of alcohol in the growth medium never exceeded 1%.

RESULTS

Isolation of Mutants and Genetic Analysis. Ten cultures containing 10^9 cells each were independently treated with 1 mM FdUrd and DCMU-resistant cells were recovered from one of these. After a second treatment with 1 mM FdUrd, one clone, DCMU-4, was selected for the studies reported here. DCMU-4 grows in the light on solid minimal medium containing 10 μM DCMU. Growth of wild-type cells is inhibited by 1 μM DCMU. Though selected for resistance to DCMU, this strain grows on solid medium containing 0.1 mM atrazine. Growth of wild-type cells is inhibited by 1 μM atrazine. Reciprocal crosses between DCMU-4 and wild-type 137c show that both DCMU resistance and atrazine resistance are inherited uniparentally.

Membrane Properties. Fluorescence-induction kinetics of dark-adapted broken-cell preparations, in which both the cellular membrane and the chloroplast envelope are disrupted, demonstrate that the *in vivo* resistance of DCMU-4 to herbicide is also reflected *in vitro*. Fig. 1 gives the fluorescence-induction curves of wild-type and DCMU-4 broken cells in the presence of atrazine or DCMU. A similar result was obtained by using azidoatrazine (data not shown). The wild-type preparations show a rapid rise in the fluorescence yield during illumination. This rise corresponds to the accumulation of Q^-, since reoxidation of Q^- is blocked by the herbicide (5). In the mutant preparations at the same concentration of herbicide, Q^- is reoxidized by the secondary acceptor. The much slower rise in fluorescence kinetics of DCMU-4 reflects a gradual filling up of the plastoquinone pool.

Azido[^{14}C]atrazine photoaffinity-labeling of polypeptides from wild-type and DCMU-4 mutant cells is shown in the fluorogram of Fig. 2. Wild-type cells treated with 0.1 μM azido[^{14}C]atrazine show one labeled polypeptide, whereas DCMU-4 mutant cells under the same conditions show no binding of azidoatrazine. By comparison with a fluorogram of thylakoid membrane polypeptides synthesized inside the chloroplast, the peptide covalently binding azidoatrazine is identified as the 32-kDa protein that comigrates with D1 (40). At 10 μM azidoatrazine, both wild-type and mutant cells show this same protein labeled with ^{14}C. Similar results were obtained when isolated thylakoid membrane polypeptides from wild-type and mutant cells were photoaffinity labeled *in vitro* with azido[^{14}C]atrazine (data not shown).

Fluorescence Induction in Whole Cells. Fig. 3 compares the fluorescence-induction kinetics of dark-adapted DCMU-4 and wild-type cells in the absence of inhibitor. A significant increase in the rapid phase of the fluorescent rise under strong illumination is observed in mutant cells and indicates slowing of electron transfer from Q to B. This phenomenon

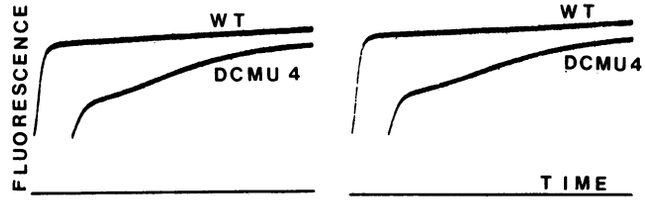


FIG. 1. Fluorescence-induction kinetics of dark-adapted broken-cell preparations of wild-type (WT) and DCMU-4 mutant *C. reinhardtii* in the presence of 4 μM atrazine (*Left*) or 1 μM DCMU (*Right*). Total sweep, 1 sec.

