Herbicide Resistance and Growth of D1 Ala_{251} Mutants in *Chlamydomonas*

Britta Förster[a], Peter B. Heifetz[c], Anita Lardans[d], John E. Boynton[a] and Nicholas W. Gillham[b]

[a,b] DCMB group, Departments of Botany[a] and Zoology[b], Box 91000, Duke University, Durham, NC, 27708-1000, USA
[c] Novartis Crop Protection Inc., Biotechnology and Genomics Center, 3054 Cornwallis Rd., Research Triangle Park, NC, 27709-2257, USA
[d] 11 La Cantellerie, 27310 Epreville en Roumois, France

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We elucidated the effects of substituting seven amino acids for Ala at residue 251 of the *Chlamydomonas reinhardtii* D1 protein on herbicide resistance and photoautotrophic growth. Ala_{251} has been suggested to play a key role in the structural integrity and function of the stromal loop between transmembrane helices IV and V of D1 and has previously been shown to affect resistance to "classical" PSII specific herbicides. Sensitive and rapid microtiter assays were employed to compare herbicide resistance and photoautotrophic growth in the various mutants. Substitution of Ala_{251} by Ile, Leu or Val conferred resistance to the PSII herbicides atrazine, bromacil and metribuzin but not to DCMU, and impaired photoautotrophic growth in high and low light. Compared to an otherwise isogenic wildtype strain, the Ile and Val mutants exhibited nearly identical levels of herbicide resistance and reduced growth while the Leu mutant had even slower growth and higher levels of herbicide resistance. In contrast Cys, Pro, Ser and Gly mutants were phenotypically indistinguishable from wildtype in terms of herbicide sensitivity and photoautotrophic doubling times. Collectively, the seven Ala_{251} mutations differed markedly from an Ala mutant (*dr-1*) at the well characterized Ser_{264} D1 residue in terms of herbicide resistance and photoautotrophic growth.

Introduction

Resistance to "classical" PSII herbicides such as atrazine, bromacil, metribuzin or DCMU results almost exclusively from amino acid substitutions between residues Phe_{211} and Leu_{275} in the D1 protein (Trebst, 1987; Ohad and Hirschberg, 1992). The domain bounded by these residues contains two transmembrane helices (IV and V) connected by a stromal loop which includes the binding site for Q_B, the second stable acceptor of photosystem II [reviewed by Bowyer *et al.* (1991)]. This binding site is the target of the aforementioned herbicides which act as competitive inhibitors of Q_B (e.g. Gronwald, 1994). Herbicide resistance is thought to result from structural modifications to the Q_B site which may alter both herbicide and Q_B binding (Trebst, 1987; Ohad and Hirschberg, 1992). Consequently, many herbicide resistant mutants also result in some phenotypic impairment in the absence of herbicide. Conflicting reports exist in the literature on the extent to which these mutants affect the efficiency of photosynthetic electron transport and crop yield or biomass accumulation (Galloway and Mets, 1984; Devine *et al.*, 1993; Gronwald, 1994; Thiemann and Barz, 1994). The degree to which herbicide resistant mutants affect overall fitness, reflected in photoautotrophic growth, has not been extensively characterized in truly isogenic lines.

High level DCMU resistance is conferred by the $Ser_{264} \rightarrow$ Ala mutation in the D1 proteins of *Chlamydomonas reinhardtii* (Erickson *et al.*, 1984) and cyanobacteria (Golden and Haselkorn, 1985) and by the $Ser_{264} \rightarrow$ Gly mutation in the D1 protein

Abbreviations: atrazine, 2-chloro-4-ethylamine-6-isopropylamine-S-triazine; bromacil, 5-bromo-3-methyl-3-(1-methylpropyl) uracil; DCMU, 3 (3,4-dichlorophenyl)-1,1-dimethyl urea; I_{50}, 50% inhibition of autotrophic biomass accumulation; metribuzin, 4-amino-6-(1,1 dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one; MLHC, minimal lethal herbicide concentration; PSII, photosystem II; Q_A, Q_B, quinones, stable electron acceptors at PSII; *spr*, spectinomycin resistance mutation.

Reprint requests to Prof. John E. Boynton.
Fax: (919) 613-8177.

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of higher plants [Hirschberg and McIntosh, 1983; reviewed in Gronwald (1994)]. However both the $Ser_{264} \rightarrow$ Ala mutants of *Synechocystis* PCC 6714 (*DCMUII-A*) and *C. reinhardtii* (*MZ1*, *DCMU4*) also show decreased photosynthetic electron transport and oxygen evolution, and exhibit altered fluorescence and thermoluminescence properties (Erickson *et al.*, 1989; Kirilovsky *et al.*, 1990; Crofts *et al.*, 1993; Sundby *et al.*, 1993; Perewoska *et al.*, 1994). This is thought to result from loss of hydrogen bonding between Q_B and Ser_{264} in the wildtype D1 protein. The herbicide binding site spatially and structurally overlaps with the Q_B site to the extent that a single amino acid change at position 264 both impairs Q_B binding and results in a DCMU resistant phenotype (Trebst, 1987; Bowyer *et al.*, 1990; Mackey and O'Malley, 1993).

