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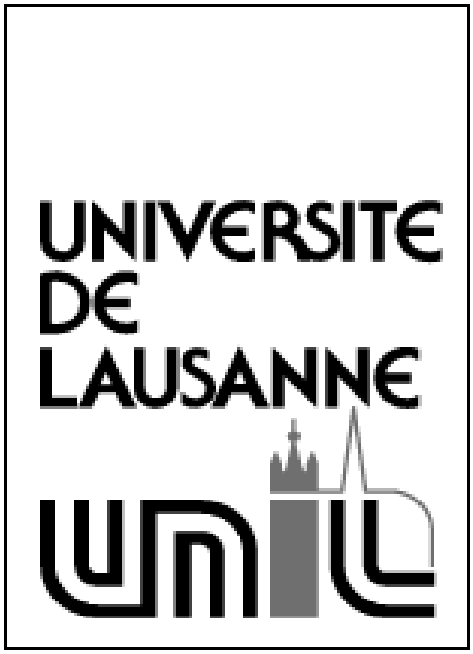

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Contact: Prof. Jean-Pierre Zrÿd, Laboratory of Plant Cell Genetics
Phone: ++41216924251, Fax ++41216924255, e-mail jzryd@lpc.unil.ch

This meeting is dedicated to

Professor **PAUL-ANDRÉ SIEGENTHALER**

for his achievements in thylakoid membrane research and his contributions to the understanding of membrane lipid metabolism

for his dedicated teaching of photosynthesis at the University of Lausanne and Neuchâtel

Programme

Session I

Thursday afternoon from 3pm-6pm; chairperson: Wollman

· STRUCTURE AND DYNAMICS OF THE THYLAKOID MEMBRANE

· *J. Joyard (Grenoble)*	Molecular cloning and functional expression of GDG synthase from spinach chloroplast envelope membranes
· *P. A. Siegenthaler (Neuchâtel)*	Lipid organization and function in thylakoid membrane.
· *A. Trémolière (Gif sur Yvette)*	Open questions on phosphatidylglycerol-protein interactions in the photosynthetic membrane
· *J. Allen (Lund)*	Structural effects of LHC II phosphorylation and redox control of chloroplast transcription in photosystem stoichiometry adjustment

Session II

Thursday evening from 8pm – 11pm; chairperson: Allen

· CONTROL OF PHOTOSYNTHETIC GENE EXPRESSION

· *C. Bowler (Naples)*	Phytochrome signal transduction pathways
· *L. Eichaker (Münich)*	Light dependent regulation of translation elongation in chloroplasts
· *M. Goldschmidt (Genève)*	Chloroplast gene expression: the role of nucleus-encoded factors
· *P. Falkowsky (Brookhaven)*	Redox control of nuclear genes in eukaryotic algae

Session III

Friday morning from 8am – 11am; chairperson: Paulsen

· IMPORT AND INSERTION OF THYLAKOID PROTEINS

· *R.B. Klösgen (Münich)*	Mechanism and phylogeny of protein transport across the thylakoid membrane
· *K Kindle (Ithaca)*	Targeting and translocation of nucleus-encoded proteins into the chloroplast
· *K. van Wijt (Stockholm)*	Synthesis, targeting and assembly of the chloroplast encoded D1 protein
· *R. Bassi (Verona)*	Chlorophyll and xanthophyll binding sites in the

photosystem II subunit CP29

Session IV

Friday afternoon from 3pm –6pm; chairperson: Bassi

- **ASSEMBLY AND TURN-OVER**

· *H. Paulsen (Mainz)*	How are light-harvesting chlorophyll a/b complexes assembled?
· *F. A. Wollman (Paris)*	The Control by Epistasy of Synthesis (CES process) during chloroplast protein biogenesis
· *B. Andersson (Stockholm)*	A new kind of immunophilin in the thylakoid lumen: significance for protein turn-over and protein phosphorylation
· *P. Matile (Zürich)*	Dismantling of thylakoid membranes

MOLECULAR CLONING AND FUNCTIONAL EXPRESSION OF GDG SYNTHASE FROM SPINACH CHLOROPLAST ENVELOPE MEMBRANES

Christine Miège[1], Eric Maréchal[1], Maryse A. Block[1], Hiroyuki Ohta[2], Ken-Ichiro Takamiya[2], Roland Douce[1] and Jacques Joyard[1]

[1]Laboratoire de Physiologie Cellulaire Végétale, URA 576 (CEA/CNRS/Université Joseph Fourier), Département de Biologie Moléculaire et Structurale, CEA-Grenoble, 38054 Grenoble-Cedex 9, France

[2]Department of Biological Sciences, Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Midori-ku, Yokohama, Kanagawa 226, Japan.

Plastid membranes contain galactolipids (monogalactosyldiacylglycerol, or MGDG, and digalactosyldiacylglycerol, or DGDG) as major constituents. MGDG is synthesized in the inner envelope membrane, at least in spinach chloroplasts, owing to a UDP-galactose: 1,2-diacylglycerol 3-β-D-galactosyltransferase (E.C. 2.4.1.46), or MGDG synthase, which transfers a galactose from a water-soluble donor, UDP-galactose (UDP-gal), to a hydrophobic acceptor molecule, diacylglycerol. MGDG synthase catalyzes the following reaction:

UDP-gal + 1,2-diacyl-*sn*-glycerol → 1,2-diacyl-3-O-β-D-galactopyranosyl-*sn*-glycerol + UDP

MGDG synthase has been recently cloned from a cucumber cDNA library (Shimojima et *al.,* 1997, *Proc. Natl. Acad. Sci. USA, 94,* 333-337). We have obtained the homologous clone from spinach. The full-length spinach cDNA was 1749 bp and contains a 1566-bp open reading frame encoding a protein of 522 AA. The deduced amino acid sequence shows a higher homology (74 % identity, 82 % similarity) to the deduced sequence from cucumber than that from *Arabidopsis* (54 % identity, 64 % similarity). We have overexpressed the protein using pET-15b and pET-3A in *Escherichia coli* (BL-21). The transformed cells were able to synthesise MGDG. We have compared the activity of the overexpressed MGDG synthase enzyme with that of the partially purified enzyme from spinach chloroplast envelope membranes, using procedures we have previously developed (Maréchal *et al.,* 1994, *J. Biol. Chem.* 269, 5788-5798; Maréchal et *al.,* 1995, *J. Biol. Chem.* 270, 5714-5722). The kinetic mechanism of the enzyme with respect to the various diacylglycerol molecules was investigated in mixed micelles.

