Lawrence Bogorad

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Personal perspective

Photosynthesis research: advances through molecular biology – the beginnings, 1975–1980s and on. . . *

Lawrence Bogorad
Department of Molecular and Cellular Biology, Harvard University, The Biological Laboratories, 16 Divinity Ave., Cambridge, MA 02138, USA (e-mail: bogorad@mcb.harvard.edu; fax: +1-617-495-4292)

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Abstract

Restriction endonuclease recognition sites and genes for rRNAs were first mapped on chloroplast chromosomes in 1975–1976. This marked the beginning of the application of molecular biology tools to photosynthesis research. In the first phase, knowledge about proteins involved in photosynthesis was used to identify plastid and nuclear genes encoding these proteins on cloned segments of DNA. Soon afterwards the DNA sequences of the cloned genes revealed the full primary sequences of the proteins. Knowledge of the primary amino acid sequences provided deeper understanding of the functioning of the protein and interactions among proteins of the photosynthetic apparatus. Later, as chloroplast DNA sequencing proceeded, genes were discovered that encoded proteins that had not been known to be part of the photosynthetic apparatus. This more complete knowledge of the composition of reaction centers and of the primary amino acid sequences of individual proteins comprising the reaction centers opened the way to determining the three-dimensional structures of reaction centers. At present, the availability of cloned genes, knowledge of the gene sequences and systems developed to genetically manipulate photosynthetic organisms is permitting experimental inquiries to be made into crucial details of the photosynthetic process.

Abbreviation: RUBISCO – ribulose bisphosphate carboxylase oxygenase

Introduction

Over the long history of photosynthesis research, a greater understanding of an aspect of photosynthesis has very often followed an advance in photophysics, cell biology, biochemistry or physiology. The ideas and tools of molecular biology, on the other hand, have been applied to many aspects of photosynthesis research and have enabled the dissection of basic photosynthetic processes such as electron transport and energy transfer at molecular and intramolecular levels. Many of the historical minireviews in these issues describe research results that have been obtained through the use of molecular biological approaches (see, e.g., Rochaix 2002; Shestakov 2002). The present paper deals mainly with results from the 'beginnings' – defined here as the period of the earliest applications of the tools and ideas of molecular biology to photosynthesis research – from about 1975 mainly through the mid-1980s and into the early 1990s.

It was only in the early 1970s that the threads of understanding and research methodologies of photosynthesis, biochemistry and molecular biology could be brought together to open the way for a molecular biology approach to photosynthesis research

More than 20 years before James Watson and Francis Crick described the structure of DNA in 1953, Cornelis B. van Niel, in 1931, proposed that the path of carbon in photosynthesis is distinct from the path of photons and electrons. Van Niel's radical proposal was

* Dedicated to Sam Granick (1909–1977) (see Figure 17).

made in the context of the emergence of biochemistry and the recognition of the occurrence and prominence of oxidation-reduction reactions in biology. Van Niel also concluded that the production of oxygen in oxygenic photosynthesis is a consequence of using water as the source of electrons. These insights have been important bases for modern studies of photosynthesis.

Andrew Benson (1998) has written that the publication of a paper entitled 'The path of carbon XXI: the cyclic regeneration of carbon dioxide acceptor' by James Al Bassham et al. in 1954 marked the conclusion of the effort to determine the path of carbon (also see Benson 2002; Bassham, this issue). In the course of this research, a few enzymes, among them notably ribulose bisphosphate carboxylase oxygenase (Rubisco; see Wildman 2002; Wildman and Bonner 1947; Ogren, this issue), were identified; intermediate compounds between CO_2 and sugars were discovered; and, the requirements for ATP and NADPH in photosynthetic carbon reduction were established.

By about 1960, many important steps had been taken toward acquiring a more complete understanding of the path of photons and electrons. The recognition of the existence of two photosystems (PS I and PS II) in oxygenic photosynthesis was a result of work stretching back to the 1930s that had been carried out mainly by Robert Emerson, William Arnold, Francis Haxo, Lawrence Blinks, Jack Myers, C. Stacy French, Robert Hill, Louis N. M. Duysens and their numerous co-workers (see Clayton 2002; Myers 2002; Borisov, this issue). Amesz (1998) and Govindjee (2000) have summarized much of this work. In the course of this and ensuing related research, a number of electron transfer components of photosynthesis were discovered mainly through optical difference spectroscopy.

Methods for making preparations enriched in PS I and PS II activities by differential centrifugation of thylakoids treated with mild non-ionic detergents were described in the 1960s (Wessels 1962; Boardman and Anderson 1964; Anderson 2002; Ogawa, this issue). Sodium dodecyl sulphate-polyacrylamide gel electrophoresis, described in 1969 by K. Weber and M. Osborn (1969), provided a means for separating denatured proteins according to size. Starting in the mid-1970s these methods were used to catalogue polypeptide components of PS I- and PS II-enriched preparations according to size (Anderson 1975; Bengis and Nelson 1975; Thornber 1975).

By the mid-1960s there was no question that plastids contained unique DNA as well as ribosomes different from those in the cytoplasm. There could be little question that this DNA included genes for components of the photosynthetic machinery. Indeed, until the early 1970s, there was no evidence that chloroplast DNA did not encode every protein in the chloroplast. By the mid 1970s the ideas and methods of molecular genetics and molecular biology were sufficiently well developed to be useful for studying photosynthesis. In roughly chronological order, methods had been developed to: determine the kinetics of reassociation of denatured DNA (the Cot Curve Era); to measure the extent of hybridization of DNA to RNA; to physically map restriction endonuclease recognition sites on DNA; to clone DNA fragments in *Escherichia coli* hosts; and to hybridize nucleic acids in solution to nucleic acids immobilized on nitrocellulose filters after separation according to size by agarose gel electrophoresis. E. M. Southern introduced the latter procedure (1975).

Thus, in the mid-1970s chloroplast DNA – expected to contain genes for photosynthetic proteins – could be isolated, cut with restriction enzymes, cloned, etc. The first international meeting on plant molecular biology was held in Strasbourg in July 1976 (Bogorad and Weil 1977a, b: Figures 1a and b). The first Gordon Conference on Plant Molecular Biology met in the summer of 1980. Later, in September 1980, the Fifth International Congress on Photosynthesis convened at Halkadiki, Greece. It was the first photosynthesis congress at which research was described that used the tools of molecular biology to study components of the photosynthetic apparatus (Akoyunoglou 1981).