In contrast, $Ala_{251} \rightarrow$ Val mutants in *Synechocystis* and *Chlamydomonas reinhardtii* differ from the $Ser_{264} \rightarrow$ Ala mutant with respect to their spectrum of herbicide cross resistance, suggesting that these two residues may have different effects on the topology of the herbicide binding niche (Pucheu *et al.*, 1984; Johannigmeier *et al.*, 1987; Kirilovsky *et al.*, 1989; Wildner *et al.*, 1990; Perewoska *et al.*, 1994). A current model of the D1 protein proposes that Ala_{251} is positioned in the IV-V loop at the border of an amphipathic parallel helix which forms a "bottleneck" at the entrance to the Q_B niche in the periphery of the lipid layer of the thylakoid (Trebst, 1987; Etienne and Kirilovsky, 1993; Perewoska *et al.*, 1994). Previous results (Etienne and Kirilovsky, 1993; Astier *et al.*, 1993; Crofts *et al.*, 1993; Perewoska *et al.*, 1994; Constant *et al.*, 1996) suggest that the single $Ala_{251} \rightarrow$ Val mutation exerts multiple effects on D1 function, leading to reduced photosynthetic yield and slower photoautotrophic growth, particularly in high light.

To analyze more specifically the role of this D1 residue in relation to susceptibility to photoinhibition and acquisition of herbicide resistance, we generated 12 different mutants of Ala_{251} in the D1 protein in *C. reinhardtii* [(Lardans *et al.*, 1997a and 1997b). Five of the amino acid substitutions (Arg, Asp, Gln, Glu, His) led to total loss of photoautotrophy (Lardans *et al.*, 1997a). In order to facilitate the quantitative comparison of the mutants we have developed a highly sensitive and reproducible assay for measuring growth of multiple genotypes in microtiter plates under carefully controlled environmental conditions. Here we show that when the Ala_{251} residue was changed to Cys, Pro, Ser or Gly, herbicide sensitivity and photoautotrophic growth were unaffected, while Ile, Val or Leu substitutions led to different degrees of herbicide resistance and impairment of growth at both low and high light. In contrast, photoautotrophic growth of the Ser_{264} (*dr-1*) mutant was limited only in high light.

Materials and Methods

Chlamydomonas strains and generation of Ala_{251} transformants and suppressors

Chlamydomonas reinhardtii wildtype strain CC-125 (Harris, 1989) and the transformed mutant strain CC-2827 *dr-1* (*dr* = DCMU resistant, *psbA* $Ser_{264} \rightarrow$ Ala) (Heifetz *et al.*, 1992) were obtained from the *Chlamydomonas* Genetics Center, Box 91000, Duke University, Durham, NC, 27708–1000.

Site-directed mutagenesis of exon 4 of the chloroplast *psbA* gene from *C. reinhardtii* (Lers *et al.*, 1992; Lardans *et al.*, 1997a) was performed using degenerate 32 base pair oligonucleotides (mutated codons in bold) containing codons 245 to 252 and part of the adjacent intron 4: psbA251–2 (5'-CTTACAAT**ATT**$_{247}$GTAGCT$^{\mathbf{G}}$/$_{\mathbf{A}}$**TT**$_{251}$CATAGTAAAAC-3') was used to generate Val_{251} (GTT, A251V*) or Ile_{251} (ATT, A251I*) mutations and psbA251–4 (5'-CTTACAAT**ATT**$_{247}$GTAGCT$^{\mathbf{G}}$/$_{\mathbf{C}}$$^{\mathbf{C}}$/$_{\mathbf{G}}$**T**$_{251}$CATAGTAAAAC-3') was used to generate Ala_{251} (GCT, A251A*) or Arg_{251} (CGT, A251R*) mutations. One base pair change in the Asn_{247} codon (ATC → ATT, in bold) created a SspI RFLP marker. Fragments carrying the *psbA* mutations Ile_{251} and Val_{251} and the linked RFLP marker were inserted into the same position in the 3.1 kb KpnI *psbA* wildtype fragment of P-300 (A. Lers, unpublished) to generate P-432 (*A251I**) and P-433 (*A251V**). Construction of plasmids carrying the Ala_{251}, Arg_{251} and Gly_{251} mutations (plasmids P-435, P-436 and P-434, respectively) and isolation of homoplasmic chloroplast transformants have been described elsewhere (Lardans *et al.*, 1997a and 1997b). Three or more independent *A251I** (CC-3388) and *A251V** (CC-3387) cotransformants, obtained using P-228 (Newman *et al.*, 1990), were selected for spectino-

mycin resistance on HSHA medium containing 100 μg/ml spectinomycin (Trobicin, Upjohn) in dim light (10 μmol m^{-2} s^{-1}). Mutants containing the Cys (*A251C** = CC-3390), Ser (*A251S** = CC-3392), Pro (*A251P** = CC-3391), Gly (*A251G** = CC-3393) and Leu (*A251L** = CC-3389) substitutions were isolated as photosynthetically competent, same-site suppressors of the nonphotosynthetic *A251R** transformant (CC-3376) (Lardans *et al.*, 1997a and 1997b).

