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Positive selection for sensitivity of *Chlamydomonas reinhardtii* to Photosystem II-inhibiting herbicides

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We have developed a method for the strong positive selection of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU or diuron) -sensitive mutants of the unicellular green alga *Chlamydomonas reinhardtii*. The method involves plating cells in the light on medium containing acetate with carefully balanced concentrations of diuron and metronidazole. We have used this method for the selection of sensitive revertants of diuron-resistant strain Dr2. Among the strains that survive the selection, the spectrum of mutant phenotypes is diverse. These include reduced or absent photosynthesis, altered pigment content or chlorophyll fluorescence induction kinetics, as well as increased herbicide sensitivity. Among the more sensitive strains characterized in detail were five with increased affinity of diuron binding to the thylakoid membranes. None of these mutants is as sensitive as wild type, and so none is an exact revertant of mutant Dr2. All of these mutants showed the non-mendelian inheritance pattern characteristic of chloroplast DNA. Thus, the results extend the previous conclusion that the Photosystem II herbicide binding site is under the exclusive control of the chloroplast genome.

Introduction

Mutants of biotypes resistant to Photosystem II-inhibiting herbicides like atrazine or diuron have been described in numerous higher plants [1–3], several different algae [4–16], cyanobacteria [17–20], and photosynthetic bacteria [21–24]. In plants, algae and cyanobacteria, these herbicides act by binding with high affinity to the thylakoid membranes on the D1 protein, which is the product of the chloroplast *psb*A gene [4,25–27]. Resistance often involves a change in the binding site so that it has a much lower affinity for the herbicide. In all resistant higher plants or green algae where an altered binding site has been demonstrated, the genetic determinant of resistance has shown maternal or non-mendelian inheritance consistent with a chloroplast location [3,7,9,11,13,16,28–31]. In *Chlamydomonas*, we have shown genetic linkage between several PS II-herbicide resistant mutants and known chloroplast gene markers [7,32]. All of the mutants map genetically to a single locus [7] marked by a deletion mutation [33] covering the *psb*A gene. DNA sequencing of the *psb*A gene from resistant and sensitive plant varieties [34–36], from *Chlamydomonas* mutants [9,12, 13,37] and from cyanobacterial mutants [19,38] has revealed single amino acid differences in the protein. In the case of the cyanobacterium *Anacystis nidulans* R2, transformation studies have provided direct proof that the resistance is determined by the amino acid substitution [38]. From these results, it is clear that the D1 protein plays a major role in structuring the herbicide binding site.

We have been attempting to identify other genes that might interact with *psb*A in controlling herbicide binding. In principle, protein–protein interactions within the PS II complex might allow alterations in other components to influence the structure of the binding site. The core components of the PS II complex are chloroplast gene products, but both oxygen-evolving complex proteins and light-harvesting proteins that interact with the core are nuclear gene products. An example of the interaction between D1 and the oxygen-evolving complex has been detected through a mutation affecting PS

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Abbreviations: PS II, Photosystem II; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

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II activity [39]. Extensive surveys of nuclear gene mutants of *Chlamydomonas* selected for herbicide resistance have not identified any that act by altering herbicide binding [7,8]. We felt that a different strategy for mutant selection might identify different kinds of mutations affecting herbicide binding and that some of these could conceivably alter different proteins. In the present study, we have attempted to identify suppressor mutations that would restore herbicide binding (and herbicide sensitivity) to a chloroplast diuron resistant mutant, Dr2. This mutant carries a Val-to-Ile substitution at position 219 of the wild-type *psb*A gene [12].

To find the suppressors, we needed a strong positive selection for the herbicide-sensitive phenotype. Our strategy is based upon the observation of Schmidt et al. [40] that metronidazole will kill *Chlamydomonas* cells engaged in linear photosynthetic electron transport, but is non-toxic to cells that are not active. We have found a level of diuron that will inhibit electron transport in wild-type (sensitive) cells and protect them from metronidazole mediated lethality while providing no protection for resistant cells. This level of diuron does not inhibit heterotrophic growth with acetate as carbon and energy source. Thus, sensitive cells are able to form colonies on the medium containing acetate, metronidazole and diuron, while diuron-resistant cells are killed. We have exploited this selection to search for revertants or suppressors of mutant Dr2 that are more sensitive to diuron. Among the mutants recovered are several that show partial restoration of herbicide binding to the thylakoid membranes. All of these are chloroplast gene mutants.

