Biochemistry

 Volume 27, Number 26 December 27, 1988

Accelerated Publications

Directed Mutagenesis Indicates That the Donor to P_{680}^+ in Photosystem II Is Tyrosine-161 of the D1 Polypeptide†

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Received October 11, 1988; Revised Manuscript Received October 31, 1988

ABSTRACT: Photosystem II contains two redox-active tyrosines. One of these, Y_Z, reduces the reaction center chlorophyll, P680, and transfers the oxidizing equivalent to the oxygen-evolving complex. The second, Y_D, has a long-lived free radical state of unknown function. We recently established that Y_D is Tyr-160 of the D2 polypeptide by site-directed mutagenesis of a *psbD* gene in the unicellular cyanobacterium *Synechocystis* 6803 [Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430]. Y_Z is most likely the symmetry-related Tyr-161 of the D1 polypeptide. To test this hypothesis, we have changed Tyr-161 to phenylalanine by site-directed mutagenesis of a *psbA* gene in *Synechocystis*. The resulting mutant assembles PSII, as judged by its ability to produce the stable Y_D^+ radical, but is unable to grow photosynthetically and exhibits altered fluorescence properties. The nature of the fluorescence change indicates that forward electron transfer to P_{680}^+ is disrupted in the mutant. These results provide strong support for our identification of Tyr-161 in the D1 polypeptide with Y_Z.

The minimal oxygen-evolving PSII complex consists of seven polypeptides plus several organic and inorganic cofactors [for review, see Babcock (1987), Andreasson and Vanngard (1988), and Michel and Deisenhofer (1988)]. The reaction center chlorophyll (P_{680}) and its immediate electron acceptors are associated with two of these seven polypeptides, D1 and D2. These two homologous polypeptides form a heterodimer that is thought to have structural and functional analogies to the reaction center complex of photosynthetic bacteria (Michel & Deisenhofer, 1988).

As opposed to the bacterial case, however, oxidizing equivalents that are generated by photooxidation of P_{680} are accumulated in a cluster of four manganese atoms where water is oxidized to molecular oxygen. A single electron-transfer intermediate, usually designated as Z, serves to link the photochemistry that occurs at P_{680} with the manganese cluster (Babcock, 1987; Hoganson & Babcock, 1988; Gerken et al., 1988). The EPR properties of Z^+ are virtually identical with those of the stable PSII free radical, Y_D^+, which has been shown to be a tyrosine residue by specific deuteriation (Barry & Babcock, 1987). The EPR analogies, together with recent UV optical absorption data (Gerken et al., 1988), indicate that Z is a second tyrosine in an environment similar to that of Y_D. Accordingly, the Z species has been designated as Y_Z (Hoganson & Babcock, 1988).

Recently, we employed site-directed mutagenesis of a *psbD* gene in the unicellular cyanobacterium *Synechocystis* 6803 to demonstrate that Y_D is Tyr-160 of the D2 polypeptide (Debus et al., 1988). This assignment has been independently confirmed (Vermaas et al., 1988). On the basis of C_2 symmetry present in the D1/D2 model for the PSII core, iodination data suggesting that Y_Z is located on D1 (Takahashi et al., 1986; Ikeuchi & Inoue, 1987), the similarities in spectral

†This research was supported by DOE Contract DE-AC02-76ER01338 (to L.M.), the National Institutes of Health (Grant GM 37300 to G.T.B.), the Photosynthesis Program of the Competitive Research Grants Office of the USDA (to G.T.B.), and the McKnight Foundation. R.J.D. was a National Science Foundation Postdoctoral Fellow in Plant Biology (Grant DMB-8608566). B.A.B. was a National Institutes of Health Postdoctoral Fellow (Grant GM-11078).

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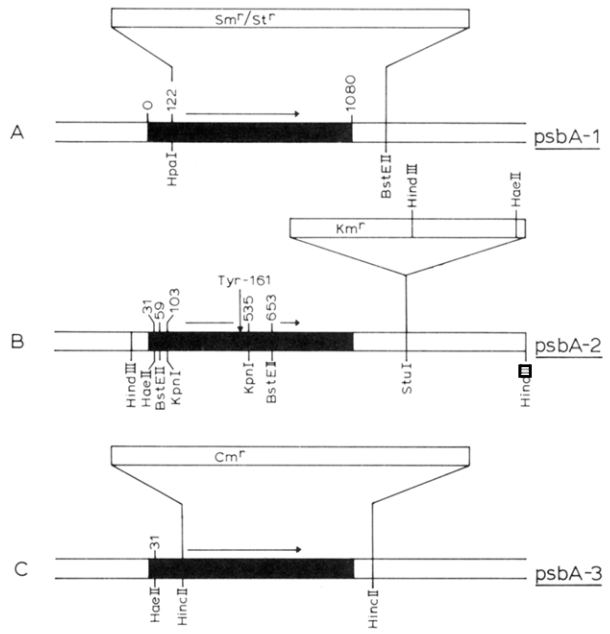

