Herbicide Resistance and Growth of D1 Ala251 Mutants in Chlamydomonas

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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. Ala251 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 Ala251 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 Ala251 mutations differed markedly from an Ala mutant (dr-1) at the well characterized Ser264 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 Phe231 and Leu275 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 Qb, 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 Qb (e.g. Gronwald, 1994). Herbicide resistance is thought to result from structural modifications to the Qb site which may alter both herbicide and Qb 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 Ser264→Ala mutation in the D1 proteins of Chlamydomonas reinhardtii (Erickson et al., 1984) and cyanobacteria (Golden and Haselkorn, 1985) and by the Ser264→Gly mutation in the D1 protein

Abbreviations: atrazine, 2-chloro-4-ethylamine-6-isopropylyamine-S-triazine; bromacil, 5-bromo-3-methyl-3-(1,1-dimethylurea) uracil; DCMU, 3 (3,4-dichlorophenyl)-1,1-dimethyl urea; I50, 50% inhibition of autotrophic biomass accumulation; metribuzin, 4-amino-6-(1,1 dimethyl-ethyl)-3-(methylthio)-1,2,4-triazin-5(4H)-one; MLHC, minimal lethal herbicide concentration; PSII, photosystem II; Qa, Qb, quinones, stable electron acceptors at PSII; spr, spectominycin resistance mutation. Reprint requests to Prof. John E. Boynton. Fax: (919) 613–8177.
of higher plants [Hirschberg and McIntosh, 1983; reviewed in Gronwald (1994)]. However both the Ser264 → 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 Qb and Ser264 in the wildtype D1 protein. The herbicide binding site spatially and structurally overlaps with the Qb site to the extent that a single amino acid change at position 264 both impairs Qb binding and results in a DCMU resistant phenotype (Trebst, 1987; Bowyer et al., 1990; Mackey and O’Malley, 1993).

In contrast, Ala251 → Val mutants in Synechocystis and Chlamydomonas reinhardtii differ from the Ser264 → 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; Johannimeier et al., 1987; Kirilovsky et al., 1989; Wildner et al., 1990; Perewoska et al., 1994). A current model of the D1 protein proposes that Ala251 is positioned in the IV-V loop at the border of an amphipathic parallel helix which forms a “bottleneck” at the entrance to the Qb 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 Ala251 → 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 Ala251 in the D1 protein in C. reinhardtii [(Lardans et al., 1997a and 1997b)]. Five of the amino acid substitutions (Arg, Asp, Gin, 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 Ala251 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 Ser264 (dr-1) mutant was limited only in high light.

Materials and Methods

Chlamydomonas strains and generation of Ala251 transformants and suppressors

Chlamydomonas reinhardtii wildtype strain CC-125 (Harris, 1989) and the transformed mutant strain CC-2827 dr-1 (dr = DCMU resistant, psbA Ser264 → 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’-CTTACAATATT247GTAGCTGATT251CATAGTAAAAC-3’) was used to generate Val251 (GTT, A251V*) or Ile251 (ATT, A251I*) mutations and psbA251–4 (5’-CTTACAATATT247GTAGCTGTT251CATAGTAAAAC-3’) was used to generate Ala251 (GCT, A251A*) or Arg251 (CGT, A251R*) mutations. One base pair change in the Asn247 codon (ATC → ATT, in bold) created a SspI RFLP marker. Fragments carrying the psbA mutations Ile251 and Val251 and the linked RFLP marker were inserted into the same position in the 3.1 kb Kpnl psbA wildtype fragment of P-300 (A. Lers, unpublished) to generate P-432 (A251I*) and P-433 (A251V*). Construction of plasmids carrying the Ala251, Arg251 and Gly251 mutations (plasmids P-435, P-436 and P-434, respectively) and isolation of homoplastic 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 A251 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 A750 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 (A750 = 0.050 to 0.120, Bausch & Lomb Spectronic 21) and diluted to A750 = 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 (A750) 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 A750 = 0.003, and regrown twice to mid-log phase. The second diluted precultures (A750 = 0.003, ca. 10⁵ 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 H₂O. Four plates were incubated together under sterile conditions in a closed transparent chamber (30.5 x 22.8 x 4.3 cm, 3000 cm³) 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% CO₂ supplied to the chamber at a flow rate of 600 cm³/min provided 12 changes of atmosphere per hour. Lower CO₂ 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), A750 was measured directly in the sterile 24 well plates at regular intervals (HL: 2 h, LL: 4 h, VLL: 6 h) until stationary phase was reached. Log A750 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: 

\[ C(t) = C_0 e^{dt}, \quad dt = \ln 2 / g \quad [C_i, A_{750} \text{ at } t_i; \quad C_0, \text{ initial } A_{750}, g, \text{ exponential growth rate}; dt, \text{ doubling time } [\text{h}]. \]

Results were averaged from at least two independent experiments involving separate pre-growth 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 I50 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, recrystallizing the herbicide from the supernatant and washing the crystals in several volumes of deionized H2O. 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 Ala251 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-
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 (A$_{251}C^*$, A$_{251}P^*$, A$_{251}S^*$, A$_{251}G^*$) 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 (A$_{251}I^*$, A$_{251}V^*$ and A$_{251}L^*$ muta-
tants and the dr-1).