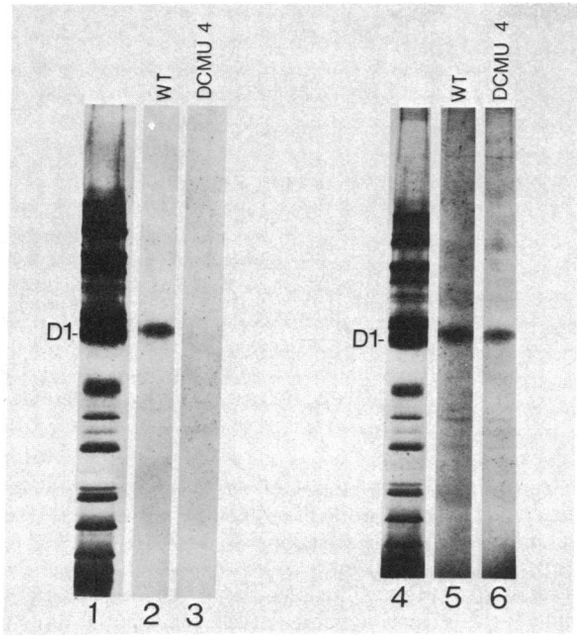


FIG. 2. Fluorography of ^{14}C-labeled proteins electrophoresed on a 12–18% gradient polyacrylamide gel containing 8 M urea. Lanes 1 and 4, thylakoid membrane polypeptides synthesized inside the chloroplast, labeled *in vivo* with [^{14}C]acetate in the presence of anisomycin (15). The diffuse band marked D1 includes the 32-kDa protein of PSII (15). Lanes 2 and 3, labeled proteins from wild-type (WT) and DCMU-4 mutant cells, respectively, after 10 min of UV treatment in the presence of 0.1 μM azido[^{14}C]atrazine. Lanes 5 and 6, as described for lanes 2 and 3, with cells irradiated in the presence of 10 μM azido[^{14}C]atrazine. In some gels, the protein labeled with azido[^{14}C]atrazine is resolved as a labeled doublet band.

is not seen under conditions of weak illumination. The fluorescence-induction kinetics of both wild-type and mutant dark-adapted cells in the presence or absence of DCMU is shown in Fig. 4. The typical increase in the initial fluorescence yield of wild-type cells in the presence of DCMU is due to the transfer of an electron from the reduced secondary acceptor B^- to the primary acceptor Q (18). This increased initial fluorescence yield induced by DCMU is significantly diminished in the DCMU-4 mutant cells. Fluorescence-induction curves of wild-type and DCMU-4 cells at varying concentrations of azidoatrazine (data not shown) indicate that blockage of Q^- reoxidation occurs with IC_{50} values of 50 nM and 5 μM, respectively.

Sequence Analysis of the DCMU-4 32-kDa Protein Gene. *psb*A, the gene for the 32-kDa protein, has been previously identified and sequenced in the wild-type *C. reinhardtii* 137c (35). This algal gene contains four introns and covers 6.7 kb. *psb*A located on restriction fragment Ba12 was initially cloned from DCMU-4 into a λ vector and subcloned into plasmid vectors. DNA fragments were sequenced according to the strategy illustrated in Fig. 5. Thus, the complete nucleotide sequence for each of the five exons was determined.

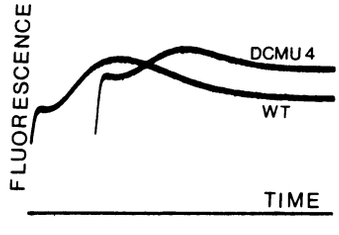


FIG. 3. Fluorescence-induction kinetics of dark-adapted cells of wild-type (WT) and DCMU-4 mutant *C. reinhardtii*. Total sweep, 2 sec.

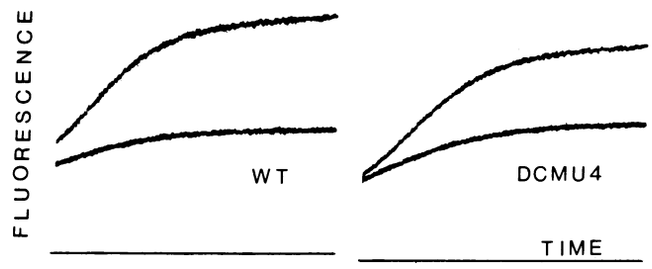


FIG. 4. Fluorescence-induction kinetics of dark-adapted cells of wild-type (WT) and DCMU-4 mutant *C. reinhardtii* in the absence (*Left*) or presence (*Right*) of 40 μM DCMU. Total sweep, 0.1 sec.

