Progress in Botany 67

67 PROGRESS IN BOTANY

Genetics
Physiology
Systematics
Ecology

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Springer

With 57 Figures

ISSN 0340-4773
ISBN 3-540-27997-0 Springer-Verlag Berlin Heidelberg New York
ISBN 03978-3-540-27997-6

The Library of Congress Card Number 33-15850

Springer is a part of Springer Sciences+Business Media
springeronline.com

Printed in Germany

Cover design: Design & Production, Heidelberg
Typesetting: SPI Publisher Services
31/3150 - 5 4 3 2 1 0 - Printed on acid-free paper

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Fascination with Chloroplasts and Chromosome Pairing

Diter von Wettstein

During the second half of the twentieth century biological research could be characterized as a period of strong convergence. Genetics, physiology, biochemistry and other sub-disciplines of biology were joined in the common goal of clarifying the molecular processes behind the function of organelles, cells, organs and organisms. The whole chain from the information contained in the genome to the properties and function of an organism was and is analysed with sophisticated methods.

It has been a pleasure and privilege to contribute to these ventures and at the outset I would like to mention and thank my mentors in the different disciplines. They taught me to carry out research and to ask important questions: Erwin Bünning and Adolph Butenandt in Tübingen, Jacob Seiler and Albert Frey-Wyssling in Zürich, Åke Gustafsson in Stockholm, Frank Stahl and Salvador Luria at Cold Spring Harbor, and Paul Stumpf at Davis and Mogens Westergaard in Copenhagen. But the results could likewise not have been achieved without the imaginative and enthusiastic efforts of co-workers, students, postdoctoral fellows and visiting scientists. They include 54 students who completed their master's degree and 65 their PhD, and I will try to review some of their work here. During my time at the Carlsberg Laboratory, the Department of Physiology hosted 115 postdoctoral fellows and visiting scientists; they provided much of the inspiration that guided innovation and progress.

In this review I would like to discuss two areas of my interests:

1. Biosynthesis of the photosynthetic membrane and chloroplast biogenesis.
2. Chromosome pairing, the mechanism of crossing-over and genome analysis.

1 Biosynthesis of the photosynthetic membrane and chloroplast biogenesis

My interest in the development of chloroplasts and chlorophyll biosynthesis began when I became an assistant to Åke Gustafsson in Stockholm in 1951

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and joined the multidisciplinary Swedish Group of Mutation Research he had created and was leading with great success. One of my tasks was to analyse mutation rates, and spectra, in the M_2 generation of barley grains treated with various ionising radiation and chemical mutagens. This was done by a test he had devised in the 1930s and consisted of counting the white, yellow, light-green or tiger-striped lethal seedling mutants emerging from thousands of spikes planted in the greenhouse during the winter season. These tests were done to find the most efficient treatments for inducing mutants suitable for barley breeding programs. Interestingly, now there is hardly a cultivar that does not contain an induced mutant among its ancestors, but at the time it was considered that all induced mutations were detrimental and therefore useless in plant breeding, a view propagated by Herman J. Muller and L. J. Stadler. Due to the tireless efforts of Åke Gustafsson and a few others like Bob Nilan in Pullman it was shown that induced mutations could yield improved cultivars – Muller and Stadler overlooked the fact that the majority of spontaneous mutations were also detrimental, and that mutations are still a major factor in the evolution of genes to organisms. The discussions for and against were not unlike the present discussions concerning the use of transgenic plants in breeding. As history repeats itself, the time will come when transformed cultivars will be as accepted and considered as "traditional" as crop plants containing induced mutations.

