NATO ADVANCED RESEARCH WORKSHOP

The Chloroplast:

From Molecular Biology to Biotechnology

PROGRAM / ABSTRACTS

LIST OF PARTICIPANTS

The Orthodox Academy of Crete, Kolymbari-Chania, Crete, Greece

August 10 - 15, 1998

The Orthodox Academy of Crete, Kolymbari, Chania, Crete, Greece

August 10 - 15, 1998

Cosponsored by

NATO
FESPP
The Intenational Society for Chloroplast Development

Under the auspices of

The National Center for Scientific Research Demokritos
Athens, Greece

Supported by

The General Secretariat of Research & Technology,
Greek Ministry of Development

Co-directors

Argyroudi-Akoyunoglou, J.H.
Institute of Biology
NCSR "Demokritos"
Aghia Paraskevi, Attiki, Greece

Karapetyan, N.
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International Organizing Committee
Argyroudi-Akoyunoglou, J.H. (Athens,Greece) (Director)
Bogorad, L. (Cambridge, MA, USA)
Karapetyan, N. (Moscow, Russia) (Co-director)
Ohad, I. (Jerusalem, Israel)
Senger, H. (Marburg, Germany) (Treasurer)

Local Organizing Committee
Argyroudi-Akoyunoglou, J.H., Athens, Greece
Kotzabasis, K., Heraklion, Crete, Greece
Papageorgiou, G., Athens, Greece
Prombona, A., Athens, Greece
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Monday August 10.

14.00	**REGISTRATION**
19.30	Welcome Adresses **ARGYROUDI-AKOYUNOGLOU, J.H.** (Greece) **SENGER, H.** (Germany) **TZANAKAKIS, G.** The Mayor of Chania, Crete, Greece
20.00	Opening lecture Chair: **SENGER, H.** (Germany) **HILLER R**. (Australia) Dinoflagellate light harvesting proteins: genes, structure and reconstitution
21.00	**-Welcome reception-**

Tuesday August 11

Molecular Structure of key photosynthetic proteins

8:30 Chair: **BARBER, J.** (UK)
Structure of Photosystem II
9.00 **KRAUSS, N.** (Germany)
X-ray crystallographic studies on the 3-D structure of Photosystem I
9.30 **MOUDRIANAKIS, E.** (USA/Greece)
Tracing the emergence of CF1 and the roots of alternating-site catalysis: is CF1 a Mazda engine?

10.00 **-Coffee-**

10.30 Chair: **KARAPETYAN, N.** (Russia)
Organization and function of Photosystem I trimeric and monomeric complexes of Cyanobacterium *Spirulina platensis*
11.00 **MARQUARDT J. (**Germany)
The photosynthetic apparatus of Prochloron-like cyanobacteria
11.30 **ROEGNER, M.** (Germany)
Membrane proteins of Photosynthesis: Structure-Function-Biotechnology

13.00 **-Lunch -**

14.30 **POSTERS - ATTENDANCE at posters on**
Structure, Import and Translocation, Pigment biosynthesis

-Coffee-

The chloroplast envelope and protein import

16.00 Chair : **CLINE, K.** (USA)
Protein transport to the thylakoid lumen
16.30 **SOLL, J.** (Germany)
The protein translocation apparatus of chloroplast envelopes
17.00 **JARVIS, P.** (USA)
Characterization of the Arabidopsis *ppi* mutant
17.30 **HOOBER, K**. (USA)
Distribution of LHCP between chloroplasts and vacuoles during chloroplast development in *Chlamydomonas reinhardtii*
18.00 **ROLLAND, N.** (France)
Disruption of the plastid YCF10 open reading frame affects uptake of inorganic carbon in the chloroplast of *Chlamydomonas*
18.30 **KRUPINSKA, K.** (Germany)
Tocopherol biosynthesis in scenescing chloroplasts. A mechanism to protect envelope membranes against oxidative stress and a prerequisite for lipid remobilization?

19.00 **-Dinner-**

20.00 **Visit to the old harbor of Chania**

Wednesday, August 12

Pigment biosynthesis- Regulation and biotechnological approaches

8:30 Chair: **SENGER, H.** (Germany)
Introduction
8.45 **RUEDIGER, W.** (Germany)
Redox-reactions in the last steps of Chlorophyll biosynthesis
9.15 **KANNANGARA G.** (Denmark)
tRNA^{glu} mediated δ-ALA biosynthesis and Biotechnology

9.45 **-Coffee-**

10.15 Chair: **HORVATH G.** (Hungary)
Introduction
10.30 **TIMKO, M.** (USA)
Light-independent and light-dependent chlorophyll biosynthesis in higher plants and algae
11.00 **GRIMM, B.** (Germany)
Tetrapyrrole biosynthesis-Prospects for pathway engineering
11.30 **ROEMER, S.** (Germany)
Carotenoid biosynthesis
12.00 **HIRSCHBERG, J.** (Israel)
Regulation of gene expression in the carotenoid biosynthesis pathway of plants.

13.00 **-Lunch-**

14.00 **EXCURSION**

-visit to the Heraklion Museum and the Palace at Knossos-

Thursday, August 13

Regulatory mechanisms in photosynthetic unit assembly

8.15 Chair: **ARGYROUDI- AKOYUNOGLOU, J.** (Greece)
Introduction

8.30 **CHITNIS, P.** (USA)
Photosystem I abundance in cyanobacteria and chloroplasts

9.00 **VAN WIJK , K.** (Sweden)
Targeting, synthesis and assembly of D1 protein

9.30 **HUNER , N.** (Canada)
Redox control of the development of the photosynthetic apparatus

10.00 **VERMAAS, W.** (USA)
Chl synthesis and biogenesis of Chlorophyll-binding proteins in Cyanobacteria

10.30 **-Coffee-**

11.00 **PAKRASI**, **H** (USA)
Proteins regulating PSI and PSII biosynthesis/function

11.30 **ADAM, Z.** (Israel)
Proteolytic enzymes in the chloroplast-Their role in maintaining a functioning organelle.

12.00 **MATTOO, A.** (USA)
Photoregulation of D1 phosphorylation and isolation of a putative D1 kinase

12.30 **ADAMSKA, I.** (Sweden)
Light stress-activated proteases of the thylakoid membranes and the thylakoid lumen

13.00 **-Lunch-**

14.30 **POSTERS - ATTENDANCE at posters on Regulatory mechanisms in PSU assembly**

-Coffee-

16.00 **POSTER DISCUSSION**
Discussion leader: SESTAK, Z. (Chech Republic)

18.00 **DISCUSSION SESSION on**
The dynamics of thylakoid protein phosphorylation: significance and regulation
OHAD, I. (Israel) Discussion leader

19.00 **Evening lecture**
BOGORAD, L. (USA)
"More lessons from RUBISCO genes"

20.30 **BARBEQUE DINNER WITH CRETAN MUSIC AND DANCERS**

Friday, August 14

Gene expression and its regulation

8.30 Chair: **ROCHAIX D-J.** (Switzerland)
Synthesis and assembly of photosynthetic complexes in *Chlamydomonas*

9.00 **BOERNER**, **T.** (Germany)
Phage type Plant RNA polymerases in higher plants

9.30 **SCHUSTER, G.**(Israel)
mRNA processing, translation, polyadenylation, degradation in the chloroplast.

10.00 **-Coffee-**

10.30 **APEL, K.** (Switzerland)
Stepping out of the dark: the effect of light on the biosynthesis and function of PORA and PORB in seedlings of angiosperms

11.00 **KLOPPSTECH, K**. (Germany)
Posttranscriptional control in the expression of the high-light-regulated messenger RNA coding for "HL#2"-proteins

11.30 **MAYFIELD, S.** (USA)
Protein/RNA interactions in light-regulated translation in the chloroplast

12.00 **EICHACKER, L.** (Germany)
Control of translation elongation in chloroplasts

13.00 **- Lunch -**

14.30 **POSTERS - ATTENDANCE at posters on**
Gene expression, biotechnological approaches

-Coffee-

16.00 **POSTER DISCUSSION**
SOFROVA, D. (Chech Republic) Discussion leader

17.00 **DISCUSSION SESSION on**
Editing, mRNA stability, processing
WEIL, J. (France) Discussion leader

18.00 **DISCUSSION SESSION on**
Circadian control of gene expression
GOLDEN, S. (USA) Discussion leader

19.00 **Evening lecture**
HERRMANN, R. (Germany)
The chloroplast as a part of the integrated genetic system of the plant cell

20.00 **-Dinner-**

Saturday, August 15

Chloroplast Genetic manipulation and biotechnological approaches

10.15	Chair: **PAPAGEORGIOU, G.** (Greece) Introduction
10.30	**MALIGA, P.** (USA) A transgenic approach to characterize the plastid transcription machinery in higher plants
11.00	**DANIELL, H. (USA)** Containment of herbicide resistance through genetic engineering of the chloroplast genome
11.30	**VALCKE, R.** (Belgium) Structure & function of the photosynthetic apparatus in transgenic tobacco
12.00	**MURATA, N.** (Japan) Genetic manipulation of membrane lipids: a clue to elucidate the molecular mechanism of cold and salinity tolerance
12.30	**GENERAL DISCUSSION** **/Closing remarks/**
13.00	**- Lunch -**
16.00	**-Departure-**

POSTERS

Structure

(Attendance at posters: **Tuesday** 14.30-16.00)

S-1. **Adir, N., Anati, R., Cheredman, V., Dobrovetzki, Y. and N. Lerner** (Israel)
Progress in the crystallization of the reaction center of Photosystem II, the 33kDa protein, cytochrome b_{559} and CP29

S-2. **Kruip J.** (Germany)
Structure and function of PSI.

The chloroplast envelope - Import and translocation

(Attendance at posters: **Tuesday** 14.30-16.00)

I-1. **Aronsson, H., Almkvist, J., Sundqvist, C., Timko, M.P. and C. Dahlin** (Sweden)
Characterization of the plastid import reaction of the pea NADPH:Protochlorophyllide oxidoreductase (POR)

I-2. **Chrost, B. and K. Krupinska** (Germany)
Alpha-galactosidases - enzymes for the remobilization of thylakoid membrane glycolipids during barley leaf senescence

I-3. **Ishida, K., Cavalier-Smith, T. and B.R. Green** (Canada)
A hypothesis for the import of nuclear-encoded proteins into chloroplasts with four envelope membranes

I-4. **Koussevitzky, S., Ne'e̱man, E. and E. Harel** (Israel)
Import and processing of *E.coli* expressed polyphenol oxidase by isolated chloroplasts

I-5. **Sonoda, M., Katoh, H., Katoh, A., Ohkawa, H., Vermaas, W. and T. Ogawa** (Japan)
Structure and function of *cemA* homologue (*PXCA*) in cyanobacteria

I-6. **Schroeder, W. P., Hagman, A., Andersson, B. and T. Kieselbach** (Sweden)
The thylakoid lumen of chloroplasts: isolation and characterization

I-7. **Sohrt, K., Heinemann, I. and J. Soll** (Germany)
The import complex of the chloroplastic outer envelope membrane

Pigment biosynthesis and its regulation

(Attendance at posters: **Tuesday:** 14.30-16.00)

P-1. **Engdhal, S., Dahlin, C., Sundqvist, C. and M. Timko** (Sweden)
Assembly of *in vitro* synthesized NADPH:Protochlorophyllide oxidoreductase into isolated prolamellar bodies and prothylakoids

P-2. **Miyashita, H., Kurano, N. and S. Miyachi, S.** (Japan)
Function of Chlorophyll d in Photosynthesis

P-3. **Oster, U., Lenz, S., Mayer, K., Klement, H. and W. Ruediger** (Germany)
Chlorophyll synthase of *Avena sativa L.*, a house keeping enzyme

P-4. **Kaidis, A. D., Argyroudi-Akoyunoglou, J. H. and A. Prombona** (Greece)
Interaction of external stimuli with the endogenous circadian rhythm in the capacity of etiolated bean to form Chl, LHCII, and Lhcb mRNA.

P-5. **Scheumann, V., Schoch, S. and W. Ruediger** (Germany)
Chl *a* formation in the Chl *b* reductase reaction requires reduced ferredoxin

P-6. **Schoch, S., Oster, U, Mayer, K., Feick, R. and W. Ruediger** (Germany)
Substrate specificity of overexpressed bacteriochlorophyll synthase from *Chloroflexus auriantiacus*

P-7. **Sperling, U, Frank, F., van Cleve, B., Frick, G., Apel, K. and G. Armstrong** (Switzerland)
Functions of the light-dependent PORA and PORB enzymes in etioplast differentiation & photooxidative protection

P-8. **Seyyedi, M., Timko, M.P. and C. Sundqvist** (Sweden)
Chlorophyll formation in the Lip1 mutant of pea

P-9. **Walmsley, J., Adamson, H., Wright, W., Wrench, P. and F. Sharples** (Australia)
Can Psilotum and Gnetum green in darkness?

Regulatory mechanisms in photosynthetic unit formation-assembly

(Attendance at posters: **Thursday** 14.30-16.00)

R-1. **Doernemann, D., Navakoudis, E. and Kotzabasis K.** (Greece)
Alterations in the plastid membrane-associated polyamines during chloroplast photodevelopment

R-2. **Droppa, M., Kota, Z., Pali, T., Szalontai, B., Horvath, L. I. and G. Horvath** (Hungary)
Structural-functional organization of thylakoids in developing chloroplasts

R-3. **Georgakopoulos, J. H. and J.H.Argyroudi-Akoyunoglou** (Greece)
The action of free radical scavengers on thylakoid protein phosphorylation and PSII core protein degradation

R-4. **Georgakopoulos, J. H., Prombona, A. and J. H. Argyroudi-Akoyunoglou** (Greece)
The abundance of Cab and psbA transcripts and of their LHCII and D1 protein products in greening etiolated leaves transferred to darkness.

R-5. **Gruber, J., Meister, A., Beyer, A. and K. Kloppstech** (Germany)
Expression of Early-Light-Inducible-Proteins (ELIPS) in mesophyll and bundle sheath cells of the C4-plant maize (*Zea Mays, L.*)

R-6. **Humbeck, K. and K. Krupinska** (Germany)
Successive degradation of the light-harvesting system of the photosynthetic apparatus during senescence of barley flag leaves

R-7. **Kotzabasis, K., Strasser B., Navakoudis, E., Doernemann, D. and H. Senger** (Greece)
The regulatory role of polyamines on the structural and functional photoadaptation of the photosynthetic apparatus

R-8. **Mueller, B. and L. A. Eichacker** (Germany)
De novo synthesis of Chla triggers assembly of Reaction Centers in barley etioplasts

R-9. **Schevchenko, O.** (Ukraine)
Changes of PSI particle characteristics at different conditions of chloroplast protein phosphorylation

R-10. **Sestak, Z.** (Czech Republic)
Chlorophyll fluorescence kinetics depends on age of leaves and plants

R-11. **Seyyedi, M., C. Sundqvist and M.P. Timko** (Sweden)
Induction of wild type morphology by application of cytokinins on dark grown Lip1 mutant of pea

R-12. **Tzivelekа, L.-A. and J.H. Argyroudi-Akoyunoglou** (Greece)
The proteolysis of LHCII in chloroplasts

R-13. **Yamamoto, Y. and K. Satoh** (Japan)
The carboxyl-terminal processing of precursor D1 protein of Photosystem II reaction center by a nuclear-encoded protease (CtpA). - An analysis of enzyme-substrate interaction using overexpressed protease

Genes and gene expression

(Attendance at posters: **Friday** 14.30-16.00)

G-1. **Eggink, L., Wolfe, G., Park, H. and K. Hoober** (USA)
Characterization of two nuclear photosynthetic mutants of *Chlamydomonas reinhardtii*

G-2. **Funk C. and W. Vermaas** (Sweden)
Small CAB-like proteins: relatives to the Chla/b binding proteins in cyanobacteria.

G-3. **Krajcovic, J., Vacula, R., Loeffelhardt, W., Belicova, A , Slavikova, S. and E. Stutz** (Slovakia)
Molecular effects of some stress factors on the chloroplast genetic apparatus of the flagellate "*Euglena gracillis D*"

G-4. **Krause K. and K. Krupinska** (Germany)
Characterization of two RNA polymerases from spinach chloroplasts

G-5. **Liere, K. and P. Maliga** (USA)
In vitro characterization of promoters for the nuclear encoded plastid RNA polymerase (NEP)

G-6. **Lino-Neto, T., Tavares, R. M., Palme, K. and M.S.S. Pais** (Portugal)
Expression of glutathione peroxidase during *Zanthedeschia aethiopica* spathe senescence and regreening.

G-7. **Maliga, Z.** (USA)
Targeted deletion of promoters for the plastid ribosomal RNA operon indicate that operon expression depends on the eubacterial type plastid RNA polymerase

G-8. **Muehlbauer S. and L.A. Eichacker** (Germany)
Translational reegulation of LSU by the photosynthetic proton gradient.

G-9. **Piques, M.C., Lino-Neto, T., Palme, K., Pais, M.S.S. and R.M. Tavares** (Portugal)
Expression of *rbcS, rbcL* and *rca* genes during senescence and regreening of *Zanthedeschia aethiopica* floral spathe

G-10. **Rolland, N., Janosi, L., Block, M. A., Shuda, M., Teyssier, E., Miege, C., Cheniclet, C., Carde, J.-P., Kaji, A. and J. Joyard** (France)
Characterization of a chloroplast homologue of the "Ribosome Recycling Factor" (RRF) from *Escherichia coli*

G-11. **Suzuki, J. and P. Maliga** (USA)
Gene expression in tobacco plastids lacking the putative transfactor interaction domain of the eubacterial-type RNA polymerase α subunit.

G-12. **Van der Kooij, T.A.W., Krause, K., Doerr, I. and K. Krupinska** (Germany)
Molecular, functional and ultrastructural characterization of plastids from seven species of the parasitic flowering plant genus *Cuscuta*

G-13. **Yurina, N.P., Oleskina, Y.P., Melnik, S.M. and M.S. Odintsova** (Russia)
DNA-binding proteins of chloroplast nucleoids

Biotechnological approaches

(Attendance at posters: **Friday** 14.30-16.00)

B-1. **Allakhverdieva, Ya., Ferimazova, N. and R. Gasanov** (Azerbaijan)
The stabilizing effect of glycinebetaine on the various oxygen evolving photosystem II complexes under heat stress

B-2. **Daenhardt, D., Falk, J., Krupinska, K., Moelleken, H. and R. Schulz** (Germany)
Tocopherol biosynthesis in *Synechocyctis* sp. PCC6803 in response to oxidative stress

B-3. **Stamatakis, K. and G. C. Papageorgiou** (Greece)
Cytoplasmic water deficit and Chl a fluorescence in cyanobacteria.

B-4. **Piletskaya, E.V., Piletsky, S. A., Sergeeva, T.A., El'skaya, A. V., Sozinov, A. A., Rouillon, R. and J.-L. Marty** (Ukraine)
Application of thylakoids in herbicide-detecting system

B-5. **Schmid, G., Radunz, A., He, P. and Ch. Kedeinis** (Germany)
Influence of a CO_2-partial pressure of 700 ppm on the lipid and fatty acid composition of higher plants

B-6. **Zacharieva, I., Busheva, M., Markova, Tz. and M. Velitchkova** (Bulgaria)
The role of membrane fluidity for primary photosynthetic processes of pea thylakoids

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ABSTRACTS

(in alphabetical order of first author's name)

The Orthodox Academy of Crete, Kolymbari-Chania, Crete, Greece

August 10 - 15, 1998

PROTEOLYTIC ENZYMES IN THE CHLOROPLAST

Z. Adam
Department of Agricultural Botany, The Hebrew University, Rehovot 76100, Israel

The maintenance of a functioning chloroplast requires an elaborate proteolytic machinery in the different compartments of the organelle. As a major cellular site of light absorption and extensive redox reactions, proteins in the chloroplast are prone to photo- and oxidative damage, and need to be removed. Adaptation to changing environmental conditions involves proteolytic degradation of specific proteins. Balancing the stoichiometry of multi-protein complexes results in degradation of unassembled proteins. Similarly, proteins lacking their prosthetic groups are rapidly degraded, and so are the products of premature termination of transcription or translation.

As a first step toward elucidation of the regulation of these processes, we sought to identify chloroplast proteases. Three criteria were employed in establishing the identity of a chloroplast protease: i. isolation of a gene; ii. characterization of its product; and iii. demonstration of proteolytic activity. The current repertoire of chloroplast proteases consists of homologues of known bacterial proteases, distributed in three different sub-compartments of the organelle.

The proteolytic subunit of Clp protease, ClpP, is the only chloroplast-encoded component of the proteolytic machinery. It is a serine-type protease, dependent on a cognate ATPase as a regulatory subunit for its proteolytic activity. The association between the two subunits requires binding of ATP, whereas protein degradation requires ATP-hydrolysis. Two different proteins can serve as regulatory subunits: ClpC, a large ATPase containing two ATP-binding domains, and a smaller one, ClpX, containing a single ATP-binding domain. The expression of subunits of Clp protease is constitutive, it is localized to the chloroplast stroma, and it can degrade unassembled soluble proteins.

The thylakoid membrane contains a homologue of FtsH protease. This is an ATP-dependent metalloprotease, anchored to the membrane by two trans-membrane α-helices. The catalytic and ATP-binding sites are exposed to the stroma, making it available for degradation of both membrane-bound and soluble stromal substrates. Unlike Clp protease, its expression is totally dependent on light. Another serine protease is associated with the lumen side of the thylakoid membrane. This is a homologue of the ATP-independent protease DegP. Its association with the membrane is mediated probably by hydrophobic interactions, and its localization suggests a role in degradation of both thylakoid membrane proteins and soluble lumenal proteins. Similar to its bacterial homologue, its expression is stimulated by heat.

ClpC and ClpX and DegP contain in their C-termini PDZ-like domains. PDZ domains were recently implicated in protein-protein interactions in a wide range of biological systems, including bacterial proteases and molecular chaperones. The implication of these on substrate binding and/or recognition in chloroplasts will be discussed.

LIGHT STRESS-DEPENDENT PROTEOLYSIS IN THE CHLOROPLASTS

Iwona Adamska
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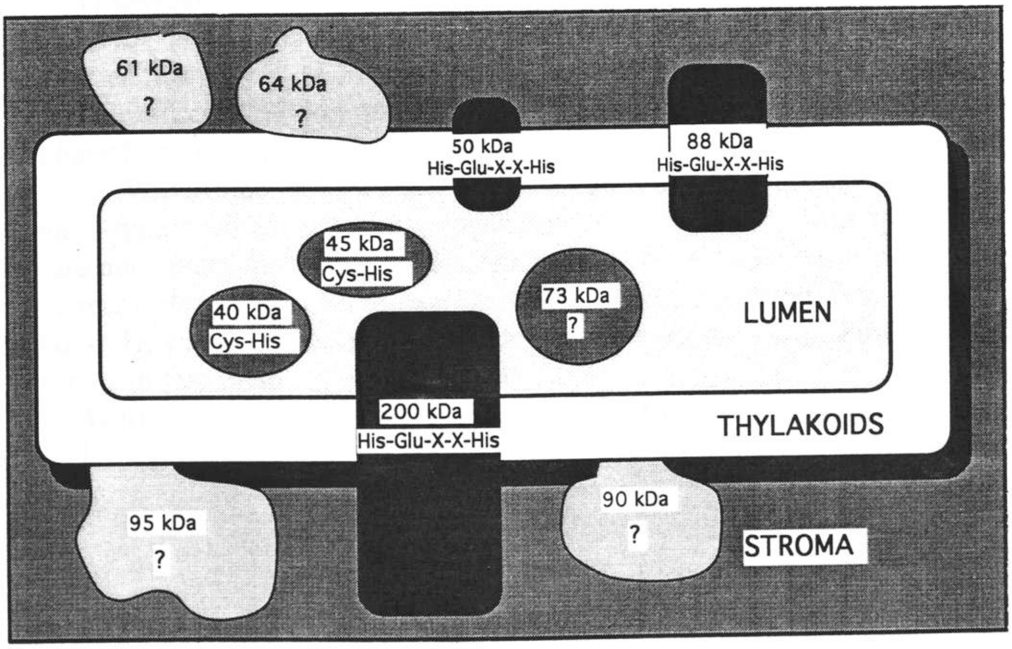
Two types of proteolysis can be distinguished in the chloroplasts: (i) protein processing which involves cleavage of signal sequences during protein import into organelles, and (ii) destructive proteolysis which leads to loss of particular proteins from various chloroplast compartments. The substrates degraded by the latter one can be grouped into three classes: (a) "non-functional" proteins, such as misfolded or mistarget proteins which accumulation is promoted under various stress conditions; (b) "timing proteins" which function is restricted to limited period of time, and (c) "proteins without partners", such as members of multiprotein complexes or protein without cofactors. My current work is related to the degradation of the proteins under ligth stress conditons and identification of the proteases which are involved in this process. Using "activity gels" and chromogenic artificial substrates I was able to identify 10 proteolytical activities which were present in various compartments of the chloroplasts under light stress conditions (for summary see schema below). All of these proteases were characterized with respect to their catalytic centers, pH-optimum, ATP- or ion-requirements. Three of these proteases were found to be localized in the thylakoid lumen. An evidence is provided that at least two processes are directly or indirectly involved in the regulation of the activity of lumenal proteases under light stress conditions. One of these processes is the change of the trasthylakoidal delta-pH, which increases under light stress conditions due to uptake of protons from the stromal side of the membrane and their release to the thylakoid lumen. The second is the change in the ion concentration in the membrane surrounding. In addition to the lumenal proteases four of the proteolytic actvities have been found to be peripheral attached to the thylakoid membranes and three to be integral membrane proteins. Mechanisms involved in the activation/inactivation of these proteases will be discussed.