The biochemical characterisation of the recombinant MGDG synthase showed that it shares the same chromatographic behaviour on Hydroxyapatite-agarose gels as the native enzyme solubilized from envelope membrane vesicles. Recombinant activity is sensitive to chemicals previously known to affect galactosylation activity (DTT, NEM, a specific agent for reduced amine residues, and orthophenanthroline, an hydrophobic chelating agent). Finally, specific recombinant galactosylation activity is in the order of magnitude expected for MGDG synthase. Moreover, the recombinant MGDG synthase precursor is imported and processed to its correct mature form in intact chloroplasts in

vitro. The enzyme was immunolocalized in the inner membrane of the spinach chloroplast envelope. Together, these results show that spinach chloroplast envelope MGDG synthase corresponds to a mature polypeptide of 45-46 kDa. Topological studies indicate that MGDG synthase is monotonic and associated with one side of the inner envelope membrane. Finally MGDG synthase was partially refolded from inactive bacterial inclusion bodies. The refolded recombinant enzyme will provide a useful tool for further characterisation of the function and structure of MGDG synthase.

LIPID ORGANIZATION AND FUNCTION IN THYLAKOID MEMBRANE.

Paul-André Siegenthaler

Laboratoire de Physiologie végétale, Université de Neuchâtel, Rue Emile Argand 13, CH-2000 Neuchâtel

Lipid composition and organization

The lipid composition of higher plant photosynthetic membranes is unique in that, instead of the commonly encountered phosphoglycerides, the major components are three glycosylglycerides (MGDG,DGDG and SQDG)* and only one phospholipid (PG). The two galactolipids (MGDG and DGDG), which represent about 80 to 85 mol% of the total membrane lipids, are characterized by an exceptionally high content of α-linolenic acid (up to 95% for MGDG) whilst PG contains a unique acid, trans-Δ^3-hexadecenoic acid located at the *sn*-2 position of the glycerol molecule in most of its molecular species. Considering the four lipid classes, the thylakoid membrane contains up to about 40 different molecular species which are largely conserved throughout the plant kingdom. Thylakoid membranes are notable in that the major glycolipid (MGDG) comprising half of the total lipid content may adopt, under defined conditions, a non-bilayer configuration, in contrast to the three other lipid classes, which will form typical bilayer structures.

The extreme diversity of thylakoid acyl lipids and their unique (physico) chemical characteristics suggest that they are arranged, at the molecular level, as distinct membrane domains. Several experimental approaches, including fractionation of subchloroplast particles, separation of appressed and non-appressed regions of the thylakoids and purification of (chlorophyll-) protein complexes, show that acyl lipids are asymmetrically distributed within the plane of the membrane and that some of them (generally those more saturated than the bulk lipids) are tightly bound to proteins. However, conclusions based only on such evidence should be viewed with caution because the use of detergents to obtain subparticles or lipoprotein complexes may result in differential displacements of acyl lipids and pigments non-covalently bound to proteins. To overcome this drawback, several types of subthylakoid vesicles (e.g. stroma lamellae and grana vesicles, grana core and margin vesicles) can be isolated in absence of detergents, thanks to a procedure combining sonication and aqueous two-phase partitioning. In addition, cyclodextrins have also been used recently as a new tool for the controlled lipid depletion of thylakoid membranes. These molecules are cyclic oligosaccharides consisting of 6 to 8 glucopyranose units linked by α (1-4) bonds which adopt a torus shape and are able to bind a range of small guest molecules of poor water solubility (e.g. lipids) within their hydrophobic cavity to form a water soluble guest-cyclodextrin inclusion complex. The advantage of such an approach is to avoid the use of detergents. Antibodies directed to membrane lipids can also achieve the detection of lipids. The transmembrane distribution of acyl lipids in the thylakoid membrane has been extensively studied. We still report on the enzymatic approach which consists of digesting and removing lipids, stepwise and selectively, in the two membrane monolayers. The results indicate that the outer monolayer is highly enriched in MGDG and PG whilst the inner one contains high levels of DGDG, thus confirming the general sidedness of thylakoid membrane components.

Role of lipids in thylakoid membranes

The great diversity of thylakoid acyl lipids and their unique (physico)chemical characteristics as well as their peculiar topology in the membrane strongly suggest that specific functions and distinct domains of acyl lipids exist in the thylakoid membrane. To investigate the functional significance of thylakoid lipid composition, several approaches have been adopted. The functional alterations occurring in aged thylakoids and during leaf senescence can be attributed, at least in part, to a release of free fatty acids and/or to the loss or modification of the parent lipids which are essential for the functioning of the thylakoid membrane. The effects of ageing are strikingly similar to those of exogenous free unsaturated fatty acids. Controlled lipolytic treatments of thylakoid membranes are another approach allowing the stepwise digestions of specific acyl lipids, first in the thylakoid outer monolayer, then in the inner one. These topological lipid depletions show that only certain lipid populations (e.g. located in one of the two membrane monolayers) are able to sustain photochemical reactions. The removal of thylakoid acyl lipids by cyclodextrins represents a potentially interesting depletion technique, since no detergents are used. The modulation of thylakoid membrane fluidity by homogenous catalytic hydrogenation in situ shows quite clearly the importance of the lipid unsaturation for optimal photochemical reactions. In addition, alteration in membrane fluidity may be the first signal in the perception of temperature changes in the plant environment. Another interesting approach is to use antibodies directed to specific membrane lipids and measure the photochemical reactions which are impaired in thylakoids. Lipids are also involved in the mode of action of herbicides by mediating the accessibility of these compounds to their binding site, for instance to the Q_B protein level. Finally, a number of *Arabidopsis* and *Chlamydomonas* mutants have been characterised as being deficient in desaturases or glycerol-3-acyltransferases. The availability of these mutants provides another tool in determining the physiological consequences of variations in lipid unsaturation. All these approaches present advantages and drawbacks.

In spite of considerable effort, the structure/function relationship of acyl lipids in the thylakoid membrane remains ambiguous and elusive. This is probably due to the fact that, in contrast to proteins, lipids have by themselves no recognised catalytic properties. They allow the maintenance of an appropriate conformation and orientation of proteins which may express their function only in the presence of specific lipids. This function is expected to require only a few **specific lipid molecules** as it appears to be the case for PG-16:1 (3t) in the formation of the trimeric LHCII and the development of grana stacks. In the literature, there is no clear distinction between the role of these specific lipids and the general physicochemical properties of the membrane **bulk lipids** which are likely to be involved in structural aspects of the membrane such as the mode of herbicide action, chilling injury, photoinhibition and, in general, responses to environmental changes.