The *A251A** transformant (CC-3394) was constructed as a "wildtype" control for assessing potential physiological effects on D1 function and photoautotrophic growth of the 16S rRNA *spr* marker used for cotransformation and the silent SspI marker in the *psbA* gene. Doubling times of *A251A** did not differ significantly from the wildtype CC-125 strain grown under high light, low light or very low light. Thus, phenotypic consequences of both markers are negligible and the phenotypes of the seven otherwise isogenic A_{251} mutants result directly from the amino acid changes at residue 251 in the D1 protein.

Culture of Chlamydomonas strains

Mutant and wildtype strains were grown photoautotrophically in HS (high salt) medium or mixotrophically in HSHA (high salt high acetate) medium for physiological experiments as described by Lers *et al.* (1992). Photoautotrophic cultures were incubated under VLL (very low light, 33 μmol m^{-2} s^{-1}), LL (low light, 70 μmol m^{-2} s^{-1}), and HL (high light, 600 μmol m^{-2} s^{-1}). Media were prepared as described in Harris (1989) except that the phosphate stock solution was autoclaved separately and added to cool sterile medium to avoid cloudy precipitates that interfere with A_{750} measurements. For spot tests and strain maintenance (1.5% w/v) agar solidified media (HS, HSHA) were used at 25 °C in VLL, LL, or HL. Antibiotics and herbicides were added from concentrated stock solutions (in ethanol) to the liquid or solid media after autoclaving.

Spot tests for semiquantitative analysis of growth and herbicide resistance and determination of minimal lethal herbicide concentrations

Cells were grown photoautotrophically in liquid culture to mid-log phase (A_{750} = 0.050 to 0.120, Bausch & Lomb Spectronic 21) and diluted to A_{750} = 0.020. Aliquots (15 μl) of cell suspension were spotted in triplicate onto solid HS medium with or without various concentrations of herbicides (up to 70 μM atrazine, 100 μM bromacil, 40 μM DCMU, 1000 μM metribuzin) and incubated at 25 °C for 4–9 days in HL or 7–15 days in LL to determine the minimum lethal herbicide concentration (MLHC).

Microtiter growth assay

Growth was measured as increase in total biomass determined spectrophotometrically at 750 nm (A_{750}) using an automated microtiter plate reader (Cambridge Technology Model 7520) and software supplied by the manufacturer. Cells of wildtype and mutant strains were pregrown autotrophically as described above, diluted to A_{750} = 0.003, and regrown twice to mid-log phase. The second diluted precultures (A_{750} = 0.003, ca. 10^5 cells/ml) were dispensed in 1 ml aliquots, the largest volume that permitted optimal mixing, into individual wells of sterile 24–well microtiter plates (Falcon #3047). Five to fifteen replicates of each genotype were randomly distributed in wells within and between plates, with the exception that the four corner wells of each plate were filled with deionized H_2O. Four plates were incubated together under sterile conditions in a closed transparent chamber (30.5 x 22.8 x 4.3 cm, 3000 cm^3) mounted on a controlled temperature water jacket and agitated constantly on a rotary shaker in the appropriate light environment. The temperature in the microtiter plate wells was maintained at approximately 26 °C by blowing cool air across the outside of the chamber and/or controlling the water temperature of the jacket. Filtered 5% CO_2 supplied to the chamber at a flow rate of 600 cm^3/min provided 12 changes of atmosphere per hour. Lower CO_2 flow rates resulted in reduced growth rates in HL under otherwise identical conditions.

After a variable initial lag phase (12 h to 60 h, depending on the culture conditions), A_{750} was measured directly in the sterile 24 well pates at regular intervals (HL: 2 h, LL: 4 h, VLL: 6 h) until stationary phase was reached. Log A_{750} values, corrected for absorption by the medium, were plotted over time for each well separately. The exponential growth rate (g) was calculated for the

cell populations in individual wells by linear regression. Doubling times (dt) based on units of biomass were calculated with following equations: $C_i = C_0\ e^{gdt}$, dt = ln2 / g [C_i, A_{750} at t_i; C_0, initial A_{750}; *g*, exponential growth rate; dt, doubling time [(h)]. Results were averaged from at least two independent experiments involving separate pregrowth cultures (minimum of 24 replicates total) and evaluated statistically by analysis of variance and the Tucker test [described in Cantatore de Frank (1980)]. Doubling times in all 20 wells of four plates in the incubator box proved to be very uniform for a given genotype between replicates in the same or separate experiments. The ability to measure many independent replicates under controlled environmental conditions rules out the possibility that differences observed in doubling times between different genotypes result from variation in assay conditions rather than genetic differences.

Herbicide resistance I_{50} assay

Atrazine (Ciba-Geigy) and DCMU (Sigma) were obtained as highly purified compounds (95%-99%). Metribuzin (Dupont) and bromacil (Dupont) were purified by dissolving the powder in acetone, pelleting insoluble particles, recrystalizing the herbicide from the supernatant and washing the crystals in several volumes of deionized H_2O. All four inhibitors were light stable in HS medium after exposure to HL over the time course of a typical experiment (data not shown). Wildtype and known resistant mutants did not respond differently when treated with herbicides pre-incubated in HL for 80 h vs. treated with freshly prepared herbicides of the same dilution (data not shown). Thus, changes in resistance patterns appear to be related to the *psbA* genotype rather than light enhanced herbicide turnover.