PROGRESS IN THE CRYSTALLIZATION OF THE REACTION CENTER OF PHOTOSYSTEM II, THE 33kDa PROTEIN, CYTOCHROME b_{559} AND CP29

Noam Adir, Rina Anati, Valeria Cheredman, Yelena Dobrovetzki and Natalia Lerner
Department of Chemistry, Technion, Technion City, Haifa 32000 Israel

Primary photosynthetic reactions occur within the large membrane protein complexes called reaction centers. Of the different reaction center types, one of the most complex is that of Photosystem II, found in all higher plants, green algae and cyanobacteria. It is the site of the initiation of linear electron flow, proton gradient formation and oxygen evolution. Reaction center II (RCII) contains 9 proteins, 7 different cofactors and has a molecular weight of about 250,000. Because of the high degree of complexity in the function of these systems, it is necessary to have structural information on both the cofactors and unique protein environment surrounding each of the cofactors. Experimental determination of large protein complex structures can be achieved only by crystallographic methods. In our lab, we are attempting to determine the structure of RCII using X-ray crystallographic methods. Data will be presented describing our latest efforts towards obtaining this goal:

A. We have crystallized the entire RCII complex in various detergent mixtures. The RCII crystals diffract to ~8Å., but due to high mosaicity and stability problems, data collections is not yet possible. We are using HPLC and gel electrophoresis to try and analyze the interaction between the RCII complex and the detergent mixes used for crystallization. This in turn will be used to try and obtain a more homogenous fraction of RCII following detergent treatment, in order to obtain higher quality crystals.

B. Because of the large size and complexity of RCII, we are also trying to isolate various protein components and determine their structure separately. These partial structures will be important to help determine those functions associated with the particular subunit and may also be useful for solving the structure of the entire complex:

1) Oxygen evolution in RCII is dependent on the presence of a tetramanganese cluster, protected by a membrane associated protein called the 33kDa protein. We have successfully isolated this protein, to apparent homogeneity. We are performing extensive searches for proper crystallization condition for this protein. Preliminary results indicate that small microcrystals have been obtained.

2) Within the core of all RCII complexes is a unique di-peptide 15kDa transmembrane b-type cytochrome called cytochrome b_{559}. The function of this component is unclear. We are attempting to isolate the cytochrome b_{559} by a variety of methods in order to crystallize it: direct isolation from RCII; overexpression of a unique fused gene; reconstitution of isolated peptides. This project has been undertaken in collaboration with Prof. I. Ohad of the Hebrew University in Jerusalem.

3) RCII is connected to the main antenna, LHCII by a number of intermediate antenna molecules. One of them, CP29, has been isolated and the first crystals have been obtained.

THE STABILIZING EFFECT OF GLYCINEBETAINE ON THE VARIOUS OXYGEN EVOLVING PHOTOSYSTEM II COMPLEXES UNDER HEAT STRESS

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The compatible solute glycinebetaine is strongly implicated as an osmoprotectant in cyanobacteria, green algae and high plants (Papageorgiou GC and Murata N [1995] Photosynth Res 44:243-252). It was shown that glycinebetaine protected the photosynthetic oxygen evolving machinery of thylakoid and subthylakoid preparations from cyanobacteria against thermally induced and salt induced inactivation.

We carried out is experiments using oxygen evolving photosystem II particles (OE PS II) and modified OE PS II particles with the different composition of oxygen evolving complex (OEC) extrinsic polypeptides. OE PS II particles were isolated by Triton X-100 treating from spinach leaves *(Spinacia oleracea)* according to Berthold DA, Babcock GT and Yocum CF (FEBS Lett [1981] 134:231236) with some modifications. OE PS II particles depleted of 17 and 23 kD extrinsic polypeptides (d PS II) were obtained as described by Kuwabara T and Murata N ([1983] Plant Cell Physiol.24:741-747). To obtain OE PS II particles lacked all three extrinsic polypeptides (dd PS II) were obtained as described by Ono T and Inoue Y [1984] FEBS Lett 168:281-286). Heat inactivation of photosynthetic activity of untreated and modified OE PS II particles was performed by samples incubation for 10 min in the dark at various temperatures in the range from 20^0C to 65^0C in the presence or absence of 0.5 M glycinebetaine. Photosynthetic oxygen evolution was measured by Clark-type oxygen electrode in the presence of 0.4 mM PBQ and 0.1 mM $K_3[Fe(CN)_6]$ as electron acceptor. Photoreduction of DCIP by the ddPSII was measured spectroscopically by monitorings absorption changes at 600 nm, with DPC as artificial electron donor. Glycinebetaine stabilized the photosynthetic activity OE PS II particles at all used temperatures and had a stimulation effect. The results presented here strongly suggest the protective role for glycinebetaine against heat destabilization of dPSII particles. At the same time, the level for 50% inactivation of dPSII OEA in the presence of glycinebetaine was the same as of OE PS II particles demonstrated in the presence of glycinebetaine. These results may be explained by the hypothesis that heat stress results in dissociation of 33 kDa protein from the OEC and glycinebetaine prevents this process. In conclusion, the results of these experiments suggest that glyinebetaine protected ddPSII particles against heat stress.

Stepping out of the dark: the effect of light on the biosynthesis and function of the protochlorophyllide oxidoreductases A and B in seedlings of angiosperms

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Angiosperms strictly couple Chl production to the light by relying exclusively on the nuclear encoded, plastid-localized and light-dependent NADPH-protochlorophyllide oxidoreductase (POR) to reduce Pchlide to Chlide. In contrast, other oxygenic photosynthetic organisms contain not only POR, but also a structurally unrelated, dark active Pchlide-reducing enzyme, whose three subunits are plastid-encoded. While in lower plants and photosynthetically active procaryots only one POR enzyme has been detected (POR B in higher plants) in spermatophyta an additional second enzyme (POR A) appears that is characterized by several rather unique properties. Both POR A and POR B are expressed in etiolated tissues. Accumulation of POR A mRNA is, however, negatively regulated by light and temporally restricted to young seedlings. Furthermore, the import of the precursor of POR A into the plastid requires Pchlide. After the beginning of illumination the concentration of Pchlide in etiolated seedlings drops sharply and soon becomes too low to support further unlimited uptake of the precursor into the plastid compartment. In contrast, the import of the POR B precursor occurs independently of the Pchlide concentration. The possible physiological significance of these striking differences between the two POR proteins will be discussed.

CHARACTERIZATION OF THE PLASTID IMPORT REACTION OF THE PEA NADPH: PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR).

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The light-dependent reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is catalyzed by the enzyme NADPH:protochlorophyllide oxidoreductase (POR). POR is nucleus-encoded and post-translationally imported into the plastids where it accumulates in the prolamellar bodies (PLBs) but decreases during illumination. This anomaly of decreasing POR levels during active chlorophyll synthesis was cleared with the discovery of two different POR protein, POR A and POR B, in barley and *Arabidopsis thaliana*. During greening, POR A is negatively regulated by light both at transcriptional as well as proteolytical levels whereas the amounts of POR B is largely unaffected. In addition, import of POR A has been suggested to require Pchlide in order to be translocated into the plastid. In this respect, POR A differs from other known nucleus-encoded plastid proteins, and as it appears, represents a novel and exclusive import characteristic.

In pea, only one POR gene has been found indicating that the situation for the regulation of POR import and accumulation is far from clear. We here present data from import reactions of pea pPOR into plastids where increased levels of Pchlide have been obtained either by added as free pigment or as a product of ALA-incubation to the import suspension prior to the translocation. The results obtained show pea pPOR to be independent upon its substrate for proper import. This indicate pea POR to be more similar to the POR B regulation than the substrate-dependent POR A regulation. To further characterize the translocation pathway of POR, we here present results from import assays into plastids with pea pPOR under different temperature conditions and with or without co-factor, ATP and illumination. Etioplasts and chloroplasts have been purified from plants of different species and used for *in vitro* translocation reactions employing radiolabelled protein. Import efficiencies have thereafter been analysed by SDS-PAGE followed by fluorography.

ELUCIDATION OF THE STRUCTURE OF PHOTOSYSTEM II AT HIGH RESOLUTION

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A combination of electron crystallography and single particle analyses is being employed to elucidate the structure of photosystem two (PSII). The latter technique has been particularly useful to obtain a 3D reconstruction of a 720 kDa LHCII-PSII supercomplex. This complex is dimeric with each monomer binding about 100 chlorophylls per reaction centre (75 chl *a* and 27 chl *b*). The positions of the major proteins in the complex have been mapped, including the extrinsic proteins of the oxygen evolving complex. Moreover, the supercomplex gives a framework in which to incorporate high resolution data recently obtained from 2D crystals composed of the reaction centre proteins, D1 and D2, and the chlorophyll binding protein, CP47. The crystal also contains other smaller polypeptides, giving in total 23 transmembrane helices. The 10 helices of the D1 and D2 proteins are arranged in a manner very similar to the 10 helices of the L and M subunits of the purple photosynthetic bacterial reaction centre. Similarly, there is a close structural homology between the 6 helices of CP47 and the N-terminal helices of the photosystem one (PSI) reaction centre proteins (PsaA/B). With 8.0Å resolution data in the x/y plane it has been possible to identify densities likely to be due to the tetrapyrrol heads of chlorophyll/pheophytin molecules. In so doing a comparison can be made between the location of the cofactors bound to the L and M subunits and the corresponding pigments in PSII (i.e. P680 accessory chlorophylls and pheophytins). Possible locations for chlorophyll molecules within C47 can also be identified. Additional data obtained from 2D crystals of oxygen evolving complexes have further identified the location of the helices of CP43 and support the concept that PSII and PSI share a structural homology indicative of a common evolutionary origin.

MORE LESSONS FROM RUBISCO GENES

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PHAGE-TYPE RNA POLYMERASES IN HIGHER PLANTS

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For more than ten years yeast mitochondria have been known to use an RNA polymerase (RNAP) similar to the single-subunit type RNAP of phage T7 and other phages for transcription of their genes. Our data strongly suggest that higher plants transcribe not only their mitochondrial but also plastid genes by phage-type RNAPs.

The nuclear genome of Arabidopsis contains three related genes (*RpoT;1;2;3*) with striking similarity to genes coding for single-subunit RNAPs of the phage type. The putative products of the three Arabidopsis genes possess all amino acids reported to be essential for the function of the T7 RNAP (Sousa et al., Nature 364, 593, 1993). The genes are interrupted by 19 introns located at identical positions. Presumably, the common ancestor of monocots and dicots had already a gene of highly similar structure since we observed a comparable exon-intron organization in an RNAP gene of barley *(Hordeum vulgare)*. Sequence analyses of partial cDNAs suggest an alternative splicing of the pre-mRNA transcribed from this barley gene.

To determine the location of the putative RNAPs within the plant cell, we have constructed recombinant plasmids containing the genetic information for the putative transit peptides of the Arabidopsis RNAPs and for the green fluoresence protein (GFP). The transit sequences of RpoT;1 and RpoT;3 were found to target GFP into isolated mitochondria and chloroplasts, respectively (Hedtke et al., Science 277: 809, 1997). Identical results were obtained with transgenic Arabidopsis plants expressing GFP fused to the respective transit peptide. We conclude that *RpoT;1* encodes a mitochondrial RNAP, whereas *RpoT;3* bears the information for a plastid RNAP. Similar experiments with the transit sequence of RpoT;2 have been initiated. In the same way we could demonstrate that the previously identified cDNA of *Chenopodium album* (Weihe et al., Nucl. Acids Res. 25, 2319, 1997) codes for a mitochondrial RNAP of the phage type.

Plastids need two RNA polymerases (RNAPs) to transcribe their genes. One plastid RNAP (PEP) is a multisubunit enzyme as known from eubacteria. Its subunits (except the sigma factors) are encoded by chloroplast genes. The other plastid RNAP (NEP) is exclusively encoded by nuclear gene(s). According to our data, the NEP of Arabidopsis is most probably the product of *RpoT;3*, i.e. an enzyme belonging to the family of single-subunit RNAPs.

We performed Northern hybridization to check the transcription of *RpoT;1* and *RpoT;3*. Transcripts of these genes were detectable in poly(A)$^+$ RNA prepared from both white cells of an Arabidopsis suspension culture and green cells of Arabidopsis leaves. This is in agreement with the observation that NEP transcribes genes in white mutant plastids lacking PEP activity and also in green chloroplasts (Hajdukiewicz et al., EMBO J. 13, 4401, 1997; Hübschmann and Börner, Plant. Mol. Biol. 36, 493, 1998).

We identified partial cDNAs corresponding to two and three different phage-type RNAP genes in spinach and tobacco, respectively. Thus, the existence of more than one phage-type RNAP gene in the nuclear genome seems to be a general phenomenon among higher plants.

PHOTOSYSTEM I ABUNDANCE IN CYANOBACTERIA AND CHLOROPLASTS

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The rate of photosynthesis is modulated by regulating the abundance and activity of photosynthetic enzymes. We investigated how the transmembrane architecture and external factors regulate photosystem I (PSI) abundance in cyanobacteria and higher plants.

To understand the roles of extramembrane loops and transmembrane helices in maintaining the stability of PSI, we generated deletion and site-directed mutations in the PsaB core protein in the cyanobacterium *Synechocystis* sp. PCC 6803. Among the deletion mutants in the H extramembrane loop of PsaB, the PSI proteins failed to accumulate in the membranes if the deletions were close to the transmembrane helices. The systematic mutations of amino acids in the interior of the *m* transmembrane helix abolished the ability of PSI proteins to accumulate in the membrane. In contrast, replacement of the residues, that are closer to the exterior of the lipid bilayer allowed accumulation of PSI proteins in the membrane. Mutations in the cofactor binding pockets of PsaB also affected the PSI accumulation. Therefore, packing of transmembrane helices and cofactor-binding are two crucial intrinsic factors that determine PSI stability.

Physiological and environmental factors regulate PSI levels in cyanobacterial membranes. When the chloramphenicol-resistance cartridge was inserted in the down-stream sequences of the *psaB* gene, accumulation of PS I proteins was reduced, suggesting a role of the 3' untranslated region in expression of the *psaB* gene. Genetic background, the presence of glucose and the light intensity also affected accumulation of the PSI proteins. Elevated temperature or high light intensity accelerated degradation of PsaA-PsaB in the PsaD-PsaE-less strain of *Synechocystis*, resulting in a rapid decrease in the PSI proteins. The levels of different PSI proteins were modulated differently by elevated temperature and PS II-mediated electron transport. Therefore the *in vivo* proteolysis of PSI proteins is regulated by multiple mechanisms.

To identify the genes that regulate PSI levels in higher plants, we investigated maize mutants that are defective in the level of photosynthetic apparatus. The zebra necrotic and zebra stripe mutants of maize are phenotypically characterized as having leaves with necrotic lesions (zebra necrotic) or bands of light green tissue (zebra stripe). Mutant tissues from both phenotypes contain diminished amounts of photosynthetic membrane proteins. The leaves of the high chlorophyll fluorescence (hcf) mutants hcf44 and hcf47 contain reduced levels of chlorophyll, decreased PSI activity and lower levels of PSI polypeptides. However, when the PSI activity is considered on an equal chlorophyll basis, no difference was found between mutant and normal seedlings, suggesting a general defect in the regulation of the PSI density in the membranes.

To conclude, stability of the wild type proteins is dependent on the protein architecture in the lipid bilayer. We have identified physiological, environmental and genetic components that affect levels of PSI in the thylakoid membranes of cyanobacteria and higher plants.

PROTEIN TRANSPORT TO THE THYLAKOID LUMEN.

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Many thylakoid lumenal proteins are encoded in the nucleus, synthesized in the cytosol and imported into chloroplasts. Localization to the lumen is a two-step process wherein precursors are imported across the envelope into the stroma and then transported across the thylakoid membrane. Two pathways have been described for thylakoid transport. The thylakoid Sec pathway employs a chloroplast homologue of the bacterial SecA protein and probably also requires chloroplast membrane proteins homologous to the bacterial SecY/E. The Delta pH pathway originally appeared to be a novel system, possibly a eukaryotic invention, because of its unique requirement for the trans-thylakoid ΔpH as sole energy source and because of the lack of a suitable counterpart in prokaryotes. However, several lines of biochemical and genetic evidence argue that it is of ancient origin and related to an unrecognized prokaryotic system. First is that hydrophobic signal peptides similar to those found in bacterial and ER precursors direct Delta pH translocation. An important difference is a conserved and essential twin arginine motif flanking the hydrophobic core. Second, precursors initiate transport via a peptide loop, a characteristic of bacterial and ER export systems. Finally, a component of the Delta pH machinery was isolated by a genetic approach in which mutants were selectively defective in transport of substrates of the Delta pH pathway. The cloned Hcf106 gene predicts a single spanning membrane protein. Biochemical studies confirm that Hcf106p is present in the thylakoids and is oriented with a large hydrophilic stromal domain, suggesting a role as a receptor. Although there is no direct evidence that Hcf106p is a receptor, recent experiments showing that antibodies to Hcf106p inhibit transport of Delta pH substrates argue strongly that it is involved in the transport reaction. Of considerable interest is that striking homologies exist between Hcf106p and predicted proteins of unknown function in a wide range of bacteria. Recent genetic studies in bacteria support the idea of an analogous system for transport of twin arginine-containing proteins across the cytoplasm membrane. We have recently subdivided the mechanism of Delta pH transport into a binding and a translocation step. Details of this process including the role of Hcf106p will be described. Supported in part by National Science Foundation grant MCB-9419287 and National Institutes of Health grant R01 GM46951.

TOCOPHEROL BIOSYNTHESIS IN *SYNECHOCYSTIS* sp. PCC6803 IN RESPONSE TO OXIDATIVE STRESS

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Tocopherols are lipophilic antioxidants whose synthesis in higher plants seems to be localized primarily in chloroplasts (Schultz *et al.*, Physiol. Plant.64, 123-129, 1985). It is supposed that tocopherols protect membranes towards oxidative stress (Fryer, Plant, Cell & Enviroment 15, 381-392). According to the endosymbiontic theory the ancestor of plastids are protists related to cyanobacteria. Therefore these organisms are widely used as models for the study of chloroplast metabolism. Indeed tocopherols have been detected in several strains of cyanobacteria (Powls and Redfearn, Biochem. Soc. Trans. 14, 969-970, 1986). By mass spectroscopy we could show that also the strain of *Synechocystis* sp. PCC 6803 contains alpha-tocopherol. To induce oxidative stress growing cultures of *Synechocystis* sp. PCC 6803 were incubated with different concentrations of methylviologen (paraquat) and norfluorazon, respectively. These herbicides are known to generate different reactive oxygen species. It is well known that in photosynthetic organisms paraquat generates superoxide anions by transferring electrons to oxygen behind photosystem I. Norfluorazon blocks carotenoid biosynthese by inhibiting phytoene desaturase and thereby generates singulet oxygen. In a recent paper it has been shown that both herbicides may induce different antioxidative protection mechanisms in cells of cyanobacteria (Thomas *et al.*, Plant Physiol. 116, 1593-1602, 1998). In contrast to our studies these investigations were however done with *Synechococcus* sp. strain PCC 7942 which lacks tocopherols.

The tocopherols of *Synechocystis* sp. PCC 6803 cultures treated with these herbicides were analyzed by HPLC and mass spectroscopy. Our data clearly show that the concentration of alpha-tocopherol increases during oxidative stress imposed by paraquat. Moreover two further tocopherols are synthesized exclusively under oxidative stress conditions. So far, one of them has been identified as gamma-tocopherol. These results clearly show that tocopherols are part of the antioxidant system in *Synechocystis* sp. PCC 6803. Further investigations with norfluorazon will clarify whether they are only induced by superoxide anions or moreover singulet oxygen.

ALTERATIONS IN THE PLASTID MEMBRANE-ASSOCIATED POLYAMINES DURING CHLOROPLAST PHOTODEVELOPMENT

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Polyamines play a role in many developmental processes in plants. Nevertheless, in almost every case the place of action and the mechanism are not yet known. In this contribution attempts are made to elucidate, at least in part, the physiological role of the four main polyamines putrescine, spermidine, spermine and norspermidine in chloroplast development. We demonstrate that the unicellular green alga *Scenedesmus obliquus* and its pigment mutants C-2A' and C-6D after heterotrophic growth in darkness exhibit, when exposed to light, an immediate and sustained decrease of the intracellular putrescine level. On the contrary, spermine, spermidine and norspermidine show only marginal changes. Many of the examined stages of the developing chloroplast reveal additionally a considerable redistribution of the polyamines among the isolated thylakoid membranes. This fluctuation and redistribution of the plastid membrane-bound polyamines during dark growth and on illumination was determined and correlations to the different ways of chloroplast development, as represented by the three different types of *Scenedesmus* cultures (wildtype, and mutants C-2A' and C-6D), are established. Finally, we discuss, in how far these polyamines might be involved in the assembly and stabilization of the different photosynthetic complexes during the light-dependent formation of the entire photosynthetic apparatus.

STRUCTURAL-FUNCTIONAL ORGANIZATION OF THYLAKOIDS IN DEVELOPING CHLOROPLASTS

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Parallel with the functional investigation of photosynthetic electron transport by measuring the O_2 evolution and fluorescence induction parameters, the structural organization of thylakoid membranes was considered at the level of both lipids and proteins investigated by FTIR and ESR spectroscopies.

After illumination of etiolated leaves the photosynthetic electron transport rapidly intensified as chlorophylls were accumulated. The protein/lipid ratio, however, was not changed during the first 24 hours and showed only slight increase at the later steps of greening. As shown by ESR, the integration of TEMPO into the membrane stepwise increased indicating the structural rearrangement of lipid bilayers. In the FTIR spectra, the Amide I band exhibited characteristic bandshift from 1652 cm^{-1} to 1656 cm^{-1} corresponding to the increase in the a-helix content. The sum of the relative area of the bands at 1636 cm^{-1} and 1625 cm^{-1} was nearly equal during greening but their proportion dramatically changed indicated definite alterations in the β-turn contents of polypeptides inserted into the developing chloroplasts.

CHARACTERIZATION OF TWO NUCLEAR PHOTOSYNTHETIC MUTANTS OF *Chlamydomonas reinhardtii*

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Random insertion of pARG7.8 into the nuclear genome of *C. reinhardtii, cw15arg7A,* generated two distinct photosynthetic mutants. One mutant, designated R25, was shown to be deficient in photosystem II (PSII) by 77K fluorescence spectra and fluorescence induction kinetics. Ultrastructural examination of light-grown R25 revealed that the thylakoids stack to form grana. Electrophoresis revealed several differences in the pattern of total protein, in particular, a deficiency in a 21 KDa protein, but LHCII apoprotein levels were normal. Exposure of dark-grown R25 cells to light caused greening typical of parental cells. The site of assembly of LHCII was investigated by confocal fluorescence microscopy. During the early phase of greening, fluorescence was detected only along the periphery of the chloroplast. Further methods for elucidating the initial site of LHCII assembly, via confocal microscopy, are being developed. The second mutant, designated MC9, is deficient in LHCII when grown at relatively high light (600 μmol photons m^{-2} s^{-1}) as shown by 77K fluorescence spectra and electrophoretic analysis. Pulse-chase experiments with MC9 show that, when exposed to light, the mutant had a reduced rate of LHCII apoprotein synthesis. Sucrose density gradients of isolated chloroplasts revealed differences in distribution of proteins within a low density fraction. Consistent with reduced LHCII levels, the chloroplast in MC9 cells contained fewer membranes than the parent strain. Mature-sized LHCII apoproteins accumulated in electron opaque material in cytoplasmic vacuoles as shown by immunoelectron microscopy. Thus the LHCII apoproteins were apparently partially translocated across the envelope but not retained by the chloroplast. We propose that an integral step in LHCII assembly within the envelope is impaired in MC9.