Plastid DNA

In 1962 Hans Ris and Walter Plaut showed that there are 25–50 thick DNase-sensitive fibers and aggregates of these fibers in *Chlamydomonas moewusii* chloroplasts (Ris and Plant 1962). (DNase-sensitive fibers and aggregates like these occur in bacteria.) This was the first unequivocal demonstration that there is DNA in chloroplasts. Similar DNase-sensitive material was afterwards observed in plastids of a number of species, including *Beta vulgaris* (Kislev et al. 1965) and *Acetabularia mediterranea* (Puiseuex-Dao et al. 1967). Subsequently, Manning et al. (1971a, b; Manning and Richards 1972) detected 40–44 μm circular DNA molecules in lysates of *Euglena gracilis* and spinach chloroplasts. Each 40 μm circle was judged

Figure 1. Above: Photograph by David Tepfer at the July 15–24, 1976, meeting arranged at Strasbourg by Jacques-Henry Weil with Lawrence Bogorad. The meeting – Nucleic Acids and Protein Synthesis in Plants – was an advanced course sponsored by NATO, FEBS and EMBO and a colloquium sponsored by the CNRS (Bogorad and Weil 1977a, b). The participants are listed in Bogorad and Weil (1977b). Jacques-Henry Weil is eighth from the left in the front row; Lawrence Bogorad is seventh from the left. *Left:* Jacques-Henry Weil (left) and Lawrence Bogorad (right) in Paris, 1976.

Figure 2. Richard Kolodner (2002).

Figure 3. John R. Bedbrook (1977).

to be capable of coding for about 280 polypeptides of 20 kDa, and each *Euglena* chloroplast could carry between 9 and 72 circles. The genetic complexity of the DNA, they said, closely approached their 'value for the molecular size of the chloroplast DNA obtained by contour length measurements of circular DNA molecules. . . .' Richard Kolodner (Figure 2) and Krishna Tewari (1975) showed that circular DNA molecules occur in preparations from isolated plastids of other higher plants as well. Their electron micrograph length measurements were also in agreement with estimates of genome size from renaturation kinetics, indicating that a single type of circle probably occurs in the plastids of each species. (They also observed what appeared to be circles that would be multiples of the basic size. These larger circles were judged to be multimers.) The conclusion that there is one kind of DNA circle in *Zea mays* chloroplasts was supported by subsequent restriction endonuclease analyses starting in 1975–1976. The presence of many copies of the same chromosome in each chloroplast has proven to be universal to date except for dinoflagellates, whose chloroplasts contain many small DNA circles each of which contains a single gene (Zhang et al. 1999).

Single sets of recognition sites for each of three restriction endonucleases (*SalI*, *BamHI* and *EcoRI*) were mapped on *Zea mays* chloroplast DNA in 1976 (Bedbrook and Bogorad 1976a, b; Figure 3). That a single map was obtained showed that all the maize chloroplast chromosomes were the same, although very small populations of variants could have gone undetected. The major structural features of the chromosome were its circular form and the presence of a pair of large inverted repeated sequences. Comparable large inverted repeated sequences have since been located on chloroplast chromosomes, of most, but not all, species (Palmer 1991; Bogorad 1998). The *Z. mays* chloroplast chromosome was found to consist of a 12.6 kbp small unique region, a 22 kbp repeat, an 80 kbp large unique region and the second copy of the 22 kbp repeat. The two 22 kbp repeated sequences were found to be in inverted orientations to one another. Restriction endonuclease recognition site maps of the chloroplast chromosomes of *E. gracilis* (Gray and Hallick 1976) and spinach (Crouse et al. 1978) were also described early. Jeffrey Palmer at Indiana University (personal communication) estimates that restriction site maps of chloroplast chromosomes of 400–500 to perhaps more than 1000 species had been made as of the end of 2001.

Genes in the chloroplast genome

In 1976 Bedbrook (Figure 3) and Bogorad (1976a) traced genes for the chloroplast ribosomal RNAs to locations on the two large inverted repeated segments of the *Z. mays* chloroplast chromosome. To accomplish this, chloroplast DNA was digested with re-

striction enzymes and the fragments were separated according to size by agarose gel electrophoresis. The DNA fragments were transferred to nitrocellulose using the method of Southern (1975). The 16S and 23S rRNAs of chloroplast ribsomes were separated from one another, labeled with ^{32}P *in vitro* and hybridized to the restriction fragments of maize chloroplast DNA immobilized on sheets of nitrocellulose. The genes for the rRNAs were mapped to within a large restriction fragment located in each of the large inverted repeats. The genes were mapped more precisely by Southern blotting against smaller fragments of the chloroplast chromosome and electron microscopy-molecular hybridization (Bedbrook et al. 1977).

The first gene for a protein involved in photosynthesis to be identified, cloned and sequenced was that for the large subunit of Rubisco on the maize chloroplast chromosome (Coen et al. 1977; Bedbrook et al. 1979; McIntosh et al. 1980; Figures 3 and 4). In 1967, A. C. Rutner and M. D. Lane (1967) reported that Rubisco consists of two types of polypeptides: a small subunit of about 15 kDa and a large subunit of about 50 kDa. The active enzyme in higher plants is comprised of eight of each of the two kinds of subunits. Sam Wildman and coworkers made reciprocal crosses between species of *Nicotiana* and showed that the small subunit is transmitted in a Mendelian fashion. Consequently the gene (later designated *rbcS*) was judged to be in the nuclear genome (Wildman et al. 1975). The gene for the large subunit, on the other hand, was found to be transmitted maternally (Chan and Wildman 1972), suggesting that it was likely to be encoded in chloroplast DNA in tobacco. C. Correns, in 1909, had found that some chloroplast traits were inherited maternally in four-o'clock plants. A very brief summary of this kind of work is in Bogorad (1998).

Donald Coen et al. (1977) (Figure 4, left) identified a cloned *BamHI* fragment of maize chloroplast DNA that supported the production of the large subunit of Rubisco in an *in vitro* transcription-translation linked system comprised of *E. coli* RNA polymerase and rabbit reticulocyte lysate. The protein product of the transcription-translation reactions was identified (a) by its reaction with an antibody made against the large subunit of Rubisco isolated from maize and (b) by the similarity of proteoloytic fragments of the authentic maize large subunit polypeptide and of the product of *in vitro* transcription-translation. The first sequence of a gene for a plant protein to be determined was that of maize *rbcL* (McIntosh et al. 1980) (Figure 4, center and right). Some proteolytic fragments of the Rubisco large subunits of barley and spinach had been sequenced earlier; the sequences of the 244 amino acid residues obtained directly could be aligned with segments of the total sequence of the protein deduced from the nucleotide sequence of *rbcL.* This provided additional evidence that *rbcL* encoded the large subunit of Rubisco.