*Abbreviations: MGDG (DGDG) = mono(di)galactosyldiacylglycerol; PG = Phosphatidylglycerol; SODS = sulphoquinovosyldiacylglycerol

OPEN QUESTIONS ON PHOSPHATIDYLGLYCEROL-PROTEIN INTERACTIONS IN THE PHOTOSYNTHETIC MEMBRANE

Antoine Trémolière

Institut de Biotechnologie des Plantes, Université Paris-Sud, Bâtiment 630
91405 ORSAY CEDEX, FRANC, Tel.: 33-1-69 33 63 19, Fax: 33-1-69 33 63 18

Phosphatidylglycerol is the phospholipid found in all photosynthetic membranes representing from around 10% of the polar lipids in eucaryotic organisms to 20% in cyanobacteria. It has been shown to undertake specific but non-covalent interactions with several pigment-protein complexes in this membrane.

The most extensively studied of them concerns it interaction with the main light-harvesting-Chlorophyll a/b-protein complex (LHCII). One molecule of phosphatidylglycerol, recognising a short sequence of five amino acids at around 15 amino acids from the N-terminal extremity of the mature polypeptide, in the region emerging at the stromal side of the membrane, induces a cascade of reactions probably starting by a stabilisation of the monomeric LHCII in a functional state, then by the trimerization of this LHCII and further, or perhaps at the same time, by the Mg^{++}-mediated formation of grana stacks. The relation between this first interaction and trimerization of LHCII, grana stacking process and ability to perform states transition is discussed. Questions of the place where and the time when this interaction is supposed to occur are addressed. Also the question of the possible significance of the presence of Δ3-trans-hexadecenoic acid in the phosphatidylglycerol is discussed.

Phosphatidylglycerol has been shown also to play a role as a functional effector and a membrane anchor of the D1-protein. The possibility that a site of recognition of phosphatidylglycerol and D1 protein showing similar properties than the site of recognition in LHCII is discussed in reference to sequences of the D1 protein.

Mutants affected in phosphatidylglycerol composition, or in *Chlamydomonas* or in *Arabidopsis*, are also affected in the organisation of the antennae around the PSI suggesting that this lipid could also play a role at this place in the photosynthetic membrane. This assertion is strengthened by the possibility to restore a normal PSI-antenna system by feeding mutants of *Chlamydomonas*, lacking phosphatidylglycerol with Δ3-trans-hexadecenoic acid, with liposomes of this lipid. A set of mutants of *Chlamydomonas* in which PSII activity is affected in strict relation with the PG-C 16:1 trans content are presented. These mutants synthesise all the proteins of the PSII center but are unable to stabilize and to assemble them in a functional state in the absence of PG-C 16:1 trans. Recovery of PSII activity is observed in mutants having recovered this lipid.

In conclusion, is discussed the paradox between the more and more clear demonstration of highly specific interactions of lipids with proteins in the photosynthetic membrane and the fact that lipid composition can be largely changed without loosing the ability to perform photosynthesis.

STRUCTURAL EFFECTS OF LHC II PHOSPHORYLATION AND REDOX CONTROL OF CHLOROPLAST TRANSCRIPTION IN PHOTOSYSTEM STOICHIOMETRY ADJUSTMENT

John F. Allen, Anders Nilsson, Thomas Pfannschmidt* and Todd Silverstein**

Plant Cell Biology, Lund University, Box 7007, S-220 07 LUND, Sweden

*Ruhr-Universität Bochum, Fakultät für Biologie, Arbeitsgruppe Pflanzliche Zellphysiologie, D-44780 BOCHUM, Germany

**Department of Chemistry, Willamette University, Salem, Oregon 97301, USA.

Functional effects of phosphorylation of chloroplast LHC II arise from changes in its 3-D structure. A synthetic peptide corresponding to the first fifteen amino acid residues of pea LHC IIb shows pronounced changes in NMR, CD and FTIR spectra when Thr-5 is replaced by phosphothreonine. LHC II isolated from pea thylakoids shows similar, though proportionally smaller, changes in CD and FTIR spectra when isolated from thylakoids that had been incubated with ATP in the light: conditions that give rise to LHC II phosphorylation. For both the model peptide and the native protein, the spectral changes indicate helix formation upon phosphorylation. Helix formation around an N-terminal phosphorylation site itself is known form X-ray crystallography to be the basis of tertiary and quaternary structural changes upon activation, by phosphorylation, of the soluble enzyme glycogen phosphorylase. The implication that such changes occur also in the intrinsic membrane protein LHC II is currently under investigation.

Redox activation of the LHC II kinase is the post-translation branch of a redox signalling pathway that also controls chloroplast gene expression. The parallel, hypothetical branch may be involved in long-term adaptations that act as counterparts to the short-term adaptations know as state transitions, which have LHC II phosphorylation as their primary mechanism. Photosystem stoechiometry adjustment, like state transitions, serves to regulate the distribution of light energy between photosystem I and photosystem II in order that the two photosystems can function in series with maximal efficiency even under different light regimes and metabolic demands. Evidence from studies of transcription of psbA (encoding the D1 protein of the photosystem II reaction center) and psaB (encoding the B subunit of the photosystem I reaction center) in mustard cotyledons reveals the same complementarity that characterizes redox control of LHC II phosphorylation: psaB transcription is induced under illumination favoring photosystem II; psbA transcription is induced under illumination favoring photosystem I. The origin of these effects as a redox signal from the plastoquinone pool is consistent with effects of site-specific electron transport inhibitors on transcription in isolated, intact pea chloroplasts carrying out CO_2-dependent oxygen evolution at physiological rates.

The precise sites at which redox control is exerted over chloroplast protein phosphorylation and gene expression, and at which the redox signalling pathways branch, are unresolved, but the midpoint of electrochemical redox titration of LHC II phosphorylation is now shown to be pH-dependent, indicating a role of the plastoquinone/plastoquinol redox couple itself.