CONTROL OF TRANSLATION ELONGATION IN CHLOROPLASTS

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Intact and lysed chloroplasts isolated from the day or night phase of seedling growth exibit a higher rate of [^{35}S]-Met incorporation in the D1 protein in light than in darkness.

In the presence of the translation initiation inhibitor lincomycin radiolabel incorporation remains unaffected for 7.5-10 min of the *in vitro* translation reaction indicating that radiolabel incorporation is regulated by translation elongation. Comparison of the translation elongation rate on the level of a polysome bound D1 translation intermediate and the membrane integrated D1 protein demonstrates that the rate of translation elongation is higher in the presence of light. In the light, less time is required to transiently radiolabel a D1 translation intermediate of about 17 kDa and to chase the translation intermediates into mature D1 proteins.

Light could regulate the rate of translation elongation by increased synthesis of ATP! Indeed, the rate of [^{35}S]-Met incorporation in D1-proteins can be increased by addition of exogenous ATP to the *in vitro* translation reactions in darkness and light; however, ATP can not replace light, and at physiologic concentrations of stromal ATP (40µM), the rate is at least 25 -fold higher in the light than in darkness. This indicates that translation elongation is nearly arrested in darkness, although the level of ATP remains high (30 µM). We propose from these data that light regulates the enzymatic process of translation elongation in chloroplasts.

We next addressed the question, how light could regulate translation elongation in chloroplasts! In darkness, translation elongation is slow, even in the presence of exogenously added ATP and dithiothreitol. In the light and presence of ATP, addition of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) inhibits translation elongation. This indicated that a redox component of the photosynthetic electron transport chain could be involved in translation regulation.

DCMU inhibition of translation can be overcome by addition of artificial electron donors; however, only in the presence of light, but not in darkness: Electron flow between photosystem II and I is shown to be sufficient for the activation of translation elongation, and activation of translation can be obtained by electron donors to photosystem I only, which transport protons into the thylakoid lumen.

Release of the proton gradient by uncouplers prevents the light dependent activation of translation elongation, whereas reduced ferredoxin and thioredoxin have no stimulatory effect. Also, induction of translation activation is switched off rapidly upon transfer from light to darkness. Hence, we propose that the formation of the photosynthetic proton gradient across the thylakoid membrane activates translation elongation in chloroplasts.

ASSEMBLY OF IN VITRO SYNTHESIZED NADPH:PROTOCHLOROPHYLLIDE OXIDOREDUCTASE INTO ISOLATED PROLAMELLAR BODIES AND PROTHYLAKOIDS.

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NADPH:protochlorophyllide oxidoreductase (POR) catalyzes the conversion of protochlorophyllide to chlorophyllide. This critical step in the chlorophyll synthesis pathway is induced by light, and the POR enzyme is shortly thereafter down-regulated.
The POR is a nuclear encoded protein synthesized in the cytosol as a preprotein of 44 kDa (pPOR), and transported into the etioplast post-translationally. The imported protein its then processed to it´s final size of 36 kDa and assembled to the etioplast inner membranes, the tubular prolamellar bodies (PLB) and the lamellar prothylakoids (PT).
POR is supposed to be a peripheral protein firmly bound at the stroma facing side of the etioplast inner membranes, and almost exclusively to the PLB. Here it is found in complex with NADPH and it´s substrate, protochlorophyllide.
From a cDNA clone containing the coding sequence for pPOR, radiolabelled protein has been transcribed in vitro, and incubated with isolated PLB and PT in order to study the membrane assembly reaktion. We here present part of the first detailed analysis on the assembly reaction of POR with etioplast inner membranes in vitro.

The assembly of POR to both PLB and PT is stimulated by hydrolyzable ATP and exhibit an absolute requirement for NADPH. On the other hand, PLB´s are more efficient than PT´s in integrating POR, but both membrane types have the capability to do so.
SDS-PAGE/fluorography analysis following post-treatment of the membranes with NaOH, Na_2CO_3 further supported the earlier observations that POR is an enzyme tightly bound to the membrane.

POR protein containing mutations in the NADPH-binding site and the active site failed to assemble into both PLB and PT membranes. This indicates that formation of the NADPH-POR-protochlorophyllide complex may be a prerequisite for physiological membrane assembly of POR.

Several levels of regulation adjust the content of POR during greening. These include transcriptional and translational control, as well as proteolytic activity.
Our results also indicate a regulation at the level of association to the inner plastid membranes. Inner membranes from dark-grown plants exihibit significantly higher integration capacity than illuminated and light-grown plants. This could be one of several ways that plants regulate their content of POR.

SMALL CAB-LIKE PROTEINS: RELATIVES TO THE CHLOROPHYLL A/B BINDING PROTEINS IN CYANOBACTERIA

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In higher plants, the chlorophyll *a/b*-binding light harvesting complex is an abundant peripheral antenna complex that harvests light to be transferred to the photosystems. LHC is encoded by a multi-gene family of at least 10 different nuclear *cab* genes in many higher plants. The chlorophyll *a/b*-binding proteins (Cab proteins) in eukaryotes are assumed to share a common evolutionary origin, and have three or four membrane-spanning regions per polypeptide, from which the first helix is similar to the third one, and the second helix is similar to the fourth (if present). Cyanobacteria contain chlorophyll *a* but, in contrast to higher plants, lack chlorophyll *b* as well as multi-helix Cab proteins. The major light-harvesting complex in cyanobacteria is the phycobilisome, which is peripheral to the thylakoid membrane and contributes to the deep blue-green color of cyanobacteria.

In the genome of the cyanobacterium *Synechocystis* sp. PCC 6803 five putative genes were identified that have significant sequence similarity to a eukaryotic CAB gene family and to *hli*A, a gene coding for a small high-light induced protein in *Synechococcus* sp. PCC 7942. Four of the five open reading frames (*scp*B, *scp*C, *scp*D, and *scp*E; scp designating **s**mall **C**ab-like **p**rotein) in *Synechocystis* sp. PCC 6803 are 174-213 bp in length; the corresponding polypeptides are predicted to have a single transmembrane helix. The first 80% of the fifth gene (*scp*A) codes for ferrochelatase, and the end of the gene codes for a Cab-like domain. Upon interruption of *scp*A between the ferrochelatase and Cab-like gene domains, in a photosystem I-less background an increased tolerance to high light intensity was observed. Moreover, the amount of phycobilisome-absorbed light needed for photosystem II-driven electron flow was increased in this mutant, suggesting that ScpA contributes to energy transfer and therefore is likely to bind antenna pigments. The genes of *scp*B, C, D, and E were expressed preferentially in the absence of photosystem I, but gene expression was not enhanced at high light intensity. Immunodetection of ScpC and ScpD indicates that either or both accumulate in mutants with a reduced number of chlorophyll binding sites per cell. These proteins are also detected in bands of about 36 kDa on denaturing gels, suggesting that they may form stable hexamers. The SCPs might represent a new group of antenna proteins or pigment scavengers that are independent of photosystem I and II. The functional relationships of SCPs to other potential chlorophyll-binding proteins as well as to ferrochelatase will be discussed.

THE ACTION OF FREE RADICAL SCAVENGERS ON THYLAKOID PROTEIN PHOSPHORYLATION AND PSII CORE PROTEIN DEGRADATION

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Under conditions of thylakoid protein phosphorylation, even under low light, a thylakoid protein, immunodetectable by a D1/D2 antibody after resolution by SDS-PAGE on non-urea gels, was found to be drastically reduced. The reduction of immunostain coincided with the increase in the fluorescence emission ratio F730/F685 (77K), it was overcome in the presence of kinase inhibitors (FSBA), and was considered to reflect D1 degradation (Georgakopoulos and Argyroudi-Akoyunoglou, Photosyn. Res. 53, 185, 1997).

Using antibodies from a variety of sources, and SDS-PAGE resolution either on non-urea-12.5% or on 6 M urea-15% gels, we found that although all antibodies immunoreacted with a protein of equal apparent molecular weight (32 kDa) on non-urea gels, they immunoreacted with proteins of slightly different molecular weight after resolution on 6 M urea gels. The antibody raised against *Oscillatoria Chalybea* D1 immunostained a band at 34-35 kDa, that raised against D1/D2 immunostained bands at 32 and 34-35 kDa, while that raised against the D-E loop of *Synechocystis* D1 immunostained a doublet at about 32 kDa. As to the reduction of immunostain during thylakoid protein phosphorylation, immunostain loss was not observed when using an antibody raised against the peripheral 33 kDa PSII protein nor against LHCII or against the β-subunit of CF1, neither when using the antibody raised against the D-E loop of *Synechocystis* D1. On the contrary the immunostain loss was drastic when using the antibody raised against *Oscillatoria Chalybea* D1. Thus, although it is not possible to say which one of the PSII-core-poroteins is involved, it is clear that the PSII core is greatly affected.

To see whether kinase activation may be involved with this degradation of PSII-core-proteins, we have further tested the effect of various free radical scavengers, known to inhibit or not D1 degradation or PSII photoinactivation, on thylakoid protein phosphorylation. We found that NaN_3, shown earlier to enhance high-light induced D1 damage, enhanced also thylakoid protein phosphorylation and the ATP-induced PSII-core-protein degradation. NaN_3 at similar concentrations was found to inhibit completely (in the light) the PSII electron transport (H_2O-DCIP), pointing to a direct effect of NaN_3 on the kinase. Imidazole, and epinephrine, on the other hand, shown earlier to protect D1 degradation or PSII photoinactivation, were found to inhibit completely thylakoid protein phosphorylation, the ATP-induced PSII core protein degradation, the reduction of the F730/F685 (77K) emission ratio, as well as the H_20-DCIP electron transport. Finally, n-propyl gallate, shown earlier to protect D1 degradation and PSII photoinactivation, but to allow H_20-DCIP electron transport, was found to inhibit thylakoid protein phosphorylation and the ATP-induced PSII-core-protein degradation. So under all conditions, thylakoid protein phosphorylation and PSII-core-protein degradation were found to be closely correlated. In addition, we found that this PSII-core-protein degradation could be detected even in the dark, provided that ATP was added and the kinase was activated (in the presence of dithionite). These data may suggest either that the phosphorylated PSII-core-proteins have low immunoreactivity to the antibodies raised against their non-phosphorylated forms (so that the loss in immunostain reflects their phosphorylation rather than their degradation), or that phosphorylation of a putative protease may be involved in the ATP-induced PSII-core-protein degradation.

THE ABUNDANCE OF *Cab* AND *psbA* TRANSCRIPTS AND OF THEIR LHCII AND D1 PROTEIN PRODUCTS IN GREENING ETIOLATED LEAVES TRANSFERRED TO DARKNESS

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Plants transferred to darkness following brief preexposure to continuous light, can accumulate new PSII units, which use preexisting Chla molecules for their assembly. These Chla molecules seem to be released from LHCII, which undergoes degradation. New PSII unit formation or LHCII degradation is not observed in plants preexposed to continuous light for long time (Argyroudi-Akoyunoglou et al. Plant Physiol. 70, 1242, 1982). To understand how PSII unit assembly may be controlled during chloroplast development, we studied how D1 and LHCII levels (immunodetection in total LDS extracted leaf proteins) are correlated with the levels of their transcripts (Northern analysis) during exposure of etiolated plants to light, but also following their transfer to the dark.

Cab mRNA was found to follow circadian oscillations in 6 day etiolated bean exposed to continuous light, the amplitude of which gradually decreased; the oscillations persisted after transfer of the plants to darkness. The level of the LHCII protein was greatly reduced in plants transferred to the dark following brief preexposure to light (conditions of high *Cab* transcript accumulation), or remained unchanged in plants transferred to the dark following prolonged light exposure (conditions of diminished *Cab* transcript accumulation). An inverse relationship was observed between LHCII level and *Cab* mRNA level: whenever degradation was high, transcript level was high. This may suggest that LHCII degradation products may in some way be involved in the transcription of its own genes (feed back?) and/or in the stabilization of the transcripts.

psbA mRNA, in contrast, followed a gradual increase during light exposure; the increase continued for at leat 48 h. The steady state transcript level remained unaffected by the transfer to the dark of plants preilluminated for 48 h, but increased in those preilluminated for shorter periods of time. The shorter the preillumination period, the larger was the increase in *psbA* transcript level. A low transcript level was detectable in 6-day etiolated bean, which was increased as etiolation was prolonged. On the other hand, D1 protein was not detected in etiolated leaves, despite the presence of *psbA* transcript; in this case only D1 degradation products (or translation-arrest products) were detected. These lower molecular weight products could also be detected after light exposure, the 17 kDa one being reduced as time in the light was prolonged. Results concerning the D1 level in plants transfered to the dark following their exposure to light will be discussed.

CONTROL OF METABOLITE DISTRIBUTION IN CHLOROPHYLL AND HEME SYNTHESIS

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The macrocyclic tetrapyrroles serve various essential biological functions in all organisms including catalysis, signalling, transport. They also serve as accessory pigments. Chlorophyll and protoheme are the main endproducts of the plant tetrapyrrolic pathway. Chlorophyll synthesis is exclusively localised in plastids. Only the last two enzymatic steps of heme synthesis take place both in plastids and mitochondria. The compartimentation opens the possibility of specifically regulated gene expression in spatially separated metabolic pathways for chlorophyll and heme synthesis. Protoporphyrinogen Oxidase (PPX) and Fe chelatase are located both in plastids and mitochondria. In plastids Mg chelatase and Fe chelatase compete for the substrate protoporphyrin IX and direct the tetrapyrrole intermediate towards chlorophyll and heme synthesis. We cloned all cDNA sequences encoding the three enzymes PPX, Mg and Fe chelatase, which are involved in distribution of metabolites in the branched pathway. We analyzed the regulation of expression and activity in tetrapyrrole biosynthesis under photoperiodic and constant light or dark conditions and focused on the elucidation of the mechanism controlling the channeling of protoporphyrin IX into the heme or chlorophyll synthesizing branch. The control of 5-aminolevulinate synthesis and of the allocation of protoporphyrin to Mg or Fe chelatase reflects the functional coordination of tetrapyrrole biosynthesis in response to daily fluctuations in tetrapyrrole requirements. Moreover, transgenic plants with deficient activities of either PPX, Mg- or Fe chelatase have been generated. These transgenic lines enable a prediction on the control of the metabolic flux in the pathway. The presentation provides evidence for the significance of Mg chelatase activity for signaling between plastids and nucleus and for the function of PPX to balance heme synthesis in both organelles

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EXPRESSION OF EARLY-LIGHT-INDUCIBLE-PROTEINS (ELIPs) IN MESOPHYLL AND BUNDLE SHEATH CELLS OF THE C4-PLANT MAIZE (ZEA MAYS, L.).

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ELIPs are stress-related nuclear encoded proteins of the thylakoid membranes. It has been suggested that ELIPs are related to photosystem II (PSII). To proof this suggestion we chose the C4-plant maize which owns dimorphic chloroplasts. Bundle sheath (BS) chloroplasts have only active PSI while mesophyll cells (MS) possess both photosystems. According to the above stated thesis, there should be no expression of ELIPs in BS. Based on the difference in chlorophyll fluorescence properties of MS and BS chloroplasts, we separated both types of thylakoids by flow cytometry. We obtained about $30*10^6$ mesophyll- and $15*10^6$ bundle sheath thylakoid particles per preparation which were analyzed by western blots. The results are that ELIPs are expressed in the same manner in both cell types, in contrast to LHCII, which is synthesised mainly in the MS. This result suggests that the function of ELIPs should not be merely related to the function of PSII.

THE CHLOROPLAST AS A PART OF THE INTEGRATED GENETIC SYSTEM OF THE PLANT CELL

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The biogenesis of the chloroplast, and implicitly of the photosynthetic machinery, is embedded into the *integrated* compartmentalized genetic system of the plant cell. It cannot be understood without knowledge of the history of that cell, which originated in an endosymbiotic cell conglomerate and developed a common metabolism and inheritance. The generation, maintenance, adaptation and senescence of the chloroplast are strictly regulated, over a wide range of time scales, at various levels, and includes, for instance, at least two RNA polymerases, posttranscriptional processes, kinases/phosphatases and a surprisingly complex spectrum of proteases.

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DINOFLAGELLATE LIGHT-HARVESTING PROTEINS: GENES, STRUCTURE AND RECONSTITUTION

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Dinoflagellates possess two light-harvesting systems, an intrinsic LHC, whose constituent peptides are related to other chl *a-b-c*-containing proteins, and the unique water soluble Peridinin-Chlorophyll *a*-Proteins (PCPs). The genes for both systems are nuclear encoded and members of multigene families. In *Amphidinium*, the LHC is synthesised as polyprotein whose individual peptides of 19kDa are generated by hydrolysis of an arginine residue at the C-terminus of the preceding peptide. A library of genomic DNA in lambda phage has been screened and part of an LHC gene sequenced. It encodes a leader sequence of 93 amino-acids in which several different motifs have been identified. It also encodes four complete 19kDa peptides. There is at least one intron interrupting each 19kDa peptide but one is at a conserved position and suggests different proportions LHC peptides could be generated by differential splicing in response to environmental variation. These introns are 200 - 400 bases in length and many are atypical. There are two distinct forms of PCP, both are synthesised as individual peptides with leader sequences of 50-60 amino acids. The genes are without introns. One of the most abundant PCPs has been crystallised and its structure determined at 2.0Å (Hoffman et al. Science 272, 1788 1996). The structural basis of the energy transfer from peridinin to chl a is the clustering of the carotenoids at van der Waals distance from the chl macrocycle. The immediate hydrophobic environment of the carotenoids is provided by lipid, the chl phytol chain and hydrophobic amino acid R-groups. To understand better the spectroscopy of PCP and mechanism of energy transfer we propose to vary the PCP structure at key conserved residues eg tryptophan 23, by site directed mutagenesis. To this end we have expressed both full length and N-terminal domain PCP apoprotein in *E.coli*. The protein is recovered from inclusion bodies by standard techniques and purified by ion exchange chromatography. In the presence of pigments extracted from purified PCP, the holoprotein can be reconstituted by simple mixing. The resulting PCP is indistinguishable from the native form as judged by absorbance, visible CD and florescence. The construct of N-terminal domain alone reassembled better than did the full length. Interestingly, it was found by size exclusion chromatography that the N-terminal construct dimerized. These studies are being extended to the second minor PCP form which has a markedly different amino acid sequence (31% identity) and only six peridinins instead of eight.

REGULATION OF GENE EXPRESSION IN THE CAROTENOID BIOSYNTHESIS PATHWAY OF PLANTS

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Carotenoid pigments are essential components of the photosynthetic apparatus. They function also in pigmentation of flowers and fruits. In plants biosynthesis of carotenoids takes place within plastids. It originates from the central isoprenoid pathway that exists in all living cells. During recent years the genes for more than twenty different carotenogenic enzymes have been cloned from various organisms, including higher plants. To study the regulation of xanthophyll biosynthesis, we have taken a map-based cloning approach to identify novel genes for lycopene cyclases. In tomato these enzymes are encoded by a small gene family. We have discovered the molecular basis for three fruit-color mutations in tomato, *Del*, *B* and *og*^{c}. It was shown that transcriptional regulation is the major mechanism that governs carotenogenesis in plants. This regulation is affected by environmental and developmental signals. To demonstrate the ability for genetic manipulation of carotenogenesis in vivo, we have focused on the production of astaxanthin, a red keto-carotenoid pigment of immense economical value. We have cloned a cDNA of the gene *crtO*, which encodes β-C-4-oxygenase (ketolase), from the alga *Haematococcus pluvialis* and functionally expressed it in transgenic tobacco plants. This induced accumulation of astaxanthin in the chromoplasts of the nectary tissue, changing its color from yellow to dark red.

DISTRIBUTION OF LHCP BETWEEN CHLOROPLAST AND VACUOLES DURING CHLOROPLAST DEVELOPMENT IN *Chlamydomonas reinhardtii*.

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The distribution of LHCP in light-grown cells of several strains of *C. reinhardtii* was examined by immunoelectron microscopy. In addition to thylakoid membranes, antibodies against LHCP also reacted with dense granules in cytoplasmic vacuoles. These granules were purified and shown by immunoblots to contain mature-sized LHCP. (The major component of the granules is polyphosphate; the major protein is a 70 kDa precursor of the cell wall). Immunoelectron microscopy was then used to determine the sites of initial accumulation of LHCP during chloroplast development in intact cells. Immunolabeling of the region adjacent to the inner membrane of the chloroplast envelope, where invaginations had previously been observed, was prominent within minutes after dark-grown cells were exposed to light. Simultaneously, the dense granules in cytoplasmic vacuoles became immunolabeled. In the chlorophyll *b*-less strain *cbn1-113arg2* that also did not synthesize chlorophyll *a* in the dark, LHCP accumulated in vacuoles and the cytosol but were not detected in the chloroplast in the dark. These cells, when grown in the light in the absence of acetate, had a *chlorina* phenotype. Less LHCP were detected in the chloroplast and a higher level of labeling of vacuolar granules was found under these conditions as compared with cells grown in the presence of acetate. Because only mature-sized proteins were detected by immunoblots after electrophoresis, the LHCP precursor may have been translocated across the chloroplast envelope sufficiently to be processed. These results suggested that LHCP accumulated in vacuoles when insufficient chlorophyll or xanthophylls were available to accommodate all newly synthesized LHCP in light-harvesting complexes. Immunoelectron microscopy detected rubisco and rubisco activase at high levels in the pyrenoid and also at a much lower level in granules within vacuoles. Antibodies against 54CP, the chloroplast stromal homolog of SRP54 in higher plants, did not label the chloroplast of *Chlamydomonas* but labeled vacuoles. To search for structures that would provide insight into the detection of LHCP and stromal proteins in granules, the ultrastructure of cells was examined after cryofixation. Protuberances of the outer envelope membrane, which contained stroma-like material, possibly were the origin of chloroplast material in vacuoles. However, these structures did not generally contain thylakoid material. The membranes of the chloroplast envelope were appressed, with no apparent intermembrane space, except where the outer membrane extended outward around protuberances or the inner membrane budded inward. Our evidence is consistent with assembly of LHCII at the level of the envelope, but the mechanism of transfer of LHCP to vacuoles is not known.

DISTURBANCE OF THYLAKOID DEVELOPMENT UNDER HEAVY METAL POISONING CONDITIONS

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Heavy metals are known as harmful environmental agents interfering with many physiological processes. They inhibit photosynthesis at the level of both electron transport and carboxylation functions. Although various heavy metals (Cd, Cu, Ni, Pb, Cr etc.) were found to induce chlorosis, only the Cd effect was extensively studied in greening and found to inhibit chlorophyll biosynthesis.

In the present work, therefore, we compared the different heavy metals induced alterations in chloroplast development. All heavy metals studied were found to inhibit dramatically the accumulation of both chlorophylls and carotenoids. Using low temperature fluorescence emission spectroscopy we clearly demonstrated that neither Cd and Ni nor Cu inhibited the chlorophyll biosynthesis. Cd was found to act by disturbing the integration of chlorophyll molecules into the stable pigment protein complexes required for normal functional photosynthetic activity. In contrast, Ni inhibited protein accumulation which resulted in the photodistruction of synthetized free chlorophyll molecules, while Cu induced oxidative stress condition, and the formed oxygen free radicals generally destroyed the newly synthetized components of greening etio-chloroplasts.

RECONSTITUTION OF CO-TRANSLATIONAL TARGETING OF POLYTOPIC MEMBRANE PROTEINS TO THE THYLAKOIDS IN A HOMOLOGOUS CHLOROPLAST TRANSLATION SYSTEM.

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Very little is know about the mechanisms of targeting, insertion and assembly of the chloroplast-encoded thylakoid membrane proteins despite there importance. Progress in the understanding of these mechanisms in chloroplasts has been hampered by the lack of a homologous in vitro initiation/translation system.

We have begun to reconstitute the targeting, insertion and assembly of the polytopic D1 protein using a homologous translation system isolated from tobacco chloroplasts (Hirose and Sugiura (1996) EMBO J. 15(7), 1687-1695). Truncated forms of the *psbA* gene were translated in this system and stable ribosome nascent chain complexes were purified.

To study the interaction of soluble and membrane components of the targeting and insertion machinery we employed so-called amber suppression crosslinking technology. Interacting components with the D1 ribosome nascent chain complexes were photocrosslinked to SRP54 by photocrosslinking groups incorporated in the D1 nascent chain at specific amino acid positions. SRP54, the 54 kDa homologue of the Signal Recognition Particle, was shown to interact strongly with the first transmembrane span of the D1 protein, only when the D1 nascent chain is still attached to the ribosome. The interaction was dependent on the length of the nascent chain that had emerged from the ribosome.