Encouraged by the successful efforts of Beadle and Tatum in analysing metabolic pathways by knock-out mutations, it seemed to me that all these hundreds of interesting mutants should be useful for a detailed analysis of the development of chloroplasts and pigment biosynthesis. I thus started to collect representatives of the different types of mutants and to conduct crosses to determine allelic relationships by complementation tests. At that time electron microscopy of thin sections started to reveal the ultrastructure of animal and plant cells. So I took my mutants and spent a few days every week at Arne Tiselius's Biochemistry Institute at Uppsala University, where Håkan Leyon had constructed a microtome and developed embedding procedures, and where I could use the third electron microscope built by Siemens in 1940. It had been acquired by The Svedberg and was installed next to his ultracentrifuges. The mutants turned out to be very useful for characterizing the development of chloroplast structure as presented in a summary (von Wettstein 1959). In higher plants, chloroplasts develop from proplastids in the light or via the etioplast pathway after an initial dark period. The primary thylakoid layers are formed by alignment of vesicles budded from the inner membrane of the plastid envelope. In contrast to the in depth knowledge obtained since then of the organization of the photo-

synthetic membrane and the import of the protein components into the chloroplast and their targeting to the thylakoids, progress in learning how the lipid bilayer membranes are formed is less apparent (von Wettstein 2001). This may change with the discovery by Kroll et al. (2001) and Westphal et al. (2001) of a function of the vesicle-inducing protein in plastids (VIPP).

In pea chloroplasts the 37-kDa VIPP protein is located both in the vicinity of the chloroplast envelope and the thylakoid membranes and was considered by Li et al. (1994) as a candidate for the transfer of galactolipids from their site of synthesis at the chloroplast envelope to the thylakoids. Daniella Kroll and co-workers (Kroll et al. 2001) studied a recessive *Arabidopsis* T-DNA insertion mutant with severe disturbances in the photosynthetic electron transport chain and the formation of the thylakoids. The insertion was identified in the gene encoding VIPP and the mutant could be rescued by transformation with the *VIPP* cDNA. The cause for the disturbed development or maintenance of the thylakoids was the failure of the mutant to bud the vesicles from the inner chloroplast envelope membrane, which transfer lipids from the inner envelope to the thylakoid membranes. In the transformants the process of vesicle budding was re-established and the thylakoid organization normalized. The companion paper by Sabine Westphal and co-workers (2001) identifies *VIPP 1* genes in the genomes of *Synechocystis*, *Anabaena, Synechococcus* and *Nostoc*. In these cyanobacteria, the protein is located in the plasma membrane, but its disruption in *Synechocystis* by insertion mutagenesis with a kanamycin cassette prevents ordered thylakoid formation and light-dependent oxygen evolution.

The photosynthetic membrane in barley and other higher plants converts solar energy into chemical energy, and as we now know, it uses six larger protein complexes for this purpose (Simpson and von Wettstein 1989; von Wettstein et al. 1995) (Fig. 1). They are called the reaction centres of photosystem I and II, the two light harvesting complexes of photosystem I and II, the cytochrome b6/f complex and the chloroplast coupling factor (synthesizing ATP). The polypeptides in these complexes bind and orient chlorophyll and carotenoid molecules and the different metals and molecules which are required for energy channelling and electron transport. Circa one half of the ~57 membrane proteins are encoded by genes in the nucleus and the other half in the chloroplast's own DNA genome. This cooperation between the two genomes in the plant cell also takes place in the assembly of the CO_2-fixing enzyme, Rubisco, that is made up of two, different-sized subunits, of which the larger one is encoded in chloroplast DNA and the smaller one in the nuclear chromosomal DNA. The following results of our research are of special significance.