FIGURE 1: The 1080-bp *psbA* genes from *Synechocystis* 6803. (A) *psbA-1*, showing the 1.2-kb *Hpa*I/*Bst*EII fragment replaced by the 1.9-kb DNA cartridge that encodes resistance to Sm. (B) *psbA-2*, showing the position of the Tyr-161 codon. The plasmid pRD1031Km^r contains 1.9 kb of *Synechocystis* DNA extending from the *Hae*II site located 31 bp 3′ of the *psbA-2* ATG codon to the *Hin*dIII site located 3′ of the coding region. This plasmid also contains a 1.25-kb fragment of DNA conferring resistance to Km inserted into the *Stu*I site 288 bp 3′ of *psbA-2*. (C) *psbA-3*, showing the 1.0-kb *Hin*cII fragment replaced by the 1.9-kb DNA cartridge that encodes resistance to Cm.

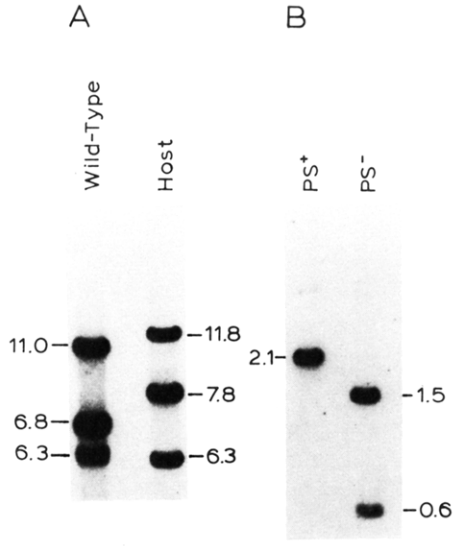

FIGURE 2: Southern blot analysis of total genomic DNA from wild-type *Synechocystis* 6803, the Sm^r/Cm^r host strain, and two Km^r/Sm^r/Cm^r transformants. Fragment sizes expressed in kb are indicated. (A) DNA digested with *Xba*I and hybridized with ^{32}P-labeled plasmids bearing *psbA-1*, *psbA-2*, and *psbA-3*. In the Sm^r/Cm^r host the 11.0- and 6.8-kb fragments bearing *psbA-1* and *psbA-3*, respectively, are larger, indicating substitution of *psbA-1* and *psbA-3* DNA by the larger Sm^r and Cm^r genes (see Figure 1). (B) DNA from one PS$^+$ and one PS$^-$ Km^r/Sm^r/Cm^r transformant was digested with *Hin*dIII + *Sst*II and hybridized with ^{32}P-labeled *psbA-2* DNA. The PS$^+$ transformant shows the expected single 2.1-kb *Hin*dIII fragment bearing *psbA-2* and part of the Km^r gene (see Figure 1B). The PS$^-$ transformant shows the presence of a *Sst*II site within *psbA-2*, indicating substitution of Phe for Tyr-161.

properties between Y_Z^+ and Y_D^+, and similarities in sequence between D1 and D2 in the Tyr-160/161 region, we proposed that Y_Z is Tyr-161 of the D1 polypeptide (Debus et al., 1988). To test this hypothesis, we have changed Tyr-161 to Phe by site-directed mutagenesis of a *psbA* gene in *Synechocystis* 6803. Because this organism possesses three nonidentical copies of *psbA* (Jansson et al., 1987), this project first required deletion of two *psbA* copies so that the mutation could be introduced into the remaining copy. The resulting mutant does not grow photosynthetically but assembles PSII and generates the Y_D^+ radical. The mutant exhibits altered fluorescence characteristics that indicate a disruption of forward electron transfer to P_{680}^+, as expected for an organism that lacks Y_Z.