Targeting of D1 ribosome nascent chains was achieved by addition of nuclease treated and washed thylakoid membranes into the translation reaction. D1 nascent chains showed a salt- and sodium hydroxide resistant association with the thylakoid membranes, especially after addition of puromycin. Based upon studies in the ER membrane, this puromycin stimulated interaction represents the movement of the D1 nascent chains from the translocon into the lipid bilayer.

Since chloroplast are originally derived from prokaryotes, it can be postulated that targeting pathways of the chloroplast encoded proteins will show strong homology to those in *E. coli.* Targeting and insertion to the cytoplasmic membrane in *E. coli* is well studied using the model protein leader peptidase (Lep) containing 2 transmembrane spans.

To compare the (co-translational) targeting and insertion pathways in the chloroplast with those in *E. coli*, we cloned the encoding region for Lep behind the D1 ribosome binding site. We found that Lep was expressed in the chloroplast translation system and also that Lep could be co-translationally (but not post-translationally) targeted to the thylakoid membrane. Based on protease protection essays we could show that the topology of Lep inserted into the thylakoid was similar to Lep inserted into the *E. coli* inner membrane.

Interaction with soluble and membrane components during targeting and insertion is in progress and will be addressed.

SUCCESSIVE DEGRADATION OF THE LIGHT-HARVESTING SYSTEM OF THE PHOTOSYNTHETIC APPARATUS DURING SENESCENCE OF BARLEY FLAG LEAVES

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Levels of light-harvesting complexes were investigated during maturation and senescence of flag leaves derived from field-grown barley plants (*Hordeum vulgare* L.). In mature flag leaves possessing photosynthetically highly active chloroplasts levels of the major light-harvesting complex LHCII and also of the minor light-harvesting complexes CP29 of photosystem II and LHCI of photosystem I were high. The high levels of the light-harvesting complexes obviously guarantee efficient light absorption and energy transfer to the reaction centers at this stage of development.

During growth of barley plants in the field in 1992 the light conditions drastically changed. Variations in photosynthetically active radiation (PAR) in the range from 200 to 1500 $\mu mol * m^{-2} * s^{-1}$ did neither affect photosynthetic capacity nor photosystem II efficiency in the mature flag leaves, indicating that the photosynthetic apparatus was sufficiently protected against photodamage. The changes in PAR did also not affect levels of LHCII, LHCI and CP29. In contrast, levels of another member of the chlorophyll a/b-binding protein family, ELIP, drastically fluctuated showing high ELIP levels at days with high PAR. In the mature flag leaves accumulation of ELIP seems to be an extra protection mechanism against harmful effects of excess light energy.

During senescence of flag leaves of field-grown barley plants levels of minor light-harvesting complexes CP29 and LHCI decreased much faster than those of the bulk LHCII. The early decline in the minor complexes coincided with a decrease in photosystem II efficiency. The decrease in the levels of minor complexes LHCI and CP29 was however retarded by a decrease in ambient light intensity.

The senescence-specific decrease in minor LHCs relative to the bulk LHCII results in a decrease in energy transfer to the reaction centers and seems to represent an additional protection mechanism against photodamage, the risk of which is increasing during senescence. In contrast, ELIP levels fluctuated independently from the stage of development, being high at days with high PAR and being low at days with low PAR, in mature as well as in senescing flag leaves. These data indicate that the protection mechanism via accumulation of ELIP is present at all stages of development. During senescence, in addition, reaction centers are protected by a specific decrease in the proportion of the minor complexes. While ELIP content was upregulated at high light conditions that of minor LHCs was downregulated during senescence.

Northern analyses using a cDNA clone specific for CP29 and another one specific for ELIP revealed that both, the senescence-specific decline in the minor complex CP29 and the light dependent fluctuations in ELIP level are regulated at the transcript level.

REDOX CONTROL OF THE DEVELOPMENT OF THE PHOTOSYNTHETIC APPARATUS

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Changes in environmental conditions such as irradiance, temperature and nutrient status result in an imbalance between light energy absorbed through temperature-independent photochemical reactions versus the energy utilized through the temperature-dependent biochemical reactions involved in photosynthetic electron transport and carbon metabolism. Such an energy imbalance may be sensed through the modulation of PSII excitation pressure, estimated as the relative reduction state of PSII, which reflects changes in the redox poise of the thylakoid intersystem electron transport chain. Modulation of this chloroplastic redox signal either by excess light or by low temperature initiates a signal transduction pathway which represses nuclear photosynthetic genes such as Lhcb but derepresses genes encoding Cbr, the carotenoid binding protein in green algae as well as ELIPs in higher plants. Furthermore, this chloroplastic signal transduction pathway also appears to co-ordinate nuclear expression of a specific cold-acclimation gene, Wcs19, as well as to influence plant morphology in monocots and dicots. It is suggested that photosynthetic acclimation in terrestrial plants, green algae and cyanobacteria is a response to changes in the redox state of a component(s) of chloroplastic intersystem electron transport. However, the mechanism by which some higher plants respond photosynthetically to modulation of this redox signal differs significantly from that observed in green algae and cyanobacteria. Thus, in addition to its traditional role in energy transduction, the photosynthetic apparatus may also be considered a primary sensor of environmental change through a redox sensing/signaling mechanism which acts synergistically with other signal transduction pathways to elicit the appropriate molecular and physiological response.

A HYPOTHESIS FOR THE IMPORT OF NUCLEAR-ENCODED PROTEINS INTO CHLOROPLASTS WITH FOUR ENVELOPE MEMBRANES.

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Chloroplasts of some major groups of algae: chromophytes, haptophytes, cryptophytes, and chlorarachniophytes, have four envelope membranes, while there are only two envelope membranes in land plants, chlorophytes and red algae. Although those chloroplasts with two envelope membranes have evolved from a cyanobacterial endosymbiont in a eukaryotic host cell, it is widely accepted that the chloroplasts with four envelope membranes have evolved from several (at least two) secondary endosymbioses, which have been established between eukaryotic host and photosynthetic eukaryotic endosymbiont. Almost all genes for chloroplast proteins encoded by the endosymbiont's nuclear genome are now found in the host's nuclear genome. Clarifying the mechanism of import of nuclear-encoded proteins into those four-membraned chloroplasts would greatly contribute to understanding the mechanism of chloroplast acquisition and how chloroplasts were integrated into the host's cell, and may lead to discovery of new protein import mechanisms.

In order to study the mechanism of protein import into the four-membraned chloroplasts, we chose a chromophyte alga, *Heterosigma akashiwo*, as a model organism because it has many chloroplasts in a cell, has no cell wall, and is easy to grow. 3D-reconstruction of cell ultrastructure and immuno-gold study using antibodies against the fucoxanthin-chlorophyll *c* binding protein (FCP) showed that: 1) the outermost membrane of the chloroplast envelope was continuous with rough ER as in other chromophyte algae (chloroplast ER), but no ribosome was attached to the chloroplast ER, 2) Golgi body may not be used for transporting FCP to the chloroplast, 3) brefeldin A, an inhibitor for the coatmer protein (COP) mediated vesicle trafficking, did not seem to affect the chloroplast envelope system. Moreover, the presequences of the FCP precursors are similar to the signal sequence of secretory proteins rather than the transit sequence of higher plant's nuclear-encoded chloroplast proteins. Our current hypothesis is that nuclear-encoded chloroplast proteins of *H. akashiwo* are synthesized by ribosomes on the ER membrane, translocated into the ER lumen, shipped to the spaces between the two outer envelope membranes through the lumen, then transported into the chloroplasts by mechanisms totally or partially specific to the chromophyte chloroplasts. To investigate how the proteins pass through the rest of membranes (three membranes), we have started to establish an in vitro protein import system for the chromophyte chloroplast.

CHARACTERIZATION OF THE ARABIDOPSIS *ppi1* MUTANT

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Genetic screens for loci involved in the positive regulation of *CAB* gene expression have identified a series of Arabidopsis mutants displaying altered plastid development. One such mutant, *ppi1* (*plastid protein import*), has a pronounced pale or yellow-green phenotype throughout its life-cycle, although older leaves frequently appear darker green than younger tissue. Molecular analyses of the *ppi1* locus revealed the presence of a T-DNA insertion within the second intron of a gene encoding a GTP-binding protein having substantial homology with components of the plastid outer envelope protein import apparatus. The *PPI1* gene encodes a protein of 33 kDa which shares 60 % identity with Toc34 (translocon, outer envelope, chloroplast, 34 kDa) from Arabidopsis and pea. Import experiments using isolated chloroplasts have demonstrated that the protein, referred to as Toc33, inserts into the outer envelope membrane in the predicted fashion. Given (1) that the majority of proteins entering the chloroplast do so through the general import apparatus (of which Toc34 is a component), (2) that the *ppi1* mutation is clearly null, and (3) that the *ppi1* mutant phenotype is not severe, we currently envisage two possible roles for Toc33. In the first of these, Toc33 would be involved in the general import process as a redundant component acting interchangeably with Toc34. Indeed, complementation of the *ppi1* genetic defect by over-expression of Toc34 has recently been demonstrated. However, Toc34 expression was found to be unperturbed in the *ppi1* background, and the import of six different proteins into mature mutant chloroplasts *in vitro* was found to be normal. One possibility is that Toc33 and Toc34 perform the same roles but at different developmental stages. The second possible role envisaged for Toc33 places it in a more specific protein import pathway. The precursor of the chlorophyll biosynthetic enzyme, PORA, is imported by the plastid only when there is an availability of the enzyme's substrate, protochlorophyllide, at the envelope membrane; it is possible that Toc33 is involved specifically in this regulated import process. Since PORA accumulates in etiolated plants and plays its primary role in the early stages of greening, it is possible that a PORA deficiency would give rise to a phenotype similar to that which we have observed for *ppi1*. The lack of phenotype severity can be explained by the presence of a second POR enzyme (PORB) which enters the plastid constitutively. Evidence in support of this latter hypothesis is as follows: (1) Toc33 is expressed at higher levels in dark-grown seedlings than in those grown in the light; (2) *CAB* is under-expressed in etiolated mutant plants; (3) the prolamellar bodies of mutant etioplasts are substantially smaller than those in the wild type; and (4) accumulation of the 44 kDa POR precursor (corresponding to non-imported protein) has been observed *in vivo* by western blotting.

INTERACTION OF EXTERNAL STIMULI WITH THE ENDOGENOUS CIRCADIAN RHYTHM IN THE CAPACITY OF ETIOLATED BEAN TO FORM CHLOROPHYLL, LHCII AND *Lhcb* mRNA

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Regulation of the expression of the nuclear coded light-harvesting protein of Photosystem II (LHCII) is exerted mainly at the transcriptional level through the action of phytochrome and the endogenous circadian oscillator. Light induces the rhythmic expression of several *Lhc* genes and causes the entrainment of the oscillator. These are important mechanisms, necessary for the adaptation of the plants to the changing environmental conditions.

In order to understand further the influence of light on the function of the circadian oscillator we examined the capacity of young etiolated bean leaves to accumulate chlorophyll and the LHCII apoprotein upon exposure to light. Thus, 8-10 day old etiolated seedlings from eight sowings every 6 hours during 1.5 days, were exposed all simultaneously to white light for 6 hours. Chlorophyll was found to follow circadian oscillations, suggesting that the rhythm is endogenous, preexisting in complete absence of light and its expression is triggered by imbibition of seeds at sowing. The accumulation of the LHCII apoprotein in etiolated bean plants from one sowing exposed to white light for six hours every six hours during 1.5 days shows also circadian oscillations, indicating the capacity of the plant to accumulate chlorophyll and the LHCII apoprotein according to an endogenous diurnally oscillating program. Similar results were obtained with etiolated pea primary leaves where a heat-shock was applied in the dark prior to total leaf RNA or protein extraction. To seedlings from one sowing grown in the dark for 8 days, a 30 min heat shock was applied in the dark; on the next day leaves were harvested every 6 hours for two consecutive days without being exposed to light. The accumulation of *Lhcb* mRNA or LHCII apoprotein formed in the dark (following the heat-shock application) followed circadian oscillations. In control, dark grown and non-light exposed leaves, accumulation of the steady-state levels of *Lhcb* mRNA was also regulated by the biological clock in a different phase than the one observed in the heat pretreated plants. These results indicate that light or heat-shock do not induce the function of the circadian oscillator, but act as enhancer and signals for elevated gene expression and protein synthesis/stabilization entraining the circadian oscillator.

In the present study we further address the question how the endogenous circadian oscillator detected in complete darkness is influenced by or interacts with signals, like phytohormones, pH or cutting.

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tRNA^{Glu} MEDIATED δ-AMINOLEVULINATE (ALA) BIOSYNTHESIS AND BIOTECHNOLOGY

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In nature, two routes exist for the biosynthesis of ALA. In one, a single enzyme condenses succinyl-CoA and glycine to produce ALA. This route is used by animals, yeast and few bacteria such as *Rhizobium.* Plants, algae, cyanobacteria and other bacteria such as *Escherichia coli* and *Bacillus subtilis* convert glutamate to ALA with the help of three enzymes. The conversion proceeds via glutamyl tRNA^{Glu} and glutamate 1-semialdehyde. Aminoacylated tRNAs are ordinarily used for protein biosynthesis where they serve as substrates in the transfer of amino acids to develop the polypeptide chains following the codons of a given mRNA. In ALA synthesis, glutamyl tRNA^{Glu} is reduced to glutamate 1-semialdehyde and this is the only known example of a reduction of an aminoacylated tRNA to an α-aminoaldehyde. The genes encoding the tRNA^{Glu} and the enzymes as well as catalytically active recombinant enzymes are now available. Several aspects of utilising the tRNA^{Glu} mediated ALA biosynthetic pathway in industry and agriculture are considered. Partial suppression of the last enzyme of the pathway produced a wide variety of inheritable chlorophyll variegation patterns. Depending on the location of insertion of the antisense gene, the variegations ranged from entire pale green leaves to only specific regions with chlorophyll reductions. Thus, new horticultural strains could be developed by silencing the aminotransferase gene. In *Salmonella typhimurium*, ALA is synthesized from glutamate via glutamyl tRNA^{Glu} and glutamate 1-semialdehyde. Since in birds, ALA is synthesized by condensation of glycine and succinyl-CoA, one way to eliminate of *Salmonella* from birds is by specifically inhibiting the tRNA^{Glu}-mediated ALA synthesis. To curtail plant pathogens, one could introduce the *tigrina-d*^{12} gene in antisense orientation under the control of a pathogen inducible-promoter. Silencing the *tigrina-d*^{12} gene lead to de-repression of ALA synthesis and overproduction of protochlorophyllide in darkness. Induction of the antisense DNA expression by the pathogen would deregulate ALA biosynthesis causing the infected leaf cells and the neighbouring cells to accumulate excessive amounts of protochlorophyllide in darkness. Daylight will then selectively photodynamically destroy the pathogen and the host cells in the areas attacked by the pathogen and thereby curtail the infection. Compounds which inhibit plant growth by inhibiting the tRNA^{Glu}-mediated ALA synthesis are potential herbicides. It has already been shown that salicylate inhibits the ligase while the glutamate analogue of puromycin inhibits the reductase of barley. Furthermore several compounds such as gabaculine, 6-amino-5-hydroxy-1,3-cyclohexadiene-1-carboxylic acid, glutamic-γ-monohydroxamate and 4-amino hex 5-ynoic acid, irreversibly inactivated glutamate 1-semialdehyde aminotransferase. Gabaculine, which is most potent, is a neurotoxin and is not suitable as a herbicide. However, elucidation of the enzyme mechanism of glutamate 1-semialdehyde aminotransferase makes it possible to design herbicides targeted to this enzyme.

ORGANIZATION AND FUNCTION OF PHOTOSYSTEM I TRIMERIC AND MONOMERIC COMPLEXES OF CYANOBACTERIUM *SPIRULINA PLATENSIS*

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Trimeric and monomeric complexes of Photosystem I (PSI) of *Spirulina* differ in the longwave forms of antenna chlorophyll (Chl) though they show a similar Chl/P700 ratio of about 90-100 (**1**, **2**). Core antenna of complexes contains the same set of bulk Chls, but only the antenna of PSI trimers has the extreme longwave Chl form absorbing at 735 nm (Chl735) and emitting at 760 nm (F760). This 77 K longwave emission band serves as an internal marker of PSI trimers *in vivo*, since the F760 band was also found in *Spirulina* cells and isolated membranes (**2**). The intensity of F760 highly depends on the redox state of P700: it is maximal when P700 is reduced and strongly decreases when P700 is oxidized ($P700^+$). Calculation of the overlap integral between the absorption bands of P700 and $P700^+$, and the emission band of F760 indicates that $P700^+$ is a much stronger quencher of fluorescence than P700 reduced. It was suggested that the quenching of F760 is due to the energy migration from Chl735 to $P700^+$ (**3**).

Using the F760 as an internal marker of PSI trimers, we confirmed the suggestion that the PSI trimer/monomer ratio within the membrane can be regulated by salt conditions. Incubation of detergent-free membranes at "high salt" (150 mM) decreases the F760 intensity because of transfer of PSI trimers into monomers. Washing of „high salt" treated membranes with 5 mM salt restored up to 50% of the initial F760, indicating the re-formation of PSI trimers (**4**). These data also show that the longwave Chl735 may originate from pigment-pigment interaction of Chl molecules, located on the surface of the monomeric subunits forming the trimeric complex: transfer of PSI trimers into monomers at „high salt" is accompanied by disaggregation of Chl735, while the trimerization of monomeric subinits at „low salt" results in the appearance of Chl735. Each monomer in the PSI trimer contains two Chl735 that is consistent with the suggestion on the origin of Chl735 (**4**). Circular dichroism spectrum of PSI trimers, in contrast to monomers, shows a pronounced carotenoid band at 512 nm (**2**). But since PSI trimers and monomers contain the same amount of β-carotene/P700, it was concluded that the trimerization of monomers may change the state of Chls and carotenoids as well. At „high salt" conditions cyanobacterial membranes show the higher PSI activity than at „low salt", i.e. probably the PSI monomers *in situ* are more active in electron transport than the PSI trimers. No difference in the PSI activity of isolated trimers and monomers was observed.

Simultaneous measurement of the kinetics of F760 quenching and P700 photooxidation in PSI trimers at 77 K revealed a delay of $P700^+$ accumulation as compared to the F760 quenching though $P700^+$ is responsible for the fluorescence quenching. The nonlinear relationship between these two parameters is considered as an indication on the energy exchange (via the longwave Chl735) between core antennae of monomeric subunits within a trimer that leads to dissipation of the excess energy into heat (**5**). No energy exchange between PSI subunits of the trimer was found that provides a gain in P700 photooxidation (**3**). Thus cyanobacterial PSI trimers have mainly the protecting role, while the cyanobacterial PSI monomers are rather involved in photosynthetic electron transport as PSI complexes of higher plants.

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(**2**) Shubin VV, Tsuprun VL, Bezsmertnaya IN, Karapetyan NV (1993) *FEBS Lett.* 334, 79-82;
(**3**) Shubin VV, Bezsmertnaya IN, Karapetyan NV (1995) *J. Photochem. Photobiol.* 30B, 153-160; (**4**) Karapetyan NV, Dorra D, Schweitzer G, Bezsmertnaya IN, Holzwarth AR (1997) *Biochemistry* 36: 13830-13837; (**5**) Karapetyan NV, Shubin VV, Bezsmertnaya IN, Strasser RJ (1995) in *Photosynthesis: from Light to Biosphere* (Mathis P, ed) Kluwer, v. 2, pp. 55-58

X-RAY CRYSTALLOGRAPHIC STUDIES ON THE 3D-STRUCTURE OF PHOTOSYSTEM I

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In water oxidising photosynthesis of cyanobacteria, algae and higher plants, the primary processes of conversion of light energy into chemical energy take place at two multi-subunit membrane embedded protein pigment complexes, the photosystems I and II (PS I and PS II). Our structure analysis using crystals of PS I isolated from the thermophilic cyanobacterium *Synechococcus elongatus* led to a structural model at 4 Å resolution (Krauß et al., *Nature Struct. Biol.* **3**, 965-973, 1996; Schubert et al., *J. Mol. Biol.* **272**, 741-769, 1997) consistsing of the α-helices including some interhelical links, the iron sulfur centres, most of the chlorophyll *a* cofactors and one phylloquinone position.

Further improvement of phases in the resolution range up to 4 Å was recently achieved by replacing the old native data set by a new one (suitable up to 3.5 Å resolution), including a new mercury derivative data set (obtained by co-crystallisation and including reasonable anomalous data) and using new procedures in heavy atom paramter refinement, calculation of MIRAS and density modification. The completē set of cofactors involved in electron transfer can be localized in the corresponding electron density map, including two phylloquinone positions which are related by the pseudo-twofold symmetry axis within the PS I core. Subunit PsaE, which is located at the stromal side of PS I, can now be modelled into the electron density map using the NMR structure of a homologous PsaE (Falzone et al., *Biochemistry* **33**, 6052-6062, 1994), indicating significant improvement of the phases.

Further improvements of the structural data obtained from these PS I crystals can be expected from diffraction data collected under cryogenic conditions. Freezing of the crystals is accompanied by a change of the space group from hexagonal $P6_3$ ($a = 286$ Å, $c = 167$ Å) at 277 K with one monomer per asymmetric unit to monoclinic $P2_1$ ($a = 277$ Å, $b = 165$ Å, $c = 283$ Å, $\beta = 119°$) at 100 K corresponding to one PS I trimer per asymmetric unit. A native data set suitable up to 2.9 Å could be collected at beam line ID2 at the ESRF (Grenoble).

THE REGULARORY ROLE OF POLYAMINES ON THE STRUCTURAL AND FUNCTIONAL PHOTOADAPTATION OF THE PHOTOSYNTHETIC APPARATUS

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In this contribution we describe the changes in structure and functioning of the photosynthetic apparatus of the unicellular green alga *Scenedesmus obliquus* induced by the inhibition or the induction of different steps of polyamine biosynthesis, respectively, under low and high, as well as under blue and red irradiation. We observed that a decrease of the intracellular putrescine level and, *vice versa*, an increase of spermine, indicated by a raised ratio of spermine to putrescine, mimiced a low light photoadapted photosynthetic apparatus. Indeed, the inhibition of putrescine biosynthesis or the addition of spermine led to an increase of the antenna size, accompanied by a decrease of the population of photosynthetic reaction centers. These changes were, as to be expected, followed by an increased rate in primary photochemistry and electron transport per reaction center, which, as final result, leads to a decreased rate of photosynthesis. Action spectra on the spermine/putrescine ratio compared to a variety of those on characteristics of photoadaptation, e.g. chlorophyll biosynthesis, antenna size, the number of reaction centers per unit area or the rate of primary photochemistry and electron transport, showed that the photoreceptors for both, the adaptation of the photosynthetic apparatus to low light conditions and for the regulation of the spermine/putrescine ratio are identical, or, at least, very similar. We show that the photoreceptor is primarily a blue light receptor with a superimposed red light receptor that absorbes at 680 nm. and speculate that the PSII reaction center could possibly play a role in this red light perception. Furthermore, the participation of the spermine/putrescine ratio in a signal transduction chain from the photoreceptor to the final acceptor of the adaptory reaction is discussed.

IMPORT AND PROCESSING OF *E. COLI* EXPRESSED POLYPHENOL OXIDASE BY ISOLATED CHLOROPLASTS

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Polyphenol oxidase (PPO), a copper containing enzyme, is targeted to the chloroplast lumen in two steps. The 67 kDa precursor (pPPO) is processed by a stromal peptidase to a 62 kDa intermediate (iPPO) and the latter traverses the thylakoid membrane and is converted by a thylakoid processing peptidase to the 59 kDa mature polypeptide.

pPPOA, encoded by a member of the tomato gene-family, was over-expressed in *E. coli* and accumulated in inclusion bodies. The precursor, dissolved in urea/DTT, was imported by pea chloroplasts, processed to iPPO and converted to the mature polypeptide. The amount of iPPO in the stroma increased during the first 30 minutes and decreased thereafter while the mature form accumulated in the thylakoid lumen. Analysis of competition with *in vitro* translated pPPO confirmed that import and processing of *E. coli* expressed pPPO proceeded normally. The *E. coli* expressed pPPO could therefor be used for competition studies. Over-expressed pPPO competed with *in vitro* translated precursors of other proteins in crossing the envelope and interacting with thylakoids. Import of *in vitro* translated pPPO was specifically inhibited by 1-5 μM Cu^{2+} . Higher concentrations of Cu^{2+} were required for inhibition of the import of over-expressed precursor. Lower concentrations increased the accumulation of the 59 kDa, suggesting that translocation across the thylakoid might be assisted by Cu^{2+}. Wheat germ extract, retyculocyte lysate and a preparation of cytosolic proteins from pea markedly enhanced the import of *E. coli* expressed pPPO.