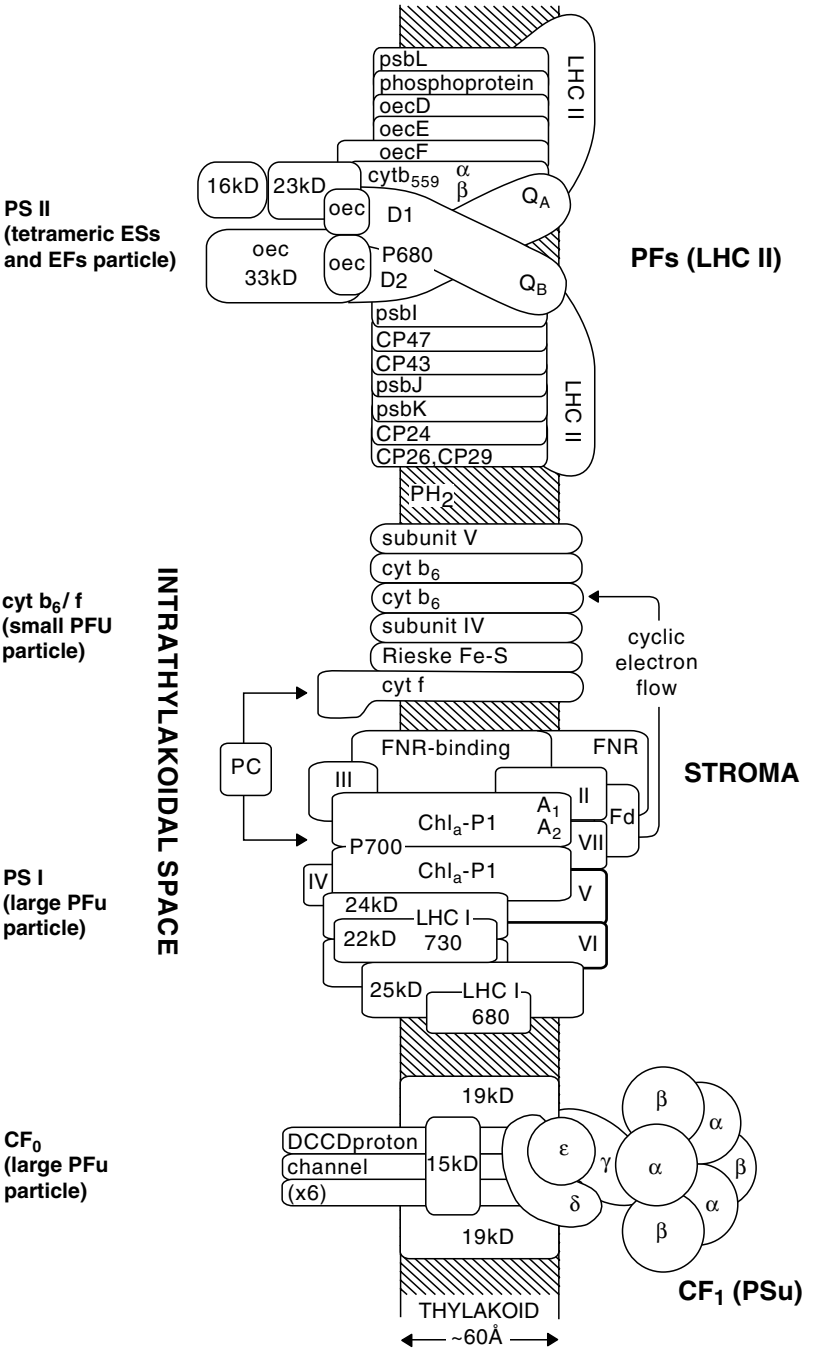