Both strands were sequenced in every exon except for the first 42 base pairs (bp) of exon 3. In this case, sequencing from the 5'-labeled *Pvu* II site was repeated several times. There is only one difference between the exon sequences of the mutant and the wild-type *psb*A. Fig. 6 shows a single base pair change of T-A to G-C, which results in the conversion of a serine residue in the wild type to an alanine residue in the herbicide-resistant mutant. No other differences were observed between the wild-type and DCMU-4 exon sequences.

Fig. 7 shows the position of the serine residue in the 32-kDa protein that is affected by the *psb*A mutation. This serine, at position 264, is the twelfth amino acid residue in the fifth exon. It is in a region of 121 predicted amino acid residues that are totally conserved between *S. oleracea* (36), *N. debneyi* (36), *A. hybridus* (41), soybean (42), and *C. reinhardtii* (35).

DISCUSSION

Sequence analysis of *C. reinhardtii psb*A, the chloroplast gene for the 32-kDa protein, has revealed a single T-A to G-C transversion in the DCMU-4 mutant that results in a deduced amino acid change of serine to alanine. This change correlates with the observation that azido[^{14}C]atrazine at 0.1 μM binds to the 32-kDa protein from wild-type, but not mutant, cells. An atrazine-resistant biotype of the pigweed *A. hybridus* shows a similar, reduced affinity of the herbicide analog for the 32-kDa protein (12). The exact site of herbicide binding to the wild-type protein has not yet been determined. Prediction of the hydrophobic helical structure of the spinach 32-kDa protein places this serine residue in, but near the edge of, a hydrophobic helix that spans the membrane (43). The serine residue that is altered in the mutant may be an amino acid residue directly involved in the binding of herbicide or may have a conformational effect on the binding site.

The deduced amino acid substitution of alanine in the mutant protein for serine in the wild type occurs in a region of one-third of the protein that is totally conserved between spinach, tobacco, pigweed, soybean, and the alga (Fig. 7). Such conservation may reflect strong functional constraints on the protein. Recently, *psb*A from wild-type and atrazine-resistant biotypes of the pigweed *A. hybridus* have been sequenced by Hirschberg and McIntosh (41). Deduced from the nucleotide sequence, a serine residue in the wild-type 32-kDa protein of pigweed is changed to a glycine residue in the herbicide-resistant biotype. This is the same serine residue that we find altered to alanine in the *C. reinhardtii* mutant. However, the wild-type serine codons differ at this position (Fig. 6). There is no way that a single point mutation can convert the algal TCT serine codon to glycine or, conversely, the pigweed AGT serine codon to alanine.

It has been proposed that the triazine binding domain shares residues in common with the plastoquinone binding site (20). Thus, one might expect that a structural change in the 32-kDa protein that alters atrazine binding would also