Although mature PPOA accumulated in thylakoids to levels easily detected in Western blots, no enzymatic activity was observed in chloroplasts following import. It is possible that pea, which lacks active PPO, is unable to activate the imported polypeptide. This possibility is being examined using thylakoids from PPO-null transgenic tomato plants in import assays.

MOLECULAR EFFECTS OF SOME STRESS FACTORS ON THE CHLOROPLAST GENETIC APPARATUS OF THE FLAGELLATE *Euglena gracilis*

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The chloroplast genetic apparatus of the flagellate *Euglena gracilis* is particularly sensitive to various chemical and physical factors which can induce irreversible mass bleaching of the cultures and cause depletion of chloroplast DNA (ctDNA). Our goals were: (i) to test whether typical structural modifications of the chloroplast genome occur when xenobiotics with different mode of action are applied; (ii) to analyse total RNA profiles from wild type cells *versus* various mutants with damaged plastids. The results are as follows. Both chemicals, ofloxacin, a representative of new quinolone antibacterial chemotherapeutics and nitrosoguanidine, a classical mutagen/carcinogen induce complete bleaching of euglena cultures, as well as the hyperthermic cultivation at 33°C for 7 days. In contrast to the quinolone ofloxacin which produce 100% white cells after 24 hours treatment in concentrations not affecting cell viability, the mutagen nitrosoguanidine acts more rapidly (6-8 hours), but only in near toxic concentrations (100% lethality after 48 hours). Treatment with both substances, quinolone ofloxacin and mutagen nitrosoguanidine results in a mass degradation of *E. gracilis* chloroplast DNA. The restriction endonuclease patterns of ctDNA from treated cells are identical with those of wild type *E. gracilis*. Significant loss in the hybridisation signal intensity is evident in all ctDNA samples from treated cells. Its decreasing follows the prolongation of cell exposure to the chemicals. No obvious evidence for chloroplast genome rearrangement could be detected in chloroplasts of chemically stressed *E. gracilis*. This result contrasts with heat bleaching experiments where we show that heat induced loss of chloroplast functions is associated with partial chloroplast genome specific restructurations. Electrophoretograms of *E. gracilis* total RNA show that there is an obvious difference between wild type cells and bleached mutants. Bands for 23S and 16S rRNA are lost in all mutants. Subsequent hybridizations with three different DNA probes for nuclear encoded chloroplast proteins (two for Rubisco and LHCPII) provided in all cases identical signals. However in the case of cytochrome c-553 (*petJ*) used as the fourth probe were obtained different hybridization signals with northern blots from an ofloxacin-bleached mutant depending on light régime. Undetected signal under light conditions indicates that there is a feedback between the state of plastids and expression of nuclearly encoded photosynthetic genes which remind the situation in higher plants. Contrary, positive signal in the darkness suggests the existence of more complicated pathways in biosynthesis of chloroplast system in *E. gracilis*.

CHARACTERIZATION OF TWO RNA POLYMERASES FROM SPINACH CHLOROPLASTS

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Chloroplasts of higher plants possess at least two biochemically different RNA-polymerases which might be differentially activated during chloroplast development (Mullet, Plant Physiol. 103:309-313, 1993).

In the work presented here two fractions with RNA polymerase activity have been isolated from the same spinach chloroplast raw extract by means of gel filtration, providing further evidence for the coexistence of at least two RNA polymerases in mature chloroplasts. One of these two RNA polymerase activities is tightly attached to the plastid DNA and has therefore been termed 'transcriptionally active chromosome' (TAC) (Hallick et al., Biochem. 15: 3039-3045, 1976). The interaction between this RNA polymerase and the plastid DNA is not affected by high salt concentrations or by the competitor molecule heparin. The other chloroplast RNA polymerase, in contrast to the TAC enzyme, is dependent on the addition of exogenous DNA and is sensitive towards heparin and high salt concentrations. The analysis and comparison of several other biochemical parameters revealed that the two enzymes share certain properties but differ with regard to others.

It is still unknown whether these two RNA polymerases represent two structually and functionally completely different enzymes or whether they only differ in additional transcriptional factors but not in the core enzyme. We further purified the two enzyme fractions in order to study their protein composition. So far we succeeded in purifying the TAC to near homogeneity from 5 kg of spinach leaves. Electrophoresis of proteins under denaturing conditions and immunological analyses have revealed that this purified extract does not only contain the RNA polymerase but at least DNA-binding HU-like proteins as well.

The core subunits of the heparin-sensitive chloroplast RNA polymerase are encoded by genes of the plastome (*rpo*A, *rpo*B, *rpo*C1 and *rpo*C2) (Hu and Bogorad, Proc. Natl. Acad Sci. USA 87: 1531-1535, 1990). Using specific antibodies we could show that the *rpo*A-encoded α-subunit (Suck et al., Curr. Genet. 30: 515-521, 1996) as well as the *rpo*B-encoded ß-subunit are both also part of TAC enzyme. This result supports the hypothesis that both polymerases share at least some core components. By use of the photoaffinity labeling technique we hope to identify other core components of the RNA polymerase in the TAC. Sequence analysis of these proteins will elucidate to what extent nuclear-encoded components contribute to the TAC core enzyme and whether they are present in the other RNA polymerase fraction, too.

TOCOPHEROL BIOSYNTHESIS IN SENESCING CHLOROPLASTS - A MECHANISM TO PROTECT ENVELOPE MEMBRANES AGAINST OXIDATIVE STRESS AND A PREREQUISITE FOR LIPID REMOBILIZATION?

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Tocopherols are lipophilic antioxidants which are supposed to be synthesized in chloroplasts (Schultz et al. Physiol. Plant. 64, 123-129, 1985). Earlier work has shown that the overall tocopherol content of chloroplasts increases during leaf senescence (Lichtenthaler Ber. Dt. Bot Ges. 79, 111-117, 1966). Analyses of tocopherols in organic extracts derived from flag leaves of barley plants cultivated in a field revealed that α-tocopherol content of senescing leaves is about twice as much as that of mature flag leaves.
We have isolated a key enzyme of the biosynthetic pathway leading to tocopherols and plastoquinones. This enzyme, 4-hydroxyphenylpyruvate dioxygenase (HPPD), catalyzes the formation of homogentisate from 4-hydroxyphenylpyruvate, a product of the shikimate pathway (Schultz et al. 1985).
A partial cDNA specific for the *hpd* gene has been isolated by differential display of RNA derived from primary foliage leaves of barley induced to senesce by darkness (Kleber-Janke and Krupinska, Planta 203: 332-340, 1997). The complete cDNA has been obtained by screening of a library representing gene expression of senescence flag leaves of barley. Northern blot analyses revealed indeed that the gene is expresssed both during dark induced senescence and during natural senescent under field conditions. Under both conditions expression of the *hpd* gene clearly precedes the degradation of chlorophylls. Expression of the *hpd* gene was further observed in barley leaves exposed to various stress factors including heat, drought and herbicide treatment. The expression data suggest that expression of the *hpd* gene is due to oxidative stress.

It is hypothesized that tocopherols during senescence and various stress situations leading to premature senescence could play an important role in differential stabilization of membranes with essential functions during senescence. Ultrastructural analyses with ultrathin sections from flag leaves of barley show that cellular membranes have different stabilities during senescence. In the case of chloroplasts it is striking that envelope membranes have a higher stability than thylakoid membranes. It is likely that these membranes are preserved preferentially due to their important functions in lipid degradation during senescence. A model explaining the putative function of tocopherols in protecting plastid envelope membranes against increasing oxidative stress during senescence and thereby ensuring remobilization of lipids is presented.

IN VITRO CHARACTERIZATION OF PROMOTERS FOR THE NUCLEAR ENCODED PLASTID RNA POLYMERASE (NEP)

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The existence of a plastid-localized, nuclear-encoded phage-type RNA polymerase (NEP), which is distinct from the plastid encoded *E.coli*-like RNA polymerase (PEP), has been established by several reports [Lerbs-Mache, S. (1993) *PNAS* USA **90**, 5509; Allison, L.A., Simon, L.D. and Maliga, P. (1996) *EMBO J.* **15**, 2802; Hedtke, B., Börner, T. and Weihe, A. (1997) *Science* **277**, 809]. In order to characterize the structure of plastid NEP-promoters, an *in vitro* NEP transcription system has been developed. The *in vitro* system utilizes plastid extracts from tobacco plants, which lack PEP. These plants were obtained by targeted deletion of the plastid *rpoA* gene encoding the PEP alpha-subunit [Serino, G. and Maliga P, (1998) Plant Physiol. in press]. Using a series of 5'- and 3'-deletion constructs of the tobacco plastid *rpoB* and *accD* promoters the core promoter element was localized within a region -15/+3 nucleotides (nt) around the transcription initiation site, resembling phage and phage-type promoters. Sequences outside the promoter core will be investigated for potential *cis* elements essential for NEP promoter activity. The core promoter will be further defined by more detailed deletions. In addition to promoter mapping, the novel *in vitro* transcription system will be useful to elucidate the enzymatic characteristics of the NEP enzyme.

EXPRESSION OF GLUTATHIONE PEROXIDASE DURING *ZANTEDESCHIA AETHIOPICA* SPATHE SENESCENCE AND REGREENING

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Even under optimal conditions many metabolic processes in plants produce active oxygen species (AOS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH°). In leaf senescence the breakdown of the photosynthetic apparatus could lead to the formation of AOS which are produced as a consequence of both inefficient transfer of electrons through the photosystems and breakdown of thylakoid components due to lipoxygenation. As a result, uncontrolled free radical chain reactions take place damaging unsaturated lipids, proteins and DNA.

Glutathione peroxidases (GPX, EC 1.11.1.9) are a family of key enzymes involved in scavenging oxyradicals in animals. The existence of this enzyme in plants has been reported only recently. This enzyme catalysis the reduction of H_2O_2, organic hydroperoxydes and lipid hydroperoxydes by reduced glutathione.

In order to be able to study the expression of this enzyme during senescence and regreening processes of calla lily (*Zantedeschia aethiopica* Spreng., Araceae) we have screened a young leaf cDNA library for GPX. After sequence analysis we studied the genetic expression of this enzyme during the senescence and regreening processes. These results will be discussed considering the alterations suffered by photosynthetic electron chain during spathe development.

POLYADENYLATION, DEGRADATION AND TRANSLATION OF mRNA IN THE CHLOROPLAST

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The addition of poly(A) tail to the 3' end of eukaryotic cell transcripts plays a key role in generating functional mRNA and in regulating mRNA decay. The discovery that polyadenylation of bacterial mRNA significantly affects transcript stability and may trigger rapid degradation promoted us to look for post-transcriptional polyadenylation of mRNA in higher plant chloroplasts. We found that poly(A)-rich sequences are post-transcriptionally attached to the plastid *psbA* mRNA at specific positions. Unlike eukaryotic and bacterial mRNAs, the sequence moiety does not consist exclusively of poly(A) but is rather composed mostly of purines, 70% adenosines and 25% gaunosines. Polyadenylated RNA was rapidly degraded when incubated in chloroplast extract.

Analysis of the initial steps in *psbA* mRNA decay revealed specific endonuclease cleavage sites that perfectly matched the sites where poly(A)-rich sequences are added. The addition of the polyadenylation inhibitor, cordycepin-triphosphate (3'-dATP), inhibited the degradation of mRNAs and degradation intermediates generated by endonucleolytic cleavages accumulated. Therefore, the addition of poly(A)-rich sequences to endonucleolytic cleavage products of chloroplast mRNA ***is required*** to target these RNAs for rapid exonucleolytic degradation. When *in vitro* transcribed RNAs were incubated with chloroplast-protein extract, competition between polyadenylated and non-polyadenylated RNA for degradation resulted in the rapid degradation of the polyadenylated molecules and stabilization of their non-polyadenylated counterparts.

We found that the chloroplast exoribonuclease 100RNP/PNPase preferably degrades polyadenylated RNA because it bound the poly(A) sequence with much higher affinity than other RNA molecules, thereby defining the poly(A)-rich RNA as a preferential substrate for this enzyme.

Our results suggest a possible mechanism for the degradation of *psbA* mRNA in which endonucleolytic cleavages are followed by the addition of poly(A)-rich sequences to the upstream cleavage products, which target these RNAs for rapid decay.

Using chloroplast transformants of the green algae *Chlamydomonas reinhardtii*, we analyzed whether 3' end processing of mRNA is required for translation in the chloroplast. We found that only 3' end processed mRNA is associated with polysomes. Unprocessed transcripts could not be detected to be associated with polysomes suggesting that 3' end processing of the mRNA is required for translation in the chloroplast.

A TRANSGENIC APPROACH TO CHARACTERIZE THE PLASTID TRANSCRIPTION MACHINERY IN HIGHER PLANTS

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Plastid genes in higher plants are transcribed by two RNA polymerases: a plastid-encoded (*rpo* genes), eubacterial-type multisubunit enzyme (PEP) and a nuclear-encoded phage-type enzyme (NEP). Deletion of the plastid-encoded subunits in tobacco revealed that the two RNA polymerases are distinct and do not share subunits. NEP promoters were mapped in tobacco plants lacking PEP activity due to targeted deletion of the plastid-encoded subunits and in the *iojap* mutant of maize which lacks plastid ribosomes, and therefore the ability to translate the mRNAs for the plastid-encoded *rpo* genes. Promoter identification revealed that Photosystem I and II genes are exclusively transcribed by PEP, a few genes (e.g. *rpo*) are transcribed by NEP, whereas most other genes are transcribed by both polymerases. Although PEP expression is dependent on NEP activity, the two RNA polymerases appear to form a parallel system with both activities present at all times during development. Individual NEP promoter activity seems to depend on promoter-specific transcription factors. *In vivo* and *in vitro* dissection of the *clpP*, *accD* and *rpoB* NEP promoters lead to the identification of two types of NEP promoters with distinct promoter elements. The Type I NEP transcription machinery is present in both monocots and dicots, whereas the Type II transcription machinery is present only in dicots. The two RNA polymerases and promoter-specific transcription factors form a sophisticated regulatory network that ensures coordinated expression of plastid and nuclear genes during development.

THE PHOTOSYNTHETIC APPARATUS OF *PROCHLORON*-LIKE CYANOBACTERIA

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Cyanobacteria usually contain unstacked thylakoids and have phycobilisomes as major light-harvesting complexes of photosystem II. In ultrastructure and pigment composition they resemble the plastids of red algae but differ significally from higher plant chloroplasts. When *Prochloron didemni*, a prokaryote with stacked thylakoids containing Chl *a* and Chl *b*, was discovered [1] it was regarded as a close relative of the ancestors of green plastids. Lateron, more organisms with similar ultrastructure and pigment composition were found [2,3] which were combined with *Prochloron* in the taxon Prochlorophyta. However, phylogenetic analysis revealed the polyphyletic origin of the prochlorophytes within the cyanobacteria [4,5]. In recent years the presence of a Chl *c*-like pigment [6] and of biliproteins [7] has been demonstrated. Furthermore, nucleotide sequences for LHC polypeptides showed no homology to higher plant LHC but to the photosystem II core polypeptide CP43 [8]. Thus the resemblance of prochlorophytes to higher plant chloroplasts seems to be more superficial.

Recently, another photosynthetic prokaryote with stacked thylakoid membranes and without phycobilisomes, *Acaryochloris marina*, was isolated. This organism has a unique pigment composition since it contains Chl ***d*** as major pigment in combination with Chl *a*, a Chl *c*-like pigment and biliproteins [9]. These biliproteins are not organized as phycobilisomes as in ordinary cyanobacteria, but as rod-shaped structures consisting of PC, AP, and traces of PE [10]. They are attached to the cytoplasmic side of certain thylakoid domains, where they are structurally and functionally coupled to photosystem II. Photosystem I, which contains Chl ***d*** in the reaction center [11], is organized predominantly in the form of trimers. Up to now, neither an LHCI nor an LHCII were found. Despite the unusual pigment composition all proteins sequenced so far have high homology with their counterparts from ordinary cyanobacteria, and also phylogenetic studies show that *Acaryochloris* falls within the cyanobacterial line.

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PROTEIN/RNA INTERACTION IN LIGHT REGULATED TRANSLATION IN THE CHLOROPLAST

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Translation of many chloroplast mRNAs is regulated in response to light, and requires both specific RNA elements and corresponding mRNA binding proteins. We have isolated a set of four proteins that bind with high affinity and specificity to the 5' UTR of the chloroplast *psbA* mRNA in the green algae *Chlamydomonas reinhardtii.* Binding of these proteins to the mRNA is light regulated and is required for translation initiation of this mRNA. Analysis of these RNA binding proteins has shown that one of these is a member of the poly(A) binding protein (PABP) family. Another of these proteins is a protein disulfide isomerases (PDI), which are enzymes involved in the oxidation and reduction of disulfide bonds associated with protein folding. The *psbA* mRNA associated PDI is capable of altering the mRNA binding activity of the PABP in a redox dependent manner, suggesting that light activated translation may involve activation of PABP binding to the *psbA* mRNA through changes in the light generated redox potential of the cell. Binding of the PABP to RNA elements contained within the 5′ UTR of the *psbA* mRNA allows for increased ribosome association and translation initiation, perhaps by changes to the RNA structure or by interacting with general translation initiation factors.

POSTTRANSCRIPTIONAL CONTROL IN THE EXPRESSION OF THE HIGH LIGHT-REGULATED MESSENGER RNA CODING FOR “HL#2“

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The derived amino acid sequence of a cDNA that has been cloned for a light stress induced protein, HL#2, of barley (Hordeum vulgare, L.) has been published (Pötter et al., Planta 199 (1996) 314-320). Computer analysis revealed considerable similarity of HL#2 with jasmonate inducible proteins, lectins as well as proteins of unknown function in rice. Despite this information the function of the protein could not be deduced. We shall report on the expression of the mRNA and the protein of two forms of HL#2 under the influence of high light and jasmonate. The mRNA of HL#2 was induced by high light and the mRNA level remained considerably elevated even after several hours of exposure to high light fluxes. The RNA concentration was determined highest in the basal segment of the leaf. In contrast, using an antibody against HL#2, we could not observe any change at the level of HL#2 proteins of 18 kDa and the related form HL#2* of 15.5 kDa during the same period of treatment. These data indicate an interesting stress-induced control of HL#2-expression at a post-transcriptional level. The HL#2* protein is transported into the apoplast the same may be the case for HL#2 itself. We have also found an induction of HL#2 together with that of the two most closely related jasmonate induced proteins (JIPs) of 32.6 and 32.7 kDa after the exposure of detached leaves of barley to jasmonate (45 μM) under normal plant growth conditions. The two JIPs cross-react with the antibody to HL#2 confirming the data obtained by sequence comparison studies. In contrast to the ELIPs whose mRNA and protein levels are upregulated by high light and cold those for HL#2 protein appear not influenced by low temperatures in green barley plants while its mRNA -at least in etiolated plants - is up-regulated as was found for the mRNA of ELIPs. Therefore the control of ELIPs and HL#2 by high light is at least partially under the control of two different signal transduction chains which diverge after transcription.

TRANSLATIONAL REGULATION OF LSU BY THE PHOTOSYNTHETIC PROTON GRADIENT

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The regulation of chloroplast translational activity was investigated by labeling isolated barley chloroplasts with ^{35}S-methionine. Translation elongation of membrane and stromal proteins is retarded in darkness, or in light in the presence of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), even in the presence of exogenously added ATP and dithiothreitol. This inhibition can be overcome by addition of artificial electron donors in the presence of light, but not in darkness. Activation can only be obtained by electron donors which release protons into the thylakoid lumen, but not by reduced ferredoxin or thioredoxin. Release of the proton gradient by uncouplers prevents the light dependent activation of translation elongation. Hence, we propose that the formation of a photosynthetic proton gradient across the thylakoid membrane activates translation elongation in chloroplasts. A pH-sensitive membrane component could link the the elongation activity of membrane-bound polysomes to the luminal pH.

We further tested whether this hypothesis could also be applied to soluble proteins like the large subunit of the ribulose-1,5-bisphosphate carboxylase (LSU). Translation reactions containing only the soluble phase of the lysed chloroplasts do not incorporate detectable amounts of radioactivity, whereas readdition of membranes leads to accumulation of labeled products in the soluble phase. Furthermore, incubation of pulse-labeled thylakoid membranes with unlabeled stroma leads to release of labeled products into the stroma; the use of membranes deprived of polysomes by high salt washing greatly reduces the amount of labeled soluble proteins. Finally, immunoprecipitation with an antibody against LSU detected a much higher amount of LSU translation intermediates in the washed membrane fraction than in the soluble fraction. We therefore conclude that translation of LSU, although a subunit of the soluble rubisco holocomplex, is performed mainly by membrane-bound polysomes, hereby allowing its regulation by the thylakoid proton gradient.

DE NOVO SYNTHESIS OF CHLOROPHYLL A TRIGGERS ASSEMBLY OF REACTION CENTERS IN BARLEY ETIOPLASTS

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Etioplasts of barley seedlings (Hordeum vulgare L., var Steffi) were used to study the chlorophyll-dependent assembly of plastid-encoded chlorophyll binding proteins of photosystem I and II (Chl-P) *in vitro*. In etioplasts, neither Chl-P nor chlorophyll (Chl) are present. The Chl precursor protochlorophyllide (Pchlid) has to be enzymatically reduced to chlorophyllide a (Chlid) by light and NADPH/H^+. For accumulation of Chl-P, light independent esterification of Chlid with geranylgeraniolpyrophosphate (GGPP) and binding of Chl to the *de novo* synthesized apoproteins is the decisive step (Eichacker et al., 1996).

In this work, sucrose density gradients of solubilized inner etioplast membranes were utilised to measure assembly of the Chl-P as shift in the molecular mass of the de novo stabilized apoproteins. In order to define the molecular mass fractions of the sucrose gradient we first isolated Chl-P complexes from chloroplasts. Protein complexes were identified by silver staining of SDS-PAGE, Western analysis and spectroscopy.
In etioplasts, assembly was kinetically followed by radiolabeling of de novo synthesized proteins in the absence and presence of increasing concentrations of Chl.

When the endogenous concentration of Chl was increased over a threshold value, stabilization of all Chl a binding proteins was observed. When the assembly kinetic of the Chl-P was followed for 80 min *in vitro*, Chl-P of PSI (P700) assembled in a complex of about 300 kDa, whereas Chl-P of PSII (D1,D2,CP47) assembled in a complex of about 100-150 kDa. Interestingly, CP43 did not assemble into the PSII-complex, although it was stabilized by de novo synthesized Chl.
The presence of Chl within the de novo assembled complexes was verified by spectroscopic analysis of the sucrose gradient fractions and by isolation of the pigment.

We therefore conclude that in etioplasts isolated from dark-grown barley seedlings *de novo* synthesis of Chl triggers not only stabilization of Chl-P, but also assembly of Chl-P to reaction center particles of PSI and PSII.

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GENETIC MANIPULATION OF MEMBRANE LIPIDS: A CLUE TO ELUCIDATE THE MOLECULAR MECHANISM OF COLD AND SALINITY TOLERANCE

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A recent study of the gene knockout of fatty acid desaturases has demonstrated the direct involvement of the unsaturation of membrane lipids in tolerance to low temperature and salt stress. *Synechocystis* sp. PCC 6803 (*Synechocystis*, here-in-after) contains four desaturases and they can be inactivated by insertional mutation of the desaturase genes in a step-wise manner [1]. After deletion of polyunsaturated fatty acids in membrane glycerolipids, this organism did not grow at 20°C, at which the wild-type strain grew well. Further study has demonstrated that this effect is caused by an increase in sensitivity of the organism to the photo-induced inactivation at low temperature [1]. The deletion of polyunsaturated fatty acids changed the cyanobacterial cells to be more sensitive to salt stress. It is likely that the saturation of fatty acids of lipids solidified the membrane which lead to the inactivation of the Na^+/H^+ antiporter.

