# The *D–E* Region of the D1 Protein Is Involved in Multiple Quinone and Herbicide Interactions in Photosystem II†

Hadar Kless,*,‡ Michal Oren-Shamir,‡ Shmuel Malkin,§ Lee McIntosh,∥ and Marvin Edelman‡

*Departments of Plant Genetics and Biochemistry, Weizmann Institute of Science, Rehovot, 76100 Israel, and MSU-DOE Plant Research Laboratories and Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824*

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ABSTRACT: The region between helices *D* and *E* (*D–E* region) of the D1 protein of photosystem II (PSII) is exposed at the stromal side of the photosynthetic membrane, contains the secondary plastoquinone ($Q_B$) binding niche, and is involved in processes at the reducing side of PSII. The role of the *D–E* region was studied in 27 site-directed mutants generated in the *psb*AII gene of the cyanobacterium *Synechocystis* sp. PCC 6803. The photochemical performance of the modified PSII reaction centers was assessed with respect to photoautotrophic growth, oxygen evolution, fluorescence induction, and herbicide inhibition. A few mutations, located at positions presumably involved in essential interactions in the $Q_B$ binding niche, greatly interfered with PSII performance. On the other hand, mutations in the presumptive loop region between helices *D* and *de* resulted in relatively minor effects, indicating a flexible region not critical for photochemical function. Indeed, although more than 80% of the *D–E* region is phylogenetically invariant, the bulk of the mutations affected the measured parameters only moderately. The significance of the conserved residues appears to be in subtle interactions that optimize the thermodynamic balance between some of the redox components of PSII, as indicated by mild changes in the steady state fluorescence. Many mutations modified tolerances to PSII herbicides. The dispersion of these mutations throughout the *D–E* region indicates the complex nature of the interactions, direct and indirect, affecting herbicide binding in the $Q_B$ niche. Mutation of codons Ser221 and Ser222 to Leu221 and Ala222 revealed a new location coordinating the herbicide diuron in the D1 protein. Mutational analysis of the *D–E* region fully supports the widely held analogy in structure and activity between the D1 protein of PSII and the L subunit of the bacterial reaction center.

The protein sequences and distribution of hydrophobic domains in the D1 and D2 proteins of photosystem II (PSII) resemble those in the L and M polypeptides of the purple bacterial reaction center (Rochaix et al., 1984; Michel & Deisenhofer, 1988). Similar cofactors and pathways of electron transfer also exist in both complexes, with specific liganded residues aligned in similar locations (Youvan et al., 1984; Trebst, 1986). The functional and, to a lesser extent, structural analogies between the two reaction centers serve as the framework for a widely accepted model of PSII, in which the D1 and D2 proteins form the core of the reaction center (Deisenhofer et al., 1985; Trebst, 1986). The homologous regions between the core proteins of the two reaction centers are mainly evident at the ends of the transmembrane helices, in the regions of the primary donors and acceptors, and near the primary and secondary quinones (cf., Michel & Deisenhofer, 1988). However, the lack of protein sequence homology in the exposed loop regions, as well as the presence of unique protein subunits in each reaction center [for a review see Mattoo et al. (1989); Vermaas & Ikeuchi, 1991], also highlights significant differences between the two.

The *D–E* region of the D1 protein is taken to include those parts of transmembrane helices *D* and *E* adjacent to the stromal side and the exposed residues in between. The exposed residues incorporate the parallel helix, *de*, and two connecting loops: *D–de* and *E–de* (Michel & Deisenhofer, 1988). The *D–E* region is involved in PSII reducing side activities, with various residues interacting with $Q_B$, herbicides (Ohad & Hirschberg, 1992), and bicarbonate (Diner & Petrouleas, 1990; Govindjee et al., 1991).

The *D–E* region of the D1 protein contains overlapping binding sites for several classes of herbicides, such as triazines, ureas, and phenylureas. These compounds block electron flow between $Q_A$ and $Q_B$ by displacing the secondary quinone from its binding niche. Herbicide-resistant biotypes show amino acid substitutions clustered in the *D–E* region, while azido-herbicide labeling experiments indicate specific residues binding these inhibitors [for reviews, see Trebst et al. (1988) and Bowyer et al. (1991)]. Mutations affecting response to these same herbicides are clustered in the *D–E* region of the L subunit as well (cf., Sinning, 1992).

The amino acid sequence of the D1 protein is phylogenetically conserved (Svensson et al., 1991), with the *D–E* region from 42 cyanobacterial, algal, and plant species being 90% similar and >80% identical (J. Hirschberg, personal communication). This suggests a strict requirement for most of these residues. The *D–E* region of the D1 protein is 17 residues longer than that of the L protein, with the extension concentrated mainly in the *D–de* loop. The extra residues were proposed to carry information for rapid, light-dependent degradation of the protein (Greenberg et al., 1987).

In this study, we investigate the role of the *D–E* region of the D1 protein by the analysis of 27 *psb*AII mutants of *Synechocystis* sp. PCC 6803. Mutants were prepared to carry either short deletions or single amino acid changes within the region. Characterization of the mutants indicates a more

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* Address correspondence to this author at the following address: Department of Botany and Center for the Study of Early Events in Photosynthesis, Arizona State University, Tempe, AZ 85287-1601. Telephone (602) 965-3698; Fax (602) 965-6899.

‡ Department of Plant Genetics, Weizmann Institute of Science.

§ Department of Biochemistry, Weizmann Institute of Science.

∥ Michigan State University.

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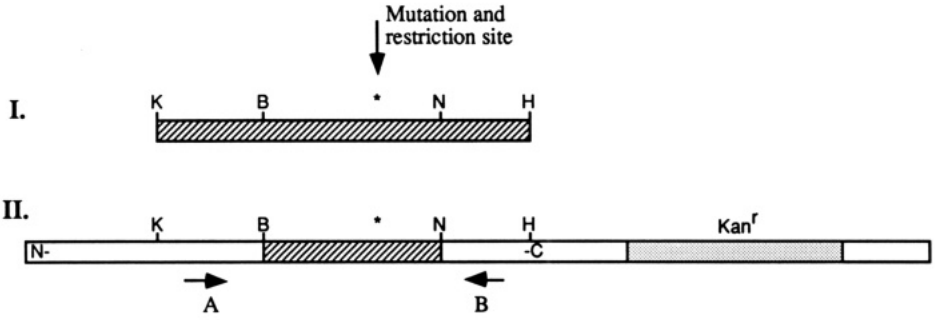


FIGURE 1: Mutagenesis scheme of the *psb*AII gene of *Synechocystis* sp. PCC 6803. (I) Site-directed mutagenesis was initially performed in a Bluescript plasmid (KS$^-$) containing a *Kpn*I–*Hinc*II (K, H) fragment of the *psb*AII gene [nucleotides 536–1074, respectively; numbering according to Ravnikar et al. (1989)]. (II) A *Bst*EII–*Nco*I (B, N) fragment bearing the introduced mutations (*) was cloned from the KS$^-$ construct to replace the wild-type sequence in the mutagenesis cartridge. This cartridge contains an additional kanamycin resistance gene (Kan$^r$). The N-terminus (truncated to a *Hinc*II site) and the C- terminus of the open reading frame of the *psb*AII gene are indicated.

comprehensive involvement of the *D–E* region in $Q_B$ and herbicide interactions than was previously recognized.