Materials and Methods

Strains, growth conditions, crosses, spot tests

C. reinhardtii diuron-resistant strain Dr2 [8] and its wild-type progenitor strain 2137 [41] were used in these experiments. Cultures were routinely grown at 25 °C, 4000 lx on Tris-phosphate minimal medium [42]. For heterotrophic or mixotrophic growth, 10 mM sodium acetate was added (acetate medium). Diuron (DuPont) was dissolved in ethanol at 1000 × the final medium concentration and added to cooled, autoclaved medium just before pouring plates. Metronidazole (Sigma) was added to media before autoclaving.

Spot tests [8] were employed to evaluate the growth potential of strains on various kinds of media. These were performed by suspending cells in liquid medium to approx. 10^6 cells/ml and placing 10 µl of each strain on the plates of media to be tested. After 10 days, the degree of growth was scored.

Colony growth rates were determined by plating approx. 50–75 cells per plate and measuring the diameter of 15–20 colonies with an ocular micrometer each day for 2 weeks. Average colony diameters were used to obtain linear regression slopes as growth rates in µm per day.

Crosses and tetrad analysis to determine inheritance patterns were as described [8]. Mutants were crossed as the mating type plus (mt +) with a wild-type diuron-sensitive mt − strain. Tetrads were replica plated to 3 µM diuron-containing media to score resistance vs. sensitivity in the progeny.

Mutagenesis of strain Dr2, mt + , was carried out as described for the selection of herbicide resistance [8,41], using successive fluroodeoxyuridine and ethylmethanesulfonate treatments. After an expression time in the dark of 10 days, 1 ml samples containing $3 \cdot 10^6$ cells were plated on acetate medium containing 6 µM diuron and 10 mM metronidazole. Two plates were prepared from each independent mutagenesis flask and all were incubated at 25 °C, 4000 lx. Colonies appeared in 1–2 weeks and were replica plated to minimal medium plus 10 mM metronidazole, minimal plus 3 µM diuron, acetate plus 3 µM diuron, and two acetate plates (one for heterotrophic growth and one for mixotrophic growth at 4000 lx). After 1–2 weeks, the replicas were scored and colonies of interest saved.

Hill reactions, fluorescence induction kinetics, chlorophyll measurements

1 l liquid cultures of cells to be used for Hill reactions were grown at 25 °C, 6000 lx with 5% CO_2 aeration to final cell concentrations ranging from $1 \cdot 10^6$ to $3 \cdot 10^6$ cells per ml. Cells were disrupted by three passages through a French pressure cell at 30 MPa and thylakoid membranes were purified by differential centrifugation and flotation on discontinuous sucrose gradients [43]. Concentrations of diuron giving 50% inhibition of the Hill reaction (I_{50}) were measured as previously described [8]. The I_{50} is equivalent to the binding constant between the herbicide and its site of action.

Chlorophyll-fluorescence induction curves were obtained from dark-adapted colonies using the apparatus described previously [7].

Concentrations of chlorophyll *a* and *b* per cell were estimated spectrophotometrically [44] on cold 80% acetone extracts of cells grown at 4000 lx.

Results

Selection conditions

Metronidazole has been used previously to kill photosynthetically competent cells growing in liquid culture [40]. We found that both wild-type and Dr2 cells were killed on solidified acetate media containing 10 mM metronidazole if they were incubated in the light at 4000 lx. When incubated in the dark, both strains grew on plates of this medium. Appropriate concentrations of diuron allow growth of wild-type (diuron-sensitive) cells

TABLE I

Comparison of wild type and Dr2 growth on acetate media containing DCMU and 10 mM metronidazole

−, originally plated cells died; (−) originally plated cells did not divide, but did not die; (+ −), one or two divisions of plated cells; + to + + +, macroscopically observable growth from minor to luxuriant. On media containing 3 μM DCMU and no metronidazole, the growth of strain Dr2 is scored + + +, while that of strain 2137 scores (−).

DCMU concn. (μM)	30	12	6	3	1.2	0.6
Strain Dr2	(−)	−	−	−	−	−
Strain 2137	(+ −)	(+ −)	(+ −)	(−)	(−)	−

on this medium in the light, while Dr2 cells are still killed (Table I). Concentrations in the range of 1 to 6 μM are most effective in producing a differential effect on resistant vs. sensitive cells, and we chose 6 μM for use to select sensitive mutants. At such a high concentration of diuron even slightly increased herbicide sensitive would confer protection from the metronidazole. Wild-type cells grow slowly on this medium, since 6 μM diuron produces significnat inhibition of heterotrophic growth rates by inhibiting cellular functions other than photosynthesis.

