

67 PROGRESS IN BOTANY

Genetics
Physiology
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Ecology

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Contents

Review

Fascination with Chloroplasts and Chromosome Pairing	3
Diter von Wettstein	
1 Biosynthesis of the photosynthetic membrane and chloroplast biogenesis	3
2 Chromosome pairing, mechanism of crossing-over and genome analysis	12
3 Perspectives	23
An Apology and Special Thanks	24
References	24

Genetics

Recombination: Cytoplasmic male sterility and fertility restoration in higher plants	31
Renate Horn	
1 Introduction	31
2 Mechanism of cytoplasmic male sterility	32
2.1 Open reading frames identified as cause of male sterility	32
2.2 CMS-specific proteins and possible functions	33
3 Mechanism of fertility restoration	36
3.1 Genetics and functions of fertility restorer genes	36
3.2 PPR genes function as restorer genes	42
4 Conclusions	45
References	45

Function of genetic material:**From genomics to functional markers in maize** 53

Chun Shi, Gerhard Wenzel, Ursula Frei, Thomas Lübberstedt

Abbreviations 53

1 Introduction 54

2 Structural genomics in maize and rice 55

3 Comparative genomics: synteny between maize and rice 59

4 Functional genomics in maize 61

5 Genomics and biodiversity: functional markers in maize 65

6 Conclusions and outlook 69

References 70

Extranuclear inheritance:**Gene transfer out of plastids** 75

Ralph Bock

1 Introduction: the evolutionary significance of gene transfer 75

2 Gene transfer from the plastid to the nuclear genome 77

2.1 Functional gene transfer from the plastid
to the nuclear genome 792.2 Non-functional gene transfer from the plastid to the
nuclear genome 80

3 Gene transfer from the plastid to the mitochondrial genome 80

3.1 Non-functional gene transfer from the plastid to the
mitochondrial genome 813.2 Functional gene transfer from the plastid to the
mitochondrial genome 834 Gene transfer from the mitochondrial
to the nuclear genome 84

5 Gene transfer from the nuclear to organellar genomes 86

6 Experimental approaches to investigate gene
transfer to the nucleus 877 Mechanisms of gene transfer from the plastid
to the nucleus 90

8 Implications for plastid biotechnology 92

References 94

Molecular cell biology:**Epigenetic gene silencing in plants** 101

Roman A. Volkov, Nataliya Y. Komarova,

Ulrike Zentgraf, Vera Hemleben

1	Introduction	101
2	Molecular mechanisms of gene silencing	102
2.1	Methylation of cytosine and DNA methyltransferases	102
2.1.1	Cytosine methylation	102
2.1.2	DNA methyltransferases	103
2.2	Histone modifications	105
2.2.1	Histones as targets for post-translational modifications	105
2.2.2	Acetylation of histones	106
2.2.3	Methylation of histones	108
2.3	Cross-talk between DNA methylation and modifications of histones	109
2.4	Chromatin remodeling	110
2.5	RNA silencing	113
3	Cellular processes regulated via gene silencing/ chromatin remodeling	117
3.1	Control of developmental processes	117
3.2	Ribosomal DNA transcriptional regulation and nucleolar dominance	119
3.3	Silencing of transposons	121
4	Conclusions and perspectives	123
	References	124

Genetics of phytopathology:**Secondary metabolites as virulence determinants****of fungal plant pathogens** 134

Eckhard Thines, Jesús Aguirre, Andrew J. Foster, Holger B. Deising

1	Introduction	134
2	Secondary metabolism and its biochemical precursors	136
3	Fungal secondary metabolites as phytotoxins and virulence determinants	137
3.1	Polyketides	138
3.1.1	Cercosporin	141
3.1.2	Zinniol	144
3.1.3	Pyriculariol and pyriculariol	144
3.1.4	T-toxin	145
3.1.5	Non-toxic polyketides essential for pathogenicity	145