## MATERIALS AND METHODS

*Strains and Growth Conditions. Synechocystis* sp. PCC 6803 cells were grown in BG-11 medium (Williams, 1988). Mutant cultures were maintained on 1.5% agar (Difco) plates in the presence of 20 $\mu$g/mL spectinomycin, 2.5 $\mu$g/mL chloramphenicol, and 5 $\mu$g/mL kanamycin. Glucose (5 mM) and 20 $\mu$M atrazine were routinely added to plates in order to maintain PSII-independent growth. Liquid cultures were grown in 250-mL flasks containing 40 mL of BG-11 medium on a gyratory shaker (100 rpm) at 30 °C and 50 $\mu$E m$^{-2}$ s$^{-1}$ fluorescent light. The growth chamber was aerated (2 L min$^{-1}$) using an aquarium pump. Glucose (5 mM) was added to the liquid cultures except where indicated. Cell density was estimated by measuring turbidity at 730 nm after diluting cultures to an OD value of <0.5. Under these conditions, the turbidity was roughly proportional to the cell concentration. Chlorophyll concentration was determined by methanol extraction (Tandeau de Marsac & Houmard, 1988). Cells for photosynthetic measurements were harvested at the end of their logarithmic growth phase, which corresponded to a chlorophyll concentration of 4–7 $\mu$g/mL. The wild-type strain for these studies lacks the *psb*AI and -III genes, which were replaced by spectinomycin (Sm$^r$) and chloramphenicol (Cm$^r$) resistance cartridges, respectively (Debus et al., 1988). The mutagenesis cartridge, containing a kanamycin resistance gene (see Figure 1), was introduced to the above strain without any mutation, thus retaining the intact wild-type *psb*AII gene.

*Transformation and Nucleic Acid Manipulations.* Transformation of *Synechocystis* sp. PCC 6803 (Williams, 1988) and site-directed mutagenesis (Kunkel et al., 1987) were performed as described. The single-stranded DNA (corresponding to the antisense strand) of the Bluescript KS$^-$ plasmid construct (Stratagene) was prepared in *Escherichia coli* CJ-236. Following second-strand polymerization using T4 polymerase (New England Biolabs) and the mutagenesis primers (Table 1), the plasmids were recovered in *E. coli* MV1190. The fragment containing the introduced mutations was cloned from the KS$^-$ construct to replace the wild-type sequence in the mutagenesis cartridge (Figure 1). The mutagenesis cartridge is a modification of pRD1031Km$^r$ (Debus et al., 1988) by deletion of its 5′ end up to the *Hinc*II site (nucleotide 174), leaving unique *Bst*EII, *Kpn*I, and *Hinc*II restriction sites (Figure 1). This cartridge contains an additional kanamycin resistance gene (Kan$^r$) just downstream of *psb*AII and was used to transform an Sm$^r$/Cm$^r$ mutant line as described (Debus et al., 1988). Following *Synechocystis* transformation, kanamycin-resistant colonies were isolated and a PCR-generated DNA fragment was amplified from the genomic *psb*AII gene. PCR amplification (30 cycles of 30 s at 93 °C, 45 s at 56 °C, and 60 s at 73 °C) was performed (Ericomp) with *Taq* DNA polymerase as recommended (U.S. Biochemical), using oligonucleotides III or VIII at the 3′ and VII or IV at the 5′ parts of the gene (Table 1). The presence of the introduced mutation was verified by the restriction pattern of the PCR product. Typically, 50–80% of the kanamycin-resistant colonies contained the change in restriction site (Table 1). Direct sequencing of the PCR products was performed (Casanova et al., 1990) using either oligonucleotides I, II, V, VI, $S_{232}A$, or $S_{264}A$ (Table 1). In the mutated regions, both strands were sequenced between the *Bst*EII and *Nco*I restriction sites to cover the whole DNA fragment used in the mutated construct (see Figure 1).

*Oxygen Evolution.* The maximal rate of oxygen evolution was determined in intact cells at a chlorophyll concentration of 4–7 $\mu$g/mL using a Clark-type electrode. Measurements were performed at 25 °C in 25 mM Hepes/NaOH (pH 7.2) in the presence of 2,5-dimethyl-*p*-benzoquinone (0.1 mM) and $K_3Fe(CN)_6$ (1 mM). The light from a halogen lamp projector was filtered through an OG 570 (Schott) glass and was saturating with respect to the electron transfer rate (light intensity was ca. 2000 $\mu$E m$^{-2}$ s$^{-1}$ in the middle of the electrode cell).

*Chlorophyll Fluorescence Induction.* PSII fluorescence induction measurements (Cahen et al., 1976; Briantais et al., 1986; Krause & Weiss, 1991) were performed using whole cells (grown in the presence of glucose) at a final concentration of 2 $\mu$g of chlorophyll/mL. The excitation light was at 620 nm and had an intensity of about 70 $\mu$E m$^{-2}$ s$^{-1}$. Following the opening of an electronic shutter, which allowed the exciting light to reach the sample, fluorescence emission resulted. The fluorescence was filtered by a cutoff glass transmitting above 665 nm, detected by an HUV-4000 (EG&G) silicon photovoltaic sensor, and recorded by a storage oscilloscope. The kinetics of chlorophyll *a* fluorescence monitors the build-up of reduced primary quinone ($Q_A$) in PSII (Briantais et al., 1986; Krause & Weiss, 1991). To obtain the basal fluorescence ($F_o$), corresponding to maximally oxidized $Q_A$, the initial fluorescence was measured following at least 20 s of dark incubation of a well-aerated sample. To allow for the shutter opening time, $F_o$ was determined more precisely by extrapolating the slope of the initial fluorescence kinetics, following full shutter opening, back to a time point equal to the half-time of the shutter opening (2.5 ms). Steady state fluorescence ($F_s$) was reached and recorded after 1 s in the light. The maximal fluorescence ($F_m$), corresponding to the maximal