PHYTOCHROME SIGNAL TRANSDUCTION PATHWAYS

Chris Bowler
Stazione Zoologica, Naples, Italy

The phytochromes are the best studied plant photoreceptors, controlling a wide variety of responses at both whole plant and single cell levels. Three signal transduction pathways, dependent on cGMP and/or calcium, are utilized by phytochrome to control the expression of genes required for chloroplast development and anthocyanin biosynthesis. The relative inputs into these different transduction pathways is highly regulated and may be a means by which plant cells can adjust their biochemical status, in particular for controlling the levels of photoprotectants (such as anthocyanins) and the relative amounts of the different photosynthetic complexes. An overview of these different cross-talk mechanisms between the different phytochrome pathways will be presented. In addition to these studies of light-activated processes, we have also performed some experiments to understand how phytochrome can down-regulate other events. For example, the expression of several genes is known to be negatively-regulated by light. We have determined how such genes are regulated by phytochrome in the context of calcium and cGMP and have identified an 11 bp cis-element present within the promoters of such genes that is both necessary and sufficient to mediate light down-regulation.

Phytochrome is only one of a series of photoreceptors present in plants. Specifically, a blue light photoreceptor, denoted cryptochrome, has been identified, and much evidence implicates the existence of photoreceptors for ultraviolet light. Light responses must therefore integrate signals originating from all these photoreceptors. We have begun to examine the interactions between phytochrome and UV signalling using a pharmacological approach in photomixotrophic soybean cell cultures. This has proved particularly interesting for the anthocyanin biosynthetic gene chalcone synthase (*CHS*). Whereas phytochrome control of *CHS* gene expression is positively regulated by cGMP and negatively regulated by calcium and calmodulin, in UV light calcium and calmodulin appear to act as positive regulators. Results from combined red and UV irradiations indicate that such apparently antagonistic functions are able to operate in the same cell because they are temporally separated from each other, with the phytochrome response being activated first and the UV photoreceptor response activated subsequently.

In our laboratory we are also attempting to elucidate the mechanisms utilized by marine algae to sense changes in light quality and quantity in the marine environment. As an experimental system we have chosen a diatom, representative of the most successful group of phytoplankton. We have cloned several light regulated genes and have utilized quantitative RT-PCR to analyse the dynamics of light activated transcription. The results are providing the first hints of the nature of diatom photoreceptors.

LIGHT DEPENDENT REGULATION OF TRANSLATION ELONGATION IN CHLOROPLASTS

Lutz Andreas Eichacker
Botanisches Institut der Universität München, 80638 München, Menzinger Str. 67, FRG

I will present a new effect of light on the general efficiency of translation elongation in barley chloroplasts, which is superimposed on a regulation of translation by redox factors or ATP, Light stimulation of translation elongation is dependent on the formation of a proton gradient across the thylakoid membrane, which arises from photosynthetic electron transport.

In higher plant chloroplasts, photons drive photosynthesis, and control biogenesis of the photosynthetic apparatus. Also, maintenance of the apparatus is under light control possibly because wear and tear of photosynthetic proteins require a controlled resynthesis of these proteins. This can be investigated on the level of translation of two plastic localised mRNAs encoding the large subunit of ribulose 1,5-bisphosphate (rbcL; LSU) and subunit one of the reaction centre 11 (psbA; D1). My presentation will concentrate on the light dependent translation regulation of the psbA mRNA.

In the first part of my talk, I will show that light controls the rata at which the D1 protein is synthesised. In particular, mature intact and lysed chloroplasts reveal a higher rate of ^{35}S-Methionine accumulation in the Dl protein in light than in darkness. Inhibition of translation initiation by lincomycin does not affect light induced radiolabel accumulation during the first 7.5 min of the *in vitro* translation reaction, indicating a light regulation of translation elongation.

A D1 translation intermediate of about 17 kDa transiently accumulates radiolabel at a higher rate in the light, than in darkness and that less time is required to chase radiolabel from the D1 translation intermediate to full-length Dl, indicating that light increases the rate of translation elongation of the D1 protein. Exogenous addition of ATP stimulates radiolabel accumulation in D1 in darkness and light, but ATP can not replace light.

These data indicate that the rate of translation elongation is regulated by light, because light activates an enzymatic activity of the translation elongation process, e.g. one of the translation elongation factors or aminoacyl-tRNA syntheses, and translation elongation is directly dependent on the rate of photosynthetic ATP production.

In the second part of my talk, I will concentrate on the mechanism of how the light signal is transformed in a biochemical control of translation elongation. Here, I will show you that in the light, addition of the electron transport inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) inhibits translation elongation even in the presence of ATP and that this inhibition can be overcome by addition of artificial electron donors in the presence of light, but not in darkness.

Electron flow between photosystem II and I induced by far red light of 730 nm is sufficient for the activation of translation elongation and that this activation can also be obtained by electron donors to photosystem I which transport protons into the thylakoid lumen. release of the proton gradient by uncouplers prevents the light dependent activation of translation elongation and that translation activation is switched off within seconds upon transfer of chloroplasts from light to darkness.

I conclude from these data that the formation of a photosynthetic proton gradient across the thylakoid membrane activates translation elongation in cbloroplasts and will present a model of how such a regulation could be achieved

CHLOROPLAST GENE EXPRESSION: THE ROLE OF NUCLEUS-ENCODED FACTORS

***M. Goldschmidt-Clermont*, K. Perron, C. Rivier, F. Vaistij and J.-D. Rochaix.**
Departments of Plant Biology and of Molecular Biology, University of Geneva, Sciences II, 1211 Geneva 4, Switzerland.

Chloroplast biogenesis is governed by two genomes, one in the organelle and the other in the nucleus. Chloroplast development, and in particular the assembly of the photosynthetic complexes in the thylakoid membrane, requires the coordinate expression of genes in the two compartments. Transcriptional, and primarily, post-transcriptional steps of gene expression are involved in this regulation. To investigate these processes, *Chlamydomonas reinhardtii* offers the combined advantages of classical genetics, of transformation and reverse genetics, and of biochemical and biophysical approaches. This unicellular green alga is a facultative phototroph, so that many mutants with defects in chloroplast function have been recovered. The mutations map either to the chloroplast genome or to nuclear loci. Some of the mutations cause defects in the structural genes for components of the chloroplast such as photosystems or enzymes involved in carbon fixation. Other mutations affect chloroplast gene expression and reveal trans-acting factors that are required in post-transcriptional steps such as RNA splicing, mRNA stability or translation. The mutations are surprisingly specific since they usually affect the expression of a single chloroplast gene or gene cluster. They reveal a large number of nuclear loci required for the expression of the chloroplast genome. We have investigated factors that are required for splicing of the *psaA* mRNA, which encodes one of the major subunits of PSI, and factors that affect the stability of the *psbB* mRNA, which encodes the 47 kDa subunit of PSII.