Gene-engineered enhancement of tolerance to low temperature and salt stress has been achieved by introducing one of the desaturase genes (the *desA* gene for the $\Delta 12$ desaturase) from *Synechocystis* into a low-temperature-sensitive strain *Synechococcus* sp. PCC 7942 (*Synechococcus*, here-in-after). The wild-type cells of *Synechococcus* contain only the $\Delta 9$ desaturase, and fatty acids of their membrane lipids are either saturated or monounsaturated. The genetic introduction of the *desA* gene to *Synechococcus* enabled this organism to synthesize diunsaturated fatty acids in the membrane glycerolipids, and to tolerate low temperature [2]. Further study has indicated that the enhancement of the tolerance is caused by protection of the photosystem II complex against the photo-induced inactivation at low temperature by acceleration of the recovery of the photosynthetic machinery from the photo-inactivated state. The increase in the unsaturation also enabled the cyanobacteiral cells to be tolerant to salt stress.

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CHLOROPHYLL SYNTHASE OF AVENA SATIVA L. , A HOUSE-KEEPING ENZYME

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We investigated the chlorophyll synthase of *Avena sativa L.* at the levels of DNA, RNA and protein.

Two chlG genes were isolated from a cDNA-bank using PCR methods. They are identical in the coding region and are different only in a short region at the 3'UTR. Both genes show homology to the known chlorophyll synthases. The homology of the Avena ChlG proteins to Arabidopsis thaliana is 73%; to Synechosystis PCC6803 is 59%; to Rhodobacter capsulatus is 33%; to Chloroflexus aurantiacus BchG is 36% and Chloroflexus aurantiacus BchG2 is 31%.

We have investigated the development of etiolated and greening Avena sativa plants from three to eight days at the RNA and protein level.

For our investigation of the RNA level we took a probe of 500bp from the middle part of the sequence. There is no light induction of RNA at any time.

To investigate the protein level we prepared peptide antibodies against the sequence YIYSAPPLKLKQNGW which is very conserved for all the chlorophyll synthases. These antibodies were used to identify the chlorophyll synthase in total protein extracts from Avena sativa. The protein proved to be a double band between the α and β subunits of the ATPase. We quantified the concentration of the chlorophyll synthase from 20 mg plant material. The protein level concentration of chlorophyll synthase is dependent on development . Until the fourth day etiolated plants show no light induction of the protein. From day 5 to 8 there is an induction of the protein at the first two hours of light exposure.

AN ABC TRANSPORTER SYSTEM FOR MANGANESE IN THE CYANOBACTERIUM *SYNECHOCYSTIS* 6803

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In photosynthetic organisms, Mn is absolutely required for oxygenic photosynthesis. Photosystem II (PSII), a pigment protein complex, contains four Mn atoms which play important redox roles during O_2 evolution. By complementation analysis of a random PSII-deficient mutant of the cyanobacterium *Synechocystis* 6803, we have identified an ABC transporter protein for Mn in this organism[1].

The ABC transporter system is encoded by an operon containing three closely linked genes, *mntC*, *mntA* and *mntB*. The *mntA* gene encodes a soluble protein containing ATP binding motifs, *mntB* encodes an integral membrane protein with eight putative transmembrane spans, whereas *mntC* encodes a periplasmic substrate-binding protein. We have recently overexpressed the MntC protein in *E. coli* cells. A soluble form of the protein has been purified in large amounts after extraction from inclusion bodies and refolding in the presence of manganese.

The newly synthesized MntC protein has a clevable N-terminal signal peptide, and the mature protein is predicted to be anchored to the cytoplasmic membrane via a lipid moiety attached to its N-terminal cysteine residue. Sequence analysis of the MntC protein suggests that it possesses two β/α domains. Similar to other known periplasmic binding proteins, the interface of the two domains of MntC may form a cleft for bind its substrate. In the predicted binding sites, the residues conserved among the proteins homologous to MntC are mainly acidic ones. We have also predicted the presence of a calcium-binding loop in this protein. *In vivo*, Ca^{2+} enhances the accumulation of $^{54}Mn^{2+}$ in *Synechocystis* 6803 cells, whereas *in vitro*, MntC has a higher affinity to $^{54}Mn^{2+}$ in the presence of Ca^{2+}. Thus, calcium binding may be involved in the stabilization of the MntC protein. We are currently testing our structural and functional hypotheses by directed mutagenesis and spectroscopic analysis.

The ABC transporter system is induced in a Mn-deficient medium (< 0.5 μM)[2]. The kinetic parameters for this transporter are K_m = 1-3 μM and V_{max} = 3-8 pmol/ min.10^8 cells. $^{54}Mn^{2+}$ uptake by this system is inhibited by other divalent cations in a distinct rank order of potency: $Mn^{2+} > Cd^{2+} > Co^{2+} = Zn^{2+}$. Based on kinetic studies, we have also concluded that *Synechocystis* 6803 cells contain a second high affinity transporter for Mn. This system is induced in μM levels of Mn. Moreover, it appears to be specific for Mn only.

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APPLICATION OF THYLAKOIDS IN HERBICIDE-DETECTING SYSTEM

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Environmental pollution by toxic chemicals has become one of the world's serious problems. The most widespread group of commercial pesticides is photosynthesis inhibiting herbicides, such as atrazine, metribuzin, diuron, bromacil, ioxynil and dinoseb. They all belong to different chemical families but have a common mode of action: blocking electron transport between Photosystem I and Photosystem II of chloroplasts.

The appearance of herbicides in groundwater and in agricultural products has posed considerable problems in the control of drinking water and products quality. Regulation governing herbicides concentration in drinking water are very strictly enforced by European Community with a maximum allowable at 0,1 μg/l level for each individual substance and 0.5 μg/l for the sum of photosynthesis inhibiting herbicides.

The main goal of the research is development of rapid and cheap method of herbicide detection in the drinking water. The assay is based on inhibition of photosynthetic electron transport in spinach thylakoids by the herbicides preventing reduction of the redox dye from its normal blue form to colorless reduced form. Herbicide concentration is determined by measuring the change in absorbance of the dye after illuminating a mixture of sample, thylakoids, and redox dye for a fixed period of time. It is proposed to improve the sensitivity of method by sample preconcentrating on the solid-phase extraction cartridges. The C_{18} solid-phase extraction cartridges provide acceptable recoveries (>85%) of atrazine, simazine and cyanazine in broad range of concentrations. The C_8 cartridges are used for diuron and metribuzin concentrating. Different types of cartridges can be employed to produce class - selective detecting system. The method proposed allows to detect photosynthesis-inhibiting herbicides in the concentration range 0.025-25 μg/l. Three thylakoid immobilization procedures are performed and compared in order to improve the thylakoid membrane stability. The chloroplasts entrapped in photocrosslinkable polyvinilalcohol bearing styrylpyridinium groups (PVA-SbQ) are the most promising for utilization in the microtiter plate format. This method permits to maintain 25% of thylakoid membrane activity at $-18°C$ for one year. The advantage of microtiter plate format is ability to assay 20 samples in triplicate (along with blanks and standards) in the very short time. Easy preparation of the test-kits and inexpensive reagent utilization permit to consider them as practical approach to detect trace concentrations of herbicides in the drinking water.

EXPRESSION OF *rbcS, rbcL* AND *rca* GENES DURING SENESCENCE AND REGREENING OF *ZANTEDESCHIA AETHIOPICA* FLORAL SPATHE

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The spathes from the ornamental plant commonly known as calla lily - *Zantedeschia aethiopica* (L.) Spreng., Araceae - suffer a senescing process soon after its formation. The spathe (floral bud stage) exhibits reduced photosynthetic activity and rapidly changes into a non-photosynthetic organ (white stage). When fructification occurs the white spathe develops into a green leaf-like structure (regreened spathe) which completely envelopes the spadix. The regreening takes place only if fructification occurs; otherwise the spathe dies. Previously, it was shown that the application of kinetin or BAP to the cut surface after spadix ablation was able to induce the regreening suggesting that spathe senescence inhibition and its regreening are due to the action of endogenous cytokinins produced at time of fructification [1]. Although having a similar ultrastructure, regreened spathe and leaf chloroplasts showed markedly differences, namely the absence of Δ^3-*trans*-hexadecenoic acid from regreened spathe thylakoid membranes [2]. Biochemical analysis, including ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) activity and concentration, suggest that during the regreening process there is *de novo* synthesis of Rubisco. The assembling of rubisco occurs in stroma chloroplast, as a consequence of a coordinate action of both nuclear genome (encoding Rubisco small subunit) and cpDNA (encoding Rubisco large subunit). In addition, under physiological conditions, besides CO_2, and Mg^{2+} concentration Rubisco activity is also regulated by a stromal protein (rubisco activase, rca).

In this work we reported the identification and characterisation of *rbc*S and *rbc*L genes coding for rubisco small and large subunits, respectively, as well as rubisco activase (*rca*) gene and their use in expression analysis by Northern blotting during time course of spathe development. *rbc*S and *rca* gene were identified by screening a cDNA library, while *rbc*L gene was identified by screening a cpDNA library. In what concerns rubisco activase, four different cDNAs were identified, and their deduced aminoacid sequence suggests the existence of two distinct rca polypetides with Mr of 46 and 42 kDa. The expression analysis results suggest that *Z. aethiopica* spathe senescence could be interpreted as an example of a programmed cell death, since no *rbc*S, *rbc*L and *rca* transcripts were detected in this earliest stage of spathe development, although floral bud stage is a photosynthetic organ. The increase of mRNAs levels from these genes during regreening, suggest that besides rubisco, rubisco activase expression is also regulated by cytokinins.

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SYNTHESIS AND ASSEMBLY OF PHOTOSYNTHETIC COMPLEXES IN CHLAMYDOMONAS

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Genetic analysis of photosynthetic mutants of Chlamydomonas has revealed a large number of loci required for the proper expression of chloroplast genes. The factors encoded by these loci appear to be targeted to the chloroplast where they act in a gene-specific manner at various post-transcriptional steps which include RNA stability, RNA processing, splicing, translation and assembly of the photosynthetic complexes. Using chloroplast transformation we have performed an extensive dissection of the *psbD* 5'UTR and identified elements which play an important role in RNA stability and translation. We have used genomic complementation of the nuclear mutant nac2-26 deficient in *psbD* mRNA accumulation to isolate the gene affected by the mutation. It encodes a large polypeptide containing nine TPR (tetratricopeptide repeat) motifs consisting of a degenerate 34 amino acid repeat which has been found in numerous proteins of different biological function and which appears to be important for protein-protein interactions. A TPR-containing protein is also required for *psbB* mRNA stability. Several proteins binding to the 5'UTR of chloroplast mRNAs have been identified by UV-crosslinking. They appear to play an important role in RNA stability, processing and translation. We have localized several of these proteins on a low density membrane system which resembles the inner chloroplast envelope membrane, but which appears to be associated with the thylakoids. These observations raise the possibility that translation of chloroplast mRNAs may occur on this membrane system.

MEMBRANE PROTEINS OF PHOTOSYNTHESIS: STRUCTURE - FUNCTION - BIOTECHNOLOGY

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Prerequisite for the structural and functional investigation of membrane proteins of photosynthesis, i.e. photosystem 1 (PS1), photosystem 2 (PS2) and the cytochrom b_6f-complex (b_6f) is the mass production of such complexes in appropriate systems. Mesophilic (i.e. *Synechocystis* PCC 6803) and thermophilic (i.e. *Synechococcus elongatus*) cyanobacteria can be grown as mass cultures under controlled conditions in a new photobioreactor especially designed for these organisms; its main feature is the use of transparent polyamid foil instead of glass which enables a safe *in situ* sterilisation.

Thylakoid membranes isolated from cyanobacteria indicate the existence of oligomeric structures of all three membrane protein complexes mentioned above which seem to be in a dynamic equilibrium with their monomers. This equilibrium can be shifted *in vitro* by changing the surface charge density, for example by changing the salt concentration of the medium. For a detailed analysis of the factors responsible for reversible oligomerization, we developed a model system consisting of PS1 from *Spirulina platensis* reconstituted into liposomes. The formation of trimers starting from purified monomers could be monitored both by HPLC analysis and by fluorescence with a long wavelength-absorbing chlorophyll being an internal „marker“ for the trimer formation.

HPLC analysis could also show the existence of a dimeric PS2 in *Synechocystis*. This PS2 dimer had a higher oxygen-evolving activity than the monomer, which is in line with results from *Synechococcus elongatus*. However, in contrast to PS2 isolated from the mesophilic organism, thermophilic PS2 shows at least 5 times higher oxygen-evolving activity and the presence of a functional Q_B binding site.

From *Synechocystis*, a homogenous and active monomeric b_6f complex could be isolated. In contrast to *Chlamydomonas*, this complex preserves the Rieske-subunit and also a single chlorophyll molecule which is shown to be highly oriented and associated with the b_6-subunit. Also, a sub-stoichiometric amount of a carotinoid can be shown to be firmly bound to the complex. The apparent absence of any energy transfer between these pigments suggests their primarily structural role in this complex. Further information on their role in assembly of the complex may be gained from studies with isolated subunits which have been successfully overexpressed in *E.coli*, i.e. subunit IV (PetD), cyt. b_6 (PetB) and the PetB/PetD fusion protein mimicking cyt. b of the bc_1-complex. Out of the small subunits present in the b6f-complex of *Synechocystis*, PetM could be shown by deletion analysis to be not essential for cyt. b_6f function.

CAROTENOID BIOSYNTHESIS IN HIGHER PLANTS AND ALGAE

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In photosynthesis carotenoids have two main functions: light harvesting and photoprotection. As essential components of the photosynthetic apparatus they serve as light collectors as well as scavengers of singlet oxygen (and free radicals) and quenchers of excess light energy. In addition, they are involved in stabilization of the thylakoid membrane and oligomerization of the light harvesting complexes. A role in prevention of lipid peroxidation has also been suggested. Whereas ß-carotene is mainly located in the core complexes of photosystem I and II, the xanthophylls are predominantly found in the light harvesting system.

Carotenoid content and composition is subjected to changes in response to environmental conditions and developmental stages. During chloroplast biogenesis etioplasts are transformed into chloroplasts containing functional photosynthetic complexes. This process involves alterations in ultrastructure and coordinated synthesis of pigments (e.g. chlorophylls and carotenoids) and proteins. Much of our understanding about carotenogenesis was derived from inhibitor studies. Recently, advances in molecular biology allowed the cloning of many carotenoid genes of various plants thus providing the basis for investigations of the regulation of carotenoid biosynthesis. Analysis of *Arabidopsis* and algal mutants has been used to identify carotenoid genes and to unravel function and importance of certain carotenoids. This approach has been complemented by genetically engineered tobacco and tomato plants.

In our group, we are investigating the xanthophyll cycle and photoprotection mechanisms on the molecular level. Therefore, we have cloned some of the relevant genes from tobacco which can now be used as probes for expression studies. Furthermore, we are constructing sense and antisense plants (e.g. ß-carotene hydroxylase and lycopene cyclase) to obtain more detailed information about the regulation of this biosynthetic pathway. An overview about the current knowledge and recent developments in carotenoid biosynthesis including our own findings will be presented.

DISRUPTION OF THE PLASTID YCF10 OPEN READING FRAME AFFECTS UPTAKE OF INORGANIC CARBON IN THE CHLOROPLAST OF *CHLAMYDOMONAS*

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The complete DNA sequences of a dozen plastid genomes have been determined and they have revealed the existence of many open reading frames of unknown function (for review, see Reardon and Price, 1995, *Plant Mol. Biol. Rep.* 13, 320-326). Those that are conserved in algae and in higher and lower plants have been designated *ycf* for "hypothetical chloroplast open reading frame". The *ycf10* gene codes for a putative polypeptide of 229 to 231 amino acids. In pea, *ycf10* has been shown to be cotranscribed with *petA*, encoding apo-cytochrome *f* (Willey and Gray, 1990, *Plant Mol. Biol.*, 15, 347-356; Nagano *et al.*, 1991, *Curr. Genet.*, 20, 431-436). The Ycf10 protein was immunolocalized in the inner membrane of the pea chloroplast envelope (Sasaki *et al.*, 1993, *FEBS. Lett.*, 316, 93-98; Gray, 1996, In: *Membranes: Specialized Functions in Plants*. M. Smallwood, J.P. Knox, D.J. Bowles, eds. pp. 441-458. *Bios. Scientific Publ.*). Accordingly, *ycf10* was renamed *cemA* (chloroplast envelope membrane). Cyanobacterial mutants affected in CO_2 transport have been shown to contain mutations within the *cotA* gene which displays significant sequence identity to *ycf10* (Katoh *et al.*, 1996a, *Proc. Natl. Acad. Sci. USA*, 93, 4006-4010). More recently a role for the *cotA* product in light-induced proton extrusion has been proposed (Katoh *et al.*, 1996b, *J. Bacteriol.*, 178, 5452-5455).

In the green unicellular alga *Chlamydomonas reinhardtii, ycf10,* located between the *psbI* and *atpH* genes encodes a putative hydrophobic protein of 500 residues, which is considerably larger than its higher plant homologue because of a long insertion that separates the conserved N and C termini. To gain further insights into the function of *ycf10,* we have disrupted this chloroplast gene in *Chlamydomonas reinhardtii*, using biolistic transformation. The recovery of homoplasmic transformants first demonstrated that the Ycf10 protein is not essential for cell viability. The *ycf10*-deficient mutants were also found to grow both photoheterotrophically and photoautotrophically under low light, thereby revealing that the Ycf10 protein is not essential for the photosynthetic reactions. However, the *ycf10*-deficient mutants are highly sensitive to light, and the transformants did not grow photoautotrophically and barely photoheterotrophically under high light. This property cannot be related to altered chlorophyll, carotenoid or quinone metabolism. Instead, the increased light sensitivity of the transformants appears to be a consequence of a limitation in photochemical energy utilization and/or dissipation which correlates with a decrease in CO_2-dependent photosynthesis, as demonstrated by oxygraphic measurements. Mass spectrometric measurements with either whole cells or isolated chloroplasts from the transformants revealed that the CO_2 and HCO_3^- uptake systems have a reduced affinity for their substrates, CO_2 uptake being more affected than HCO_3^- transport (Rolland *et al.*, 1997, *EMBO. J.* 16, 6713-6726). The results suggest the existence of a *ycf10*-dependent system within the plastid envelope which promotes efficient inorganic carbon uptake into chloroplasts.

CHARACTERIZATION OF A CHLOROPLAST HOMOLOGUE OF THE « RIBOSOME RECYCLING FACTOR » (RRF) FROM *ESCHERICHIA COLI*

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Protein synthesis in eukaryotic and prokaryotic cells requires a wide variety of molecules such as initation, elongation and termination factors, ribosomes, mRNA, tRNA, and enzymes (aminoacyl-tRNA synthetases...). Chloroplast translational system has been widely studied (reviewed in Stern *et al.*, 1997, *Trends Plant Sci.*, 2, 308-315) and its similarities with that of *Escherichia coli* are so extensive that initiation and elongation factors and even ribosomal subunits can be interchanged. In bacteria, two proteins are involved in the disassembly of the termination complex: the Ribosome Recycling Factor (RRF) and the elongation factor G (EF-G). RRF, which is essential for bacterial growth, was first purified from *E. coli* and its gene was cloned (Janosi *et al.*, 1996a, *Adv. Biophys.*, 32, 121-201; 1996b, *Biochimie*, 78, 959-969). RRF is responsible for the release of ribosomes from mRNA during the termination of protein synthesis preventing ribosomes from reading beyond a termination codon without the initiation signal. It also prevents translational errors during peptide chain elongation. RRF increases translation efficiency by recycling ribosomes from one round of translation to another. To date, no functional characterization of other RRF-like proteins has been provided. In plants, and especially in chloroplasts, little is known on the final steps in protein synthesis, i.e. termination and disassembly of the termination complex.

We report here the identification of an eukaryotic reading frame whose predicted product displays high sequence similarity to the bacterial RRF. These data provide the first evidence for the presence of a nuclear-encoded RRF homologue in an eukaryotic cell. The spinach RRF homologue is a chloroplast protein (cpRRFH) as demonstrated (a) by purification of the mature protein from a soluble extract from spinach chloroplasts, (b) by immunolocalization using antibodies raised against the overexpressed protein, (c) by the import of the precursor protein into isolated chloroplasts and (d) by immunocytolocalization. cpRRFH is mostly present in the chloroplast stroma, but was also found to be associated with the chloroplast envelope. We never detected cpRRFH in isolated thylakoids, but a functional association with this membrane system cannot be ruled out as shown by immunolocalization within isolated chloroplasts. We also found that transcription of the single *frr* gene was restricted to photosynthetic tissues.

Complementation studies using mutant *E. coli* provide functional characterization of this factor. Expression of the plant *frr* homologue had an allele-specific effect on the host bacteria since (a) it clearly inhibited the growth of *E. coli* carrying the gene encoding temperature-sensitive RRF (ts-*frr*), even at the permissive temperature, (b) the plasmid containing the plant *frr* homologue was specifically eliminated from the host bacteria carrying ts-*frr*. cpRRFH also inhibits the *E. coli* RRF activity *in vitro* (the release of ribosomes from naturally occurring puromycin-treated *E. coli* polyribosomes). The results show that chloroplasts contain a protein homologous to the Ribosome Recycling Factor from *E. coli*. cpRRFH exerts an allele-specific inhibitory effect on the functionning of the bacterial protein, thus suggesting a similar role in chloroplasts and *E. coli*, i.e. the regulation of the translation of polycistronic mRNA.

REDOX REACTIONS IN THE LAST STEPS OF CHLOROPHYLL BIOSYNTHESIS.

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The last steps of chlorophyll biosynthesis are formation of chlorophyllide *a* (Chlide *a*) from protochlorophyllide *a* (Pchlide *a*), esterification to chlorophyll *a* (Chl *a*) and formation of Chl *b*. Redox reactions are the reduction of ring D (Pchlide → Chlide), the oxidation of the methyl group at C-7 to a formyl group (Chl *a*→ Chl *b*), and the reverse reaction, reduction of the formyl group at C-7 to a methyl group (Chl *b* →Chl *a*).

We investigated these redox reactions either with the enzymes that were (partially) purified from oat and barley etioplasts or with recombinant enzymes overexpressed in E. coli. Reduction of ring D, catalyzed by NADPH: protochlorophyllide oxidoreductase (POR), requires light. Several modified Pchlide derivatives were applied. The results allow a tentative model for the enzyme mechanism. The oxidation of Chl *a* derivatives to Chl *b* compounds with the recombinant chlorophyll *a* oxidase will be described here for the first time. The in vitro reaction allows to investigate the requirement for cofactors. Likewise, the cofactor requirement for the reverse reaction, Chl *b*→ Chl *a*, will be described.

CHLOROPHYLL *A* FORMATION IN THE CHLOROPHYLL *B* REDUCTASE REACTION REQUIRES REDUCED FERREDOXIN

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The Tanaka group detected the *in vitro* reduction of chlorophyllide *b* to chlorophyll *a* when they investigated the esterification of chlorophyll *b* in etioplasts of cucumber and barley (Ito et. al. 1994). The natural substrate chlorophyllide *b* can be exchanged by its zinc-complex, zinc pheophorbide *b*. Zinc 7^1OH-pheophorbide *a* is an intermediate of the reduction of zinc pheophorbide *b* to zinc pheophorbide *a* both in the chemical way and in etioplasts of barley. Therefore the reaction can be divided into two steps, the reduction of the formyl to the hydroxy methyl group and the final reduction to the methyl group. NADPH acts as the reducing agent of the first step in broken etioplasts (Scheumann et al. 1996).

The second reduction step was restricted to intact etioplasts and required ATP. We now demonstrated the reduction of chlorophyllide *b* and zinc pheophorbide *b* to chlorophyll *a* and zinc pheophytin *a*, respectively, in lysed etioplasts with ferredoxin as the reducing agent. Glucose-6-phosphate, glucose-6-phosphate-dehydrogenase, NADPH and $NADP^+$-ferredoxin-oxidoreductase were added to maintain the reduced state of ferredoxin. In contrast to the system with intact etioplasts no ATP was necessary. The reduction occured both with and without phytyl-pyrophosphate, i.e. it was independend from esterification. To investigate only the second reduction step, zinc pheophorbide *b* was replaced by zinc 7^1OH-pheophorbide *a* and phytyl pyrophosphate was omitted. Linear reaction kinetics were observed up to 90 min. There are evidences that the enzyme, responsible for the second reduction step, is localized in the plastid membranes. We showed by immunoblotting that ferredoxin and ferredoxin-$NADP^+$-oxidoreductase are already present in etioplasts. We therefore suggest that ferredoxin is the natural cofactor not only *in vitro* but also *in vivo*.

Ito, H., Takaichi, S., Tsuji, H., Tanaka, A. (1994)
Properties of synthesis of chlorophyll *a* from chlorophyll *b* in cucumber etioplasts, *J. Biol. Chem.* **269** (35), 22034 - 22038.