Table 1: List of Oligonucleotides Used for D1 Mutagenesis

| mutation[a] | oligonucleotide sequence[b] | corresponding *psbA*II position[c] | restriction site modification[d] |
|---|---|---|---|
| $SS_{221-2}LA$ | GGTAACCTTGGCCTTGGTGC | 654–673 | *Hae*III |
| $V_{224}R$ | CTCCTCCTTGCGCCGTGAAACC | 660–681 | *Hha*I |
| $R_{225}L$ | CTCCTTGGTACTGGAAACCACC | 663–684 | *Rsa*I |
| $TT_{227-8}AA$ | GTGCGTGAAGCTGCAGAAGTTG | 674–695 | *Pst*I |
| $V_{230}T$ | CCACCGAAACCGAAAGCCAGAAC | 680–702 | –*Hinf*I |
| $\Delta EVESQ_{229-33}$ | GTGAAACCACC–AACTACGGTTAC | 674–711 | –*Hinf*I |
| $S_{232}A$ (s) | GAAGTTGAAGCCCAGAACTAC | 685–705 | –*Hinf*I |
| $S_{232}D$ | GAAGTTGAAGACCAGAACTAC | 685–705 | –*Hinf*I |
| $\Delta NYG_{234-6}$ | GTTGAATCCCAG–TACAAATTCG | 688–718 | *Rsa*I |
| $Y_{237}F$ | AACTACGGTTTTAAATTCGG | 700–719 | *Dra*I |
| $K_{238}V$ | ACGGTTACGTATTCGGTCA | 704–722 | *Sna*BI |
| $\Delta YKF_{237-9}$ | CAGAACTACGGT–GGTCAAGAAGAA | 697–729 | –*Eco*RI* |
| $\Delta GQ_{240-1}$ | GTTACAAATTC–GAAGAAGAAAC | 707–734 | *Bst*BI |
| $\Delta EEE_{242-4}$ | CAAATTCGGCCAA–ACCTACAAC | 711–741 | *Hae*III |
| $\Delta YNIV_{246-9}$ | GAAGAAGAAACG–GCCGCCCAC | 724–756 | *Hae*III |
| $Y_{246}F$ | GAAGAAACCTTTAACATCGTT | 724–744 | *Mse*I |
| $V_{249}D$ | CTACAACATCGATGCCGCC | 735–753 | *Cla*I |
| $\Delta AA_{250-1}$ | TACAACATCGTT–CACGGCTAC | 736–762 | –*Fnu*4HI |
| $H_{252}V$ | ATCGTTGCTGCCGTCGGCTACT | 742–763 | –*Fnu*4HI |
| $Y_{254}T$ | GCCCACGGTACCTTTGGTCG | 751–768 | *Kpn*I |
| $\Delta YFGR_{254-7}$ | GCCCACGGC–CTGATCTTCCAA | 751–783 | *Hae*III |
| $R_{257}V$ | CTACTTTTGAGTCTTGATCTTC | 759–780 | *Hinf*I |
| $S_{264}A$ (s) | CAATATGCTGCTTTCAAC | 781–798 | *Fnu*4HI |
| $\Delta NN_{265-7}$ | GCTTCTTTC–AGCCGGTCCTTG | 787–813 | *Hpa*II |
| $\Delta SRS_{268-70}$ | TTCAACAAC–CTGCACTTCTTC | 793–822 | *Bsp*MI |
| I (s) | TGCAGATTATTCAGTTGGC | 2016–1098 | |
| II (s) | CCAGATGCCGATTACAGG | 852–835 | |
| III (p) | GGCATTGCGTTCGTGCAT | 1008–991 | |
| IV (p) | CTTCCACATGTTAGGTGT | 588–605 | |
| V (s) | CGTGTTCCAAGCTGAGCAC | 552–570 | |
| VI (s) | CTGATGTATTGAACCGAGCC | 953–972 | |
| VII (p) | CCGTATTCTTGATCTACCC | 467–485 | |
| VIII (p) | GGATTAATTCTCTAGACTC | 1245–1227 | |

[a] Oligonucleotides used for site-directed mutagenesis, PCR amplification (p), and sequencing (s) are indicated (see Materials and Methods). For a description of the mutants, see Table 2 (however, note that additional modifications appear there.) [b] Sequences are listed in the 5′ to 3′ direction. Modified nucleotides (boldface) and locations of the deleted sequences (–) are indicated. [c] Numbering for *psb*AII is according to Ravnikar et al. (1989) and L. McIntosh (unpublished). [d] Restriction sites modified in the oligonucleotides used for mutagenesis are indicated.

amount of $Q_A^-$ under the applied conditions, was determined as the $F_s$ value in the presence of 0.1 mM diuron (Kless et al., 1992; Ohad & Hirschberg, 1992). The excitation light used was within the light limiting range in all mutants, and the position of the steady state fluorescence yield, $F_s$, relative to $F_o$ and $F_m$ was indeed independent of the light intensity (measured for a range of 7–120 $\mu$E $m^{-2}$ $s^{-1}$). The kinetics of $Q_A^-$ accumulation in the photoautotrophic mutants, monitored by the fluorescence induction in the presence of diuron (0.1 mM), was similar to that of the wild type (ca. 50 ms to reach the level of $F_m$).

*Membrane Preparation.* Oxygen-evolving membranes were prepared according to Burnap et al. (1989) and Vermaas et al. (1990). Cells were harvested in the late logarithmic growth phase, concentrated (10 min, 5000*g*), and washed with 50 mM sodium phosphate, pH 7. The pellet was resuspended gently with a paint brush in 5% of the initial volume T-buffer (50 mM Hepes (pH 7), 5 mM $MgCl_2$, 50 mM $CaCl_2$, 5% (v/v) glycerol, and 0.5% (v/v) DMSO) and kept on ice for 60 min. Ten milliliters of cell suspension and 10 mL of 0.1-mm glass beads (suspended in T-buffer) were disrupted in a 35-mL plastic bottle using a Mini Bead Beater (Medi Mix-02, Medical) vibrated four times for 20 s at moderate speed. The supernatant was concentrated (12 000 rpm, 20 min), and the membrane pellet was resuspended in T-buffer and frozen at −80 °C.