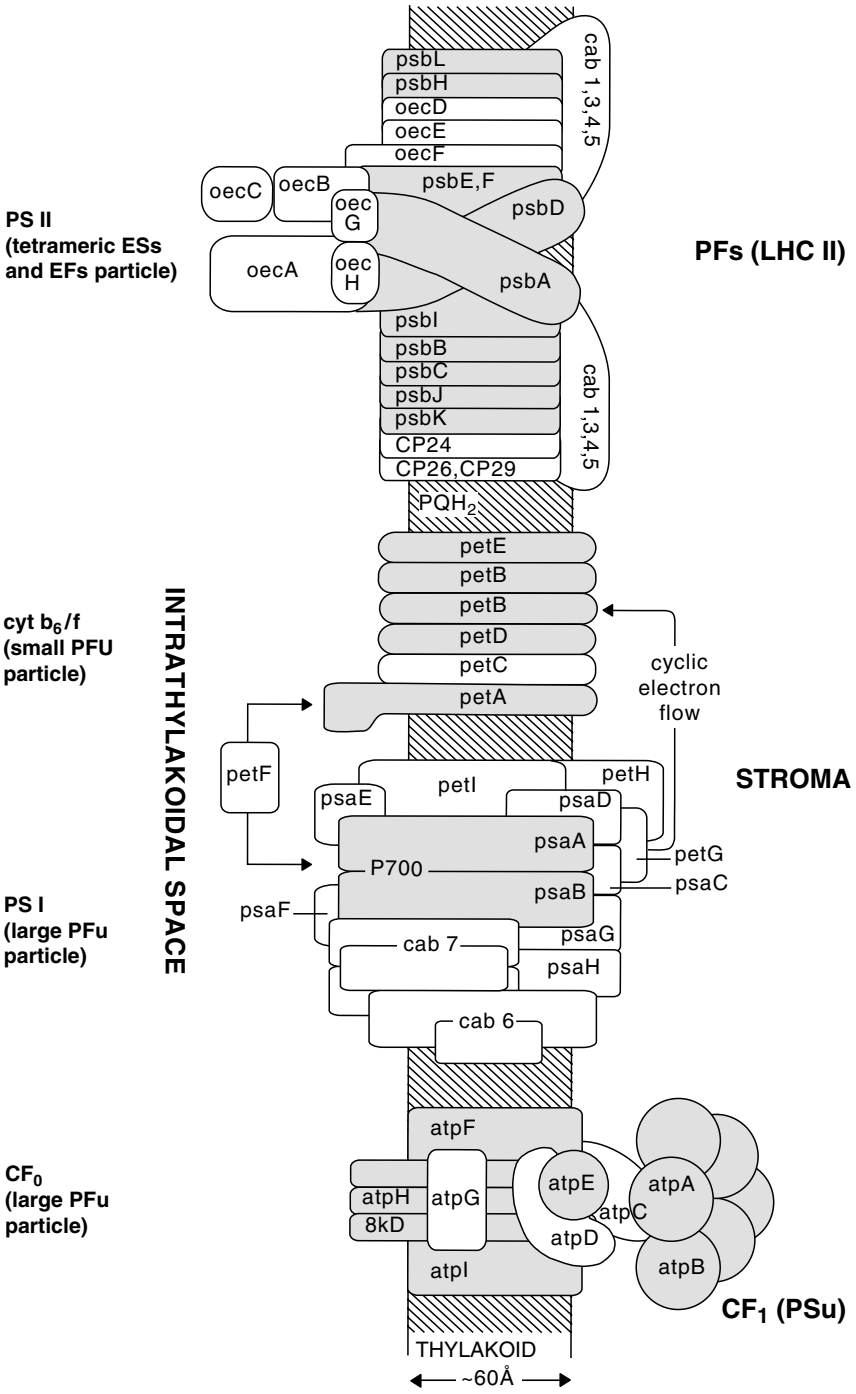