3.2	Isoprenoids and terpenoids	146
3.2.1	Trichothecenes	147
3.2.2	Ophiobolin A	148
3.2.3	Colletotrichin	149
3.2.4	Fusicoccin	149
3.3	Aromatic compounds and peptides	150
3.3.1	Victorin	150
3.3.2	HC-toxin	151
3.3.3	Tentoxin	151
3.3.4	Fusaric acid	152
4	Regulation of secondary metabolism during pathogenic development	152
5	Concluding remarks	154
	References	155

Plant Breeding:

MADS ways of memorizing winter: vernalization

in weed and wheat	162
Günter Theißen	

Abbreviations:	162	
1	Introduction	162
2	Vernalization in <i>Arabidopsis</i>	164
2.1	The major genes	164
2.2	The central role of <i>FLC</i>	165
2.3	Complex regulation of <i>FLC</i> expression	167
2.4	Role of other <i>FLC</i> -like genes	168
3	Vernalization in winter varieties of wheat	169
4	Evolution of the vernalization requirement	170
5	Future prospects	173
6	Concluding remarks	174
	References	174

Biotechnology:

Engineered male sterility in plant hybrid breeding	178
Kerstin Stockmeyer and Frank Kempken	

1	Introduction	178
2	Natural male-sterility systems in plants	179
2.1	Cytoplasmic male sterility (CMS)	179
2.2	Nuclear male sterility	180

3	Methods of producing male-sterile plants	181
3.1	Selective destruction of tissues important for the production of functional pollen	181
3.2	Changing of levels of metabolites needed for the production of viable pollen	182
4	Strategies for the multiplication of male-sterile lines	183
4.1	Herbicide application for selection of male-sterile plants	183
4.2	Reversible male sterility	183
4.3	Use of maintainer lines	184
5	Conclusion	185
	References	185

Physiology

Membrane turnover in plants	191
Ulrike Homann	

1	Introduction	191
2	Membrane turnover during polarized cell growth	191
2.1	Regulation of exocytosis	192
2.2	Endocytosis during tip growth	192
2.3	Coupling between exo- and endocytosis	194
3	Guard cell functioning and tension modulated exo- and endocytosis	195
3.1	Exo- and endocytosis during osmotically driven surface area changes	196
3.2	Surface area regulation and membrane tension	197
3.3	Role of tension modulated exo- and endocytosis	198
4	Constitutive exo- and endocytosis	200
4.1	Constitutive turnover of K ⁺ channels	200
4.2	Cycling of PIN proteins	200
	References	202

Besides water:

Functions of plant membrane intrinsic proteins and aquaporins	206
Ralf Kaldenhoff	

1	Aquaporins in plants	206
2	Plant aquaporins and water transport	207

2.1	Characterization of aquaporin function in <i>Xenopus</i> oocytes	207
2.2	Other single cell systems	208
2.3	Plant protoplasts for functional analysis of aquaporins	209
3	Effects of aquaporin water conductivity in plants	209
4	Permeability to small non-ionic molecules	210
4.1	Glycerol	210
4.2	CO ₂	211
4.3	NH ₃	214
4.4	Boron	215
	References	215

New insight into auxin perception, signal transduction and transport 219

May Christian, Daniel Schenck, Michael Böttger,
Bianka Steffens, Hartwig Lüthen

1	Growth stimulation: the classical effect of auxin	219
2	Auxin receptors	219
2.1	Auxin binding protein 1 (ABP1)	220
2.1.1	3-D structure of ABP1	221
2.1.2	ABP1 mutants	223
2.1.3	Indirect evidence for ABP1 as a growth relevant receptor at the single cell level	224
2.2	Other receptor candidates	225
2.2.1	ABP57	225
2.2.2	Receptor-like kinases (RLKs)—novel players in auxin perception?	226
3	Auxin-induced gene expression	226
3.1	Transcriptional regulators	227
3.2	Protein degradation—an essential step in auxin signalling	229
3.3	How does auxin regulate gene expression?	232
4	Auxin-upregulated genes and their functions	233
4.1	Plasma membrane ATPase	233
4.2	K ⁺ -inward channels	233
4.3	Others	234
5	Polar auxin transport	235
5.1	How does auxin efflux work?	236
5.1.1	The PINs	236
5.1.2	ABC transporters as efflux carrier candidates	238
5.2	How does auxin influx work?	238
5.3	Auxin transport depends on K ⁺	239