*Herbicide Inhibition Measurements.* The effect of several PSII herbicides on the rate of electron transfer from $H_2O$ to the electron acceptor 2,6-dichlorophenolindophenol (DCPIP) was measured using 1 mL of thylakoids (in T-buffer) at a chlorophyll concentration of 2 $\mu$g/mL. Measurements were performed using a dual-wavelength spectrophotometer (Aminco) at room temperature and in the presence of 0.3 mM DCPIP (Ohad & Hirschberg, 1990). The light from a halogen projector was filtered through RG 645 (Schott) glass and was saturating for the rate of DCPIP reduction. DCPIP reduction was followed at 590 nm, the major absorption peak for the oxidized form, and normalized to the absorbance at 480 nm. Chemically pure herbicides were used and were obtained from the following sources: diuron and atrazine, Riedel-dehaën; ioxynil, Chem Service; bromonitrothymol, gift of A. Trebst.

## RESULTS

*Mutant Preparation.* Twenty-seven site-directed mutants of *Synechocystis* sp. PCC 6803 were prepared having amino acid modifications in the *D–E* region of the D1 protein (Table 2). All changes in the DNA fragment used for mutagenesis (see Figure 1) were verified by sequencing both strands. Nonplanned changes occurred in a few cases (indicated by * in Table 2). These originated in the plasmid mutagenesis step ($KS^-$ construct; Figure 1) before *Synechocystis* transformation; thus, no selection pressure favoring a functional advantage was involved. Two of these changes involved the insertion of a codon that maintained the reading frame. Both occurred in a piece of the *psb*AII gene coding for amino acids 245–247 (Table 2). These abnormal insertions may be explained by duplication of the 5′ nucleotides of the annealed primers used for mutagenesis (see Table 1). Secondary structural element(s) of the DNA that can be stably formed in that region may induce such modifications during the synthesis of the second strand.

Table 2: Mutations in the *D–E* Region of the D1 Protein

| cell line[a] | growth rates[b] | saturated rate of $O_2$ evolution[c] (Mut/WT) | $\phi = (F_m - F_o)/F_m$[d] (Mut/WT) | $f = [F_m - F_s]/[F_m - F_o]$[d] (Mut/WT) |
|---|---|---|---|---|
| WT (wild type) | +++ | 1.0 ± 0.06 | 1.00 ± 0.17 | 1.00 ± 0.05 |
| TT3 | – | 0.00 | 0.00 | nd |
| $SS_{221-2}LA$ | +++ | 0.6–1.2 | 0.93 | 0.91 |
| $V_{224}R+S_{264}A$ | ++ | 0.6–0.9 | 1.07 | 0.62 |
| $R_{225}L+S_{264}A$ | ++ | 0.3–0.7 | 0.85 | 0.62 |
| $TT_{227-8}AA$ | +++ | 0.9–1.3 | 0.99 | 0.95 |
| $V_{230}T+S_{264}A$ | ++ | 0.7–1.0 | 1.13 | 0.72 |
| $\Delta EVESQ_{229-33}$ | +++ | 0.8–1.2 | 0.95 | 0.91 |
| $S_{232}A$ | +++ | 0.8–1.2 | 1.10 | 0.90 |
| $S_{232}D$ | +++ | 0.6–1.0 | 0.98 | 0.94 |
| $\Delta NYG_{234-6}$ | +++ | 0.8–1.1 | 0.83 | 0.93 |
| $Y_{237}F$ | +++ | 0.6–1.0 | 1.10 | 0.91 |
| $K_{238}V$ | +++ | 0.7–0.9 | 1.10 | 0.97 |
| $\Delta YKF_{237-9}$ | ++ | 0.7–1.0 | 1.06 | 0.76 |
| $\Delta GQ_{240-1}$ | ++ | 0.6–0.7 | 0.95 | 0.85 |
| $\Delta EEE_{242-4}$ | ++ | na | na | na |
| $*\Delta YNIV_{246-9}::E_{244-5}$ | – | 0.0 | 0.30 | 0.00 |
| $Y_{246}F$ | ++ | na | na | na |
| $*V_{249}D+N_{266}Y$ | +++ | 0.9–1.2 | 1.00 | 0.82 |
| $*\Delta AA_{250-1}::Y_{246-7}$ | + | 0.3–1.0 | 0.87 | 0.00 |
| $*AH_{251-2}PV$ | – | 0.0 | 0.95 | 0.00 |
| $Y_{254}T$ | + | 0.7–0.8 | 0.63 | 0.96 |
| $\Delta YFGR_{254-7}$ | – | 0.0 | 0.00 | nd |
| $R_{257}V$ | ++ | 1.0–1.1 | 1.23 | 0.78 |
| $*L_{258}F+Y_{237}F$ | – | 0.0 | 0.00 | nd |
| $*L_{258}I+Y_{237}F$ | +++ | na | na | na |
| $S_{264}A$ | ++ | 0.2–0.5 | 1.02 | 0.66 |
| $\Delta NN_{266-7}$ | – | 0.0 | 0.27 | 0.00 |
| $\Delta SRS_{268-70}$ | – | 0.0 | 0.15 | 0.00 |

[a] Cell lines are designated as follows: original residue(s), location(s), modified residue(s). Δ signifies a deletion mutation. + signifies a double mutation in noncontiguous locations. :: signifies an insertion mutation. * signifies nonplanned modifications originating in the mutagenesis step. The TT3 mutant lacks all three copies of the *psb*A gene and does not assemble any of the PSII proteins (Jensson et al., 1987). [b] Photoautotrophic growth rates were assayed without glucose and are indicated as – or by 1–3 + signs as shown in Figure 2. [c] Saturated rates of oxygen evolution were measured using whole cells in the presence of electron acceptors. The range of values for 3–5 measurements is normalized to wild-type activity (183 ± 11 μmol of $O_2$ (mg of Chl)$^{-1}$ h$^{-1}$). [d] Parameters of fluorescence: The basal fluorescence ($F_o$) was evaluated as the initial value of the fluorescence in the fluorescence induction curve. The steady state fluorescence, $F_s$, is taken as the final steady value of fluorescence obtained at the end of the induction. The maximal fluorescence ($F_m$) was evaluated as the final level in the fluorescence induction curve in the presence of an oversaturating concentration of diuron (0.1 mM). This high concentration was chosen to inhibit the electron transfer activity in all mutants, including diuron-tolerant ones. These parameters were used to derive the ratio $f$ (Kless et al., 1992), measured under limiting light. $\phi$ indicates the photochemical quantum yield. Values are averages for 3–5 determinations and are normalized to the wild-type numbers. Standard error values obtained for the mutants are <10%. The wild-type value for $\phi$ was 0.37 ± 0.062 and that for $f$ was 0.876 ± 0.08. na, not analyzed. nd, not defined. When there is no variable fluorescence, $F_o = F_m$, $\phi = 0$, and $f$ is not determined mathematically.