Some other proteins involved in photosynthesis, besides the large subunit of Rubisco, were known at the time as bands on gels or as isolated proteins. These proteins were used as antigens. The latter were employed to identify genes through their protein products made *in vitro*. Some of these genes are enumerated in a later section.

The second gene for a protein involved in a photosynthetic process (besides *rbcL*) was mapped on the maize chloroplast chromosome in 1978 (Bedbrook et al. 1978) (see photograph of G. Link in Figure 5). It is now designated *psbA*. This gene was mapped by a different approach than had been used to identify and map *rbcL* on the maize chloroplast chromosome.

Angiosperm seedlings germinated and grown in darkness are yellow. They have immature plastids and lack chlorophyll as well as many proteins of the photosynthetic apparatus. Upon illumination, the seedlings produce photosynthetic pigments and the proteins required to complete the photosynthetic apparatus. It was known in the early 1970s (L. Haff and L. Bogorad, unpublished) that transcripts representing 14% more of the maize chloroplast chromosome were present in the chloroplasts of green leaves than in etioplasts. [Subsequent, more detailed, analyses showed this number to be about 19% (Rodermel and Bogorad 1985; Figure 6).] RNAs isolated from plastids of unilluminated and illuminated dark-grown maize seedlings were labeled *in vitro* with ^{32}P and hybridized against restriction fragments of maize chloroplast DNA separated by size using gel electrophoresis and transferred to nitrocellulose filters by the method of Southern (1975). From this analysis (Bedbrook et al. 1978) and a concurrent study of thylakoid membrane protein synthesis during light-induced development of plastids in dark-grown maize seedlings (Grebanier et al. 1978; Figure 7, top), it was concluded that a 34.5 kDa polypeptide, which is a precursor of a 32 kDa thylakoid protein, corresponded to the product of the RNA that increased in abundance most conspicuously upon illumination of etiolated seedlings. This most conspicuously increased RNA hybridized to *BamHI* fragment 8 that had been mapped on the maize chloroplast chromosome.

Figure 4. Donald M. Coen (1978) (left). Lee McIntosh (1978) (center). Carsten Poulsen (1979) (right).

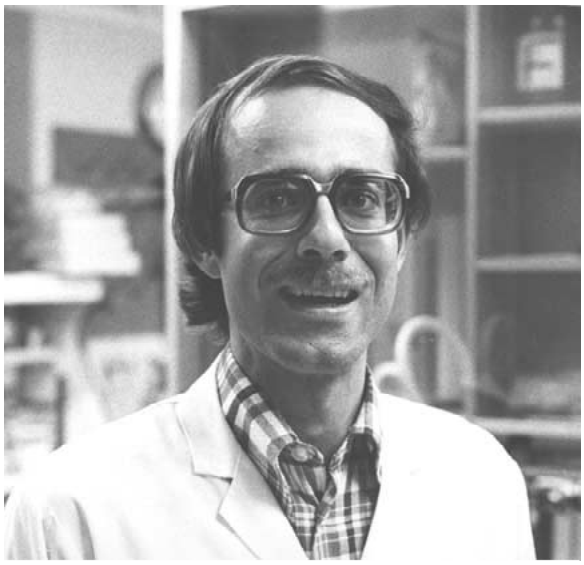

Figure 5. Gerhard Link (1977).

Figure 6. Steven R. Rodermel (1989).

The protein product of *psbA* was subsequently shown to bind azido-atrazine (Steinback et al. 1981; Figure 7, bottom) and to function in electron transport at the Q_B site in the PS II electron transport chain. The gene was sequenced by Zurawski et al. (1982a).

The early 1980s – more plastid genes for photosynthesis

Chloroplast genes for a number of proteins in addition to *rbcL* and *psbA* that were known to be components of the photosynthetic apparatus were identified, cloned and sequenced in the early 1980s. The kinds of procedures outlined above – including analysis of products of *in vitro* transcription-translation, immunochemical reactions and comparisons of proteolytic fragments as well as comparisons of predicted and experimentally determined amino acid sequences, etc. – that were employed to identify the chloroplast DNA sequence encoding the large subunit of Rubisco were used often subsequently to identify genes from their protein products. This was the era before significant gene and protein sequence databases existed. Among the genes

Figure 7. Top: Alice E. Grebanier (1978). *Bottom:* the late Katherine E. Steinback (1973).

identified and sequenced in the early to middle 1980s were those for the β and ϵ subunits of CF_1 (Krebbers et al. 1982, Figure 8; Zurawski et al. 1982b); the α subunit of CF_1 (Deno et al. 1983); subunit 3 of CF_0 (Howe et al. 1982) genes for the components of the cytochrome b_6/f complex including cytochrome f (Willey et al. 1984); cytochrome b_6 and subunit 4 (Alt et al. 1983; Heinemeyer et al. 1984); cytochrome$_{559}$ (Herrmann et al. 1984); genes for core components of PS II in addition to the *psbA* gene; the D2 polypeptide (Rochaix et al. 1984); the 47 kDa (Morris and Herrmann 1984) and the 43 kDa apoproteins of PS II (Alt et al. 1984).

Putative amino acid sequences deduced from DNA sequences were the starting points in another approach used to match proteins of the photosynthetic apparatus with unidentified open reading frames. As outlined above, transcripts of *psbA* become more abundant after dark-grown seedlings have been illuminated (Bedbrook et al. 1978). Sequencing of another region of the maize chloroplast chromosome for which transcripts also become more abundant after illumination of etiolated seedlings (Rodermel and Bogorad 1985) revealed two homologous adjacent genes (Fish et al. 1985a; Figure 9), now designated *psaA* and *psaB*. These genes encode large maize proteins of 83.2 and 82.6 kDa, respectively. The deduced amino acid sequences of the two proteins revealed high contents of hydrophobic helical regions (i.e., predicted membrane spanning regions as would be expected for a thylakoid protein). At that time the largest protein that had been detected by polyacrylamide gel electrophoresis of denatured proteins of thylakoid membranes, or of PS I or II preparations, was the PS I CPI protein of apparent molecular weight of about 70 kDa. The predicted amino acid compositions of the two large maize proteins resembled the values for barley CPI. An antibody prepared against a synthetic peptide predicted to be present in both of the proteins reacted with material in a 67,000 M_r band in a polyacrylamide-lithium dodecyl sulfate electrophoresis gel of maize thylakoid proteins. The gel band was further characterized as CPI because of its reaction with an antibody against barley CPI (Fish et al. 1985a). Through the use of antibodies against short synthetic peptides predicted to be different in the two proteins, both proteins were shown to be present in PS I reaction center preparations from maize (Fish and Bogorad 1986). Homologs of these two maize genes were identified subsequently in the plastid genomes of pea, spinach (Lehmbeck et al. 1986; Kirsch et al. 1986) and other plants. The proteins encoded by the *psaA* and *B* classes of genes are about 45% identical in sequence, and 55% similar including conservative amino acid replacements.