Herbicide resistance and cross resistance were determined in microtiter growth assays based on A_{750} reached at the end of the exponential growth phase (approximately 55 h for CC-125, *A251A** and 60 h for *A251I**, *A251V**, *A251L**, *dr-1*). Replicate 1 ml cultures were inoculated in 24 well microtiter plates as in autotrophic HL growth assays (A_{750} = 0.003). Aliquots (10 µl) of ethanol (control) or 100x herbicide stock solutions (experimental) were added and the plates immediately shaken. A_{750} was measured at 2–5 h intervals beginning 42 h after inoculation. Final herbicide concentrations were 0 to 10 µM atrazine, 0 to 15 µM bromacil, 0 to 2 µM DCMU and 0 to 22 µM metribuzin. Average A_{750} was plotted against log herbicide concentration and the value corresponding to the half-maximum A_{750} (50% in reference to A_{750} of untreated control) was derived graphically from the resulting curve for each genotype / inhibitor combination. This value was defined as the I_{50}. Herbicide concentrations leading to 100% growth inhibition (MLHC) were determined by extrapolation of the curves to the *X*-axis when data points were within the interval A_{750} = 0–0.005.

Results

Analysis of autotrophic growth

The wildtype control *A251A** as well as the Ala_{251} mutants *A251C**, *A251P**, *A251S**, *A251G**, *A251I**, *A251L** and *A251V** grew photoautotrophically in both LL and HL on solid HS minimal medium (spot tests, data not shown). To compare photoautotrophic growth of these seven mutants more precisely, we measured biomass accumulation (A_{750}) in HL using the quantitative microtiter growth assay. Doubling times obtained for independently isolated transformants of the same genotype were identical as shown in Fig. 1 for *A251V**. Therefore, a single transformant was randomly chosen to represent each mutation in all subsequent experiments.

These seven A_{251} mutants and *dr-1* can be divided into three phenotypic classes based on their autotrophic doubling times under different light intensities (Fig. 2). 1) Equivalent to wildtype in HL and LL (CC-125, 3.5±0.0 h and 8.7±0.2 h, respectively): transformed control *A251A**, *A251C**, *A251P**, *A251S**, *A251G**. 2) Increased relative to wildtype only in HL: *dr-1* (S264A). 3) Increased relative to wildtype at all light intensities: doubling times of *A251I** and *A251V** were increased by 80% in VLL and by 40–50% in LL and HL, respectively. In contrast, doubling times of *A251L** were increased by 100% in VLL, 40% in LL and 220% in HL.

Herbicide resistance

All of the photosynthetically competent D1 mutants were tested for inhibition of photoautotro-

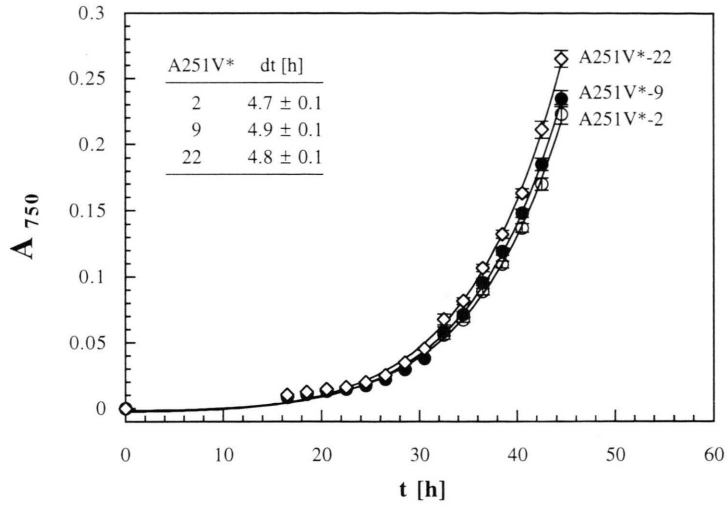


Fig. 1. Comparison of photoautotrophic growth and calculated doubling times of three independently isolated *A251V** transformants grown under HL in microtiter plates. Biomass accumulation was measured in liquid 1 ml cultures in microtiter plates. Differences in doubling times are not significant at 95% confidence level by the Tucker test.

phic growth on solid and in liquid media by four PSII herbicides representing chemically different inhibitor classes. The minimum lethal herbicide concentration (MLHC) for all eight mutants was first determined in spot tests on solid HS medium under HL. The four A_{251} mutants showing wild-type-like photoautotrophic growth rates (*A251C**, *A251P**, *A251S**, *A251G**) were also identical to wildtype in their spectrum of herbicide sensitivity (MLHC = 5 μM atrazine, 3 μM bromacil and DCMU, 1 μM metribuzin). MLHC and I_{50} resistance levels were then quantified in liquid HS culture for those mutants showing higher than wild-type herbicide resistances on plates (*A251I**, *A251L**, *A251V** and *dr-1*).