*Functional Evaluation of the Mutants.* Photoautotrophic growth rates were determined in liquid cultures at standard conditions of aeration, temperature, light, and nutrition (without glucose). Growth rates were weighted as follows: the same as wild type (+++ *type*; e.g., $\Delta EVESQ_{229-33}$); no photoautotrophic growth (– *type*; e.g., $\Delta SRS_{268-70}$); and intermediate growth rates (++ *type*; e.g., $R_{257}V$; or + *type*; e.g., $\Delta AA_{250-1}::Y_{246-7}$) (Figure 2). All of the mutants were able to grow in the presence of glucose, indicating intact photoheterotrophic capabilities. Photoautotrophic growth rates relative to wild type are presented in Table 2 for all mutants.

Maximal rates of photosynthetic oxygen evolution were measured in the presence of electron acceptors that mediate electron transfer from the reducing side of PSII. Values varied among experiments; thus a range is presented (Table 2). The variability in oxygen evolution suggests that this assay is very sensitive to the physiological state of the cells [see also Vermaas et al. (1988); Nixon & Diner, 1992]. In several photoautotrophically competent mutants (e.g., $\Delta AA_{250-1}::Y_{246-7}$, $R_{257}V$), the relative rates of oxygen evolution did not correlate with rates of growth. This indicates that in these cases oxygen-evolving capacity is not a rate-limiting factor under the conditions used.

Chlorophyll fluorescence kinetics and steady state measurements are directly related to PSII reaction center function

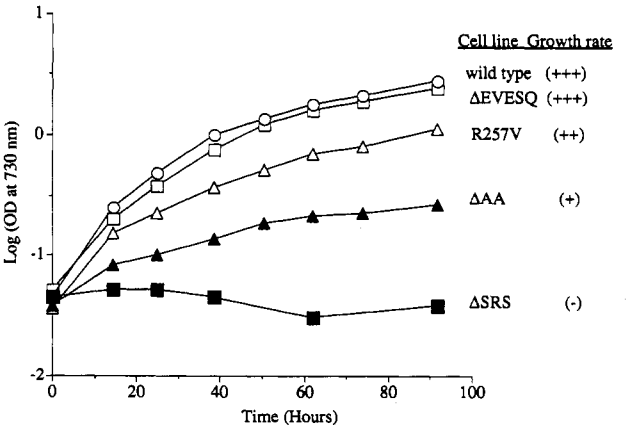


FIGURE 2: Photoautotrophic growth rates of representative mutants. Wild-type and D1 mutants $\Delta SRS_{268-70}$, $\Delta AA_{250-1}::Y_{246-7}$, $\Delta EVESQ_{229-33}$, and $R_{257}V$ were grown in the absence of glucose. Cell turbidity at 730 nm was measured as a function of time.

(Briantais et al., 1986; Krause & Weiss, 1991) through two parameters: the ratio $\phi = (F_m - F_o)/F_m$, indicating the photochemical quantum yield of an open center, and the ratio $f = (F_m - F_s)/(F_m - F_o)$, which gives an indication of the relative electron transfer flux. Inspection of Table 2 shows that in all mutants capable of photoautotrophic growth the values of $\phi$ were quite close to the value of the wild type. The

Table 3: Effect of PSII Herbicide on D1 Protein Mutant[a]

| cell line[b] | diuron | atrazine | BNT | ioxynil |
|---|---|---|---|---|
| WT[c] | 1 | 1 | 1 | 1 |
| $SS_{221-2}LA$ | 560 | 24 | 2 | 6.2 |
| $TT_{227-8}AA$ | 14 | 3 | 2.4 | 3 |
| $\Delta EVESQ_{229-33}$ | 2.6 | 1.5 | 1 | 1.5 |
| $\Delta NYG_{234-6}$ | 8.6 | 5 | 1.4 | 6 |
| $Y_{237}F$ | 4.6 | 2 | 0.2 | 5 |
| $K_{238}V$ | 1.3 | 0.6 | 0.4 | 2.3 |
| $\Delta YKF_{237-9}$ | 23 | 6 | 0.4 | 4 |
| $\Delta GQ_{240-1}$ | 26 | 21 | 0.5 | 1.4 |
| $\Delta AA_{250-1}::Y_{246-7}$ | 280 | 80 | 2 | 5 |
| $R_{257}V$ | 38 | 30 | 0.3 | 0.8 |
| $S_{264}A$ | 207 | 30 | 0.1 | 1.2 |

[a] Tolerances are given as relative $I_{50}$ values (mutant/wild type). [b] Designation of mutant cell lines is as described in Table 2. [c] Molar concentrations of $I_{50}$ values of wild type are $1.3 \times 10^{-8}$, $3.25 \times 10^{-7}$, $1.2 \times 10^{-7}$, and $1.3 \times 10^{-7}$ for diuron, atrazine, BNT, and ioxynil, respectively.

steady state values of $f$ gave a more delicate indication of the tuning of the reaction centers, possibly related to the various redox states of $Q_A$ and $Q_B$ (Crofts & Wraight, 1983).

Mutants incapable of photoautotrophic growth were also incapable of evolving oxygen (Table 2) and had zero (when $F_m - F_s = 0$) or undefined (when $F_m - F_o = 0$) values of $f$. The $\phi$ values of these mutants were also very low except in one case: mutant $AH_{251-2}PV$ still retained a high level of relative variable fluorescence (0.95 of that of wild type), indicative of $Q_A$ reduction activity while $f$ was equal to zero. This implies that assembly of the PSII reaction center was not basically impaired in this mutant. Rather, direct functional interactions or stability features (probably in the $Q_B$ niche) were damaged, interfering with electron transport to $Q_B$ and/or electron transfer from $Q_B$ to the plastoquinone pool.

All of the mutants contained the D1, D2, CP43, and CP47 proteins in their membranes, as determined by immunoblotting (not shown). This suggests that synthesis and insertion of the PSII reaction center components in the thylakoids still take place in the photosynthetically incompetent mutants. Therefore, specific functions and/or interactions, rather than the stability of the complex, were impaired in these mutants.