The work described above showed that the core of the maize (and presumably other species) PS I reaction center is comprised of two homologous proteins, encoded by *psaA* and *psaB*. By 1984 it was known that the core of the PS II reaction center was comprised of two homologous proteins, encoded by *psbA* and *psbD*. The parallel in the composition of the two reaction centers was striking.

Figure 8. Enno Krebbers (1978) (left). Ignacio M. Larrinua (1979) (center). Andre Steinmetz (1979) (right).

Among those active in identifying and sequencing chloroplast genes early in the history of the subject were the laboratories of Warwick Bottomley and Paul Whitfield, T. A. Dyer, John Gray, Richard Hallick, Reinhold Hermann, Hans Kossel, Jean-David Rochaix, Masahiro Sugiura, Erhard Stutz and Jacques-Henry Weil.

The landmark sequencing of the complete chloroplast chromosome of *Nicotiana tobacum* by Masahiro Sugiura's group in Nagoya (Shinozaki et al. 1986; see Sugiura, this issue) and of the chloroplast chromosome of *Marchantia polymorpha* by Ohyama et al. (1986) in Kyoto revealed genes for unidentified polypeptides that might be components of the photosynthetic apparatus. This information changed the landscape. Knowing the predicted sizes and properties of such previously unrecognized polypeptides directed searches for their presence in, e.g., PS I or II preparations, and analyses of their roles.

From about 1976, when the first chloroplast chromosome restriction maps were established, through about the first half of the 1980s, previously accumulated knowledge of photosynthetic polypeptides and processes permitted rapid progress toward identifying DNA sequences for encoding genes. As sequencing of chloroplast DNAs continued, open reading frames were found that encoded proteins that had not been identified as components of the photosynthetic apparatus by polyacrylamide gel electrophoresis of plastid proteins. The inventory of proteins involved in photosynthesis grew through research in molecular biology. An example of previously unrecognized proteins revealed in this way was the discovery of the two PS I reaction center core proteins. Other examples are the unidentified open reading frames for very small proteins revealed in the course of sequencing entire chloroplast chromosomes.

Nuclear genes for photosynthesis

As in the case of the chloroplast gene *psbA*, where the difference in abundance of a mRNA in leaves of illuminated and unilluminated dark-grown seedlings was used to identify the gene and track it to its location on the chloroplast chromosome (Bedbrook et al. 1978), the first nuclear genes for proteins involved in photosynthesis were identified by taking advantage of light-induced changes in cytoplasmic mRNA populations.

The first nuclear gene encoding a protein for a photosynthetic process to be studied and subsequently sequenced was *rbcS*. It encodes the small subunit of Rubisco. In 1978, P. E. Highfield and R. John Ellis (1978) isolated poly [A^+] mRNA from polysomes prepared from dark-grown unilluminated and illuminated pea seedlings. Messenger RNA for the small subunit of Rubisco was judged, by translation *in vitro* in wheat germ systems, to be much more abundant in preparations from illuminated than from unilluminated dark-grown seedlings. The protein made *in vitro* was identified by its interaction with an antibody against the small subunit of Rubisco. John Bedbrook et al. (1980) used polysomal poly [A^+] mRNAs as prepared by Highfield and Ellis as templates to prepare cDNAs that they cloned in *E. coli*. Bacteria carrying

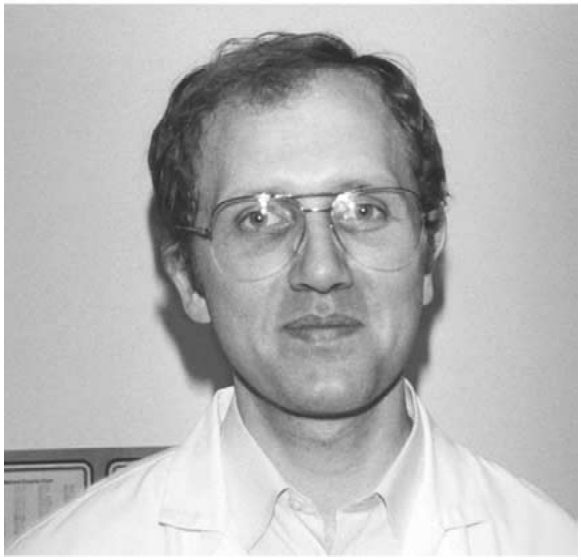

Figure 9. Top: Leonard E. Fish (1982). *Bottom:* Ulrich Kuck (1982).

the cloned cDNAs were screened with a probe population enriched in mRNAs encoding the small subunits of Rubisco. DNA prepared from a bacterial clone identified in this way was used to isolate a class of mRNA from pea leaf preparations by hybrid selection. The translation product of this hybrid-selected mRNA reacted with an antibody against the authentic Rubisco small subunit protein. The Rubisco small subunit gene, *rbcS*, protein-coding sequence was determined from the cloned cDNA.

Klaus Apel (Figure 10) and Klaus Kloppstech (1978) found that illumination of dark-grown barley

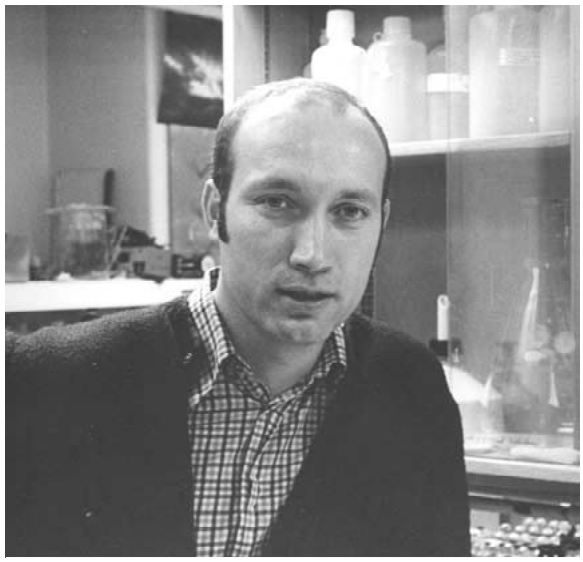

Figure 10. Klaus Apel (1974).

plants induces the appearance of a prominent mRNA species that codes for a polypeptide of Mr 29,500 that they identified as a precursor of the apoprotein of the light harvesting chlorophyll *a/b* protein of PS II. Broglie et al. (1981) identified a cDNA clone prepared from a pea leaf poly [A+] mRNA preparation that encoded a light-harvesting chlorophyll *a/b* protein. This protein had been identified previously as a band on a polyacrylamide gel using antibodies against the light-harvesting protein. The cDNA was subsequently sequenced (Coruzzi et al. 1983). Five cDNA clones for chlorophyll *a/b* proteins of PS II were also prepared from petunia leaf mRNA; sequences of the cDNAs were reported in 1983 by Pamela Dunsmuir et al. (1983).