Materials and Methods

Manipulations of DNA were performed according to standard protocols (Maniatis et al., 1982), except as noted. Growth and transformation of *Synechocystis* 6803, as well as extraction of genomic DNA, were performed as previously described (Debus et al., 1988; Jansson et al., 1987), except that 5 mM glucose was included in all cyanobacterial growth media. Measurements of EPR spectra, fluorescence induction kinetics, and oxygen evolution were performed as previously described (Debus et al., 1988; Jansson et al., 1987).

The cloning of *psbA-1* and *psbA-2* from *Synechocystis* 6803 has been described (Jansson et al., 1987), and the sequences of both genes are available (Osiewacz & McIntosh, 1987; Ravnikar et al., 1988). To allow deletion of *psbA-1* from the *Synechocystis* genome, a plasmid was constructed having the 1.2-kb *Hpa*I/*Bst*EII fragment bearing the 3′ 90% of *psbA-1* replaced by the 1.9-kb *Sma*I fragment of pHP45Ω (Prentki & Krisch, 1984) that encodes resistance to Sm (see Figure 1A). To construct a selectable vector for mutagenesis of *psbA-2*, a 1.9-kb *Hae*II/*Hin*dIII fragment bearing the 3′ 97% of *psbA-2* was inserted into the *Sma*I/*Hin*dIII sites of pUC119 (Vieira & Messing, 1987) following blunting of the *Hae*II site. The 1.25-kb *Hin*cII fragment of pUC4K (Vieira & Messing, 1982), which encodes resistance to Km, was inserted into the *Stu*I site located 288 bp 3′ of *psbA-2*, thus generating the plasmid pRD1031Km^r (see Figure 1B). For removal of *psbA-3*, the 3′ 97% of *psbA-3* was cloned as a 4.7-kb *Hae*II fragment into the *Bbe*I site of pUC119, and the 1.0-kb *Hin*cII fragment bearing the 3′ 85% of *psbA-3* was replaced by the 1.9-kb *Hin*cII fragment of pRL171 (Elhai & Wolk, 1988), which encodes resistance to Cm (see Figure 1C).

A Sm^r/Cm^r host strain of *Synechocystis* 6803 lacking the 3′ 90% of *psbA-1* and the 3′ 85% of *psbA-3* was constructed by transformation of the glucose-tolerant wild-type strain as described (Debus et al., 1988). Southern blot analysis of genomic DNA isolated from the wild-type and Sm^r/Cm^r host strains (Figure 2A) confirmed that the wild-type *Xba*I fragments bearing *psbA-1* and *psbA-3* increased from 11.0 to 11.8 and from 6.8 to 7.8 kb, respectively. The larger fragment sizes reflect replacement of the wild-type DNA by the larger Sm^r and Cm^r DNA cartridges.

To generate the Tyr-161 → Phe-161 mutation in *psbA-2*, a synthetic 32-base oligonucleotide (5′-GCCACCGCGGTATTCTTGATCTTCCCCATTGG-3′) extending from positions 459–491 of *psbA-2* was synthesized as described (Debus et al., 1988). The mutation TAC → TTC at position 481 changes the codon of Tyr-161 to that for Phe. A second mutation (GCC → GCG at position 467), not affecting the amino acid sequence, was included to introduce a site for the restriction endonuclease *Sst*II. Double-stranded plasmid DNA bearing the A → T and C → G conversions was generated as described (Debus et al., 1988) with template DNA obtained from a pUC119 derivative bearing *psbA-2*. Sequence analysis confirmed the presence of both desired

mutations and no others within the 594-bp *Bst*EII fragment of *psbA-2* (see Figure 1B).

To introduce the Tyr-161 → Phe-161 mutation into *Synechocystis*, the wild-type 594-bp *Bst*EII fragment of *psbA-2* within pRD1031Km^r (see Figure 1B) was replaced with the *Bst*EII fragment containing both mutations. The resulting mutation-bearing plasmid was then transformed into the Sm^r/Cm^r host, and transformants were selected for ability to grow on solid media containing Km. DCMU (10 μM) was included in the solid media to remove selective pressure for maintaining wild-type *psbA-2*, in the event that the mutation introduced should prove deleterious to PSII function (Jansson et al., 1987).

The resulting Km^r/Sm^r/Cm^r transformants carried either the mutant *psbA-2* or the wild-type *psbA-2* depending on whether the A → T mutation was cotransferred with the DNA encoding resistance to Km (Debus et al., 1988). Transformants with the mutated gene can be distinguished from those with the wild-type on the basis of the *Sst*II site introduced into the mutant.