*Interactions with PSII Herbicides.* Herbicide tolerance is used to study structure–function relationships in the $Q_B$ binding niche and to evaluate interactions of the protein moieties with various herbicide and quinone compounds (Trebst, 1987; Tietjen et al., 1991; Ohad & Hirschberg, 1992; Jansen et al., 1993). The molar concentration of an inhibitor that reduces electron transfer from water to DCPIP by 50% (the $I_{50}$ value) is characteristic for a herbicide and related to its binding affinity in the $Q_B$ binding niche (Tischer & Strotmann, 1977). $I_{50}$ values relative to wild type were measured for diuron, atrazine, ioxynil, and bromonitrothymol (BNT) in 11 of the D1 mutants (Table 3). Most of the mutations modified the $I_{50}$ values of the tested herbicides, many at sites not previously analyzed. For example, the results with mutant $SS_{221-2}LA$ indicate a new location strongly influencing diuron binding; those with mutants $\Delta NYG_{234-6}$, $Y_{237}F$, and $\Delta YKF_{237-9}$ indicate tolerance to ioxynil. Increased sensitivity toward BNT, a phenol-type herbicide, was generated by mutations in the region 237–241 and at positions 257 and 264 (Table 3). These results resemble the cross-resistance behavior of other mutations in the D1 (Ohad & Hirschberg, 1992) and D2 (Kless et al., 1993) proteins. The mutation $\Delta EVESQ_{229-33}$ had no effect on herbicide interactions, suggesting minimal contribution of that loop region to herbicide binding.

## DISCUSSION

*Interactions in the D–E Region That Affect PSII Activities.* Several mutations, located at positions presumably involved in essential interactions in the $Q_B$ binding niche of the D1 protein, greatly interfered with PSII performance.

*Mutations That Abolish Photoautotrophy.* In analogy to the L protein, Phe255 (Trebst, 1986; Michel & Deisenhofer, 1988), Asn266, and Ser268 (Tietjen et al., 1991) of D1 were presumed to be closely associated with $Q_B$, while His252 and Asn247 were theorized to be involved in $Q_B$ binding (A. Trebst, personal communication) or protonation (see later discussion). If these presumptions are valid, then mutation of these sites is likely to affect essential interactions in PSII. We found that D1 mutations at these locations (e.g., $AH_{251-2}PV$, $\Delta YNIV_{246-9}::E_{244-5}$, $\Delta YFGR_{254-7}$, $\Delta NN_{266-7}$ and $\Delta SRS_{268-270}$) abolished photoautotrophy, thus experimentally validating the central analogy between the L and D1 proteins in the *D–E* region.

*Mutants Modified in the D–de Loop.* The minor effects on the photosynthetic parameter of *D–de* loop mutants $TT_{227-8}AA$, $\Delta EVESQ_{229-33}$, $S_{232}A$, $\Delta NYG_{234-6}$, $Y_{237}F$, and $K_{238}V$ question the significance of the proposed EST region within the loop (Greenberg et al., 1987) as a primary determinant for rapid degradation (Rogers et al., 1986). Moreover, D1 protein degradation rates for these mutants under low light conditions (50 $\mu E\ m^{-2}\ s^{-1}$) seem to be comparable to those of the wild type (Kless, 1993). It should be mentioned, however, that PEST type signals recently have been reevaluated and are now considered *conditional* determinants for rapid protein degradation (M. Rechsteiner, personal communication). Thus, the EST signal in the *D–de* loop may become rate limiting for D1 protein degradation only under conditions of stress, such as high irradiance or temperature. Further experiments are needed to resolve this issue.

The carboxy half of the *D–de* loop was previously proposed as the site of primary cleavage for the rapidly degrading D1 protein under physiological conditions *in vivo* (Greenberg et al., 1987). Association of the *D–de* loop region as a whole with rapid D1 protein degradation *in vivo* has recently been verified (Jansen et al., 1993). Also, the phototrophically null mutant $\Delta YNIV_{246-9}::E_{244-5}$, when grown heterotrophically, shows reduced rates of D1 protein degradation (Kless, 1993). In our study, we found that, in contrast to the minor effects on growth in the amino half of the extended *D–de* loop, carboxy half mutants (viz., $\Delta YKF_{237-9}$, $\Delta GQ_{240-1}$, $\Delta EEE_{242-4}$, $\Delta YNIV_{246-9}::E_{244-5}$, and $Y_{246}F$) were impaired in growth, thus supporting a role for the carboxy portion of the *D–de* loop in $Q_B$ niche activities.

*$\Delta AA_{250-1}::Y_{246-7}$.* In this mutant, the saturation rate of oxygen evolution can reach that of the wild type, while $f$ is very small (Table 2). This implies that changes affecting the steady state concentration of $Q_A^-$ at limiting light need not be rate limiting for linear electron transfer under high light intensity. At light limiting conditions, the smallness of $f$ probably indicates some limitation in electron transport around PSII. The growth rate at limiting light intensities was indeed impaired (Figure 2). Michel and Deisenhofer (1988) aligned Ala251 of D1 with Glu212 in the L protein of the bacterial reaction center (see Figure 3). There, together with Asn213 and Ser223, it is involved in the protonation of $Q_B^{2-}$ (Paddock et al., 1989; Takahashi & Wraight, 1992). The limitation in PSII activity may be due to impaired protonation of $Q_B^{2-}$, which could lead to a high electron back-pressure and increase in the concentration of $Q_A^-$ under steady state electron