Opening the way to functional genomics

S. V. Shestakov and N. T. Khyen (1970) observed that the unicellular cyanobacterium *Synechococcus R2* and other cyanobacteria, including *Synechocystis* PCC strain 6803 (Grigorieva and Shestakov 1982; see Shestakov 2002), could be transformed with exogenous DNA. The latter species proved to be especially easy to transform (Dzelzkalns and Bogorad 1988; Figure 11). Both *Synechococcus R2* and *Synechocystis* PCC 6803 are facultative heterotrophs and have very active homologous recombination systems. One major objective of the organizers of a 1985 conference at Cold Spring Harbor entitled ‘Molecular Biology of the

Figure 11. Valdis A. Dzelzkalns (1989).

Figure 12. Alan D. Blowers (1989).

Photosynthetic Apparatus' (Steinback et al. 1985) was to bring to the attention of students of oxygenic photosynthesis in eukaryotes the possibilities for studying the functions of photosynthetic proteins by genetic manipulations of cyanobacteria. Clones and sequences of genes for photosynthesis discovered in eukaryotes were used to identify, clone and sequence homologous genes in cyanobacteria. The cyanobacterial homologs could be inactivated or be replaced with genes mutated *in vitro* in order to investigate the operation of photosynthetic proteins. The sequence of the entire genome of *Synechocystis* PCC 6803 was published by Kaneko et al. (1996). This was an important step for photosynthesis research for two reasons. First, the genome includes a full set of the genes required for oxygenic photosynthesis. In eukaryotic plants, the sequence of a full chloroplast genome includes genes for some – not all – photosynthetic proteins. Second, the availability of the cyanobacterium's genome sequence greatly facilitated genetic manipulation of the organism's photosynthetic apparatus.

The ability to study functions of altered proteins of the photosynthetic apparatus *in vivo* increased substantially with the development of biolistic methods for introducing DNA into the chloroplast of *Chlamydomonas* (John Boynton et al. 1988). As in the case of cyanobacteria, genetic material is inserted into the chromosome by homologous recombination (Boynton et al. 1988) and foreign DNA sequences are maintained stably (Blowers et al. 1989; Figure 12). Daniell et al. (1990) demonstrated that foreign DNA can be expressed transiently in tobacco chloroplasts *in vivo* and Svab et al. (1990) reported the regeneration and propagation of tobacco plants with genetically altered chloroplasts.

Outlines of the early history of the molecular biology of photosynthetic reaction centers – getting to the heart of photosynthesis

PS II and purple photosynthetic bacteria

Sequences of chloroplast *psbD* genes, which proved to be homologous to *psbA* sequences (Zurawski et al. 1982a), were reported in 1984 (Rochaix et al. 1984; Alt et al. 1984; Holschuch et al. 1984). Inasmuch as the protein product of *psbA* had been shown to be the target of triazine herbicides (Steinback et al. 1981; Figures 4, bottom, and 7, bottom) and thus to be a part of the PS II reaction center, the product of the homologous *psbD* was judged to also be part of the PS II reaction center core. The predicted protein products of *psbA* and *D* (also designated D1 and D2) in higher plants and the products of the homologous L and M encoding genes of the photosynthetic bacteria *Rhodopseudomonas sphaeriodes* (Williams et al. 1983) and *Rhodopseudomonas capsulata* (Youvan et al. 1984; Youvan and Marrs 1985) were found to be, overall, 15% identical in alignments. This mutually reinforced the notions that these two pairs of proteins constituted the cores of the PS II reaction center and the *R. capsulata- R. sphaeroides* reaction centers, and

that these two types of reaction centers had much in common. Johann Deisenhofer et al. (1985) used the L, M and H (Youvan et al. 1984) sequence information to guide sequencing of the homologous reaction center proteins of *R. viridis* and the interpretation of X-ray diffraction patterns of the reaction center. The observed folding pattern of the L subunit in the bacterial reaction center corresponded to the folding pattern that was ascertained experimentally by Richard Sayre et al. (1986) (Figure 13) for the *psbA* product in spinach thylakoids, although the orientation of the five membrane spanning helices is reversed – the loops protruding from the stroma of the spinach thylakoid correspond to loops of the L subunit that protrude into the lumen (cytoplasm) in *R. viridis.* Thus, the three-dimensional folding patterns of the *psbA*-encoded D1 protein and the bacterial L protein are the same although the overall primary sequence identity of L, m, D1 and D2 is only 15%. The match between the structural data obtained by Deisenhofer et al. (1985) regarding the orientation of the *R. viridis* reaction center core proteins and the experimentally determined information on the folding and orientation of D1 in spinach thylakoids firmly established D1 – and by extension D2 – as core reaction center proteins. This information is also a strong statement about the evolutionary and functional relationships of the two reaction centers.