Scheumann, V., Ito, H., Tanaka, A., Schoch, S., Rüdiger, W. (1996b)
Substrate specificity of chlorophyll(ide) *b* reductase in etioplasts of barley (*Hordeum vulgare* L.)
Eur. J. Biochem. **242**, 163 - 170.

CHANGES OF PHOTOSYSTEM I PARTICLES CHARACTERISTICS AT DIFFERENT CONDITIONS OF CHLOROPLAST PROTEIN PHOSPHORYLATION.

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It is known, that the proteins of light-harvesting complex (LHC II) and photosystem II (PS II) are being phosphorylated in the higher plants chloropasts. Phosphorylated LHC II and PS II are transferred to stroma thylakoids and may interact with stroma PS I. It is possible to estimate interaction with PS I by oxidation and reduction rate changes of light-induced signal reaction centre of PS I - P 700. The oxidation and reduction rates changes of P 700 signal of PS I particles from Pisum sativum chloroplasts were observed at chloroplast protein phosphorylation, phosphorylation at high reduction conditions and DBMIB presence. It was shown, that light phosphorylation induces oxidation rate increasing only, since phosphorylated LHC II migrates to stroma thylakoids and interactes with PS I. Phosphorylation at high reduction conditions don't change oxidation and reduction rates of P 700 signal, although chlorophyll a/b ratio decreases stronger in comparison to control and light phosphorylated PS I particles and electrophoresis data demonstrate LHC II transfer to PS I particles. In PS I particles from phosphorylated chloroplasts at DBMIB presense strong increasing of P 700 reduction rate was observed. It is supposed, that phosphorylated PS II may donate electrons to PS I.

INFLUENCE OF A CO_2-PARTIAL PRESSURE OF 700 PPM ON THE LIPID AND FATTY ACID COMPOSITION OF HIGHER PLANTS.

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Cultivation of *Nicotiana tabacum* and *Aleurites montana* (Chinese Tung Oil Tree) under a CO_2-partial pressure of 700 ppm leads to molar changes in the polypeptide composition of photosystem II and I (LHCP-complexes, corepeptides and OEC-peptides) [A. Makewicz, A. Radunz, K.P. Bader and G.H. Schmid, Z. Naturforsch. 50c, 511-520, 1995; P. He, A. Radunz, K.P. Bader and G.H. Schmid, Z. Naturforsch. 51c, 441-453, 1996], as well as of electron transport components (plastoquinone, cytochrome f, plastocyanin, ferredoxin, ferredoxin-NADP-reductase). [A. Radunz and A. Schmid, unpublished]. As lipids are not only components of biomembranes but are also bound as prosthetic groups to functional proteins and thus may stimulate the activity of enzymes, it was to be expected that under these cultivation conditions lipids were also subject to quantitative changes.
In *Nicotiana tabacum* as a representative of an annual plant and in *Aleurites montana,* a representative of a multiannual plant, we were able to show that corresponding to the changes in photosystem I- and II-peptides the concentration of chlorophyll and carotenoids with respect to total lipids increased. The glycolipids (monogalactosyldiglyceride, digalactosyldiglyceride and sulfoquinovosyldiglyceride) as components of the thylakoid membrane were reduced, and phospholipids (phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and cardiolipin) components of the mitochondrial and cell membranes such as tonoplasts, plasmalemma and endoplasmatic reticulum occur in higher concentrations [P. He, A. Radunz, K.P. Bader and G.H. Schmid, Z. Naturforsch. 51c, 833-840, 1996]. Corresponding to this shift in the lipid composition a change in the fatty acid composition occurs. Saturated fatty acids with 16 and 18 carbon atoms increase and the unsaturated fatty acids decrease under these conditions. This represents a decrease in membrane fluidity not only of the thylakoid membrane but also of the cell membranes.

SUBSTRATE SPECIFICITY OF OVEREXPRESSED BACTERIOCHLOROPHYLL SYNTHASE FROM CHLOROFLEXUS AURIANTIACUS

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Two genes from *Chloroflexus auriantiacus* were reported to code for synthase protein (Niedermayer et al. (1994) EMBL accesion no. Z34000; Lopez J.C., S. Ryan and R.E. Blankenship (1996), J. Bacteriol. *178*, 3369-3373). One, bchG, is located near genes coding for proteins associated with the bacterial reaction center, whereas the other, bchG2, is clustered with two genes coding for proteins associated with the antenna system. Lopez et al. (1996) suggested, that the first one codes for the synthase that produces bacteriochlorophyll *a* and the second one for the synthetase that esterifies bacteriochlorophyllid *c*.

We cloned both bchG genes into the pT7-7 and the pQE60 vectors. The BChG protein was only active if it was overexpressed using the pT7-7 vector. Whereas the activity of the protein from the overexpressed bchG2 gene was much better with the pQE60 vector. For all the presented results the optimal vectors were used. Both proteins were separately used for synthase reaction with zinc derivatives of pheophorbide *a* and bacteriopheophorbide *a*, *c*, *d*, and *e* as substrates. BChG esterified only zinc bacteriopheophorbide *a*, whereas BChG2 accepted zinc bacteriopheophorbide *c*, *d*, and *e*. Both synthases did not react with zinc pheophorbide *a*. BChG synthase accepted also zinc-3-acetyl-pheophorbide *a*, meaning that this protein recognizes the 3-acetyl group and does not discriminate between the chlorin- or bacteriochlorin-type pigment, whereas BChG2 synthase is specific for the chlorin-type pigment. To determine other essential substituents of the tetrapyrrol ring, derivatives of zinc pheophorbide and bacteriopheophorbide with exchanged substituents at C-3 and C-13^2 of the tetrapyrrol ring were synthetized and tested. The results allowed a definition of essential substituents for substrates of both BChG and BChG2. Further we used the diphosphates of several alcohols. Most esterification was found with geranylgeraniol, followed by phytol, farnesol and geraniol. Cetyl- and stearyl-alcohols were almost not accepted.

THE THYLAKOID LUMEN OF CHLOROPLASTS: ISOLATION AND CHARACTERIZATION

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The chloroplast compartment enclosed by the thylakoid membrane, the "lumen", is poorly characterized. The major aims of this work were to design a procedure for the isolation of the thylakoid lumen and to apply it for a general characterization of the lumenal proteins and enzymatic activities. The preparation was a stepwise procedure involving isolation of thylakoids, removal of loosely-assoziated thylakoid surface proteins, Yeda press fragmentation and a final centrifugation to recover the supernatant containing the thylakoid lumen. The purity and yield of the lumen fraction was determined by appropriate marker proteins of the various chloroplast compartments. Quantitative immunoblot analyses showed that the recovery of soluble lumen proteins was 60-65 % (judged by plastocyanin), while the contamination of stroma-enzymes was less than 1 % (RUBISCO) and negligable for thylakoid integral membrane proteins (D1-protein). Roughly 25 polypeptides were recovered in the lumen fraction, of which several were identified for the first time. Enzymatic measurements and/or *N*-terminal sequencing revealed the presence of proteolytic activities, violaxanthin de-epoxidase, polyphenol oxidase, peroxidase and a novel prolyl cis-trans isomerase. The presence of nucleotides will be discussed.

CHLOROPHYLL FLUORESCENCE KINETICS DEPENDS ON AGE OF LEAVES AND PLANTS

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Recently, parameters of chlorophyll fluorescence kinetics have often been used as characteristics of stress effects on plant photosynthesis. In these determinations, age of tested leaves and plants is only exceptionally taken into account. Nevertheless, photosynthetic activities markedly change during life span of leaves and plants, they even vary at individual places of the leaf blade that differ in relative age of cells and chloroplasts. These changes are reflected in chlorophyll fluorescence kinetics, and the ontogenic differences in its parameters may be larger than those induced by stress. Determinations of stress effects without respecting leaf and plant developmental phases may thus be erroneous.

INDUCTION OF WILD TYPE MORPHOLOGY BY APPLICATION OF CYTOKININS ON DARK GROWN LIPl MUTANT OF PEA.

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In dark-grown plants plastid development becomes blocked at the stage of etioplasts but light or cytokinins can remove the block. A darkgrown mutant of *Arabiodopsis* (*amp1*) with elevated levels of cytokinin display the phenotype of wild-type plants grown in low light. Dark-grown *det1* mutants *of Arabidopsis* plants develop rosette leaves and chloroplasts. Interestingly, exogenous application of cytokinin to wild-type (WT) seedlings mimics the phenotype of the *det1* mutant. The level of some light-induced chloroplast proteins is enhanced by cytokinins. In contrast, certain plant genes are also negatively regulated by cytokinin. However, cytokinins cannot induce the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) a reaction catalyzed by the protochlorophyllide oxidoreductase (POR) which is a major regulatory step in the biosynthesis of chlorophyll. The *lipl* mutant of pea, which has a light-independent photomorphogenesis, shows many of the characteristics normally associated with light-grown seedlings even when grown in complete darkness. The dark-grown *lip1* mutant exhibit short epicotyls, expanded leaves, partially developed agranal plastids and absence of prolamellar bodies. Transcripts of some light-regulated genes such as LHCII are present in the dark-grown mutant seedlings.

Cytokinins exogenously supplied to the dark- and light-grown WT and *lip1* mutant showed that the epicotyl and root length were increasingly shorter with increasing cytokinin concentrations. The presence of cytokinins inhibited the hook formation in etiolated WT. With increasing cytokinin concentrations the leaf development was also increasingly inhibited and with 100 μmol/l cytokinin the morphology of leaves in the *lip1* mutants were similar to WT. The level of phytochrome in *lipl* mutant is 10% of that of the WT (Frances et al., *Plant Cell,* 1992, 4: 1519-1530). *In vivo* spectrofluorometric measurements showed that in hormone treated WT the level of total phytochrome decreased to 74% of the control plants, in contrast in the *lip1* mutant it increased with 37% in comparison to untreated seedlings. As measured with immunoblots, the levels of phytochrome protein in WT slightly decreased in presence of cytokinins, but in the *lip1* mutant it increased considerably.

In vivo fluorescence spectroscopy also showed that cytokinins can induce the formation of Pchlide forms fluorescing at 657 nm, which is typical for PLBs. Northern blot results showed that cytokinins strongly decreased the mRNA levels of POR and PBGD in etiolated WT and *lipl* mutant plants whereas the protein levels of POR only slightly decreased. Also the mRNA levels for LHCII decreased in dark-grown *lip1* after application of cytokinins, and no LHCII protein was detected.

CHLOROPHYLL FORMATION IN THE LIP1 MUTANT OF PEA.

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Chlorophyll formation and plastid development are closely connected with the growth of the angiosperm leaf. In the absence of light the leaf development is inhibited and the plastids develop to etioplasts instead of chloroplasts. The etioplasts have two structurally distinct inner membrane systems, the prolamellar bodies (PLBs) and the prothylakoids (PTs). The prolamellar bodies have a high content of the protochlorophyllide (Pchlide) reducing enzyme NADPH-protochlorophyllide oxidoreductase (POR). The genes for two different POR enzymes (PORA and PORB) have been isolated from barely and *Arabidopsis*. PORA seems to be present mostly in the dark-grown plant and is rapidly down regulated in light. PORB is more persistent and is suggested to be the main chlorophyll-forming enzyme during greening. However, in pea only one type of POR seems to be present.

The *lip1* mutant of the Alaska wild type of pea has a light-independent photomorphogenesis, giving seedlings with short epicotyls and well-developed leaves when grown in darkness. Leaves from Alaska pea plants grown in darkness are characterised by nearly equal fluorescence emission of Pchlide at 630 and 655 nm. Leaves of the *lip1* mutant have a fluorescence emission maximum at 632 nm with only a small shoulder at 655 nm. The spectral properties are compared with those of *Arabidopsis* mutants with light independent photomorphogenesis. Flash irradiation of Alaska pea leaves gave Chlide exhibiting a Shibata shift whereas the *lip1* leaves showed a diminutive amount of Chlide with a maximum at 677 nm. In contrast with continuous irradiation a considerable amount of Chlide is formed in the *lip1* leaves but the esterification of this Chlide is considerably delayed.

SDS-PAGE and immuno-blots indicated the presence of POR in leaves and epicotyls in both dark-grown wild type and *lip1* mutant seedlings. Just one band at 36 kD was detectable. The levels of the enzyme both in Alaska and the *lip1* mutant were almost stable during 6 hours of irradiation. The high content of POR was unexpected as *lip1* lacks both the 655 nm fluorescing Pchlide form and PLBs. The immuno-localisation of the POR is under way. The possibility for prothylakoids to stabilise the POR enzyme is discussed.

THE IMPORT COMPLEX OF THE CHLOROPLASTIC OUTER ENVELOPE MEMBRANE

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The vast majority of chloroplast proteins is found to be nuclear encoded, cytoplasmically expressed and posttranslationally imported. Plastidal precursor proteins contain an N-terminal transit peptide necessary and sufficient to direct the proteins into the chloroplasts. Furthermore, transit peptides undergo phosphorylation by a Ser/Thr-protein kinase, which is unique compared to precursor proteins targeted to other organelles, such as endoplasmatic reticulum peroxisomes, and mitochondria. The poster will present a protease sensitive protein inserted to the outer envelope membrane, which could function as a precursor phosphatase. The phosphorylation/dephosphorylation cycle of transit peptides is considered to be an additional, regulatory mechanism for chloroplast protein import. Interestingly, the C-terminus of the putative protein phosphatase has tetratricopeptide repeats known to coordinate protein-protein interactions. Next to a variety of other biological functions, these TPR motifs mediate assembly of components of the mitochondrial import apparatus. Moreover, the outer envelope import complex was analyzed by blue native gel electrophoresis for the first time. Recent data exhibit that the translocon at the outer envelope of chloroplasts contains Toc34, Toc75, and Toc86 in complex with presumable new members of the outer envelope import apparatus.

Protein Transport into Chloroplasts: Development and Regulation

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The evolution of the chloroplast from a photosynthetic prokaryote has resulted in the displacement of most of the prokaryote genes to the nucleus of the host eukaryote. Accordingly, the new organism has evolved targeting and translocation mechanisms on the organellar membranes for nuclear-encoded proteins. In plastids, the protein-import machinery is distinct from that of other organelles, in both composition and mechanism. Recently, proteins homologous to several subunits of the chloroplast import machinery were identified in the cyanobacterium Synechocystis PCC6803. It appears that parts of the protein-import machinery of chloroplasts are derived from ancient transport systems in cyanobacteria. These observations open up new avenues for elucidating the origin of the chloroplast membranes and functional properties of the protein-import machinery.

STRUCTURE AND FUNCTION OF CEMA HOMOLOGUE (PXCA) IN CYANOBACTERIA

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pxcA (formally known as *cotA*) is a cyanobacterial gene homologous to *cemA* in chloroplast genomes which encodes a protein localized in chloroplast envelope membrane. Proteins encoded by *cemA* in higher plants consist of 229-231 amino acids whereas those in *Marchantia* and *Chlamydomonas* are much larger (proteins of 434 and 500 amino acids, respectively). *pxcA* cloned from *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7942 encoded proteins of 440 and 421 amino acids, respectively. Both CemA and PxcA contain 4 membrane spanning domains. Western analysis of membrane fractions of *Synechocystis* PCC6803 using an antibody raised against PxcA (partial) indicated that PxcA is located in the cytoplasmic membrane. Mutants constructed by deleting *pxcA* in the two cyanobacterial strains were unable to grow at pH6.5 in BG11 medium or at any pH when Na^+ in the medium was replaced by K^+. Light-induced Na^+-dependent proton extrusion was not observed in these mutants, indicating that PxcA is involved in this reaction. Thus, the proton extrusion is essential for the growth of cells at acidic pH or in low Na^+ medium. In the presence of 2,5-dimethyl-p-benzoquinone (DMBQ), net proton extrusion by *Synechocystis* ceased after a minute of illumination and a postillumination influx of protons was observed, suggesting that the PxcA-dependent, light-dependent proton extrusion equilibrates with a light-independent influx of protons. A PS I-deletion mutant(*psaAB*$^-$) extruded a large amount of protons in the light. Thus, PS II-dependent electron transfer and proton translocation are major factors in light driven proton extrusion, presumably mediated by ATP. However, PxcA does not have an ATP-binding motif, suggesting that PxcA forms a complex with other protein(s). We are now trying to isolate and analyze such protein complex.

At pH 8.0, NO_3^- uptake activity was very low in *pxcA*$^-$ strain at low $[Na^+]$ ($\sim$100 μM). At pH 6.5, *pxcA*$^-$ strain did not take up CO_2 and NO_3^- at low $[Na^+]$ and showed very low CO_2 uptake activity even at 15 mM Na^+. A possible role of PxcA-dependent proton exchange in charge and pH homeostasis during uptake of CO_2, HCO_3^- and NO_3^- is discussed.

FUNCTIONS OF THE LIGHT-DEPENDENT PORA AND PORB ENZYMES IN ETIOPLAST DIFFERENTIATION & PHOTOOXIDATIVE PROTECTION

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The etioplast plastid type of dark-grown angiosperms is defined by the accumulation of protochlorophyllide (Pchlide), a chlorophyll (Chl) precursor and potent photosensitizer, and the presence of a paracrystalline inner membrane known as the prolamellar body (PLB). Both features correlate with the presence of large amounts of NADPH:Pchlide oxidoreductase (POR; EC 1.3.1.33). This strictly light-dependent enzyme reduces photoactive Pchlide-F655 to chlorophyllide and plays a key role in chloroplast differentiation during greening. Two structurally related POR enzymes, PORA and PORB, have recently been discovered in angiosperms. Interestingly, the nuclear-encoded *PORA* and *PORB* genes display divergent regulation patterns in response to light and developmental state. *PORA* expression rapidly becomes undetectable during photomorphogenesis of young seedlings, whereas *PORB* is expressed throughout all stages of plant development. Furthermore, plastid import of the cytosolic precursor of PORA but not of PORB is Pchlide-dependent. These and other observations have led to proposals of specific roles for PORA in etioplast differentiation and photooxidative protection.

We have used the dicotyledonous angiosperm *Arabidopsis*, our model system for molecular and genetic studies of light-dependent Chl biosynthesis and plastid differentiation, to directly address the *in vivo* functions of PORA and PORB. To investigate the hypothesis that etioplast differentiation specifically requires PORA, we have constitutively overexpressed PORA and PORB in the wild type and in the constitutive photomorphogenic *cop1-18* (previously *det340*) mutant (Sperling et al. (1998) *Plant Cell*, 10:283-296). Plastids of dark-grown *cop1* mutant seedlings characteristically lack PLBs and Pchlide-F655. In the wildtype genetic background, POR overexpression increased PLB size, the ratio of Pchlide-F655 to nonphotoactive Pchl[ide]-F632, and the absolute amount of Pchlide-F655. Dramatically, expression of either PORA or PORB in the *cop1* mutant led to the formation of etioplasts containing an extensive PLB and large amounts of photoactive Pchlide-F655. We therefore conclude that POR, supplied as either PORA or PORB, suffices to cause etioplast differentiation.

In order to examine the postulated role of PORA in photooxidative protection, *Arabidopsis* seedlings that overexpress either PORA or PORB in a wildtype genetic background were grown in nonphotooxidative continuous far-red light (cFR) (Sperling et al. (1997) *Plant J.*, 12:649-658). Wildtype seedlings grown in cFR exhibit the typical white-light (WL) seedling morphology due to the activation of phytochrome A-mediated developmental processes. Such seedlings are, however, severely depleted in POR, contain only traces of Chl and do not green upon subsequent transfer to WL. In contrast, cFR-grown PORA- or PORB-overexpressing seedlings shifted to WL developed thylakoid membranes, accumulated large amounts of Chl and were viable at fluence rates lethal to the wild type. Furthermore, the WL fluence rate that permitted maximal Chl accumulation increased from 8 $\mu E \bullet m^{-2} \bullet s^{-1}$ in the wild type to 125 $\mu E \bullet m^{-2} \bullet s^{-1}$ in transgenic seedlings. Constitutive POR overexpression during growth in cFR also resulted in a 4-fold decrease in the steady-state content of Pchlide, the putative photosensitizer for photooxidative damage. These data demonstrate that both PORA and PORB provide photooxidative protection when reintroduced into a POR-depleted background.

CYTOPLASMIC WATER DEFICIT AND CHLOROPHYLL *A* FLUORESCENCE IN CYANOBACTERIA.

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Hyper-osmotic depletion of cytoplasmic water causes quenching of chlorophyll *a* (Chl *a*) fluorescence in light-acclimated cyanobacterial cells (light state 1) but not in dark-acclimated cells (light state 2; Papageorgiou GC and Alygizaki-Zorba A, *Biochim Biophys Acta* 1335 [1997] 1-4; Papageorgiou GC, Alygizaki-Zorba A, Ladas N and Murata N *Physiol Plant* [1998] in press). In the present research we used PAM fluorometry in order to examine the effects of hyper-osmotic media on Chl *a* fluorescence of the unicellular cyanobacterium *Synechococcus* sp. PCC7942 under the following conditions: (a) Blocking of excitation transfer from phycobilisomes (PBS) to photosystem (PS) I Chl *a* with N-ethyl maleimide (Glazer AN, Gindt YM, Chan CF and Sauer K *Photosyn Research* 40 [1994] 167-173). (b) Selective actinic excitation of PBS with narrow band 620 nm light. (c) Selective actinic excitation of PS I with far-red light (λ . 695 nm). (d) Selective actinic excitation of Chl *a* in PS II and PS I with broad-band blue light. Our results suggest that the osmolality of the cyanobacterial cell suspension regulates the electronic excitation transfer from PBS to the Chl *a* molecules of PS I. In hypo-osmotic suspension excitation transfer from PBS to PS I Chl *a* is blocked resulting in enhanced Chl *a* fluorescence from PS II. In hyper-osmotic suspension excitation transfer from PBS to PS I Chl *a* is active resulting in lower excitation supply to PSII and lower Chl *a* fluorescence. These results complement conclusions based on 77 K fluorescence spectra according to which cyanobacterial cells in hypo-osmotic suspension emit more fluorescence from PBS (666 nm) and from PS II (685 nm and 695 nm) than than in hyper-osmotic suspension (Papageorgiou GC, Govindjee, Govindjee R, Mimuro M, Stamatakis K, Alygizaki-Zorba A and Murata N, unpublished experiments).

GENE EXPRESSION IN TOBACCO PLASTIDS LACKING THE PUTATIVE TRANSFACTOR INTERACTION DOMAIN OF THE EUBACTERIAL-TYPE RNA POLYMERASE α SUBUNIT

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In *E. coli* and other eubacteria, genetic and biochemical evidence points to the importance of the RNA polymerase α subunit carboxyl terminus in transcriptional activation by interaction with transacting factors as well as with DNA sequences such as the "UP element". The core subunits of the plastid-encoded plastid RNA polymerase (PEP) of plants are homologues of the eubacterial enzyme. To address whether the carboxyl terminus of the plastid RNA polymerase α subunit plays a role in transcriptional activation, tobacco plants were constructed lacking the region of α corresponding to the transfactor interaction domain of the *E. coli* homolog. The transgenic tobacco were obtained by transformation with plasmid pJYS58 which resulted in targeted C-terminal truncation of *rpoA*. To date, five independent, homoplasmic lines have been obtained. Promoter activity will be characterized *in vivo* by comparing steady-state levels of mRNAs for a battery of plastid genes in wild-type and pJYS58-transformed plants and *in vitro* in transcription assays. The deletion should decrease activity of promoters which depend on activation *via* the α subunit C-terminus. Through this approach we shall identify plastid promoters which are targets of nucleus-encoded promoter-specific transcription factors.

REGULATION OF D1 PHOTOSYSTEM II REACTION CENTER PROTEIN: CONTROL OF D1 PHOSPHORYLATION BY AN ENDOGENOUS CIRCADIAN RHYTHM AND ISOLATION OF A PUTATIVE D1 KINASE

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Light regulates several aspects of the metabolism of the D1 reaction center protein of the Photosystem II. We have been studying light involvement in reversible phosphorylation of D1. Both the ratio of phosphorylated to unphosphorylated D1 and the rate of D1 metabolism are very sensitive to light intensity, based on studies in which plants or thylakoid membranes were illuminated with radiance of constant intensity. To gain insight into the physiological function of D1 phosphorylation, we have characterized this process with naturally-fluctuating solar intensity throughout the day. Antibodies were raised against phosphorylated and unphosphorylated synthetic peptides containing the D1 phosphorylation site and against synthetic peptide from a D1 segment lacking the site, in order to selectively detect both forms of D1 and measure the relative proportion of protein phosphorylated under specific conditions. In photoautotrophic *Spirodela* plants entrained to a light/dark cycle under greenhouse conditions, the ratio of phosphorylated versus unphosphorylated D1 protein (D1-P index) of photosystem II is shown to undergo diurnal oscillation. The D1-P index maximum occurs 4 hours into the light period, well before the maximum in light intensity. The D1-P index maximum is not affected by temperature, the rate of D1 protein synthesis, or photoinhibition. However, when the dark period in a normal diurnal cycle is cut short artificially by illuminating plants, the D1-P index maximum shifts, to maintain its maximum 4 hours into the light period. This behavior is characteristic for circadian-controlled biological rhythms. Using as substrate a synthetic peptide mimicking the N-terminus where D1 undergoes phosphorylation, we have isolated, identified and characterized a thylakoidal protein kinase. The putative D1 kinase is an extrinsic membrane protein of 50 kD with following characteristics: optimum pH of 7.4, isoelectric point of 5.5, and preference for manganese cations.