Mutant isolation and characterization

Cells of strain Dr2 were mutagenized, plated on diuron-metronidazole-acetate medium, and the viable colonies replica plated as described in Materials and Methods. Although a variety of mutant phenotypes were recovered, none of the mutants were simply resistant to metronidazole. As expected from the results of Schmidt et al. [40], numerous mutants were recovered that were unable to grow on minimal medium. Several mutants unable to grow in the light on either minimal or acetate medium were also observed. Since we expect diuron-sensitive revertants of Dr2 to be able to grow on minimal medium, mutants in these categories were discarded. No normally pigmented mutants were found that were unable to grow on minimal medium plus 3 μM diuron, as would be expected for true revertants of Dr2 to wild type. However, numerous mutants grew poorly on this medium when compared with Dr2. Several pigment-deficient mutants appeared very sensitive to diuron in minimal medium. 42 of the diuron-sensitive mutants and 6 of these leaky acetate-requiring pigment-deficient mutants were retained for further characterization.

Spot tests on a range of diuron concentrations in minimal medium confirmed that none of the mutants was a true revertant. This rescreening also reduced the number of mutants requiring detailed characterization. Only 16 fully green strains showed weaker growth than Dr2 at any diuron concentration. These are listed in Table II, along with the pigment-deficient strains. In an attempt to obtain a more sensitive and quantitative assay for the level of phenotypic resistance remaining in these strains, we measured colony growth rates in the presence and absence of diuron. It is possible that some of the mutants were unstable or were too similar to Dr2 to be of value, since several of the strains proved to be indistinguishable from Dr2 in this test. We have chosen the ratio of growth rates in the presence and absence of diuron as an indicator of relative resistance of the strains, since the absolute growth rate in the presence of diuron is not a sufficient criterion. Mutant Ds423 is an interesting example. Although it shows slower growth than Dr2 on diuron, it is also slower on minimal medium. The ratio of diuron inhibited to uninhibited growth is not lower than that of Dr2 (Table II). This mutant has reduced chlorophyll per cell and a chlorophyll *a/b* ratio in the range of 4–6 (compared with 2.2–2.4 for wild type and Dr2). It has a selective loss of about

TABLE II

DCMU resistance of wild-type and Dr2 strains compared with Ds mutants

Spot tests and colony growth measurements in the presence or absence of DCMU in minimal medium were assessed as described in Materials and Methods. Colony growth rates are given in μm/day ± 95% confidence interval.

Strain	Spot test 3 μM DCMU	Colony growth rates		Relative inhibited rate [a] (%)
		3 μM DCMU (μm/day)	minimal (μm/day)	
Wt	−	0	119.5 ± 11.4	0
Dr2	+ + +	43.5 ± 5.1	101.1 ± 7.6	43.0
Ds111 [b]	−	n.d.	n.d.	−
Ds112 [b]	−	n.d.	n.d.	−
Ds113 [b]	−	n.d.	n.d.	−
Ds212	+ +	46.3 ± 5.1	103.3 ± 4.3	44.8
Ds221	+ +	29.9 ± 3.6	119.5 ± 12.3	25.0 [c]
Ds311	+ +	40.6 ± 3.2	96.1 ± 3.5	42.2
Ds415	+ +	41.2 ± 3.5	97.1 ± 2.8	42.4
Ds416	+ +	33.0 ± 1.7	110.7 ± 5.4	29.8 [c]
Ds421	+ +	47.0 ± 6.5	84.9 ± 8.1	55.4
Ds423 [b]	(+ −)	22.5 ± 1.6	33.3 ± 1.9	67.6
Ds424	+ +	32.8 ± 4.5	118.2 ± 12.6	27.7 [c]
Ds426	+ + +	n.d.	n.d.	−
Ds427	+ +	44.8 ± 4.4	108.1 ± 11.1	41.4
Ds428	+ +	58.6 ± 8.6	120.2 ± 11.0	48.8
Ds513	+	16.8 ± 2.3	96.6 ± 15.6	17.4 [c]
Ds514	+ +	47.4 ± 4.4	115.0 ± 12.3	41.2
Ds521 [b]	−	n.d.	n.d.	−
Ds522 [b]	(+)	n.d.	n.d.	−
Ds523	+	25.5 ± 2.9	90.7 ± 11.2	28.1 [c]
Ds524	+ +	47.9 ± 5.8	126.9 ± 12.7	37.7
Ds611	+ +	44.1 ± 5.7	108.8 ± 13.7	40.5
Ds721	(+)	21.4 ± 0.6	96.6 ± 10.2	22.2 [c]
Ds1213	+	22.2 ± 2.3	91.2 ± 10.7	24.3 [c]

[a] DCMU inhibited growth rate/uninhibited growth rate.
[b] Pigment-deficient strains.
[c] Mutants significantly less resistant than Dr2.
[d] n.d., not determined.