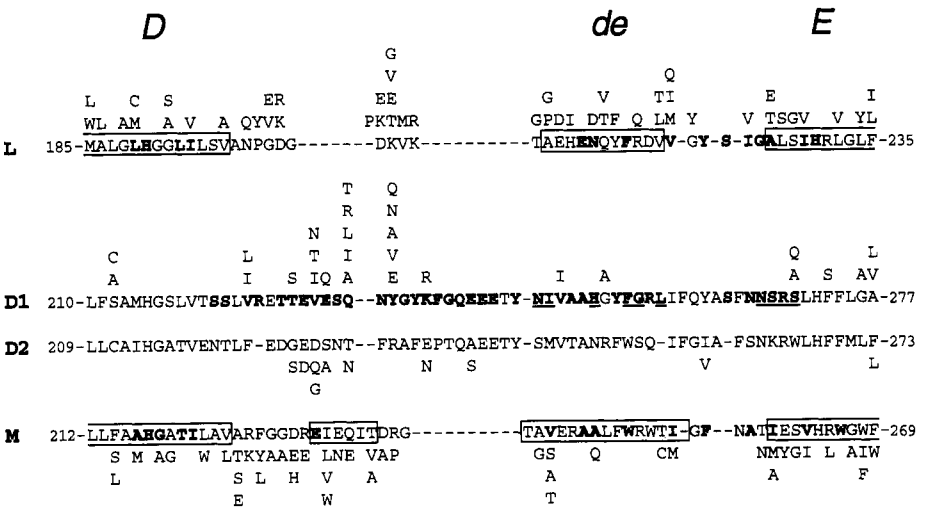


FIGURE 3: Protein sequence alignment at the *D–E* region. Protein sequences in the *D–E* region of D1 and D2 proteins of *Synechocystis* sp. PCC 6803 (Ravnikar et al., 1989; Williams & Chisholm, 1987) are compared to those of the homologous regions of the L and M proteins of *Rhodopseudomonas viridis* (Deisenhofer et al., 1985). Similar residues and proposed $\alpha$-helices and loops of D1 and D2 are aligned according to Michel and Deisenhofer (1988), except that the *de* helices of D1 and D2 are assumed to have the dimensions suggested by Trebst (1986). Amino acid residues of L and M found to contribute to quinone binding in the reaction center of *Rhodopseudomonas viridis* (Deisenhofer et al., 1985; Michel & Deisenhofer, 1988) are indicated by boldface type. Phylogenetically variable residues found in D1 (42 sequences; J. Hirschberg, personal communication), D2 (12 sequences; Svensson et al., 1991), and the L and M (5 sequences; Komiya et al., 1988) proteins are indicated above or below each position. $\alpha$-helices in L and M are boxed. Amino acids of D1 that were modified in this study are indicated in boldface type. Modified residues in the photoautotrophically incompetent mutants are underlined.

transport. Possibly His252, which is adjacent to Ala251 in the D1 protein, is involved in the protonation process. However, it cannot be excluded that the tyrosine introduced in mutant $\Delta AA_{250-1}::Y_{246-7}$ is responsible for the observed effect.

*Arg257.* This residue is located at the C-terminal end of the *de* helix and has been proposed to balance the negative charge formed by the dipole moment of the $\alpha$-helix (Michel & Deisenhofer, 1988). A change of Arg to Val at residue 257 reduces photoautotrophic growth and electron transfer yield, but not the saturated rate of oxygen evolution (Table 2). $R_{257}V$ has a marked effect on the electrophoretic mobility of the D1 protein in LDS–polyacrylamide (Kless, 1993), suggesting involvement of this arginine in conformational changes in the *D–E* region of the protein.

*Leu258.* D1 residue 258 is thought to interface between the *de* helix and the *E–de* loop (Trebst, 1986; Michel & Deisenhofer, 1988). It can accommodate a photoautotrophically competent change to Ile ($L_{258}I+Y_{237}F$) but not to Phe ($L_{258}F+Y_{237}F$). Since the change of Tyr to Phe at codon 237 has only a minor effect on the measured parameters (Table 2), we conclude that Phe in position 258 is likely to impair crucial interactions in PSII.

*Complex Herbicide Interactions in the $Q_B$ Binding Niche.* It is evident from our study that interactions of varying intensities occur between herbicides and peptide residues throughout the *D–E* region, including the *D–de* loop. This is in agreement with the labeling of Tyr237 by azidomonuron (Dostatni et al., 1988) and the protection against trypsin cleavage at Arg238 of the D1 protein by triazine and urea herbicides (Trebst et al., 1988; Jansen et al., 1993). It may be that interactions leading to higher inhibitor tolerances result from direct ligand binding, while those of lower tolerances involve secondary structural rearrangements in the $Q_B$ niche. An example of the latter is shown by Sinning (1992) for the L subunit of the reaction center of purple bacteria. In oxygenic phototrophs, an interesting example is the low tolerance for atrazine and diuron generated by mutations in the *D–de* loop of the D2 protein of *Synechocystis* (Kless et al., 1993). As the $Q_B$ niche is formed mostly, if not exclusively, from residues of the D1 protein (Tietjen et al., 1991; Ruffle et al., 1992; Sobolev and Edelman, unpublished), this case may be useful as a baseline for herbicide tolerances resulting from secondary structural rearrangements.

An example of direct binding may be the high tolerance toward diuron seen in the $SS_{221-2}LA$ mutant. Notably, however, the effect on oxygen evolution and *f* is relatively small. This suggests that the 221–2 location in the D1 protein is mainly involved with herbicide interaction and less with $Q_B$. Ser221 is conserved both in the D1 protein (Svensson et al., 1991) and in its corresponding position (i.e., Ser196) in the L protein (Komiya et al., 1988; also see Figure 3). In the L protein, Ser196 has no direct interaction with the quinone. It is located, however, at the stromal end of the *D* helix above Leu193, which does bind $Q_B$.

*Overall Organization and Conservation of the D–E Region.* The arrangement of structural elements in the *D–E* region of the D1 protein as revealed by mutational analysis, phylogeny, and analogy to the L subunit is summarized in Figure 3. In the bacterial reaction center, the *D–de* loop of the L protein contributes little to $Q_B$ binding, while the *de* helix and *E–de* loop regions are more closely associated with $Q_B$ (El-Kabbani et al., 1991; Sobolev and Edelman, unpublished). Similarly, for the D1 protein, most of the changes and deletions made by us in the C-terminal end of the *D* helix and in the *D–de* loop were found to be dispensable for photoautotrophy. The most deleterious changes were located in the *de* helix, the *E–de* loop, and the N-terminal end of the *E* helix. Overall, in the region of the D1 protein modified by us (residues 221–270), 40 out of 49 residues are phylogenetically conserved and an additional 4 are similar (Figure 3). However, at least 25 of these conserved residues (many located in the *D–de* loop region) can be functionally modified. Thus, residue conservation and PSII competence do not correlate. On the other hand, all of the modifications affected the measured parameters to some extent, mainly as changes in the relative level of the steady state fluorescence. These changes are taken to indicate variations in a steady state thermodynamic balance between redox components near $Q_A$ (most likely between $Q_A$ and $Q_B$). We conclude that many of the residues in the *D–E*

region are involved in subtle interactions that may provide functional advantage under various environmental conditions, and thus have been conserved.