In *C. reinhardtii*, the *psaA* gene is composed of three exons widely scattered in the circular chloroplast genome. They are transcribed separately as precursors which are then assembled by a process that requires two steps of splicing *in trans*. The split introns share many of the conserved structural features of group II introns, but an additional small chloroplast RNA (product of *tscA*) is required for *trans*-splicing of the first split intron. The *tscA* RNA is part of a complex that can be detected by gradient sedimentation. At least 14 nuclear loci are required specifically for the *trans*-splicing of one, the other or both of the *psaA* split introns. Using insertional mutagenesis or mutant rescue with cosmid libraries, we have identified and cloned three of the nuclear genes required for *psaA* trans-splicing, and progress in their characterization will be presented.

The *C. reinhardtii psbB* gene is part of a gene cluster that also contains *psbT* and *psbH*. In the nuclear mutant 222E, the mRNAs of the three genes fail to accumulate, but other chloroplast mRNAs are unaffected. The *psbB* gene is transcribed, indicating that the mutation causes a defect in RNA stability. Chimeric genes introduced into the chloroplast show that the 5'UTR of *psbB*

mediates the effect of the 222E mutation on RNA stability. We have cloned the corresponding nuclear locus which encodes a novel protein that belongs to the tetratricopeptide repeat (TPR) family.

REDOX CONTROL OF NUCLEAR GENES IN EUKARYOTIC ALGAE

Paul Falkowsky
Brookhaven National Laboratory, Upton NY, U.S.A.

Physiological acclimation to variations in light, temperature, carbon dioxide concentration and nitrate assimilation are well documents for both procaryotic and eucaryotic photosynthetic organisms. For example, acclimation to changes in growth irradiance (photoacclimation) in the algae normaly leads to an enhanced level of LHCs when irradiance is low and and conversely lower levels of LHCs when irradiance is high. The acclimation to irradiance is readily reversible and, for LHCII is regulated primarily by transcription of the nuclear-encoded lhcb gene family. In *Dunaliella tertiolecta* and *Chlamydomonas reihhardtii*, we found levels of LHCII were enhanced under high irradiance when the plastoquinone pool was oxidized by sub-lethal levels of herbicides or by site-directed mutations in D1 that restrict electron flow from Qa to Qb. Conversely, when the pool is reduced, either by inhibitors such as DBMIB or by mutations in the cyt b6/f complex that lead to a lower affinity of PQH2 for its docking site, LHCII levels are repressed under low irradiance. Using gel mobility shift assays, we found that the redox state of the pool is related to the induction of specific DNA binding proteins, and have identified the binding region as a motif near a G-box. We have extended the search for redox control of nuclear genes to carbonic anhydrase and nitrate reductase. The results of these studies will be discussed.

MECHANISM AND PHYLOGENY OF PROTEIN TRANSPORT ACROSS THE THYLAKOID MEMBRANE

Ralf Bernd Klösgen, Ivan Karnauchov, Jürgen Berghöfer

Botanisches Institut der Ludwig-Maximilians-Universität Menzinger Str. 67, D-80638 München, Germany - phone: ++49 - 89 - 17 861 288, Fax: ++49 - 89 - 17 82 274, e-mail: rabe@botanik.biologie.uni-muenchen.de

Protein transport into and across the thylakoid membrane of higher plant chloroplasts proceeds *via* at least four different pathways that are protein-specific and mechanistically distinct. The Sec-dependent pathway requires nucleoside triphosphates and a SecA protein in the stroma, the ΔpH-dependent route does not demand for stromal factor but strictly depends on a transthylakoidal proton gradient, the SRP-dependent pathway needs GTP and a chloroplastic homologue to SRP-54 in addition to the transthylakoidal ΔpH, and finally, there is spontaneous protein integration for which no physiological prerequisite has so far been found (reviewed in Klösgen, *J. Photochem. Photobiol. B: Biology* 38, 1-9, 1997). Except for the latter, all pathways involve proteinaceous receptor or translocase, which is exposed on the stromal side of the thylakoid membrane.

Prokaryotic origin is undisputed only for the thylakoidal Sec- and SRP-pathways, since mechanistically very similar protein transport routes exist in *E. coli*. Also spontaneous membrane integration like that described for CFo-II (Michl et al., *EMBO J.* 13, 1310-1317, 1994) finds an equivalent in prokaryotes, notably in the membrane insertion of M13 procoat (Kuhn, FEMS Microbol. Rev.17, 185-190, 1995). However, neither the prokaryotic homologues of CFo-II nor its plastome encoded sister protein CFo-I seem to utilise this mechanism for integration which implies an unusual phylogeny of this pathway.

The phylogenetic origin of the thylakoidal ΔpH-dependent pathway is particularly ambigous. Until recently, it was widely considered to be a new development of the chloroplast, because a translocation mechanism that is independent of nucleoside triphosphates and relies exclusively on the membrane potential as energy source had not been described elsewhere. In line with that, the proteins targeted by this pathway were not found in cyanobacteria. However, data will be presented demonstrating that there exists an ancient component of the thylakoid membrane, found in both chloroplasts and cyanobacteria, that is targeted by the ΔpH-dependent route which suggests that a similar pathway might exist also in the prokaryote. In contrast to the "typical" ΔpH-type mechanism, membrane transport in this case involves stromal component(s). Furthermore, the targeting signal differs significantly from that of other ΔpH-type transport signals. These data suggest that the protein might represent a missing link in the development of the ΔpH-dependent transport route.

TARGETING AND TRANSLOCATION OF NUCLEUS-ENCODED PROTEINS INTO THE CHLOROPLAST

***K. L. Kindle* and S. D. Lawrence**

Plant Science Center/Biotechnology Program, Biotechnology Building, Cornell University, Ithaca, NY 14853 USA

Nucleus-encoded proteins that are localized to the chloroplast are synthesized in the cytosol as precursors with N-terminal extensions, termed transit peptides, which contain the information necessary for targeting proteins to and within the chloroplast. With the advent of facile nuclear transformation techniques, it has become possible to apply molecular genetic approaches to study import and intra-chloroplast localization of nucleus-encoded proteins in the unicellular eukaryotic alga, *Chlamydomonas reinhardtii*. We have undertaken experiments to examine the function of the transit peptides of two nucleus-encoded chloroplast precursors in *Chlamydomonas*, using both *in vivo* and *in vitro* analyses. Now we are applying genetic selections to isolate mutants with altered chloroplast envelope translocation.