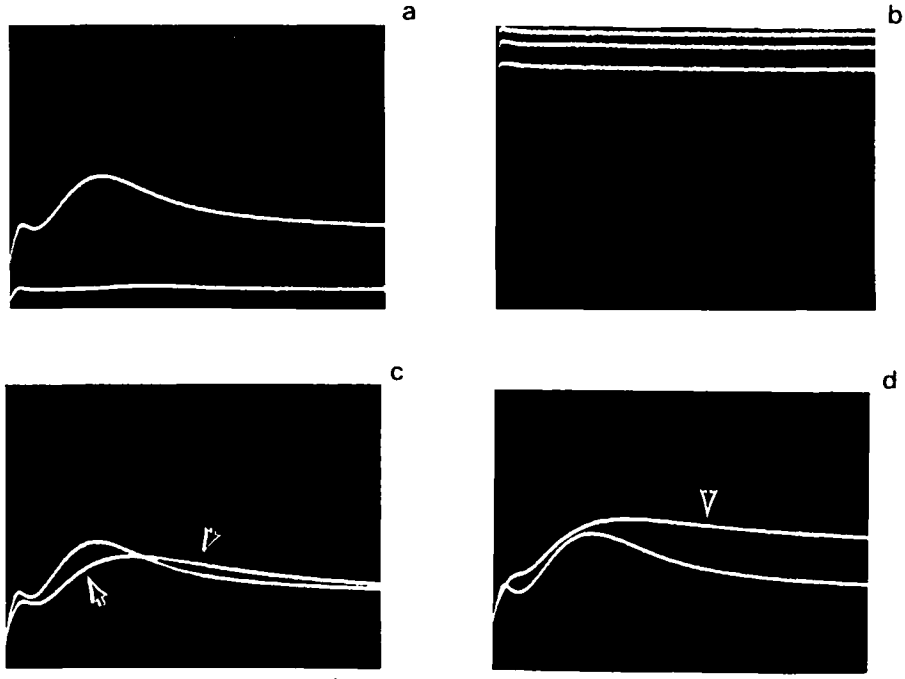

Fig. 1. Chlorophyll fluorescence induction transients. Small colonies of various *Chlamydomonas* strains growing on agar surfaces in petridishes were dark-adapted for at least 10 min and then exposed to wide-band blue actinic light for 2 s as described [7]. The relative chlorophyll fluorescence (y-axis) was recorded as a function of time (x-axis) during the light exposure on a storage oscilloscope, and the tracings were photographed. The relative fluorescence scale in panels a, c and d are the same, and that in panel b is 10-fold amplified. (a) Wild-type strain 2137 (upper trace) and mutant Ds423 (lower trace). (b) Mutant strains Ds111, Ds112 and Ds113. Because the fluorescence scale is amplified 10-fold, the trace of a wild-type strain would be off scale. (c) Mutant Ds426 (arrows) and wild type strain 2137. (d) Mutant Ds524 (arrow) and wild type strain 2137.

60–70% of the Chl a/b light-harvesting complex. Presumably, its reduced phototrophic growth rate reflects its deficiency in light-harvesting capability. At a given light intensity, its slower electron-transport rate might allow it to survive the selection conditions, independent of a change in diuron sensitivity. Mutant Ds521 is similar, with chlorophyll a/b ratio in the range of 6–8. It is deficient in about 95% of the Chl-a/b protein complex and has even slower phototrophic growth. The Chl-a/b protein deficiency in each of these mutants is determined by a nuclear gene, and the two mutations are in different genes, since they can recombine with each other (data not shown).

TABLE III

I_{50} values for DCMU-'sensitive' strains

I_{50} values were determined as described in Materials and Methods. The Ds523 tetrad was obtained by crossing Ds523, mt+ with wild-type strain 2137, mt− as described in Materials and Methods.