6	Prospects	240
	References	240

New insights into abiotic stress signalling in plants 248

Margarete Baier, Andrea Kandlbinder, Karl-Josef Dietz, Dortje Golldack

1	Light and elevated temperature	248
1.1	Saturating light intensities and moderate excess light	249
1.2	Excess high light	250
2	Perception, signalling and transcriptional regulation in response to UV-B and ozone	253
2.1	Ozone and UV-B induced gene expression	255
3	Signalling and transcriptional regulation in response to drought, cold, and salt stress	257
3.1	bZIP transcription factors responsive to abiotic stress treatment	259
3.2	Stress-induced regulation of NAC transcription factors and zinc finger proteins	261
4	Heavy metal toxicity and tolerance	262
5	Perspectives	266
	References	266

**Genetically transformed root cultures – generation,
properties and application in plant sciences** 275

Inna N. Kuzovkina and Bernd Schneider

1	Introduction	275
2	Some genetic and historical remarks	276
3	Transformation process and cultivation conditions	277
4	Morphological and physiological aspects of hairy root cultures	280
5	Secondary metabolites from hairy root cultures	282
5.1	Secondary products extracted from hairy root tissue	282
5.2	Secondary products in hairy root exudates	286
6	Use of hairy root cultures in biosynthetic and metabolic studies	288
6.1	Biosynthesis	288
6.2	Biotransformation	290
7	Hairy roots for biotechnological production of secondary metabolites	291
7.1	“Wild-type” hairy roots	291
7.2	Conventional strategies to affect production of secondary metabolites	292
7.3	Genetic engineering of secondary metabolite biosynthesis	295

8 Hairy roots for genetic improvement and clonal propagation 297
 8.1 Experimental systems 297
 8.2 Plant regeneration and clonal propagation 298
 9 Proteins from hairy root cultures 300
 10 Phytoremediation and environmental detoxification 301
 11 Conclusions 302
 References 303

Molecular chaperones—holding and folding. 315

Christoph Forreiter

1 Introduction 315
 2 Molecular chaperones and other elements of the stress response . . . 316
 3 How do molecular chaperones work? 317
 4 Structure and function of the different chaperone classes 320
 4.1 Hsp70 (DnaK) chaperone machine 320
 4.2 Hsp60 (GroE) chaperone system—the chaperonins 323
 4.3 The Hsp90 (HtpG) chaperone system 326
 4.4 Hsp 100 (Clp) family 329
 4.5 Hsp20 (Ibp) family 331
 4.6 Other proteins with chaperone function 334
 5 Résumé 336
 References 336

Systematics

Recent progress in floristic research in Korea 345

Chong-Wook Park

1 Introduction 345
 2 Taxonomic diversity and endemism 346
 3 Floristic affinities 348
 4 Historical background of floristic research 348
 5 Herbarium collections 349
 6 Current status of floristic research 350
 7 Recent progress: the New Flora of Korea Project 353
 8 Conclusion 353
 References 354

Recent progress in systematics in China	361
Jinshuang Ma	
1 Introduction	361
2 Basic information	361
2.1 Herbaria	361
2.2 Library	362
2.3 Collections	362
2.4 Research	363
3 Publications	363
3.1 Flora	363
3.1.1 Flora Reipublicae Popularis Sinicae (FRPS)	363
3.1.2 Flora of China	364
3.1.3 Local flora of China	364
4 Journals	365
5 Papers, monographs and revisions	366
5.1 Paleobotany	366
5.2 Ferns	366
5.3 Gymnosperms	366
5.4 Araliaceae	367
5.5 Asteraceae	367
5.6 Brassicaceae	367
5.7 Burmanniaceae	368
5.8 Celastraceae	368
5.9 Cornaceae	368
5.10 Corsiaceae	368
5.11 Cyperaceae	368
5.12 Euphorbiaceae	368
5.13 Gesneriaceae	369
5.14 Hamamelidaceae	369
5.15 Labiatae	369
5.16 Lauraceae	369
5.17 Orchidaceae	369
5.18 Paeoniaceae	370
5.19 Phrymataceae	370
5.20 Poaceae	370
5.21 Primulaceae	370
5.22 Ranunculaceae	370
5.23 Rhamnaceae	370
5.24 Schisandraceae	371
5.25 Scrophulariaceae	371