Absolute and relative resistance to the four different herbicides varied between the *A251I**, *A251V** and *A251L** mutants and the *dr-1* mutant in HL (Table I). Resistance between genotypes was not significantly affected by growth in liquid or on solid medium, but in every case resistance was lower in liquid medium where exposure to herbicide would not be expected to be diffusion limited. MLHC values were similar between *A251I**, *A251V** and *A251L** mutants, but relative to wildtype all mutants showed 6–7.5x increased resistance to atrazine, 10–13x (27x on solid medium) increased resistance to bromacil and 100–200x increased resistance to metribuzin. All three mutants remained as sensitive to DCMU as wild-type. The S264A mutation in *dr-1* conferred the highest relative resistance to atrazine, DCMU and metribuzin and similar levels of bromacil resistance as *A251I**, *A251V** and *A251L**. In comparison to the A_{251} mutants, *dr-1* grew on 2.5–5x higher concentrations of atrazine, 13–18x concentrations of DCMU and 2.5–6x concentrations of metribuzin.

Table I. Minimal lethal herbicide concentration in μM (MLHC) on solid HS (s) and in liquid HS (l) medium under HL. MLHC for *A251C**, *A251P**, *A251S** and *A251G** mutants on solid medium were comparable to the values shown for CC-125 and *A251A**. Therefore, these mutants were not analyzed in liquid medium.

Strain	Atrazine			Bromacil			DCMU			Metribuzin		
	s	l	s/l	s	l	s/l	s	l	s/l	s	l	s/l
CC-125	5	0.5	10	3	1	3	3	1	3	1	0.1	10
*A251A**	5	0.8	6	3	1	3	3	0.5	6	1	0.1	10
*A251I**	30	6	5	80	12	7	3	0.5	6	100	20	5
*A251V**	30	4	7.5	80	10	8	3	0.5	6	100	20	5
*A251L**	30	7	4	80	15	5	3	1.5	2	100	20	5
dr-1	70	20	3.5	80	13	6	40	9	4	600	50	12

We also compared the impact of LL and HL on MLHC in the *A251I**, *A251V** and *A251L** mutants and the *dr-1* mutant on solid and in liquid media. A nine fold difference in light level had no effect on resistance patterns of any of the four mutant genotypes (data not shown). Because the same degree of resistance was observed in LL and HL, we assumed that differential light sensitivity associated with the different mutations had no influence on responsiveness to herbicides. Quantitative I_{50} assays were therefore carried out only in HL.

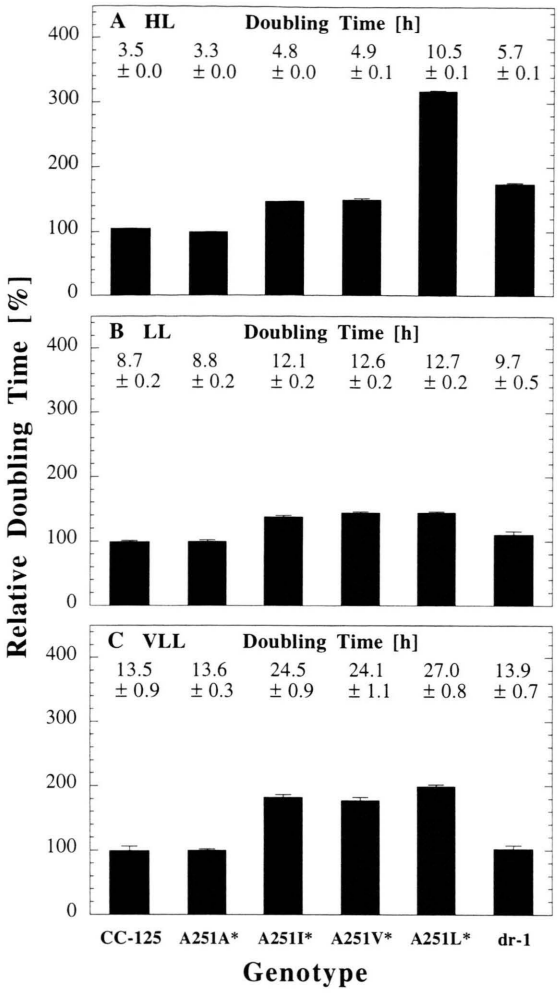


Fig. 2. Relative average photoautotrophic doubling times of wildtype CC-125 and the *A251A** control, seven A_{251} mutants and the S_{264} *dr-1* mutant determined by the microtiter plate assay and standardized to doubling times of *A251A**. Cells were grown under constant irradiance at HL (panel A), LL (panel B) and VLL (panel C). Standard error bars for the means of 5 to 15 replicates are shown. In certain cases errors are so small that they are not visible above the columns. HL and LL doubling times of *A251C** (3.2±0.0 h, 8.7±0.1 h), *A251P** (3.2±0.1 h, 8.9±0.1 h), *A251S** (3.2±0.1 h, 8.7±0.2 h), *A251G** (3.3±0.0 h, 8.8±0.1 h) are not plotted as they are identical to those for CC-125 and *A251A** control.