Fig. 1a, b. Model of the photosynthetic membrane showing the polypeptide components of the major complexes. The site of coding is indicated by shading (chloroplast DNA) or is unshaded (nuclear gene). (Modified from Simpson and von Wettstein 1989)


PS II
(tetrameric ESs
and EFs particle)
PFs (LHC II)
cyt b6/f
(small PFU
particle)
PS I
(large PFu
particle)
CF0
(large PFu
particle)
INTRATHYLAKOIDAL SPACE
STROMA
CF1 (PSu)
THYLAKOID
~60Å
cyclic
electron
flow
cab 1,3,4,5
cab 1,3,4,5
psbL
psbH
oecD
oecE
oecF
oecC
oecB
oec G
psbE,F
psbD
oecA
oec H
psbA
psbI
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psbJ
psbK
CP24
CP26,CP29
PQH2
petE
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petD
petC
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petI
petH
psaE
psaD
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P700
psaB
psaF
psaG
cab 7
psaH
cab 6
petG
psaC
atpF
atpH
atpG
8kD
atpI
atpE
atpC
atpD
atpA
atpB

In cooperation with the Biological Laboratories of Harvard University the nucleotide sequence of the first plant gene, the structural gene for the large subunit of Rubisco, was determined in 1980 (Mc Intosh et al. 1980; von Wettstein 1981). Carsten Poulsen, Anthony Holder, Brian Martin and Ib Svendsen had produced peptide maps of the large subunit of Rubisco of barley and the genus *Oenothera* and obtained partial amino acid sequences (von Wettstein et al. 1978; Holder 1978; Poulsen et al. 1979). Lawrie Bogorad called me one day in 1977 to say that he had heard that we had amino acid sequences of the large subunit and to ask if I would share them with him, since he wanted to sequence the maize gene and this would be of great help to his project. "Sure," I said, "and I would also like to send you Carsten Poulsen with his Carlsberg fellowship to help with the sequencing." After supplying Carsten with a large supply of liquorice, he and Lee completed the task. Peptide mapping of the large and small Rubisco subunits also led to the identification of the pomato, the first somatic hybrid between potato and tomato produced by Georg Melchers in Tübingen (Melchers et al. 1978; Poulsen et al. 1980; von Wettstein 1983). The most interesting aspect of the analysis of these generic hybrids was the finding that they only retained the tomato or potato chloroplast genome at an equal frequency, but not both. We still do not know how this happens.

Over the years 357 barley mutants with defects in chloroplast development and chlorophyll synthesis have been assigned to 105 gene loci. Together with Albert Kahn, Ole Frederik Nielsen, Simon Gough and Naomi Avivi-Bleiser (von Wettstein et al. 1974; Kahn et al. 1976) structural and regulatory genes of chlorophyll synthesis were identified. Knud Henningsen, John Boynton, David Simpson, Otto Machold, Gunilla Høyer-Hansen, Roberto Bassi, Bob Smillie and Torsten Fester analysed the different categories of the mutants with regard to their ultrastructure, pigment levels, thylakoid polypeptide composition and photosynthetic capacity (Henningsen et al. 1993; Simpson and von Wettstein 1980; Simpson et al. 1985; Smillie et al. 1978).

The mutants were used to localize the macromolecular photosynthesis complexes, as recognized by freeze-fracture particles, to the different domains of the chloroplast membranes (e.g. Simpson et al. 1989; Simpson and von Wettstein 1989). Birger Lindberg Møller analysed the function of the grana and stroma membranes by isolating and purifying these membrane types, by separating the membrane polypeptides and reconstituting them to give photosynthetically active membranes (e.g. Henry et al. 1982; Møller and Høj 1983; Møller 1985). The gene family encoding the light-harvesting proteins of photosystem I was also identified (e.g. Knoetzel et al. 1992). The first transcription map of a chloroplast genome was established for barley (Poulsen 1983) and alternative transcription was demonstrated

for the gene encoding the large subunit of Rubisco. The longer transcript is used by the plant when a large amount of protein is synthesized in the light (Poulsen 1984).

A single molecule of chlorophyll and haem is synthesized from eight molecules of 5-aminolevulinic acid. In 1975 it was shown by isotope labelling that higher plants, in contrast to animals and humans, synthesize this non-protein amino acid from the intact carbon skeleton of glutamate (Beale et al. 1975). Gamini Kannangara, Simon Gough, postdoctoral fellows, students and visiting scientists have elucidated this three-step pathway at the biochemical and molecular level over a period of 19 years (cf. Kannangara et al. 1994; von Wettstein et al. 1995; von Wettstein 2000a, b). This pathway is used by higher plants, algae, cyanobacteria, *Escherichia coli* (not recognized for over 30 years), as well as a number of other bacteria. Animals and humans, yeast and photosynthetic bacteria form 5-aminolevulinate by condensation of glycin and succinate. Entirely surprising was the discovery that the glutamic acid has to be activated by ligation to a glutamyl tRNA before it can be reduced to glutamate-semialdehyde and thereafter transaminated by an aminomutase to 5-aminolevulinic acid (Schön et al. 1986). It is so far the only known case in which a tRNA participates in the conversion of a low molecular weight compound. In higher plants this tRNA is encoded in chloroplast DNA and also has to serve for the translation of mRNA on chloroplast ribosomes. The three enzymes are encoded in nuclear DNA, and have to be translated on cytosolic ribosomes and imported into the chloroplast.