## REFERENCES


Bowyer, J. R., Camilleri, P., & Vermaas, W. F. J. (1991) in *Herbicides* (Baker, N. R., & Percival, M. P., Eds.) pp 27–85, Elsevier, Amsterdam.

Briantais, J.-M., Vernotte, C., Krause, G. H., & Weis, E. (1986) in *Light Emission by Plants and Photosynthetic Bacteria* (Govindjee, Amesz, J., & Fork, D. C., Eds.) pp 539–577, Academic Press, New York.

Burnap, R., Koike H., Sotiropoulou, G., Sherman, L. A., & Inoue, Y. (1989) *Photosynth. Res. 22*, 123–130.

Cahen, D., Malkin, S., Shochat, S., & Ohad, I. (1976) *Plant Physiol. 58*, 257–267.

Casanova, J.- L., Pannetier, C., Jaulin, C., & Kaurilsky, P. (1990) *Nucleic Acids Res. 18*, 4028.

Crofts, A. R., & Wraight, C. A. (1983) *Biochim. Biophys. Acta 726*, 149–185.

Debus, R. J., Barry, B. A., Sithole, I, Babcock, G. T., & McIntosh, L. (1988) *Biochemistry 27*, 9071–9074.

Deisenhofer, J., Epp, O., Miki, K., Huber, R., & Michel, H. (1985) *Nature 318*, 618–624.

Diner, B. A., & Petrouleas, V. (1990) *Biochim. Biophys. Acta 1015*, 141–149.

Dostatni, R., Meyer, H. E., & Oettmeier, W. (1988) *FEBS Lett. 239*, 207–210.

El-Kabbani, O., Chang, C.-H., Tiede, D., Norris, J. & Schiffer, M. (1991) *Biochemistry 30*, 5361–5369.

Govindjee, Schwarz, B., Rochaix, J.-D., & Strasser, R. J. (1991) *Photosynth. Res. 27*, 199–208.

Greenberg, B. M., Gaba, V., Mattoo, A. K., & Edelman, M. (1987) *EMBO J. 6*, 2865–2869.

Jansen, M. A. K., Depka, B., Trebst, A., & Edelman, M. (1993) *J. Biol. Chem. 268*, 21246–21253.

Jensson, C., Debus, R. J., Osiewacz, H. D., Gurevitz, M., & McIntosh, L. (1987) *Plant Physiol. 85*, 1021–1025.

Kless, H. (1993) Structural and functional aspects of the PS II reaction center as revealed by site-directed mutagenesis in *Synechocystis* sp. PCC 6803, Doctoral Thesis, Weizmann Institute of Science, Rehovot, Israel.

Kless, H., Vermaas, W. F. J., & Edelman, M. (1992) *Biochemistry 31*, 11065–11071.

Kless, H., Oren-Shamir, M., Ohad, I., Edelman, M., & Vermass, W. F. J. (1993) *Z. Naturforsch. 48c*, 185–190.

Komiya, H., Yeates, T. O., Rees, D. C., Allen, J. P., & Feher, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 9012–9016.

Krause, G. H., & Weis, E. (1991) *Annu. Rev. Plant Physiol. 42*, 313–349.

Kunkel, T. A., Roberts, J. D., & Zakour, A. (1987) *Methods Enzymol. 154*, 367–382.

Mattoo, A. K., H. Marder, J. B., & Edelman, M. (1989) *Cell* 56, 241–246.

Michel, H., & Deisenhofer, J. (1988) *Biochemistry 27*, 1–7.

Nixon, P. J., & Diner, B. A. (1992) *Biochemistry 31*, 942–948.

Ohad, N., & Hirschberg, J. (1990) *Photosynth. Res. 23*, 73–79.

Ohad, N., & Hirschberg, J. (1992) *Plant Cell 4*, 273–282.

Paddock, M. L., Rongey, S. H., Feher, G., & Okamura, M. Y. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6602–6606.

Ravnikar, P. D., Debus, R., Sevrinck, J., Saetaert, P., & McIntosh, L. (1989) *Nucleic Acids Res. 10*, 3991.

Rochaix, J.-D., Dron, M., Rahire, M., & Malone, P. (1984) *Plant Mol. Biol. 3*, 363–370.

Rogers, S., Wells, R., & Rechsteiner, M. (1986) *Science 234*, 364–368.

Ruffle, S. V., Donnelly, D., Blundell, T. L., & Nugent, J. H. A. (1992) *Photosynth. Res. 24*, 287–300.

Sinning, I., (1992) *Trends Biochem. Sci. 17*, 150–154.

Svensson, B., Vass, I., & Styring, S. (1991) *Z. Naturforsch. 46c*, 765–776.

Takahashi, E., & Wraight, C. A. (1992) *Biochemistry 31*, 855–866.

Tandeau de Marsac, N., & Houmard, J. (1988) *Methods Enzymol. 167*, 318–328.

Tietjen, K. G., Kluth, J. F., Andree, R., Haug, M., Lindig, M., Müller, K. H., Wroblowsky, H. J., & Trebst, A. (1991) *Pestic. Sci. 31*, 65–72.

Tischer, W., & Strotmann, H. (1977) *Biochim. Biophys. Acta 460*, 113–125.

Trebst, A. (1986) *Z. Naturforsch. 41c*, 240–245.

Trebst, A. (1987) *Z. Naturforsch. 42c*, 742–750.

Trebst, A., Depka, B., Kraft, B., & Johanningmeier, U. (1988) *Photosynth. Res. 18*, 163–177.

Vermaas, W. F. J., & Ikeuchi, M. (1991) in *The Molecular Biology of Plastids; Cell Culture and Somatic Cell Genetics of Plants* (Vasil, I., & Bogorad, L.. Eds.) Vol. 7, pp 25–112, Academic Press, San Diego.

Vermaas, W. F. J., Rutherford, A. W., & Hansson, Ö. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8477–8481.

Vermaas, W. F. J., Charité, J., & Shen, G. (1990) *Biochemistry 22*, 5325–5332.

Williams, J. G. K. (1988) *Methods Enzymol. 167*, 766–779.

Williams, J. G. K., & Chisholm, D. A. (1987) in *Progress in Photosynthesis Research* (Biggins, J., Ed.) Vol. IV, pp 809–812, Martinus Nijhoff, Dordrecht, The Netherlands.

Youvan, D. C., Bylina, E. J., Alberti, M., Begusch, H., & Hearst, J. E. (1984) *Cell 37*, 949–957.