How the sequence and structural information was used to probe electron paths in PS II at the molecular level is an especially illuminating example of an important step forward in photosynthesis research through the use of molecular biology. Two electron donors, designated Z (Y_Z) and D (Y_D), had been identified by their light-induced EPR signals. Bridgett Barry and Gerry Babcock (1987) established that the D^+ signal arises from a tyrosine radical and that, most likely, Z^+ also arises from a tyrosine radical. By applying gene knockout and replacement technology, Rick Debus et al. (1988a) and Wim Vermaas et al. (1988) produced *Synechocystis* PCC*6803* strains that contained only a mutated form of the *psbD* gene. The mutation converted TYR-160 to PHE-160. Both groups found that the resultant mutants grew photosynthetically but lacked the D^+ EPR signal. From this they concluded that the TYR-160 residue is the source of the D^+ signal. Debus et al. (1988b) suggested that, because of the symmetry between *psbA* and *psbD* protein products, TYR-161 of the *psbA* product could be the source of the Z+ signal. Debus et al. (1988b) converted TYR-161 to PHE by mutagenesis of *psbA*

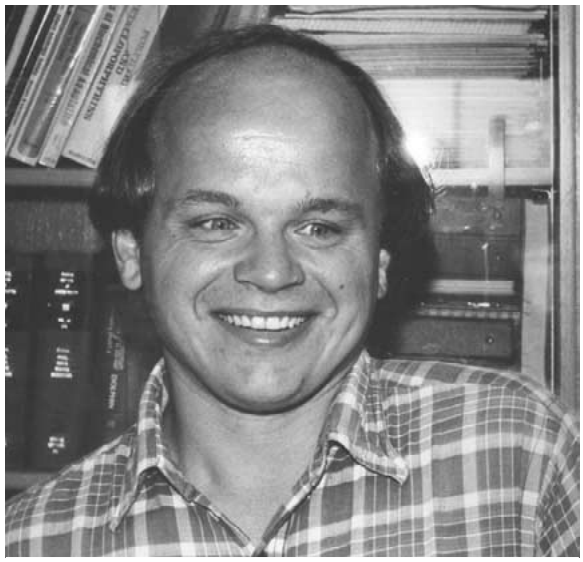

Figure 13. Richard T. Sayre (1982).

and introduced the mutated gene into *Synechocystis* PCC 6803. The altered chlorophyll fluorescence properties of the mutant led Debus et al. to conclude that TYR-161 is indeed the donor Z^+. Metz et al. (1989), who made and studied the same kind of mutant as had Debus et al. (1988b), also concluded that the Z^+ signal arises from TYR-161 of the D1 polypeptide. Not surprisingly, the roles of other PS II reaction center proteins have been investigated through comparable site-directed mutagenesis experiments.

PS I

In the mid-1970s PS I preparations were judged to contain four smaller polypeptides and a 70 kDa polypeptide, which came to be known as CPI, that associated with chlorophyll *a* molecules (Bengis and Nelson 1975, 1977; Chitnis and Nelson 1991; see Nelson and Ben-Shem 2002). But there were some reports that CPI was, in fact, comprised of two poorly resolved high molecular weight polypeptides in the 58 to 70 kDa range; these were determined to have similar amino acid compositions and to produce similar peptide fragments upon protease digestion. The prevailing view was that, if there were two large proteins in the PS I reaction center, one was a modified form of the other. As discussed above, CPI, the P700 Chl *a*-protein from the PS I reaction center, of maize has an apparent molecular weight of 66 to 69 kDa based on migration in SDS-PAGE (Steinback 1977; Metz et al. 1983). But, in fact, starting from maize chloroplast DNA gene sequence data the core of the PS I reaction

center was shown to be comprised of two homologous distinct polypeptides of 83.2 and 82.6 kDa (Fish et al. 1985a; Fish and Bogorad 1986; Figure 9). The folding of the proteins and their orientations in the thylakoid membrane proposed by Fish et al. (1985b) proved to be close to the three dimensional structure of the cyanobacterial PS I reaction center determined by X-ray crystallography (Jordan et al. 2001). The picture of PS I provided by the X-ray structure included a number of smaller proteins, many of which had been identified initially through molecular biological analyses.

As in the case of the study of PS II, deletion and mutagenesis experiments on the core and peripheral proteins became an active area of PS I research. Among early examples were experiments in which genes for PS I reaction center proteins in *Synechocystis* PCC 6803 were inactivated or deleted including: *psaF* (Chitnis et al. 1991), *psaAB* (Shen et al. 1993) or *psaB* (Smart and McIntosh 1993; Smart et al. 1993) or *psaE* (Rousseau et al. 1993). In comparable experiments, Webber et al. (1993) analyzed the effects of specific mutations in *C. reinhardtii psaB*. An especially productive result of PS I research using site-specific mutants came recently from Guergova-Kuras et al. (2001). Both the PS I and PS II cores appear to have two possible electron transfer branches. Earlier experiments with mutants had shown that only one of these branches is active in PS II. Guergova-Kuras et al. asked whether the same is true for PS I. From analyses of strains of *C. reinhardtii* with single amino acid substitutions in *psaA*- or *B*-encoded proteins, they concluded that – in contrast to electron transfer in PS II – both electron transfer branches of PS I are active.

Molecular biology and the evolution of things photosynthetic

The notion that chloroplasts might have originated from blue-green algae (cyanobacteria) dates back to observations at the end of the nineteenth century that (a) chloroplasts *in vivo* were seen to divide, and (b) that leaf chloroplasts and cyanobacteria looked about the same under the microscope – both were little green dots. In the first decade of the twentieth century K. S. Merezhkovsky and A. S. Famintsyn advanced the idea that symbionts could become integrated into other simple, but phylogenetically distinct, organisms. Merezhkovsky was particularly interested in the origin of what he called 'chromatophores' (chloroplasts). He published a paper in 1905 entitled 'The nature and origins of chromatophores in the plant kingdom' (see Khakhina 1992).

Following advances in microbiology, biochemistry and genetics, it was determined in the late 1960s and early 1970s that some aspects of plastid biochemistry (e.g., sensitivity of protein synthesis in plastids to the same antibiotics as affected bacterial processes) were more 'prokaryotic' than 'eukaryotic.' Boardman et al. (1971) edited the proceedings of a symposium held in Canberra, Australia at the end of 1969 entitled 'Autonomy and Biogenesis of Mitochondria and Chloroplasts.' The notion that chloroplasts might be autonomous organelles was considered quite seriously at that time. Just a few years later, combined biochemical and transmission genetics studies showed that some components of chloroplast ribosomes of *C. reinhardtii* are encoded in chloroplast genes and others in nuclear genes: initially Mets and Bogorad (1971, 1972; Figure 14); continued by Davidson et al. (1974; Figure 15) and Hanson and Bogorad (1977; Figure 15, bottom); and that, in tobacco, the Rubisco small subunit is inherited as expected for a nuclear gene whereas the large subunit is inherited maternally (Chan and Wildman 1972; Kawashima and Wildman 1972). Inasmuch as endosymbionts would have entered a pre-eukaryote partnership as independent complete organisms with, e.g., full sets of ribosomal rRNAs and proteins, these observations led to the conclusion that if plastids did indeed arise as endosymbionts, some of their genes had been transferred to the nucleus and/or some nuclear-encoded cytoplasmic proteins that originated in the host came to substitute for organelle or chloroplast proteins (Bogorad 1975, 1982; Figure 16). Examples of both transfer and substitution of chloroplast ribosomal proteins have since been found (reviewed in Bogorad 1998).