I_{50} for biomass accumulation derived from inhibitor response curves of the *A251I**, *A251V** and *A251L** mutants are compared to that for *dr-1* in Fig. 3, panels A-D. *A251I** and *A251V** showed enhanced resistance to atrazine and metribuzin, but resistance measured by I_{50} was less than that demonstrated by MLHC. In the case of bromacil, both I_{50} and MLHC were equally increased. The DCMU I_{50} values of the two mutants (0.3–0.6x wildtype) may indicate slight hypersensitvity to the compound. Unlike resistance levels determined by MLHC, *A251L** proved to be more resistant (about 2x) to atrazine, bromacil and metribuzin by I_{50} than *A251I** and *A251V**. *A251L** also showed no hypersensitivity to DCMU. The *dr-1* mutant had the highest I_{50} and MLHC of the four mutants tested on all four inhibitors. Relative differences in resistance between the A_{251} mutants with respect to wildtype and *dr-1* were more apparent when I_{50} rather than MLHC results were compared. The relative resistance pattern of the four mutants is reflected in the different slopes and shapes of the inhibition curves in Fig. 3.

Discussion

Replacing Ala_{251} in the D1 protein with either Ile, Leu or Val (*A251I**, *A2251L** and *A251V**) resulted in resistance to atrazine, bromacil and metribuzin but not DCMU and reduced photoautotrophic growth over a broad range of light intensities (Fig. 2) in the absence of herbicides. In contrast, Cys, Pro, Ser and Gly substitutions (*A251C**, *A251P**, *A251S** and *A251G**) produced a growth and herbicide resistance phenotype indistinguishable from that of wildtype. Comparative analysis of these otherwise isogenic mutants under controlled conditions of light, temperature, agitation and CO_2 permits precise measurement of the effects of the amino acid substitutions at the Ala_{251} D1 residue on herbicide resistance and photoautotrophic growth.

A251V mutations have been isolated previously in *C. reinhardtii* (*MZ2*, Pucheu *et al.*, 1984; Johanningmeier *et al.*, 1987), in the cyanobacteria *Synechocystis* PCC 6803 and PCC 6714 [as a double mutant *AzV* (F211S/A251V, Kirilovsky *et al.*, 1989) and as the single mutant *M35* (A251V, Perewoska *et al.*, 1994)]. Initial characterization of the *Chlamydomonas MZ2* mutant reported no significant effect on photosynthetic net O_2 evolution, chlorophyll content and thylakoid proteins (Pucheu *et al.*, 1984). In contrast, our isogenic *A251V** transformant as well as the *A251I** and *A251L** transformants showed increased photoautotrophic doubling times when grown under VLL,

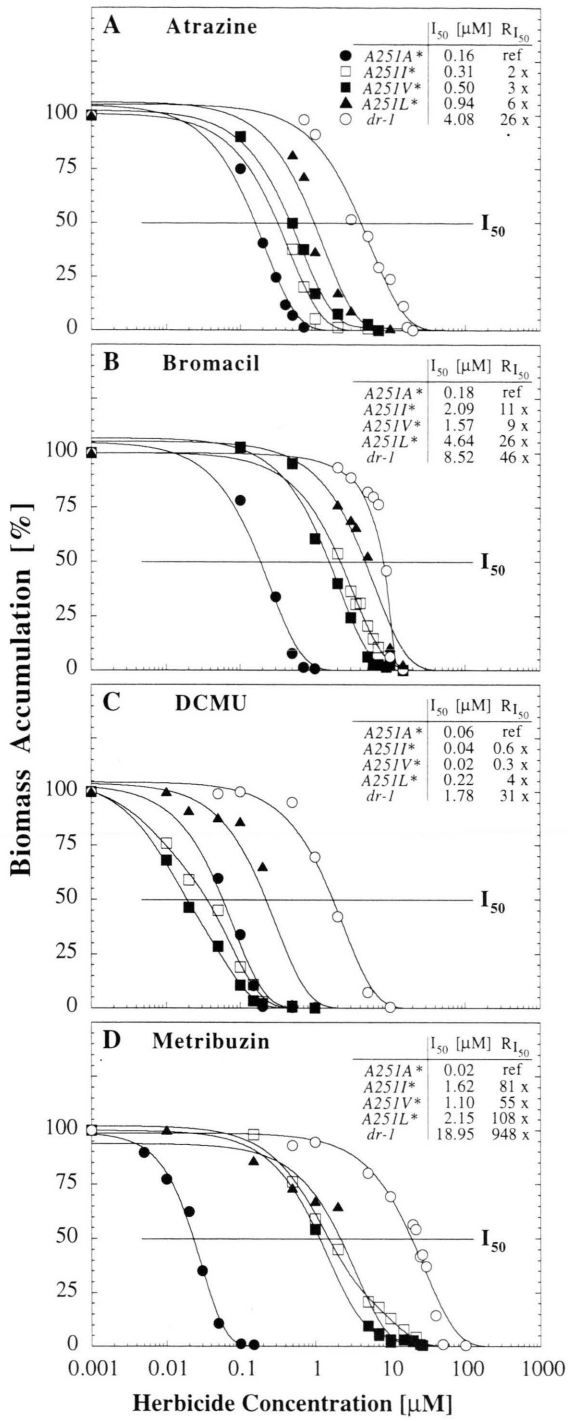


Fig. 3. Relative average photoautotrophic biomass accumulation of genotypes *A251A**, *A251I**, *A251V**, *A251L** and *dr-1* in the presence of various concentrations of PSII herbicides. Panel **A**: atrazine, **B**: bromacil, **C**: DCMU, **D**: metribuzin. Each curve has been standardized to the corresponding untreated control which was considered to represent 100% biomass accumulation. The I_{50} is marked by the horizontal line. Absolute I_{50} concentrations and relative resistances compared to *A251A** are shown in the inset tables. The intercept of individual curves with the Y-axis varies around the 100% value because optimal fits to the data points were used for generation of the curves.