We have created a number of deletion mutations in the transit peptides of plastocyanin, which is normally localized to the thylakoid lumen, and the gamma subunit of the chloroplast ATPase (encoded by *atpC*), which is an extrinsic, stroma-facing thylakoid membrane protein.

Mutant plastocyanin genes bearing deletions in the transit peptide were introduced into the null mutant Ac-208. Some transit peptide regions were required for accumulation of plastocyanin, while others appeared to be dispensable. Particularly dramatic effects were seen with short deletions at the very N-terminus of the transit peptide (amino acids 2-8) and in the C-terminal part of the transit peptide, (amino acids 29-36). Since plastocyanin is normally localized to the lumen, it probably contains a bipartite transit peptide, in which the C-terminal part contains a signal sequence for transfer across the thylakoid membrane. Since RNA blot analyses and pulse labeling of cytosolic proteins indicated that the mutant plastocyanin genes were expressed, even though accumulation of the mature protein was almost abolished, we suggest that the protein is synthesized, but unstable if the protein cannot be imported and localized to the thylakoid lumen. Deletions in the central region of the transit peptide, between amino acids 9 and 18, had a much less severe effect *in vivo*. However, a deletion near the junction between the presumed signal for envelope translocation and the signal sequence for thylakoid membrane translocation, led to the accumulation of a slightly higher molecular weight form of plastocyanin, which was localized in the lumen, suggesting that this region is important for correct processing and/or intra-organellar sorting. The same series of mutations that had been tested *in vivo* were cloned into a cDNA context and tested with the *in vitro Chlamydomonas* chloroplast protein import assay. In general, *in vivo* and *in vitro* results correlated fairly well, with two exceptions. First, very low levels of protein accumulation (1-2%) could be detected *in vivo* for some deletion mutations that failed to show any *in vitro* import activity. Second, the N-terminal deletion mutation that removed amino acids 2-8 was imported at ~25% the wild-type rate *in vitro*, but accumulated less than 5%the wild-type level of protein *in vivo*. Since transformants accumulated wild-type levels of mRNA and synthesized a stable precursor, we suggest that the defective plastocyanin precursor may complete

poorly with wild-type precursors *in vivo*.

When *atpC* genes that lacked most of the transit peptide coding region were transformed into an *atpC* mutant, photosynthetic transformants were recovered, which accumulated apparently wild-type levels of chloroplast ATPase. This suggests that the removal of more than 80% of the transit peptide does not inhibit import of the ATPase gamma subunit severely enough to limit accumulation of the complex. A deletion that removed amino acids 6-29, resulted in the accumulation of a slightly higher molecular weight gamma subunit, suggesting that this mutation prevents normal stromal processing. Two mutations that had no effect on protein accumulation *in vivo* completely inhibited import in a homologous *in vitro* chloroplast protein import assay, suggesting that the transit peptide is important for envelope translocation *in vitro*. Transformants that contained a chimeric gene in which the *atpC* transit peptide replaced to stroma-targeting domain of the plastocyanin transit peptide, accumulated nearly wild-type levels of plastocyanin, suggesting that the *atpC* transit peptide contains sufficient targeting information to direct import of plastocyanin *in vivo*. Furthermore, when mutant and wild-type transit peptides were fused to the coding region for the bleomycin resistance protein, the wild-type transit peptide caused a substantial fraction of the protein to be chloroplast-localized, while the mutant precursors were mostly cytosolic. Together, these results suggest that an intact transit peptide is important for efficient chloroplast protein import *in vivo* and *in vitro*, but that import of the gamma subunit does not limit accumulation of the ATPase complex unless it is very severely affected.

There are fundamental differences in transit peptide analysis using *in vitro* and *in vivo* approaches. The *in vivo* assay can detect a low level of import activity more readily than the *in vitro* assay, while the *in vitro* assay is more sensitive to subtle changes in import efficiency. Furthermore, for chloroplast ATPase, which is known to be an exceptionally stable complex, very low efficiency protein import of the gamma subunit may be sufficient for wild-type accumulation of the complex.

We are developing a number of genetic selections to identify loci that may encode proteins that are involved in precursor recognition and import. Prospects for isolating *Chlamydomonas* mutations that affect chloroplast targeting will be discussed.

SYNTHESIS, TARGETING AND ASSEMBLY OF THE CHLOROPLAST ENCODED D1 PROTEIN

K.J. van Wijk.
Dept. of Biochemistry, Arrhenius Lab, Stockholm University, S-10691 Stockholm Fax: +46-8-153679; email klaas@biokemi.su.se

Photosystem II (PSII) is a multiprotein complex of more than 25 different proteins in the thylakoid membrane of oxygenic photosynthetic organisms. PSII catalyses the light-driven reduction of plastoquinone by electrons derived from water. The centre of the PSII complex is formed by a heterodimer of two proteins, D1 and D2 with each 5 transmembrane spans. The D1 protein binds, together with the homologous D2 protein, all essential redox components of PSII and is thus crucial for PSII function. The turnover rate of the D1 protein is much higher than of the other PSII proteins and is a consequence of light-induced damage, followed by specific proteolysis of the D1 protein. In order to maintain PSII in a functional state, the D1 protein must be replaced and is therefore constantly synthesised and assembled into PSII. Although the replacement of the D1 protein must be very efficient and well regulated, very little is known about the mechanisms and regulation of this process. We have studied the synthesis and assembly of the D1 protein during the PSII repair process. Replacement of the D1 protein was followed by pulse and chase experiments in isolated chloroplasts and during run-off translation of ribosome-bound nascent chain complexes, using endogenous psbA mRNA. PSII assembly intermediates and their localisation in the heterogeneous thylakoid membrane were identified through biochemical analysis and the assembly process was kinetically resolved. Regulatory mechanisms by e.g. light and processing of the C-terminal extension of D1 were analysed (1-6).

To study the targeting and insertion pathways of the D1 protein, we have now optimised a novel homologous initiation/translation system isolated from tobacco chloroplasts in which plasmid derived messages can be faithfully translated (7). Excellent translation rates are obtained for the D1 protein, allowing us to investigate the (possibly co-translational) targeting and insertion pathway(s) of the D1 protein by reconstitution with purified thylakoid membranes. To facilitate the search for components of the targeting machinery, photocrosslinkers are introduced into the D1 nascent chain during translation using an amber suppressor tRNA approach (8). We are currently in the process of identifying crosslinked products to the D1 nascent chain and also to optimise the targeting and insertion of the D1 protein. New data will be presented. The D1 protein, is only one of the 30 or so chloroplast encoded membrane proteins. Virtually nothing is known about the targeting of these proteins. Therefore, in parallel to the studies on the D1 protein, we are currently also studying the targeting of other thylakoid proteins. Preliminary results will be presented.