As discussed above, it has been known for a long time that oxygenic photosynthesis of cyanobacteria and eukaryotic plants has features in common with anoxygenic photosynthesis by bacteria. Furthermore, differences in types of anoxygenic photosynthesis among bacteria – i.e., quinone type electron acceptors in purple bacteria vs. iron-sulfur centers in green sulfur bacteria – had also been recognized. The homologies of proteins encoded by the plastid genes *psbA* and *psbD* to protein products of L and M genes of purple bacteria and the involvement of quinones in both made it quite clear that the reaction centers of PS II and of these bacteria resembled one another. What about PS I reaction centers? The reaction center cores of *Heliobacteriaceae* and green sulphur bacteria

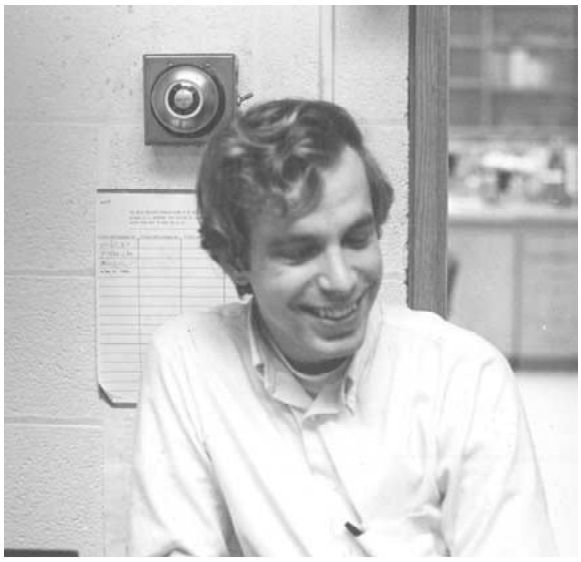

Figure 14. Laurens J. Mets (1971).

Figure 15. Top: Jeffrey N. Davidson (1973). *Bottom:* Maureen R. Hanson (1974).

of the genus *Chlorobium* are composed of two copies of a single large polypeptide. However, unlike cyanobacteria and higher plants, these green bacteria contain a single gene for the 82-kDa-reaction center protein (e.g. *Chlorobium limicola* f. *thiosulfatophilum*: Büttner et al. 1992; *Heliobacillus mobilis:* Liebl et al. 1993). The gene sequence predicts a protein that is about 15% identical in amino acid sequence to PS I-A (reviewed by Golbeck 1993). Also, iron-sulfur clusters are present in both PS I and *Chlorobium /Heliobacteriaceae* reaction centers. Among the interesting tracks revealed by such analogies are: (a) the evolutionary pathways of *psaA* and *psaB* in relation to the reaction center proteins of the *Heliobacteriaceae* and the green sulphur bacteria and (b) the track leading to the evolution and assembly of the PS I and II reaction centers for oxygenic photosynthesis in cyanobacteria and eukaryotic plants.

Systematists and evolutionary biologists have produced and made extensive use of sequences of photosynthetic genes and proteins. GenBank has over 2000 *rbcL* sequences for angiosperms plus about 600 more for other land plants. It is estimated that at least another 2000 have been mentioned in published papers but are not yet in GenBank. Approaching 5000 *rbcL* sequences! Also, it is expected that soon there will be over 100 fully sequenced chloroplast genomes.

Summary and conclusions

In 1909, Carl Correns demonstrated that some plastid traits are inherited maternally in *Mirbilis jalapa* var. *albomaculata.* Attempts to demonstrate the presence of DNA in isolated chloroplasts and *in situ* began to be made in the early 1950s, after it had been established that DNA is genetic material, but only in 1962 did Hans Ris and Walter Plaut show unequivocally that *C. reinhardtii* chloroplasts contain DNA. The mid-19th century observations of dividing chloroplasts, experiments of Correns and others, plus the observations of Ris and Plaut brought to prominence the possibility that chloroplasts might be autonomous (e.g., Board-