Results

Of those Km^r/Sm^r/Cm^r transformants examined, 40% were found to be incapable of photoautotrophic growth (PS$^-$). Southern blot analysis of total genomic DNA isolated from PS$^+$ and PS$^-$ transformants (Figure 2B) showed the presence of the introduced *Sst*II site within *psbA-2* of the PS$^-$ cells, but not within *psbA-2* of the PS$^+$ cells. Sequence analysis of *psbA-2* isolated from the PS$^-$ transformant confirmed the presence of both the C → G and A → T mutations. The gene was cloned from total genomic DNA as a *Hae*II fragment that was inserted into the *Bbe*I site of pUC119 and was selected for ability to confer Km resistance. No other mutations were found within the 432-bp *Kpn*I fragment of *psbA-2* (see Figure 1B). To verify that no other mutations *outside* this fragment might exist and account for the PS$^-$ phenotype, the PS$^-$ transformant was transformed with the wild-type *Kpn*I fragment cloned into pUC119. PS$^+$ transformants were recovered at a frequency 100-fold greater than that in a control transformation performed with pRD1031Km^r containing both introduced mutations. This result demonstrates that the inability of the PS$^-$ transformant to grow photoautotrophically arises solely from the A → T conversion at position 481, which gives rise to the substitution of Phe for Tyr-161.

Oxygen evolution rates were measured in BG-11 medium with 1 mM DCBQ and 1 mM potassium ferricyanide as acceptors. The cells of the PS$^+$ transformant evolve O_2 at a rate of 400 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}, while there was no detectable O_2 evolution [less than 50 μmol of O_2 (mg of chl)$^{-1}$ h^{-1}] in cells of the PS$^-$ transformant.

To determine whether the PS$^-$ transformant assembles PSII, both transformants were examined for the presence of Y_D^+. Both gave rise to the EPR signal corresponding to this species [Figure 3; cf. Barry and Babcock (1987) and Debus et al. (1988)]. In the experiment reported in Figure 3, the signal observed in the PS$^-$ transformant corresponded to 60% of the spins present in the PS$^+$ cells on a chl basis. In a second series of experiments on different batches of the two transformants, we saw equal numbers of Y_D^+ spins in the two. The presence of Y_D^+ in the PS$^-$ transformant is conclusive proof that partially functional PSII complexes are assembled in spite of the Tyr-161 → Phe mutation in the D1 polypeptide.

To characterize further the nature of the lesion in the mutant PSII complex, the room temperature chl fluorescence induction kinetics of both PS$^+$ and PS$^-$ transformants were examined (Figure 4). Upon illumination, cells of the PS$^+$ transformant (Figure 4A) show a rapid rise in fluorescence yield to an initial level (F_0), followed by slow variations in yield characteristic of intact cyanobacterial cells (Jansson et al., 1987; Fork & Mohanty, 1986). Addition of DCMU caused the yield to rise rapidly to a maximal level (F_{max}). Cells of the PS$^-$ transformant (Figure 4B) show an initial rise to an F_0 level somewhat higher than that for the PS$^+$ cells (perhaps reflecting a higher antenna/PSII ratio in the mutant) but did not show the slow variations in yield. As with the wild-type cells, addition of DCMU to the PS$^-$ transformant caused the fluorescence yield to increase to a maximal level, but the rate of increase was much slower, extending over a period of several seconds. Addition of the PSII electron donor hydroquinone (Yerkes & Babcock, 1980) to mutant cells in the presence of

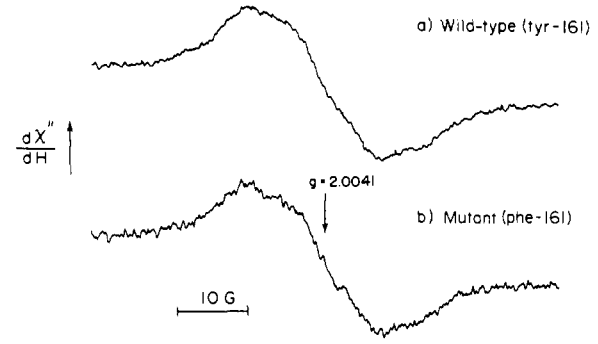

FIGURE 3: EPR spectra of photoheterotrophically grown Km^r/Sm^r/Cm^r *Synechocystis* cells recorded at room temperature following illumination. (a) PS$^+$ transformant having wild-type *psbA-2*. (b) PS$^-$ transformant having Tyr-161 of the D1 polypeptide replaced by Phe. The spectra have been normalized to facilitate comparison of the spectral line shapes. Conditions were as follows: frequency, 9.4 GHz; power, 20 mW; gain, 4.0 × 10^6; field modulation, 3.5 G; amplifier time constant, 500 ms; sweep time, 200 s. The chl concentration was 1.3 mg/mL in (a) and 1.2 mg/mL in (b).