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CHLOROPHYLL AND XANTHOPHYLL BINDING SITES IN THE PHOTOSYSTEM II SUBUNIT CP29

***Roberto Bassi*, Dorianna Sandonà, Daniela Cugini e Roberta Croce.**
Biotecnologie Vegetali, Università di Verona - Italy

Light energy for photosynthesis of green plants is collected by an antenna system, composed of many homologous proteins belonging to the Lhc multigene family (1). A detailed understanding of energy transfer processes in the antenna and reaction centres requires recognition of the topological organisation of subunits (2, 3) and knowledge of the distances between chromophores, of the mutual transition dipole orientation and of the absorption/fluorescence energy levels. While the resolution of LHCII structure at 3.4Å resolution (4) has allowed identification of chlorophyll binding sites and their relative distances, identification of transition dipole orientation and energy levels are precluded by insufficient resolution of the structure obtained or are not accessible to structural methods. Here we used an alternative approach to the identification of the chlorophyll a and chlorophyll b molecules within the antenna protein and the determination of their individual absorption spectra. The *Lhcb4* cDNA was overexpressed in *E.coli* yielding CP29 apoprotein which was reconstituted in vitro into a pigment-binding complex having biochemical and spectroscopical properties identical to the native protein extracted from leaves (5,6). A series of mutant proteins was constructed in which individual chlorophyll-binding residues (4) were substituted for by residues unable to coordinate porphyrins thus yielding proteins missing individual chromophores. Biochemical analysis and differential spectroscopy allowed determination of the chemical nature and of the spectral properties of individual chromophores.

From this study it is concluded that five chlorophyll binding sites are selective for chlorophyll a while three can be occupied by either chlorophyll a or chlorophyll b although with different relative affinity. Chlorophyll-protein interactions, rather than chlorophyll-chlorophyll interactions, determine modulation of the absorption spectra of individual chromophores.

Two xantophyll binding sites are identified in CP29. One chlorophyll a mutant and a chlorophyll b mutant are also affected in carotenoid binding suggesting that chlorophylls are intact xantophyll ligands. Violaxanthin is only bound to one of the sites while lutein and neoxanthin can occupy both sites. Violaxanthin is bound, through a chl b, to a dicichlohexilcarbodiimide(DCCD) binding residue (7) . Since DCCD is an inhibitor of Non-Photochemical Quenching (NPQ), this Chlorophyll-Carotenoid interaction may be involved in NPQ.

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HOW ARE LIGHT-HARVESTING CHLOROPHYLL A/B COMPLEXES ASSEMBLED?

S. Hobe, K. Kosemund, A. Kuttkat, and H. Paulsen
Institut für Allgemeine Botanik, Universität Mainz, Germany

We have only limited knowledge of how the chlorophyll-a/b antenna of the photosynthetic apparatus is assembled. Neither do we know how light-harvesting chlorophyll a/b proteins are inserted into the thylakoid membrane and complexed with pigments, nor do we understand how a newly made subunit of a light-harvesting complex is then assembled with other pigment-protein complexes into a functional and structurally well-defined holo photosystem. I will present some of the knowledge that has been accumulated on the membrane insertion of one of the major chlorophyll a/b-binding protein (Lhcb1) and its pigmentation as well as on the trimerisation of the resulting pigment-protein complex as a first step in the assembly of multi-subunit complexes of the photosynthetic apparatus.

Folding experiments with recombinant Lhcb1 in detergent solution indicated that protein folding is triggered by the binding of pigments (Paulsen et al., 1993). It may be speculated that, as the protein inserts into the thylakoid membrane, its folding is also induced by pigment binding. Thus, the pigmentation of Lhcb1 (and other, closely related chlorophyll a/b proteins) may be the driving force for the insertion of these proteins into the membrane. Consistently, attempts to insert recombinant Lhcb1 into etioplast membranes in vitro do not result in measurable amounts of membrane-located protein. This does not prove, though, that chlorophylls are necessary for protein insertion, as we cannot distinguish at this point whether in fact membrane insertion fails because of chlorophylls missing, or whether the protein does become inserted into the membrane but then is instable towards thylakoid-bound proteases, again because of the lack of chlorophylls.

However, we could show that recombinant Lhcb1 does get stably inserted into isolated etioplast membranes that have been complemented with chlorophylls a and b (Kuttkat et al., 1997). This observation proves that chlorophylls are the only components missing in etioplast membranes that are essential for the stable insertion of Lhcb1. If there are other components necessary for this process, they are either already present in etioplasts or not essential for the insertion step in vitro.

Thornber's group demonstrated several years ago by pulse-chase labelling that monomeric Lhcb1,2-pigment complexes are formed during early greening of barley and then assembled into trimeric complexes (Dreyfuss and Thornber, 1994). Consistently, when we insert recombinant Lhcb1 into isolated pea or barley thylakoids isolated from leaves at various stages of greening, we find the protein assembled into monomeric complexes in early, and trimeric complexes in later greening stages. We do not know whether trimerisation of the inserted protein is due to the higher amounts of endogenous light-harvesting complexes accumulated after extended greening, or whether trimerisation is regulated by a developmental factor that appears only during the greening process.

Trimerisation of recombinant Lhcb1-pigment complexes in vitro showed that an amino-proximal domain is involved in this process: When the "trimerisation motif" WYxxxR in position 16-21 of the amino acid sequence was (partially) deleted or altered by amino acid exchanges, the resulting monomeric

Lhcb1-pigment complexes would not trimerise any longer, neither in detergent solution nor upon the insertion of the mutated protein into isolated thylakoid membranes (Hobe et al., 1995). Additionally, a hydrophobic amino acid in position 11 from the C terminus seems to be essential for trimerisation: when W in this position was exchanged with a non-hydrophobic amino acid (but not when it was exchanged with F), trimerisation was lost, again both in detergent solution and in isolated thylakoids (Kuttkat et al., 1996). The protein also failed to trimerise when the C terminus was extended with a His6 tag. There may be further determinants for complex trimerisation in hydrophilic terminal or loop domains of Lhcb1. In order to identify these (and amino acids involved in other functions), we are generating Lhcb1 mutants carrying random mutations in well-defined domains of the protein.

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THE CONTROL BY EPISTASY OF SYNTHESIS (CES PROCESS) DURING CHLOROPLAST PROTEIN BIOGENESIS

Francis-André Wollman

CNRS-UPR9072, IBPC, 13 rue P.&M. Curie, 75005 Paris, FRANCE.