The importance of the pathway for chlorophyll synthesis is demonstrated by transgenic tobacco plants expressing an antisense gene for the glutamine semialdehyde aminotransferase (Höfgen et al. 1994). The barley enzyme that requires the glutamyl tRNA as substrate was purified and a partial amino acid sequence obtained (Pontoppidan and Kannangara 1994). This work identified the structural gene for this enzyme as the *HemA* gene, already cloned and sequenced in many organisms but not recognized as encoding glutamyl RNA^{Glu} reductase. Finally this interesting enzyme was expressed as a fusion protein in *E. coli* (Vothknecht et al. 1996, 1998). It turned out that haem, a prominent inhibitor of chlorophyll synthesis, binds to the N-terminal extension of the protein that is characteristic for plant enzymes, but absent in bacteria.

In 1994 Lucien Gibson, Ph.D. student with Neil Hunter, University of Sheffield, arrived and brought with him plasmids that contained the *bchH*, *bchD* and *bchI* genes from *Rhodobacter spheroides*. Lucien, Robert Willows and Gamini Kannangara expressed the proteins of these three genes in *E. coli* and demonstrated for the first time that the association of these three pro-

teins *in vitro* inserts the Mg atom into protoporphyrin IX (Gibson et al. 1995; Willows et al. 1996). Reconstitution of Mg chelatase activity required only ATP, Mg^{2+} and protoporphyrin. This opened the way to learn more about how the metal ion is incorporated into the porphyrin ring. The insertion of Mg^{2+} into protoporphyrin IX proceeds in two stages. In the first stage subunits BchD (70 kDa) and BchI (40 kDa) undergo activation by complex formation in the presence of ATP and Mg^{2+}. The protein–protein interaction of these two subunits was subsequently confirmed for the tobacco subunits with the yeast two-hybrid system (Gräfe et al. 1999). Thereafter Mg^{2+} is inserted into the protoporphyrin IX substrate that is bound to the large subunit BchH (140 kDa).

The information of the nucleotide sequence of the *Rhodobacter* genes permitted the identification, cloning and molecular characterization of the corresponding barley and other higher plant genes and their mutants (Jensen et al. 1996; Kannangara et al. 1997; cf. von Wettstein 2000b). That three different gene products are required for the insertion of Mg^{2+} into protoporphyrin IX was originally found with xantha mutants at three gene loci in barley that accumulate protoporphyrin IX when fed 5-aminolevulinate (Gough 1972; von Wettstein et al. 1974; cf. von Wettstein 2000a). They belong to the first mutants isolated and analysed in 1953. Gene *Xantha-f* corresponds to *bchH*, *Xantha-g* to *bchD* and *Xantha-h* to *bchI*.

One of the post-genomic challenges is to determine the function of the genes discovered in genome sequencing projects. Usually > 50% of the genes uncovered in the sequenced genomes have no significant matches to proteins or cloned genes in the databases for other organisms. Furthermore, while such matches can hint at similar functions they do not prove the function of the gene in question. To determine the precise function of a gene its cloning is required, frequently carried out by positional cloning. While this is expedient with small genomes like that of *Arabidopsis* it is difficult with large sequenced or un-sequenced genomes like those of small grain cereals. Due to the availability of the transcript-deficient barley mutant *xantha-h*[57] we were able to develop the microarray method for transcript-based cloning of genes only known through their mutant phenotype (Zakhrabekova et al. 2002).

Libraries of genomic clones or cDNA clones or expressed sequence tag clones representing several thousand genes are microarrayed on glass slides. Each clone occupies a round spot on the slide. cDNAs made from the mRNAs of the transcript-deficient mutant and its wild type is differentially labelled with green and red fluorescing nucleotides, respectively, and hybridized in equal amounts to the microarrayed clones. Because of the absence of the mutant transcripts, pure red fluorescence from a spot will

result from wild type DNA and identify the gene sought This technique also worked with the xantha-f 27 and xantha-f 40 mutants, which display nonsense-mediated mRNA decay, a surveillance system developed by organisms to reduce the abundance of mRNA with nonsense codons (Gadjieva et al. 2004). It can be exploited to clone genes through mutants with reduced transcript abundance. This then will allow functional identification of a majority of the ca. 1,000 barley genes for which > 8,322 mutants have been identified through Åke Gustafsson's and Udda Lundqvist's efforts and are conserved in the Nordic Gene Bank.