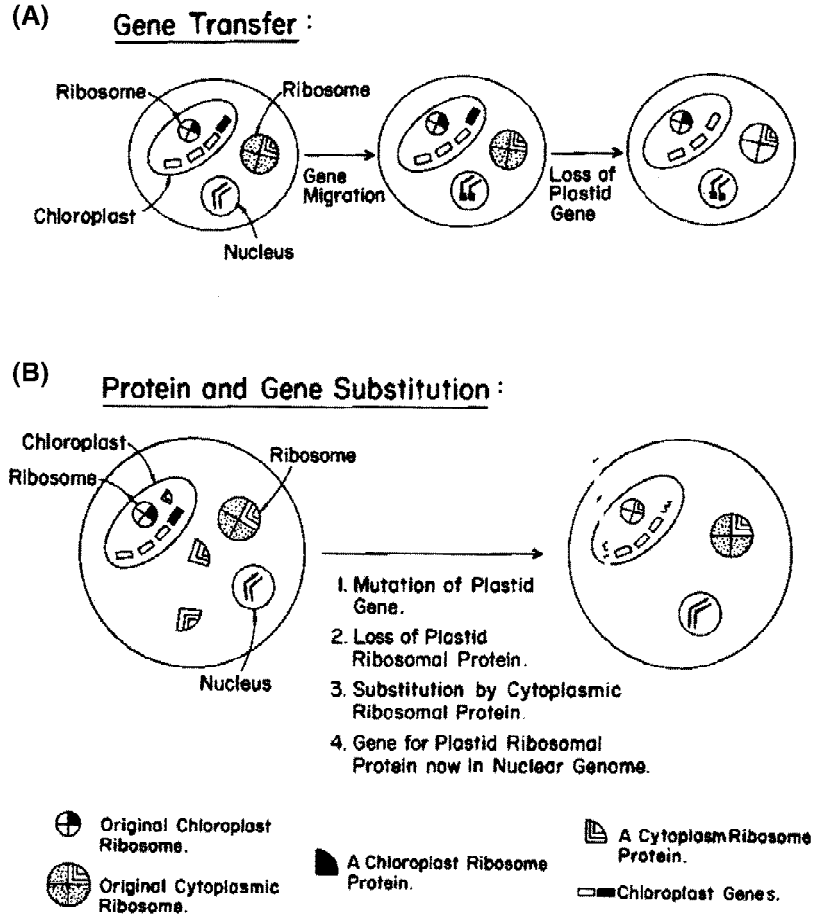

Figure 16. If plastids arose from previously free-living organisms that became endosymbionts, the free-living predecessor had to have had the genes for all of its RNAs and proteins. The discoveries that some genes for multimeric components of plastids such as ribosomes and Rubisco are dispersed – some in the plastid genome, some in the nuclear genome – together with less direct evidence regarding other proteins led to the realization (Bogorad 1975) that if plastids arose as endosymbionts, genes coding for certain of their components had moved to the nucleus or that nuclear gene products had come to substitute for chloroplast gene products. In addition, original nuclear genes – or their descendents – could have come to encode proteins for components of plastids unrelated to products of genes of endosymbiont origin. Two mechanisms proposed for dispersal of genes for plastid proteins are shown in this figure taken from Bogorad (1975). (A) A schematic representation of gene dispersal by gene transfer: The rectangles in the chloroplast or its evolutionary predecessor represent genes. The 'solid black' gene in this example codes for the 'solid black' protein of the chloroplast ribosome in the illustration. In this representation a copy of the gene becomes integrated into the nuclear genome. After acquiring appropriate DNA sequences, the gene is transcribed. The ribosomal protein is synthesized in the cytoplasm and is transported into the plastid where it is incorporated into chloroplast ribosomes. The cell and its plastids survive the loss of the chloroplast gene because the transferred gene, now in the nuclear genome, is expressed and functional. By the time of this proposal, in 1975, non-sexual gene transfer had been demonstrated in bacteria and the integration of viral genes into nuclear genomes of mammalian cells had been demonstrated. These observations of the results of gene dispersal were the first examples of extensive horizontal gene transfer between genomes – and genomes of independent species, indeed transfer between genomes of two different domains – that is now an important part of evolutionary considerations. Extensive horizontal gene transfer has been important in evolution. It has also confounded evolutionary biologists trying to draw a tree of life. (B) A schematic representation of gene dispersal by protein and gene substitution: A mutation in a gene for a chloroplast ribosomal protein renders its protein product useless. However, a ribosomal protein of cytoplasmic ribosomes might substitute. In this way, a nuclear gene and its protein product come to substitute for a chloroplast gene and its product. The original chloroplast gene that had mutated is shown here to be withering, on its way to being lost.

man et al. 1971) and contain all the genes encoding photosynthetic proteins, i.e., genes for all the proteins required for photosynthesis. Serious attempts were made to culture chloroplasts *in vitro*.

In 1971 and 1972 (Mets and Bogorad 1971, 1972; Chen and Wildman 1972; Kawashima and Wildman 1972) it was shown that some multimeric components of chloroplasts – ribosomal proteins and the small

Figure 17. Sam Granick (1909–1977) on a hiking trip in 1942. Photograph by Merrill Chase.

subunit of Rubisco, respectively – were encoded in nuclear genes. Other elements of these components were found to be encoded in the chloroplast genome. These observations led to the idea that intracellular-intergenomic gene transfer and substitution had occurred in the course of eukaryote evolution (Bogorad 1975), and the realization that photosynthetic genes are dispersed: some are in the nuclear genome and others are in the plastid genome in eukaryotic plants.

Chloroplasts were isolated first in 1938 (Granick 1938, Figure 17; W. Menke 1938). Intact chloroplast DNA prepared from isolated plastids provided the entry into the molecular biology of photosynthesis. Restriction endonuclease recognition site maps were made first in 1976. The first genes to be mapped physically on chloroplast DNA were those for the plastid 16S and 23S rRNAs (Bedbrook and Bogorad 1976a; Bedbrook et al. 1977). Then, segments of chloroplast DNA were cloned and tested for their capacity to serve *in vitro* as templates for known photosynthetic proteins. Cloned DNA segments that supported the production of the protein, initially often identified immunochemically, could then be assigned to a location on the DNA in relation to known restriction endonuclease recognition sites. This was first done for the *rbcL* gene, encoding the large subunit of maize Rubisco (Coen et al. 1977). The first sequence for a photosynthetic gene, *rbcL,* was published in 1980 (McIntosh et al. 1980). During the first half of the 1980s plastid genes for a number of other known photosynthetic proteins were identified and mapped.

As to nuclear genes for photosynthetic proteins, cDNAs for the nuclear genes encoding the small subunit of Rubisco (Bedbrook et al. 1980) and the light harvesting chlorophyll protein of PS II (Broglie et al. 1981) were obtained.

Up to about the mid-1980s the main, not inconsiderable, contribution made to photosynthesis research through molecular biology was to determine the amino acid sequences of proteins known to participate in photosynthetic processes through the sequences of the genes encoding them. Chloroplast DNA sequencing continued in the period of 1980 to the middle of the decade. Unidentified open reading frames were revealed. The deduced amino acid sequence information could then be used to identify the encoded protein. Among the latter were hitherto unrecognized photosynthetic proteins. In 1986 the complete sequences of the chloroplast genomes of tobacco (Shinozaki et al. 1986) and *Marchantia polymorpha* (Ohyama et al. 1986) were published, leading to the discovery of additional proteins of the photosynthetic apparatus.

The sequence of the entire genome of *Synechocystis* PCC 6803 (Kaneko et al. 1996) exposed – among other things – the genes for all the proteins for oxygenic photosynthesis in this cyanobacterium.

The information about photosynthetic genes and their encoded proteins that has become available through molecular biology has been used with tools for 3-dimensional structure determinations, site-directed mutagenesis and genetic transfomation-gene replacement techniques to make it possible to probe reaction mechanisms at intra- and intermolecular levels: a truly new era in photosynthesis research!

Acknowledgments

This paper is dedicated to the memory of Sam Granick (1909–1977), my post-doctoral teacher, from whom I learned enormous amounts about many kinds of things.

For my education after the time with Sam Granick, I am indebted to my many collaborators – especially to those who worked with me in my laboratory. I have included in this paper photographs that I had available of co-workers whose research is cited. If the editors had permitted, I would have slipped in photographs

of additional co-workers who contributed greatly to the advancement of subjects related to those addressed here but at times outside the period discussed. I would also have included photographs of those collaborators who studied matters not directly relevant to the subjects of this minireview. I am very grateful to all of these people regardless of whether their interesting and important work was cited. I am also indebted to J. T. Beatty and Govindjee, the editors of this volume, for their invaluable help.

All photographs except 1, 2, Donald Coen in Figure 4, and Figure 17 are by Lawrence Bogorad.

This paper was edited by J. T. Beatty and Govindjee.

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