Absolute and relative resistance to the four different herbicides varied between the A$_{251}I^*$, A$_{251}V^*$ and A$_{251}L^*$ 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 A$_{251}I^*$, A$_{251}V^*$ and A$_{251}L^*$ 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 wildtype. The S264A mutation in dr-1 conferred the highest relative resistance to atrazine, DCMU and metribuzin and similar levels of bromacil resistance as A$_{251}I^*$, A$_{251}V^*$ and A$_{251}L^*$. 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.

We also compared the impact of LL and HL on MLHC in the A$_{251}I^*$, A$_{251}V^*$ and A$_{251}L^*$ 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 A25i mutants and the S264 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*, A251P*, A251S* and A251G* (3.2±0.0 h, 8.9±0.1 h, 8.7±0.2 h and 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.

I50 for biomass accumulation derived from inhibitor response curves of the A251I*, A251V* and A251L* mutants are compared to that for dr-I in Fig. 3, panels A-D. A251I* and A251V* showed enhanced resistance to atrazine and metribuzin, but resistance measured by I50 was less than that demonstrated by MLHC. In the case of bromacil, both I50 and MLHC were equally increased. The DCMU I50 values of the two mutants (0.3–0.6x wildtype) may indicate slight hypersensitivity to the compound. Unlike resistance levels determined by MLHC, A251I* proved to be more resistant (about 2x) to atrazine, bromacil and metribuzin by I50 than A251I* and A251V*. A251I* also showed no hypersensitivity to DCMU. The dr-I mutant had the highest I50 and MLHC of the four mutants tested on all four inhibitors. Relative differences in resistance between the A25i mutants with respect to wildtype and dr-I were more apparent when I50 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 Ala251 in the D1 protein with either Ile, Leu or Val (A251I*, A251L* 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 CO2 permits precise measurement of the effects of the amino acid substitutions at the Ala251 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, Perekowska et al., 1994)]. Initial characterization of the Chlamydomonas MZ2 mutant reported no significant effect on photosynthetic net O2 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 \( M22 \). 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 \( M22 \) (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 \( \text{Ala}_{251} \) 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 \( \text{Ala}_{251} \) 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 \( \text{dr-1} \) (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 \( \text{dr-1} \) (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 \( \text{Ala}_{251} \) 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 \( A251I^* \) which has the slowest HL growth rate and concomitantly highest resistance to bromacil, metribuzin and a slight increase in resistance to DCMU (Table II).

Another possible explanation for the effect of the \( \text{Ala}_{251} \) 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 Ala251. Growth rates = ln2 / doubling time. Increasing resistance levels are indicated by + to ++++.

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<tr>
<th>D1 251 R group</th>
<th>HL growth rate</th>
<th>Herbicide resistance based on I50</th>
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<tr>
<td>CH$_3$-CH$_2$-CH$_3$</td>
<td>.146</td>
<td>+</td>
</tr>
<tr>
<td>CH$_3$-CH$_2$-CH$_3$- Val</td>
<td>.143</td>
<td>+</td>
</tr>
<tr>
<td>CH$_3$-CH$_2$-CH$_3$- Leu</td>
<td>.066</td>
<td>+</td>
</tr>
<tr>
<td>CH$_3$- Ala</td>
<td>.212</td>
<td>-</td>
</tr>
<tr>
<td>HS-CH$_2$- Cys</td>
<td>.217</td>
<td>-</td>
</tr>
<tr>
<td>Pro</td>
<td>.217</td>
<td>-</td>
</tr>
<tr>
<td>HO-CH$_2$- Ser</td>
<td>.217</td>
<td>-</td>
</tr>
<tr>
<td>H- Gly</td>
<td>.211</td>
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</table>

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$_2$O 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$_2$O 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$_2$O access to the Q$_B$ pocket. In Xiong et al.’s model, Ala251 is thought not to participate directly in bicarbonate binding or proton transfer onto Q$_B$, but replacement of Ala by larger, hydrophobic 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 Ala251 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...
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