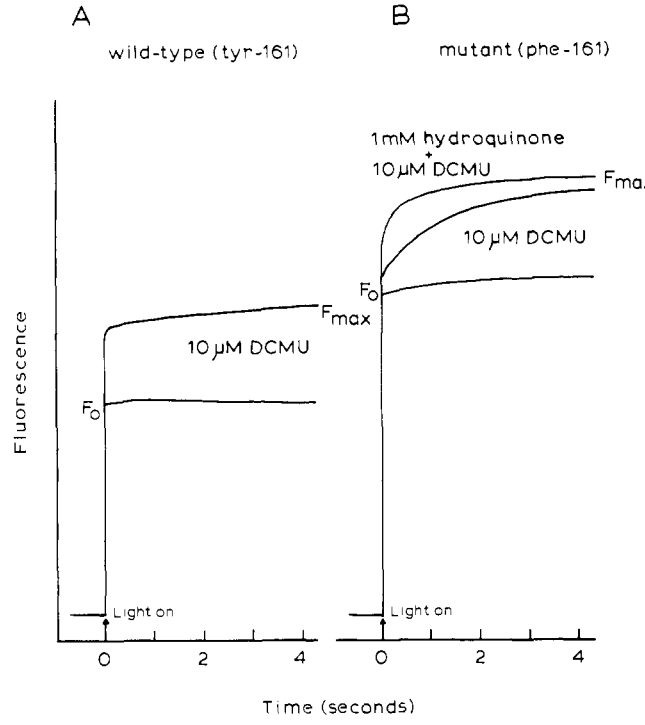

FIGURE 4: Room temperature fluorescence induction kinetics of Km^r/Sm^r/Cm^r *Synechocystis* cells. (A) PS$^+$ transformant having wild-type PSII. (B) PS$^-$ transformant having mutant PSII with Phe substituted for Tyr-161 in the D1 polypeptide. Cells were suspended at a chl concentration of 5 μg/mL in growth media. Excitation was through a CS-4-96 blue filter, and fluorescence was detected through a 690-nm interference filter (12-nm band-pass).

DCMU produced a rapid increase in yield (Figure 4B). Hydroquinone (1 mM) had no effect on wild-type cells, either in the presence or in the absence of DCMU.

The increase in fluorescence yield produced by the addition of DCMU to the PS^- transformant is further evidence that partially functional PSII complexes are assembled in spite of the Tyr-161 → Phe mutation; addition of DCMU to cells lacking PSII has no effect on the fluorescence yield (Jansson et al., 1987). The rapid increase in fluorescence observed with hydroquinone is evidence that hydroquinone donates electrons to P_{680}^+ in the mutant cells. Donation may be direct or indirect, perhaps involving cytochrome *b*-559 (Whitmarsh & Cramer, 1978).

Discussion

In this study we have described a system for site-directed mutagenesis of a *psbA* gene from the unicellular cyanobacterium *Synechocystis* 6803. This system will facilitate further investigation of electron transfer, O_2 evolution, herbicide action, and other aspects of structure/function in PSII. The particular advantage of *Synechocystis* 6803 is that it is a facultative photoheterotroph which is able to grow on glucose in the absence of functional PSII.

We have used this system to test our hypothesis that Y_Z is Tyr-161 of the D1 polypeptide by substituting Phe for that Tyr. The resulting mutant does not grow photoautotrophically or evolve O_2 but does assemble PSII, as shown by the presence of Y_D^+ (Figure 3) and by the increase in fluorescence yield produced by DCMU (Figure 4). The much slower rise in fluorescence yield observed in the mutant in the presence of DCMU is noteworthy. A high fluorescence yield (F_{max}) arises as a result of the trapping of the primary quinone acceptor, Q_A, in its reduced form, producing the state $P_{680}\,Q_A^-$ [for recent treatments of fluorescence in PSII, see Schatz et al. (1987) and van Gorkom (1986)]. This state forms rapidly in wild-type cells in the presence of DCMU because forward electron transfer from Q_A^- is inhibited by the herbicide and because reduction of P_{680}^+ by Y_Z is much faster than charge recombination between Q_A^- and P_{680}^+ [20–250 ns versus 100–200 μs, respectively (Babcock, 1987; Andreasson & Vanngard, 1988)]. The much slower rise in fluorescence observed in the mutant indicates either that the forward reduction of P_{680}^+ is much slower or that the rate of charge recombination between Q_A^- and P_{680}^+ is much faster in the mutant than in wild-type cells.