Recent advance in gene transformation has opened the way to a comparative analysis of the phenotypes of cyanobacterial mutants and *Chlamydomonas* chloroplast mutants which bear similar defects in the expression of a single gene. Regarding photosynthesis proteins, a pronounced pleitropic effect has been consistently reported in *Chlamydomonas* chloroplast but not in cyanobacteria : preventing production of only one subunit most often leads to a dramatic decrease in the content of the whole set of subunits of the same protein complex. It has been concluded that the biogenesis of photosynthesis proteins in *Chlamydomonas* chloroplast results from a concerted accumulation of their constitutive subunits.

Two processes are involved in the control of an assembly-mediated concerted accumulation of subunits. On the one hand, a subset of subunits are highly protease-sensitive when non-assembled. On the other hand, a subset of subunits, the CES subunits (Controlled by Epistasy of Synthesis), cease to be made at high rates when their assembly partnairs are missing. These observations point to a possible hierarchy among thylakoid protein synthesis, with the synthesis of dominant subunits, highly protease-sensitive, taking precedence over that of CES subunits which are poorly protease sensistive. Thus the CES subunits designate those, whose synthesis appears assembly-dependent. In summary, the CES process, and a post-translational degradation process, both contribute to control subunit production in the stoichiometry required for their functional assembly in an oligomeric protein.

It should be emphasized that the CES process could be acheived in a variety of ways at the molecular level and may indeed occur through distinct mechanisms for the various CES subunits identified so far. In Chlamydomonas the CES subunits encompass, D1 and apoCP47 in PSII, the PSAA subunit in PSI, subunit a in the ATP synthase, LS in the RubisCo and cytochrome f in the cytb6f complex. There is circumstancial evidence that LS, cytochrome f and apoCP47 are also CES subunits in higher plant chloroplasts.

We have studied in some details the molecular mechanism underlying the CES behaviour of cytochrome f in *Chlamydomonas*. Evidence will be presented that it is due to an autoregulation of translation which involves both the C-terminal domain of the unassembled polypeptide and the 5' untranslated region of the corresponding mRNA. That a similar mechanism prevails for another CES subunit, subunit a, will also be discussed.

A NEW KIND OF IMMUNOPHILIN IN THE THYLAKOID LUMEN: SIGNIFICANCE FOR PROTEIN TURN-OVER AND PROTEIN PHOSPHORYLATION

Alexander V. Vener[1], Hrvoje Fulgosi[2], Lothar Altschmied[2], Reinhold G. Herrmann[2] and Bertil Andersson[1]

[1]Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91 Stockholm, Sweden.
[2]Botanisches Institute der Ludwig-Maximilians-Universität, Menzinger Strasse 67, D-8000 München, Germany.

The first high molecular weight immunophilin-like plant protein of the cyclophilin type has been identified in chloroplasts as a 40 kDa component co-purifying with a thylakoid protein phosphatase activity. This protein was shown to be located in the thylakoid lumen, originate from a single copy nuclear gene and possess several structural domains including a cyclophilin-like C-terminal segment of 20 kDa, a predicted N-terminal leucine zipper as well as a putative phosphatase-binding domain. The immunophilin is made as a precursor of 49.2 kDa including a bipartite lumenal targeting transit peptide of 104 residues. The mature protein can attach to the inner membrane surface with a binding strength intermediate to plastocyanin and the 23 kDa extrinsic protein (PsbP). Furthermore it has a heterogeneous location along the membrane system being confined predominantly to the non-appressed thylakoid regions, the site of protein integration. Biochemical measurements gave direct experimental support to the structural prediction that the protein possesses a peptidyl-prolyl *cis-trans* isomerase (PPIase) protein folding activity typical for immunophilins but is not inhibited by cyclosporin A. Despite a pronounced enzymatic activity the cyclophilin domain of the protein has a greatest divergence among of other known cyclophilins with only about 25% of identity to human cyclophilin A. Furthermore, an intriguing multifunctionality was implied from enzymatic measurements demonstrating that this protein, designated TLP40 (Thylakoid Lumen PPIase), possesses a regulatory effect on thylakoid phosphoprotein dephosphorylation. The TLP40, representing a type of proteins previously not thought to be associated with chloroplast thylakoids, is suggested to be part of a transmembrane signal transduction mechanism that appears to link turnover, folding and phosphorylation of photosynthetic proteins. The TLP40 protein is present in etiolated tissue which gives further straight to a role in thylakoid biogenesis. The presence of a protein with functional and structural features such as TLP40 in the thylakoid lumen suggests that this often overlooked chloroplast compartment as well as the light-mediated protein phosphorylation process of the photosynthetic membranes are significantly more complex than presently anticipated.

DISMANTLING OF THYLAKOID MEMBRANES

Philippe Matile
Institute of Plant Biology, University of Zürich

In the development of plants, leaf senescence plays a most important role regarding the recycling of nutrients. The main sources of N-export from senescent leaves are the chlorophlasts which, in the course of senescence, develop into gerontoplasts. This type of plastid differentiation is characterized by the disappearance of thylakoids. Thus, membranes are dismantled and membrane components such as pigments, apoproteins of pigments and galactolipids are degraded. Whereas the degradation of thylakoidal proteins is still an unsolved riddle, there has recently been some progress with regard to chlorophyll and galactolipids.

The catabolic pathway of galactolipids seems to be initiated by the conversion into phosphatidylcholine which is exported from developing gerontoplasts. Experiments employing barley leaves containing galactolipids labeled in the acyl residues have yielded evidence for the metabolism of fatty acids via beta-oxidation, glyoxylic acid cycle and gluconeogenesis. The bulk of label was ultimately released as carbon dioxyde, suggesting that galactolipids may contribute to the supply of metabolic energy required for driving of the catabolic processes in senescent leaves.

The state-of-the-art regarding the breakdown of chlorophyll will briefly be summarized. Although the catabolic pathway has now been largely elucidated, an important problem has not been solved so far. It concerns the mechanism by which chlorophyll molecules are lured out of the pigment-protein complexes and fed into the catabolic machinery residing in the gerontoplast envelope. Studies with stay-green genotypes suggest that the apoproteins of chlorophyll are not degradable as long as they are complexed with pigments. The apoproteins account for a substantial proportion of the total protein of chloroplasts and, therefore, dismantling of the complexes is an important prerequisite of N-recycling from senescent leaves.

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