LIGHT-DEPENDENT AND LIGHT-INDEPENDENT CHLOROPHYLL BIOSYNTHESIS IN HIGHER PLANTS AND ALGAE

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During evolution, two distinct mechanisms evolved for the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide), a key step in the chlorophyll biosynthesis pathway. One mechanism, catalyzed by the unique photoenzyme NADPH: protochlorophyllide oxidoreductase (POR), is completely dependent upon light for its activity. POR is present in the cyanobacteria, non-vascular and vascular plants, and is the only mechanism found in angiosperms. The second mechanism, established in the photosynthetic bacteria and present in the cyanobacteria, non-vascular plants, lower vascular plants and gymnosperms, is capable of reducing Pchlide to Chlide in a light-independent manner. While a significant amount of information is now available on the regulation of POR synthesis and activity, little is known about the nature of the enzyme mediating light-independent Pchlide reduction (designated LIPOR) and its requirement for function. Both the light-dependent and light-independent mechanisms for Pchlide reduction operate in the green alga *Chlamydomonas reinhardtii,* making it an ideal system to study these enzymatic processes and their integrated operation. Previous studies have shown that the products of three chloroplast genes (chlL, chlN, and chlB) and at least seven loci (designated y or yellow) are required for LIPOR. Mutations in either the plastid- or nuclear-encoded genes result in cells with identical "yellow-in-the-dark" phenotypes, reflecting a loss of chloropyll formation in the dark and the accumulation of the biosynthetic precursor Pchlide. Although direct biochemical evidence is lacking, the products of the plastid-encoded chlL, chlN, and chlB genes are thought to constitute subunits of an oligomeric LIPOR complex. No structural information is available for any of the known y genes, and no function has yet to be ascribed to their products. The overall goal of our research is to define a role for the various chl and y genes in the process of light-independent Pchlide reduction and to determine how the products of these different genes interact to bring about this important biosynthetic process. We present here the current findings of the biochemical and genetic factors involved in regulating the synthesis and activity of POR and LIPOR and provide possible mechanisms for how nuclear and organellar compartments coordinate their genetic and biosynthetic activities during this crucial biosynthetic process.

PROTEOLYSIS OF LHCII IN CHLOROPLASTS

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In intact plastids LHCII is degraded by the stroma (degradation of exogenously added LHCII) and by thylakoids (degradation of endogenous and exogenous LHCII). The distribution of activity between stroma and thylakoid membranes depends on divalent cation concentration added to the lysis medium (in the presence of 2 mM Mg^{++} it is mainly thylakoid-bound). In intact chloroplasts LHCII degradation in TX-100 solubilized thylakoids can be observed even at 0^0C when thylakoids are exposed to high light (8 % degradation in the dark at 0^0C, as compared with 19% in high light, and 15% at 37^0C). The thylakoid-bound protease is heat stable (up to 60^0C), highly specific for LHCII (and POR), and fully removed by NaBr washing. The proteolytic activity in the stroma (against exogenous LHCII) or in TX-100-solubilized thylakoids (against endogenous LHCII), is enhanced by high light; under these conditions ATP further activates the proteolysis.

SDS-solubilized thylakoid proteins, analyzed by SDS-PAGE show 5 peaks of proteolytic activity against exogenous azocoll substrate. Thylakoids heated at 56^0C for 4 days, showing almost no coomassie detectable proteins following SDS-PAGE , also show the 5 peaks of azocoll proteolysis, and can actively degrade exogenously added LHCII. Following SDS-PAGE, both NaBr-wash or the 56^0C thylakoid extract, show one peak of activity against LHCII, which is located at the upper part of the gel, capable to degrade LHCII up to the 17 kDa degradation product. This suggests that the stroma exposed protease is of high molecular weight, it degrades specifically the LHCII protein to the 17 kDa product, and implicates other proteases in thylakoids or lumen for further degradation of LHCII.

To understand how this proteolytic mechanism may be involved in the stabilization of LHCII and the assembly of PSII units we have studied the development of proteolytic activity during greening of etiolated plants. It was found that the proteolytic activity is developmentally regulated. In intact etioplasts, the activity is low in 6 day plants and increases up to 10 days of age. The increase in activity comes from the thylakoid fraction where it gradually increases, and not from the stroma, where the activity is low and remains more or less constant. In thylakoids the activity is low in 6-day etiolated bean, but increases as etiolation proceeds, while it is drastically enhanced after exposure of plants to a white light pulse or to ImL cycles. In contrast, in etiolated plants greened in continuous light the proteolytic activity in thylakoids diminishes in a manner inversely proportional to Chl accumulation; this is not due to a direct effect of Chl on the protease, but it rather reflects the shielding effect of Chl on the protease substrate (LHCII), since solubilization of thylakoids overcomes the inhibition. Using plants exposed to ImL with cycles of short or long dark phase duration, it was further possible to distinguish between the effect of Chl or development on the protease; higher proteolytic activity was found in ImL plants at more advanced developmental stage, even under conditions of equal Chl accumulation. This gives a clue as to why ImL plants exposed to an equal number of light dark cycles (equal Chl accumulation) do not accumulate LHCII when exposed to cycles with long dark intervals, but can do so when exposed to cycles with short dark intervals.

STRUCTURE AND FUNCTION OF THE PHOTOSYNTHETIC APPARATUS IN TRANSGENIC TOBACCO.

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The structure and function of the photosynthetic apparatus is under the co-ordinated control of different endogenous phytohormones and of external environmental constraints. Due to the pleitropic action, the understanding of the physiological function of phytohormones is very difficult. To study the interaction between the phytohormones and the plastids, the response at the level of the gene expression and photosynthetic activity was usually analyzed by applying exogenous hormones. Moreover, well defined mutants in biosynthesis or perception of hormones on the one hand and chemically inhibitors of hormone action on the other hand are not available. Therefore the model of transgenic plants in which for instance the *ipt*-gene was introduced using the *Agrobacterium tumefaciens* transformation system is studied. The *ipt*-gene, coding for the isopentenyl transferase, a key enzyme in the biosynthesis of cytokinin is under the control of different promotors.

The model systems used were the P*hsp70-ipt* and the P*ssu-ipt* chimeric constructs. Repeated heat shock treatment of the P*hsp-ipt* transgenic plants resulted in an increase of the endogenous cytokinin content. The treated plants were characterized by an inhibition of the apical dominance, short internodes, strongly reduced root system and a changed ultrastructure of the chloroplasts. The morphology of the P*ssu-ipt* transgenic line is more pronounced. This study shows very clearly that an increased endogenous cytokinin content affects very strongly the capacity of the photosynthetic apparatus *i.e.* inhibition of the electron transport system. Analysis of the *in vivo* fluorescence induction kinetics and of the photochemical and non-photochemical quenching indicates that the efficiency of both the reaction centres and redox intermediates of the electron transport system are strongly influenced. The absence of oscillation patterns in the oxygen evolution and fluorescence induction after a "wake up" procedure in transgenic plants indicates that the regulatory mechanisms between the electron transport and the Calvin cycle as well as the sink-source relations are strongly affected by the increased content of endogenous cytokinin. Analysis of the polypeptide patterns on two-dimensional electrophoretic gels show significant changes in this regions where the polypeptides of the pigment-protein complexes and the ATP-ase enzyme are localized.

Stress treatments such as long-term darkening or drought stress have differential effects in P*ssu-ipt* transgenic grafts and seedlings with high contents of endogenous cytokinin compared to the wild-type and heat treated P*hsp70-ipt* plants which did not differ much in their cytokinin content. An overwiew of the results on the electron transport, fluorescence characteristics, polypeptide analysis on gel electrophoresis, stomatal behaviour and analysis of antioxidant enzymes will be given.

MOLECULAR, FUNCTIONAL AND ULTRASTRUCTURAL CHARACTERIZATION OF PLASTIDS FROM SEVEN SPECIES OF THE PARASITIC FLOWERING PLANT GENUS *CUSCUTA*

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Some parasitic plant species do completely rely on their host for survival. It has been observed that without the necessity for photosynthesis these plants have lost genes in the plastid genome related to the photosynthetic machinery , e.g. *Epifagus virginiana* (dePamphilis & Palmer, 1990, Nature 348: 337-339). Members of the large parasitic plant genus *Cuscuta* do neither posses leaves nor roots and completely rely on their hosts for water, nutrient and carbohydrate supply. However, without any necessity for photosynthesis, within this genus a complete range of almost fully green to almost colorless species does exist. *Cuscuta odorata*, a species without green pigmentation, lacks the plastid rpoA gene while the 16 S rRNA could still be detected. *Cuscuta reflexa*, representing one of the green species, still has both genes. In order to get more insight into the plastome reduction and chloroplast functioning, the pigment content, photosynthetic capacity and plastid ultrastructure of the *Cuscuta* species *reflexa*, *subinclusa*, *gronovii*, *campestris*, *chinensis*, *grandiflora* and *odorata* were studied.

C.odorata and *C. grandiflora* lack chlorophyll a and b, whereas *C. reflexa*, *C. subinclusa*, *C. gronovii*, *C. campestris* and *C. chinensis* do have these compounds. However, the total amount of chlorophyll is up to five times lower than in comparable stem tissue of the non-parasitic *Ipomea tricolor*, a member of the same plant family. The chlorophyll a to b ratios are comparable to stem tissue of *I. tricolor*. All the *Cuscuta* species that possess chlorophyll a and b also have plastids with clearly recognizable thylakoid structures. These structures are, however, completely missing in the plastids of the chlorophyllless *C. odorata* and *C. grandiflora*.

All the chlorophyll containing species show light driven electron transport measured as O_2-production and determined by chlorophyll fluorescence studies. The maximal photosynthetic capacity of most species was reached at rather low photon fluency rates below 200 µmol $m^{-2}s^{-1}$, except for *C. reflexa* reaching maximal capacity around 500 µmol m^{-2} s^{-1}. The photosytem II efficiency of different *Cuscuta* species, estimated by the fluorecence parameter Fv/Fm, is much lower than was observed for *I.tricolor* (0.778). The highest values (0.687) were measured for *C. reflexa* and the lowest (0.501) for *C. campestris*.

From the present data it seems as though there exists a range from almost intact chloroplasts in *C. reflexa* to plastids which completely lost chlorophylls and photosynthetic activity in species like *C. odorata* and *C grandiflora*. However, among those *Cuscuta* species still capable of using light energy driven electron transport, none reaches the light compensation point for O_2-production. The significance and advantage of this residual photosynthetic activity is unknown and needs to be further examined.

CATION DEPENDENCE OF TRYPSIN EFFECT ON THE ENERGY DISTRIBUTION BETWEEN BOTH PHOTOSYSTEMS IN THE PEA THYLAKOID MEMBRANES

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The mild trypsin digestion is known to affect the thylakoid membrane stacking as well as the main photosynthetic events that take place at photosynthetic membranes. This is believed to occur via removal of a small polypeptide which belongs to the LHC2 and which is responsible for the thylakoid stacking. The loss of granal structure is followed by the changes of energy distribution, changes of the rate of electron transport and so on. The trypsin action depends not only on the enzyme concentration but to the high extent on the cation valence and concentration in the medium during the treatment. The influence of mild trypsin digestion on the distribution of excitation energy between Photosystem I and Photosystem II at different concentrations of mono-($Na+$) and divalent-($Mg2+$) cations was investigated. The ratios of fluorescence maxima at 735 nm and 685 nm were used for estimation of the energy interaction and spillover between both photosystems. The ratios F735/F685, obtained from 77K fluorescence emission spectra were calculated at concentrations 10- 100mM NaCl and 0-5mM $MgCl_2$ for pea thylakoid membranes treated 10 min with different trypsin concentration (2-10mg trypsin/mg chl). The excitation spectra in the red (600-700 nm) and blue (400-500 nm) regions of the fluorescence at 735 nm, attributed to PSI, were used to determine the different chlorophyll species involved in the energy supply of PSI. The data obtained showed that after the mild enzyme treatment an increase of PSI absorption cross section is observed and more chlorophyll molecules belong to the LHC2 transferred energy to PSI. This is in line with the observed increase of the rate of PSI mediated electron transport and of the amount of photooxidizable P700 (measured by the size of associated ESRI signal) observed in the presence of 5 mM $MgCl_2$. The calculated rate constant of P700 photooxidation under nonsaturating light conditions suggests an increase of the PSI antenna also. The results are discussed in terms of possible stabilizing role of cations during enzyme action and rearrangement of pigment-protein complexes after trypsin-induced detachment of granal thylakoids.

CHLOROPHYLL SYNTHESIS AND BIOGENESIS OF CHLOROPHYLL-BINDING PROTEINS IN CYANOBACTERIA

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One unsolved problem in chloroplast biogenesis is the regulation of chlorophyll synthesis in relation to the biosynthesis and assembly of chlorophyll-binding proteins. No free chlorophyll accumulates, and chloroplast-encoded chlorophyll-binding proteins are not found in thylakoids if insufficient chlorophyll is available for stabilization. To study how chlorophyll biosynthesis and biogenesis of reaction centers are apparently co-regulated, we have chosen a cyanobacterial system, in which chlorophyll is associated mostly with the reaction centers and core antenna of the two photosystems, and in which the number of chlorophyll-binding proteins is limited.

Using the transformable cyanobacterium *Synechocystis* sp. PCC 6803, chlorophyll biosynthesis can be brought under strict light control by deletion of *chlL*, encoding a component of the light-independent protochlorophyllide reduction pathway (Wu and Vermaas, *Plant Mol. Biol.* 29 (1995) 933-945). In strains lacking both photosystem I (PS I) and *chlL*, chlorophyll synthesis is faster by several hours than synthesis and assembly of active photosystem II (PS II) complexes. This suggests that chlorophyll is not used immediately for PS II assembly, and that chlorophyll is temporarily associated with other proteins (free chlorophyll is very toxic in the light).

To test this further, biogenesis of the D1 protein of PS II was followed upon chlorophyll biosynthesis. Even though the *psbA* transcript level remains high during chlorophyll biosynthesis, the D1 protein is not synthesized at high rates until a considerable amount of chlorophyll has accumulated (He and Vermaas, *Proc. Natl. Acad. Sci. USA* (1998) in press). Therefore, translation of reaction center proteins appears to be regulated by chlorophyll availability.

To determine the protein(s) potentially involved with this regulation, two complementary approaches were taken: (1) proteins associated with "green bands" on non-denaturing gels of thylakoids of PS I-less/PS II-less mutants were determined and corresponding genes were identified and deleted, and (2) the genomic sequence of *Synechocystis* sp. PCC 6803 was searched for similarity with genes for known chlorophyll-binding proteins, and candidate genes were identified. The first approach yielded the identification of an N-terminal sequence that resembled that of a type-IV pilin. The remainder of the corresponding gene, however, had little in common with a pilin. The gene was deleted along with two similar genes in the *Synechocystis* sp. PCC 6803 genome. The resulting phenotype was normal in a fully greened system, but the rate of light-dependent chlorophyll synthesis (measured upon illumination of degreened *chlL*$^-$ cells) was 30% lower than in the control. Moreover, the 77K fluorescence emission spectra during chlorophyll biosynthesis revealed that in the mutant lacking the pilin-type protein conversion from a 683-nm component to a 685-nm component was slowed down considerably. These results suggest that the pilin-type protein is a chlorophyll transporter, bringing chlorophyll from the site of synthesis to the site of PS II assembly.

The *Synechocystis* sp. PCC 6803 genome has five open reading frames for polypeptides that resemble helix I and III of the light-harvesting complex II (LHCII) of higher plants. Four of these putative polypeptides are small (about 7 kDa) whereas one is the C-terminal extension of ferrochelatase. Deletion of some of these small open reading frames has not led to drastic phenotypic effects. However, genes for several of these polypeptides are expressed more strongly in strains with less photosynthetic reaction centers per cell as compared to in wild type, suggesting that these polypeptides may have a role in temporarily binding newly synthesized chlorophyll.

The results obtained thus far suggest that several polypeptides in cyanobacteria are involved in transfer of newly synthesized chlorophyll to nascent reaction centers, and in regulation of reaction center synthesis as a function of chlorophyll availability. In addition to polypeptides that resemble single LHC helices, surprisingly also pilin-type proteins are found to affect chlorophyll transport. These results obtained in cyanobacteria suggest that the process of chlorophyll synthesis and biogenesis of chlorophyll-binding proteins in chloroplasts is more complex than assumed.

CAN *PSILOTUM* AND *GNETUM* GREEN IN DARKNESS?

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Most plants including angiosperms can synthesise chlorophyll in darkness as well as in light (Adamson, Hiller, Walmsley, Protochlorophyllide reduction and greening in angiosperms: an evolutionary perspective. J. Photochem. Photobiol. B: Biology 41(1997) 201-221). For this they require a light-independent reductase to catalyse the conversion of protochlorophyllide to chlorophyllide. Genes coding for the individual subunits of such an enzyme have been identified in purple bacteria (*bch*L *bch*N, *bch*B) and a wide variety of oxygenic phototsynthetic organisms, ranging from cyanobacteria to gymnosperms (*chl*L, *chl*N, *chl*B). However, there is as yet, no evidence for them in angiosperms. This could be a consequence of sequence divergence following gene transfer from the chloroplast to the nucleus. Alternatively, it might reflect gene deletion during evolution.

Psilotum (a primitive vascular plant related to ferns) and *Gnetum* (a gymnosperm with several angiosperm-like features) are interesting in the present context because, like angiosperms, they have so far reacted negatively to light-independent reductase gene probes. The question is can they, like angiosperms, synthesise chlorophyll in darkness (lack of evidence for *chl*L, *chl*N, *chl*B notwithstanding)? Or, do they exhibit yellow-in -the-dark phenotypes and accumulate protochlorophyllide instead of chlorophyll? This paper demonstrates that, like other researchers, we have so far been unable to recover *chl*L, *chl*N, or *chl*B homologous sequences from a variety of angiosperms or *Psilotum*. It also provides quantitative data on changes in chlorophyll, chlorophyllide and protochlorophyllide levels in light-grown *Psilotum* and *Gnetum* when light is withheld. The significance of these findings is briefly discussed.

THE CARBOXYL-TERMINAL PROCESSING OF PRECURSOR D1 PROTEIN OF PHOTOSYSTEM II REACTION CENTER BY A NUCLEAR-ENCODED PROTEASE (CtpA) — AN ANALYSIS OF ENZYME-SUBSTRATE INTERACTION USING OVER-EXPRESSED PROTEASE

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The carboxyl-terminal (C-terminal) processing of precursor D1 protein (pD1) in the integration of oxygen-evolving machinery of photosystem II (PSII) is a typical example of nuclear control of chloroplast gene expression at the post-translational level. In eukaryotic organisms, the D1 protein of PSII reaction center is encoded by chloroplast DNA. The protein is synthesized at the stromal side of thylakoid membrane as a precursor form with C-terminal extension which is co-translationally translocated into the lumenal space. The post-translational cleavage of the extension takes place in the lumenal space by a nuclear-encoded endo-peptidase imported from cytosol. This process is essential in the integration of Mn-cluster of PSII. The protease involved in the processing has been identified and a nuclear gene coding for the protease (*ctpA* gene) has been sequenced. The predicted amino acid sequence revealed that the protease possesses a bipartite pre-sequence, consistent with the biochemical analysis for its location in chloroplasts. So far, no specific inhibitor could be found for the protease and this enzyme is classified into a novel type of serine protease, which uses hydroxyl/amine dyad as the catalytic center.

In this study, we have established a system for over-expressing spinach CtpA in *E. coli* in the soluble fraction in active state. The enzyme was purified in homogeneity by Ni-chelating chromatography utilizing His-tag consisted of six consecutive histidines at the N-terminus. The enzyme catalyzes the C-terminal processing of pD1 *in vitro* in a manner similar to that of isolated spinach protease, although the over-expressed enzyme has six additional amino acids, GSHMLE, at the N-terminus. In order to analyze the molecular interaction in the C-terminal processing of pD1, we utilized three classes of substrate, *i.e.*, (i) the oligopeptide corresponding to the C-terminal sequence of pD1, (ii) the *in vitro* translated full-length pD1, (iii) the pD1 in PSII complex in thylakoid membrane from LF-1 mutant of *Scenedesmus obliquus* which is deficient in the processing activity. For detecting the C-terminal processing of pD1, we prepared an antibody which specifically recognizes the C-terminus of mature D1 protein. The result of analysis indicated that the pH optimum of the catalytic reaction, as well as the affinity, was largely influenced by the incorporation of pD1 into the PSII complex, suggesting the presence of specific interaction(s) at the membrane surface.

DNA-BINDING PROTEINS OF CHLOROPLAST NUCLEOIDS

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Investigation of DNA-binding proteins of chloroplast nucleoids allowed us to reveal structural proteins directly bound to chloroplast DNA participating in chloroplast nucleoid organization. To identify proteins bound to chloroplast DNA *in situ*, an approach, which is based on fixation of DNA-protein contacts directly in whole chloroplasts by their chemical covalent cross-linking was applied. The analysis of the structure of DNA-protein cross-linking revealed five proteins directly in contact with chloroplast DNA, and the molecular masses of these proteins were 14, 17, 21, 26 and 31 kDa. By the "protein image" procedure it was shown that these proteins interact by all chloroplast genome regions. These data suggest that the chloroplast DNA is organized with these proteins as a regular structure. Isolation and characterization of some individual proteins of chloroplast nucleoids, as well as the comparative analysis of proteins from plastid nucleoids at various differentiation stages (etioplasts and chloroplasts) were performed. Using RP-HPLC, the component composition of the basic proteins from pea chloroplast nucleoids was studied. Five major and 20 minor fractions were detected. SDS-PAGE of major HPLC-fractions showed that the basic nucleoid proteins are heterogeneous with molecular masses of components from 17 to 30 kDa. One polypeptide with molecular masses of 28 kDa (P28) were obtained by RP-HPLC in purified form (fraction N12). The sequencing of tryptic peptides of P28 showed that it is homologous to the ribosomal proteins L19 of *Saccharomyces cerevisiae*, respectively.

THE ROLE OF MEMBRANE FLUIDITY FOR PRIMARY PHOTOSYNTHETIC PROCESSES OF PEA THYLAKOIDS

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The intrinsic complexes involved in energy capture and electron transport processes are laterally separated from each other - PSII and LHCII are mainly located in the appressed membranes of the grana, whilst PSI complexes are preferentially situated in the exposed thylakoid membranes-stromal and end-granal lamellae. This model raises questions about long-range diffusion processes both at the energy transfer and electron-transport levels. The regulation of energy distribution between PSI and PSII involves the physical movement of the light harvesting chlorophyll a-b complex between appressed and non-appressed membranes. These concepts emphasise the role of the thylakoid membrane lipid matrix and in particular its fluidity. The membrane fluidity can be alterred by varying the growth temperature or the temperature of chloroplast suspension and by changing the lipid composition. In the present work the influence of fluidity changes of thylakoid membranes on energy distribution between both photosystems, on the photochemical activities of PSI and PSII and on the heterogeneity of PSII was investigated. Two different membrane perturbing agents were used for alteration of the fluidity of isolated pea thylakoid membranes - cholesterol and benzyl alcohol. The changes of membrane fluidity have been estimated by the polarization of fluorescence of hydrophobic probe diphenyl hexatriene. The treatment with cholesterol and benzyl alcohol reflects on the photochemical activities of PSI and PSII as well as on the cation dependence of energy distribution between both photosystems. At the mean time the rigidification and fluidization of thylakoid membranes lead to the alteration of the antenna size geterogeneity of PSII whilst the donor side heterogeneity was not affected. The artificial alterations of membrane fluidity modulate the response of isolated thylakoid membranes to short time heat stress. The temperature dependencies of the 77K fluorescence ratio F735/F685 as a measure for spillover and of rate of PSI-mediated electron transport were also altered by manipulation of membrane fluidity. The data presented clearly show the role of the fluidity of thylakoid membranes in the processes of energy distribution and electron transport activities and its sensitivities to temperature.

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