Our previous work, which established that Y_D^+ is Tyr-160 of the D2 polypeptide (Debus et al., 1988), is strong evidence in favor of the D1/D2 model for the PSII core. This model places Tyr-161 of the D1 polypeptide near the lumenal side of the membrane, and it seems unlikely that there would be any significant alteration in the rate of charge recombination between Q_A^- and P_{680}^+ upon replacement of this Tyr by Phe. In support of this contention, we find that addition of the electron donor hydroquinone to mutant cells largely restores the rapid increase in fluorescence observed in DCMU-poisoned wild-type cells. Therefore, we conclude that substitution of Phe for Tyr-161 has slowed the forward rate of P_{680}^+ reduction by several orders of magnitude, to a rate significantly slower than the 100–200-μs rate of charge recombination from Q_A^-. Substitution of Phe for Tyr-161 has thus created a lesion on the oxidizing side of PSII that disrupts normal electron transfer into P_{680}^+. This result provides strong evidence in support of our hypothesis that Y_Z is Tyr-161 of the D1 polypeptide.

Acknowledgments

We thank N. Bowlby, J. Dekker, J. DePaula, P. D. Ravnikar, and C. F. Yocum for helpful discussions and K. Rorrer for general technical assistance.

Registry No. P_{680}, 53808-91-6; Tyr, 60-18-4.

References

Andreasson, L.-E., & Vanngard, T. (1988) *Annu. Rev. Plant Physiol. Plant Mol. Biol. 39*, 379–411.

Babcock, G. T. (1987) in *New Comprehensive Biochemistry: Photosynthesis* (Amesz, J., Ed.) pp 125–158, Elsevier, Amsterdam.

Barry, B. A., & Babcock, G. T. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7099–7103.

Debus, R. J., Barry, B. A., Babcock, G. T., & McIntosh, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 427–430.

Elhai, J., & Wolk, C. P. (1988) *Gene 68*, 119–138.

Fork, D. C., & Mohanty, P. (1986) in *Light Emission By Plants and Bacteria* (Govindjee, Amesz, J., & Fork, D. C., Eds.) pp 451–496, Academic Press, Orlando.

Gerken, S., Brettel, K., Schlodder, E., & Witt, H. T. (1988) *FEBS Lett. 237*, 69–75.

Hoganson, C. W., & Babcock, G. T. (1988) *Biochemistry 27*, 5848–5855.

Ikeuchi, M., & Inoue, Y. (1987) *FEBS Lett. 210*, 71–76.

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

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

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

Osiewacz, H. D., & McIntosh, L. (1987) *Nucleic Acids Res. 15*, 10585.

Prentki, P., & Krisch, H. M. (1984) *Gene 29*, 303–313.

Ravnikar, P. D., Debus, R. J., Sevrinck, J., Saetaert, P., & McIntosh, L. (1988) *Nucleic Acids Res.* (in press).

Schatz, G. H., Brock, H., & Holzwarth, A. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8414–8418.

Takahashi, Y., Takahashi, M.-a., & Satoh, Ki. (1986) *FEBS Lett. 208*, 347–351.

van Gorkom, H. (1986) in *Light Emission By Plants and Bacteria* (Govindjee, Amesz, J., & Fork, D. C., Eds.) pp 267–289, Academic Press, Orlando.

Vermaas, W. F. J., Rutherford, A. W., & Hansson, O. (1988) *Proc. Natl. Acad. Sci. U.S.A.* (in press).

Vieira, J., & Messing, J. (1982) *Gene 19*, 259–268.

Vieira, J., & Messing, J. (1987) *Methods Enzymol. 153*, 3–11.

Whitmarsh, J., & Cramer, W. A. (1978) *Biochim. Biophys. Acta 501*, 83–94.

Yerkes, C. T., & Babcock, G. T. (1980) *Biochim. Biophys. Acta 590*, 360–372.