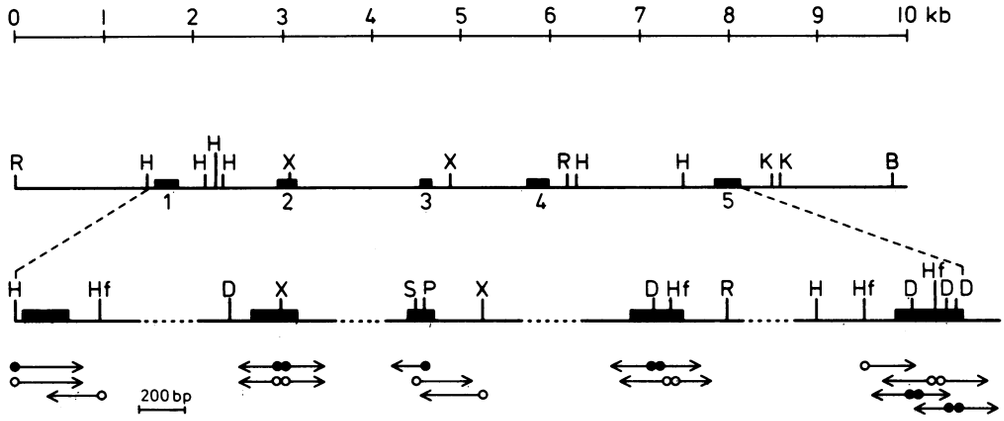


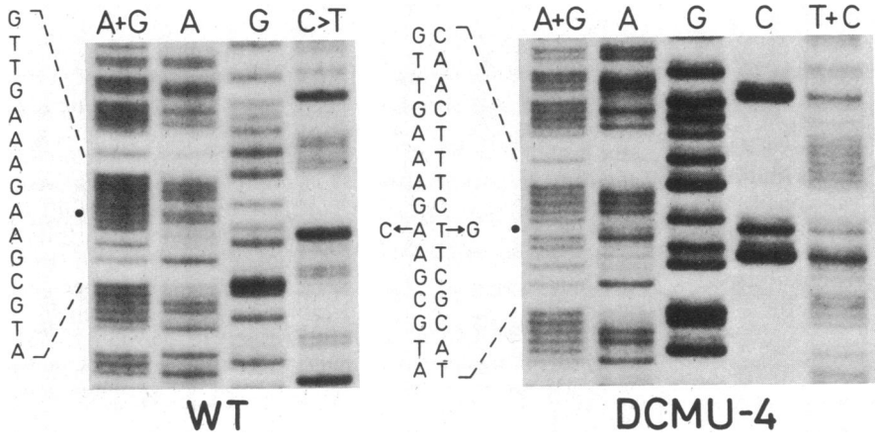
FIG. 5. Strategy for Maxam–Gilbert sequencing (39) of the *psb*A exon regions (■) from the DCMU-4 mutant *C. reinhardtii*. Fragments recovered from acrylamide gels were labeled at the 5′ (●→) or 3′ (○→) end. The restriction map for the chloroplast *Eco*RI fragment R14 (9) and the adjacent *Eco*RI–*Bam*HI fragment from R24 is given, indicating the positions of the five exons. Restriction enzyme sites for *Bam*HI (B), *Dde* I (D), *Eco*RI (R), *Hin*dIII (H), *Hin*fI (Hf), *Kpn* I (K), *Pvu* II (P), *Sau*3A (S), and *Xba* I (X) are indicated.

cause a change in the rate of electron transfer in PSII in the absence of herbicide. Fluorescence-induction kinetics of DCMU-4 show that this is, in fact, the case. In the absence of DCMU or atrazine, the secondary acceptor site of PSII is impaired in the mutant cells as compared to wild type (Fig. 3). A similar change in PSII electron transfer was previously reported for the atrazine-resistant biotype of pigweed (44). A change in plastoquinone binding in DCMU-4 is also reflected by the lower amount of stable B^- in the mutant as compared to the wild-type cells (Fig. 4). Galloway and Mets have reported four classes of uniparental herbicide-resistant mutants of *C. reinhardtii* (45). These are resistant to DCMU, to atrazine, to DCMU and bromacil, or to DCMU, atrazine, and bromacil. Only the latter mutant shows an alteration in PSII electron transport in the absence of herbicide. This mutant is 84-fold more resistant to atrazine than wild-type cells, compared to 15-fold for the mutant resistant to atrazine alone. Sequence analysis of *psb*A from these mutants should reveal much about the residues affecting binding of various herbicides and the plastoquinone.