The three-dimensional structure of the ATP-binding subunit BchI of *Rhodobacter capsulatus* solved at a resolution of 2.1 Å by Michel Fodje and Salam Al-Karadaghi in combination with the molecular genetic analyses of Mats and Andreas Hansson has allowed remarkable insights into the molecular basis of the insertion of Mg^{2+} into protoporphyrin IX (Fodje et al. 2001). It provides the starting point for clarifying the mechanism by which Mg^{2+} is inserted into the chlorophyll molecule.

BchI belongs to the chaperone-like "ATPase associated with a variety of cellular activities" (AAA) family of ATPases. Its structure could be compared with those of other members of this protein family, such as the heat shock protein 100 of *E. coli*, the delta-prime subunit of DNA polymerase III clamp loader complex and the hexamerization domain D2 of the *N*-methylmaleimide-sensitive membrane vesicle fusion protein. The domains of these proteins are highly conserved, but are located in different ways in the overall structure. BchI also contains loop structures forming a deep positively charged groove that might be involved in interaction with the other subunits of Mg-chelatase. Electron microscopy of BchI in solution in the presence of ATP revealed that it forms in the same way as hexameric ring structures of other AAA proteins. The primary structure of the BchD subunit consists of an AAA module at the N-terminal portion and an integrin I domain in the C-terminal half. An acidic, proline-rich region links the two domains and is predestined to bind to the positively charged cleft of BchI. Both BchI and BchH (the protoporphyrin-binding subunit) contain integrin I domain-binding amino acid sequences. Most likely the hexamer ring of BchI is connected to a hexameric ring of the BchD-AAA module via the proline-rich domain. The integrin BchI domains bind to BchH linking porphyrin metallation by BchH to ATP hydrolysis by BchI.

Among the seven mutant alleles of the barley *xantha*-h gene encoding the smallest subunit of magnesium chelatase (corresponding to BchI) four are recessive and three are semi-dominant. The homozygous mutants are yellow, because of a lack of chlorophyll. The heterozygotes of the recessive mutants are fully green whereas the heterozygotes carrying the semidominant allele

are pale to yellow-green. The recessive mutations prevent transcription of the gene (Jensen et al. 1996), while the semidominant alleles are mis-sense mutations leading to single amino acid substitutions (Hansson et al. 1999). Identification of the mutated residues in the BchI three-dimensional structure located all three of them in the interface between two neighbouring subunits in the AAA^+ hexamer and close to the region forming the ATP-binding site. The three amino acid changes were made by site-directed mutagenesis in the BchI gene of *R. capsulatus* and the subunits expressed in *E. coli*. Combination of wild type BchD and BchH subunits with modified BchI subunits were deficient in ATP hydrolysis and Mg-chelatase activity. However mixtures of the mutated and wild type BchI subunits could form oligomeric complexes with the D and H subunits. The oligomerization is ATP dependent but results in complexes lacking Mg-chelatase activity. Furthermore the presence of mutant BchI subunits in the oligomer did not inhibit the ATPase activity of the wild type subunits but prevented the insertion of Mg^{2+} into prototoporphyrin IX. It is suggested that a small amount of hexamers consisting only of wild type subunits rescues the heterozygous plants. It remains to be seen if disruption of ATP hydrolysis in the mixed hexamers prevents the conformational change expected to permit chelation of Mg^{2+}.