Strain	Hill reaction I_{50} for DCMU (μM)
Dr2	1.7
Ds416	1.0
Ds513	1.0
Ds523	0.9
Ds721	1.3
Ds1213	0.9
Ds523 tetrad 1–10	
1.	1.0
2.	1.1
3.	1.1
4.	1.0

Of the seven mutants that show a reduced ratio of diuron inhibited to uninhibited growth rate, five were tested for diuron inhibition of the in vitro Hill reaction. As shown in Table III, all five have membranes with slightly increased affinity for diuron (about 2-fold) when compared with Dr2, but still retain substantially lower affinity than wild type (about 10-fold; wild type $I_{50} = 0.1$ μM). These binding-site properties would explain the considerable resistance retained by these strains relative to wild type as well as their slight sensitivity relative to Dr2. These strains were crossed to determine the pattern of inheritance of the increased sensitivity, and all showed uniparental inheritance. An example of I_{50} measurements on a complete tetrad of mutant Ds523 is shown in Table III. Thus, the increased sensitivity of these strains arises from a binding-site alteration that is determined by a chloroplast gene mutation.

All of the mutants were grown on acetate medium and subjected to fluorescence induction analysis. The curves for the less resistant strains identified in Table II were all indistinguishable from wild type (data not shown). However, a large proportion of the other Ds strains had altered fluorescence induction kinetics. Some

examples are shown in Fig. 1. The low, flat fluorescence of Ds111, Ds112, Ds113, and Ds521 are characteristic of the pigment deficient mutants. Other Ds mutants frequently showed a delayed drop in fluorescence from the peak and a high final level. This is illustrated for Ds426 and Ds524 in Fig. 1. These mutants have almost normal phototrophic growth rates, and so they do not have major defects in photosynthesis. However, the altered fluorescence induction curves may indicate a slight defect that would slow electron transport sufficiently to allow these mutants to survive the presence of metronidazole plus diuron. In the mutants we have tested genetically, the altered fluorescence induction patterns are determined by nuclear gene mutations.

Discussion

Although the initial screen of the selected mutants demonstrated conclusively that true revertants with wild-type herbicide-sensitive phenotypes were not obtained, the metronidazole-diuron selection did prove of value in enriching for mutations with altered herbicide binding affinity. The sensitive mutants obtained had about a 2-fold increase in affinity relative to the progenitor Dr2 strain, but were still about 10-fold less sensitive than wild type. Thus, it seems that the selection conditions are very discriminating, and subtle differences in electron-transport rates make the difference between growth and no growth. Because none of the mutants with altered binding sites are exact revertants, we expect them all to retain the Val-219-Ile substitution seen in the *psb*A gene of Dr2 [12]. This would be coupled with a change elsewhere in *psb*A or another chloroplast gene that helps configure the herbicide-binding site. It is also possible that the mutants might carry a change in Val-219 that substitutes an amino acid other than Ile.

The frequency with which we recovered chloroplast mutants with more sensitive binding sites than Dr2 is approx. $7 \cdot 10^{-7}$ (at least five mutants in $7 \cdot 10^{6}$ cells plated). While this is low relative to the frequency of non-photosynthetic mutants, it is higher than the frequency with which we obtained resistant mutants from wild type by positive selection on herbicide-containing media (about $2 \cdot 10^{-8}$ in several experiments [7,8]). It is possible that the selection for resistance is more stringent than the selection for sensitivity or that the exposure to metronidazole is mutagenic. Also, it is possible that phenotypic dominance of sensitivity over resistance [45,46] suppresses the recovery of resistant mutants and enhances the recovery of sensitive ones.

We had hoped to recover a secondary mutation affecting thylakoid membrane resistance that resided in a gene other than *psb*A. None of the evidence in the literature indicates any effect of other genes on the herbicide-binding site, but the possibility is not excluded. It was plausible that a different, more subtle type of selection might reveal the influence of gene products that interact with the D1 herbicide binding site to influence it to some degree. As in our previous attempts to detect nuclear genes that might influence herbicide binding [7,8], the mutagenesis we employed in these experiments was effective in generating numerous nuclear gene mutants with a spectrum of other phenotypes. In view of the high discrimination achieved by the sensitivity selection, it now seems very unlikely that any nuclear gene can influence the structure of the herbicide-binding site. The experiments described here do not exclude the possibility that these sensitive mutations reside in some chloroplast gene other than *psb*A.

Metronidazole has been used successfully in selecting for numerous mutants with stringent blocks in photosynthetic electron transport [40,47]. We have found that our comparatively gentle selection protocol can enrich for mutants that have altered photosynthetic properties, yet retain some capacity for phototrophic growth. These had alterations in fluorescence induction kinetics, chlorophyll *a/b* ratios, lower chlorophyll content per cell, or generally slower growth rates. In fact, the majority of mutants recovered fall into these categories. Thus, metronidazole-diuron selection provides a valuable method for obtaining mutants with minor modifications of the photosynthetic apparatus. The chlorophyll *a/b* protein deficient mutants have already proven valuable in studies of photosynthetic light-harvesting and trapping dynamics [48].

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