An inverted repeat, containing the DNA encoding rRNA, is found in most of the chloroplast genomes studied to date (46). The *C. reinhardtii psb*A, unlike that of higher plants, is located within the inverted repeat and hence present at two copies per genome (35). Recent evidence obtained with mutants deleted for a portion of the inverted repeat suggests that gene conversion occurs between the two segments of the repeat (47). Both copies of the *C. reinhardtii* wild-type *psb*A have a serine codon at amino acid residue 264 (Fig. 6). The mutant sequence we report is from one copy of *psb*A. Sequence analysis of both copies of the mutant gene is required before any conclusions can be reached regarding the dominant or recessive nature of the *psb*A mutation and the possible occurrence of gene conversion at this locus.

In addition to its implications for the mechanism of herbicide resistance, the DCMU-4 mutant provides an excellent chloroplast marker. Such a mutation is important in relating the genetic and physical maps of the chloroplast genome. One correlation site was recently established for a mutation in *rbc*L, the gene for the large subunit of ribulose bisphosphate carboxylase/oxygenase (48). Genetic mapping of the *psb*A mutation relative to other chloroplast markers will provide an additional site of reference between the genetic and physical chloroplast maps. Another DCMU-resistant uniparental mutant of *C. reinhardtii* mapped with respect to erythromycin and streptomycin markers has established a preliminary genetic location for that mutation (49). The DCMU-4 mutant will most likely be at the same locus.

The mutant gene for the 32-kDa protein has the potential to produce a selectable phenotype in the transformation of chloroplasts. Since *C. reinhardtii* has a single large chloroplast, it is more amenable to chloroplast transformation than are higher plants. Such a transformation system, using *psb*A from the herbicide-resistant mutant, would provide an in-



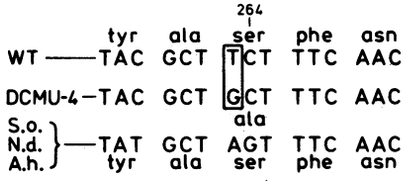


FIG. 6. Comparison of the nucleotide sequencing gels for the *C. reinhardtii* wild-type (WT) and DCMU-4 *psb*A showing the T-A to G-C transversion. The position of the mutation is marked with a dark circle. The wild-type and mutant amino acid codons for this region (mutation affects amino acid residue 264) are given and compared to those from *Spinacia oleracea* (S.o.) (36), *Nicotiana debneyi* (N.d.) (36), and *Amaranthus hybridus* (A.h.) (41).

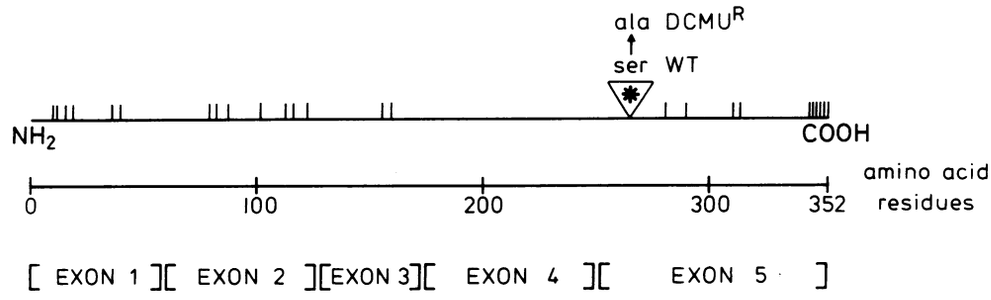


FIG. 7. Comparison of the amino acid sequence of the 32-kDa protein of *C. reinhardtii* (35) and *S. oleracea* (36). The positions of the amino acid residues that differ are indicated with vertical bars. The single mutation site in DCMU-4 is indicated with an asterisk. The mutation results in an amino acid residue change between the wild-type (WT) and mutant (DCMU^R) strains as indicated. Exon boundaries in the *C. reinhardtii* gene are indicated by brackets.

valuable tool for studying the function and regulation of chloroplast genes and their products.

Note Added in Proof. Both copies of *psb*A from DCMU-4 have the same mutant codon at amino acid residue 264.

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