LL and HL. Photosynthetic capacity in these three mutant transformants, measured as quantum yield, maximum rate of oxygen evolution and fluorescence yield, were also reduced (Lardans *et al.*, 1997b). Our data are consistent with biophysical measurements showing impaired $Q_A \rightarrow Q_B$ electron transfer (Crofts *et al.*, 1993) and equilibrium constants indicative of slower reoxidation of Q_A- and weaker binding of Q_B in *MZ2*. The cyanobacterial mutant *M35* showed a similar disturbance of the Q_A-/Q_B redox couple (Etienne *et al.*, 1990; Perewoska *et al.*, 1994; Constant *et al.*, 1996). With regard to herbicide resistance, *A251V** showed nearly the same resistance pattern based on whole cell growth inhibition to that reported earlier for *MZ2* (Pucheu *et al.*, 1984; Wildner *et al.*, 1990) and for the cyanobacterial *M35* mutant (Kirilovsky *et al.*, 1989; Perewoska *et al.*, 1994) when resistance was assayed as inhibition of electron transfer in thylakoid fractions.

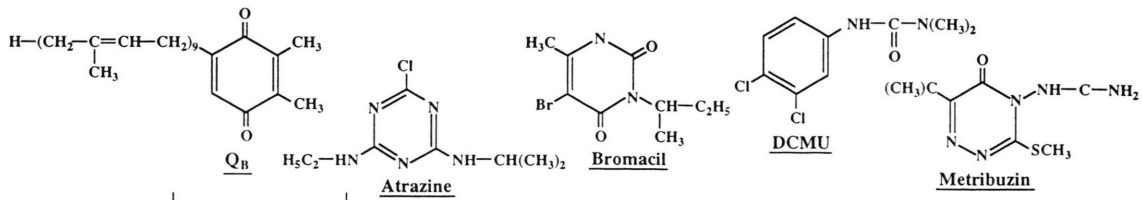
Based on these observations, the Ala_{251} residue of D1 was suggested to play a key role in structural integrity of the Q_B / herbicide binding site as well as in the conformation of the PSII donor side, both of which are changed when Ala is replaced by bulkier residues like valine (Astier *et al.*, 1993; Perewoska *et al.*, 1994; Constant *et al.*, 1996). In this model Ala_{251} is thought to be close to or embedded in the thylakoid membrane, dividing the helix IV-V loop into two functionally different domains involved in Q_B / herbicide binding and D1 structure and / or stability.

In contrast, the presence of Ala or Gly instead of Ser at position 264 of D1 directly eliminates H-bonding to Q_B and DCMU (Trebst, 1987; Crofts *et al.*, 1993). As a result, acceptor side electron transfer from Q_A to Q_B is severely impaired, leading to photoinhibition at higher light intensities that are permissive for wildtype. This is consistent with our observation that growth of *dr-1* (S264A) is reduced in HL but not in LL where the rate of photon capture is not sufficient to saturate photosynthetic electron transport in either wildtype or *dr-1* (Heifetz *et al.*, 1997). The *A251I**, *A251V** and *A251L** transformants, on the other hand, show neither this light dependent growth response nor DCMU resistance, and D1 function is chronically disabled at all light intensities. The relative bulkiness of the side chains of Ile, Val and Leu may therefore disturb the conformation of the IV-V loop of D1. Consequently, electron transfer capacity of D1 at the level of Q_B as well as binding of atrazine, bromacil and metribuzin could be reduced in these mutants without directly affecting the specific bonds ligating Q_B or DCMU to residues within this domain of the D1 protein [reviewed in Xiong *et al.*, (1996)].

We find that substitution of Ile or Val for Ala_{251} results in equivalent phenotypic changes whereas substitution of Leu increases both herbicide resistance and impairment of autotrophic growth relative to the Ile and Val mutations (Table II). Val, Ile, Leu and Ala are all classified as hydrophobic aliphatic residues and hence differ primarily in bulkiness of their side chains. Compared to Ala, the larger Val, Ile and Leu residues might interfere with both Q_B and herbicide access to their respective overlapping binding sites on the D1 protein (Trebst and Draber, 1979; Wildner *et al.*, 1990). Alternatively, the mutations may alter the energetic / steric binding properties of these sites. Either situation could explain the impaired $Q_A \rightarrow Q_B$ electron transfer observed in these mutants (Lardans *et al.*, 1997b), and therefore the reduction in photosynthetic efficiency and photoautotrophic growth under various light regimes, as well as variations in levels of resistance to different herbicides. Thus, impaired growth, possibly linked to Q_B function, and resistance to "Serine-type" herbicides (Trebst, 1987) appear to be correlated with the R group structure at residue 251. However, differences in side chain structure between Ile, Val and Leu associated with slightly different attachments of a methyl group to the carbon backbone in the R group do not provide an obvious explanation for our observation that the Leu substitution results in higher levels of herbicide resistance and increased doubling times compared to the Val and Ile substitutions. Our results also suggest that growth rates may be inversely correlated, at least in certain cases, with increased levels of herbicide resistance as is inferred for *A251L** which has the slowest HL growth rate and concommitantly highest resistance to bromacil, metribuzin and a slight increase in resistance to DCMU (Table II).