2 Chromosome pairing, mechanism of crossing-over and genome analysis

The ascomycete *Neottiella rutilans* turned out to be an excellent object with which to study the assembly and disassembly of the synaptonemal complex, the 200-nm-wide ribbon between the paired pachytene chromosomes, by electron microscopy of serial sections. Jane Mink Rossen and Mogens Westergaard had shown that the DNA replication in this organism before meiotic prophase occurs in the crozier nuclei prior to karyogamy, which laid to rest the textbook theory of chromosome pairing in connection with a DNA replication at meiotic prophase. In this ascomycete the chromosomes are always at a condensed chromatin stage, also during mitosis and meiosis, which makes it a highly favourable subject for ultrastructural studies (Westergaard and von Wettstein 1966). In a study of all stages of meiosis of *Neottiella* (Westergaard and von Wettstein 1970; von Wettstein 1971, 1977) it was demonstrated that after a rough alignment of the homologous chromosomes to within 300 nm, the lateral components (protein and RNA) are laid down between the two sister chromatids of each chromosome (Fig. 2). This causes the appearance of the leptotene chromosome as undivided in the light microscope. At the same time the central region pre-assembles in the nucleolus and is then transported together with recombination nodules into

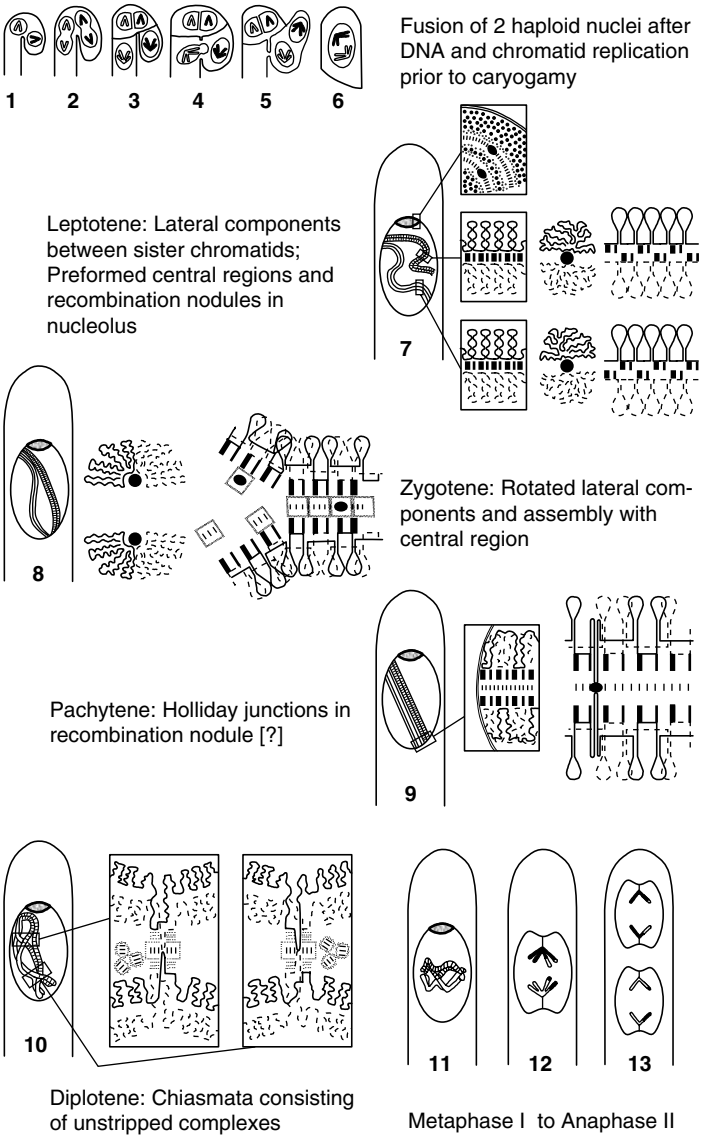

Fig. 2. Formation of the synaptonemal complex and chiasmata during meiosis in an ascomycete (*Neottiella*)

the space between the roughly aligned homologues. The two sister chromatids relocate, so that that the lateral components are positioned lateral to the chromatin of the chromosome. In the pairing fork the central region material is organized alternately on one or the other lateral component, and the synaptonemal complex is completed by attachment of the free lateral