Another possible explanation for the effect of the Ala_{251} substitutions on growth may be impairment of proton access to the Q_B site. Models of the photosynthetic bacterial reaction center (Deisenhofer *et al.*, 1985; Breton and Vermeglio,

Table II. Comparison of growth rates and relative resistance levels to four different "Serine-type" PSII herbicides in seven mutants with different amino acid substitutions at the D1 residue Ala_{251}. Growth rates = ln2 / doubling time. Increasing resistance levels are indicated by + to ++++.



D1 251 R group	HL growth rate	Herbicide resistance based on I_{50}			
$CH_3-CH_2-CH(CH_3)-$ Ile	.146	+	+	-	+++
$(CH_3)_2CH-$ Val	.143	+	+	-	+++
$(CH_3)_2CH-CH_2-$ Leu	.066	+	++	+/-	++++
CH_3- Ala	.212	-	-	-	-
$HS-CH_2-$ Cys	.217	-	-	-	-
Pro	.217	-	-	-	-
$HO-CH_2-$ Ser	.217	-	-	-	-
H- Gly	.211	-	-	-	-

1988), reveal proton transfer pathways which connect the Q_B niche buried in the thylakoid membrane with the stromal aqueous phase. Various amino acids in this niche interact with H_2O molecules in the two step protonation of Q_B^{2-} (Hanson *et al.*, 1993; Paddock *et al.*, 1994; Stowell *et al.*, 1997). A similar but not identical mechanism of H^+ delivery to Q_B^{2-} was proposed for PSII that includes bicarbonate in addition to H_2O and amino acid-bound H in the proton shuttle (Xiong *et al.*, 1996). Bicarbonate has also been shown to be essential for electron transfer from Q_A- to Q_B in association with the non-heme iron in the PSII reaction center, presumably functioning as ligand to Fe in analogy to the bacterial system (Xiong *et al.*, 1996). Hence, PSII function requires both bicarbonate and H_2O access to the Q_B pocket. In Xiong *et al.*'s model, Ala_{251} is thought not to participate directly in bicarbonate binding or proton transfer onto Q_B, but replacement of Ala by larger, hydrdophobic residues is likely to interfere with either accessibility of these cofactors to their binding sites in the vicinity of Q_B or to disturb the conformation and electric properties of this domain of the D1 protein, which may lead to the growth impairment seen in *A251I**, *A251V** and *A251L**.

We also found that replacement of Ala_{251} with Cys, Pro, Ser or Gly residues has no effect on herbicide binding or photoautotrophic growth, suggesting that the overall conformation of the Q_B / herbicide binding pocket in these mutants is not altered to a degree that would interfere with D1 function required for normal photoautotrophic growth. Cys, Ser and Gly are structurally similar to Ala with regard to size and hydrophobicity. Their aliphatic side chains are relatively small and unbranched, consisting of either a one-carbon R group (Ala, Cys and Ser) or a simple hydrogen R group (Gly) (Table II). Cys and Ser side chains differ from that of Ala by a SH or OH group, respectively, which adds polarity to these residues depen-

dent on the reduction state of the particular side group. Pro is remarkably different in structure from either group of amino acids mentioned here because of its 5-memberring that includes the amino group at C_1. Nevertheless, Pro is also considered to be relatively small and hydrophobic and appears to replace Ala in the D1 protein with the same minimal consequences as Cys, Ser or Gly. Overall, none of these "small hydrophobic" substitutions at D1 residue 251 seem to have any phenotypic consequences on herbicide resistance or photoautotrophic growth. This stands in contrast to the marked effects of larger, more complex and charged side chains that lead to loss of photosynthetic function (Arg, Asp, Gln, Glu and His) (Lardans *et al.*, 1997a) or reduced function with physiological consequences (Ile, Val and Leu) (this paper). Taken together, these results suggest that for normal function with regard to photoautotrophic growth and herbicide sensitivity, the D1 protein can tolerate a variety of small residues at position 251 regardless of charge or hydrophobicity. However, conservation of the Ala_{251} residue during evolution implies that it may be important for optimal D1 function. Our results suggest that the degree of herbicide resistance and increase in photoautotrophic doubling times are correlated properties in specific A_{251} mutants. A detailed assessment of the effect of these seven A_{251} mutations on O_2 evolution, photosynthetic electron transfer measured by fast real rate fluorimetry and thermoluminescence, D1 synthesis and carbon accumulation will be published elsewhere (Lardans et al., 1997b).

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