5.26 Styracaceae 371
 5.27 Umbelliferae/Apiaceae 371
 5.28 Zingiberaceae 371
 6 Floristic work 371
 7 Angiosperm system 372
 8 Higher plants of China 372
 9 New developments and trends 375
 10 Conclusion 375
 References 375

Ecology

Structural determinants of leaf light-harvesting capacity and photosynthetic potentials 385

Ülo Niinemets and Lawren Sack

1 Introduction 385
 2 Structural limitations of leaf light-harvesting efficiency 386
 2.1 Tissue-Scale Limitations of Light Interception 387
 2.2 Thickness (I) and Inclination Effects on Light Harvesting . . . 388
 2.3 Light Capture Dependence on the Flatness of the Leaf Lamina 390
 2.4 Modification of Light Harvesting by Leaf Size 391
 3 How Structure Determines Leaf Photosynthetic Capacity 392
 3.1 Photosynthetic Capacity in Relation to Leaf Tissue Types and Thickness 392
 3.2 Does the Efficiency of Intraleaf Diffusion Vary for Leaves of Differing Structure? Role of D and M_A 393
 3.3 Structural Determinants of Leaf Water Transport Efficiency 396
 3.4 Leaf Size and Shape Effects on Photosynthesis: Only a Matter of Leaf Energy Balance? 398
 3.5 A Further Linkage Between Leaf Size and Amass 400
 3.6 Variation in PNUE due to Leaf Structure 401
 4 Structural Acclimation of Leaf Photosynthesis to Environment 402
 4.1 Adjustments to Light 402
 4.2 Modifications due to Varying Moisture Supply 404
 5 Age-Dependent Changes in Leaf Photosynthetic Capacity 405
 5.1 Increases in the Functional Activity in Young Leaves: Biochemical Versus Structural Limits 405
 5.2 Time-Dependent Deterioration of Leaf Physiological Activity in Mature Leaves and During Senescence 406

6	Outlook: a Network of Coordinated Leaf Traits	407
7	Conclusions	411
	References	412

**Recent trends in plant-ecological modelling: Species dynamics
in grassland systems** 420
Eckart Winkler

1	Introduction	420
2	Markov and Matrix Models	421
	2.1 Markov Models of Vegetation Change	421
	2.2 Matrix Models of Population Development	423
3	SEIB Models	425
4	Analytical Models: Explanation of Coexistence as an Example	428
	4.1 Coexistence: The Basic Problem	428
	4.2 Competition and Coexistence	428
	4.3 Seedling Lottery	429
	4.4 Plant Performance under Trade-offs	430
	4.5 Limits of Analytical Models	432
	4.6 Theory and Empirical Work	433
5	Outlook	435
	References	436

**Atmospheric carbon dioxide enrichment effects
on ecosystems – experiments and the real world** 441
Martin Erbs and Andreas Fangmeier

1	Introduction	441
2	Experimental Manipulation of Atmospheric CO ₂	443
	2.1 The History of CO ₂ Enrichment Experimentation	443
	2.2 FACE Technology	444
	2.3 Deviations From the Real World Despite FACE Technology	446
	2.4 The Hohenheim Mini-FACE System	448
3	Conclusions	453
	References	455

**Quaternary Palaeoecology:
Central and South America, Antarctica
and the Pacific Ocean Region** 460
Burkhard Frenzel

1	The Problem of Exactly Dating Palaeoecological Processes	460
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2 Upper Pleistocene and Holocene Palaeoecology of Central America and the Surrounding Islands 461

3 Quaternary Glaciation History of South America and the Problem of the Younger Dryas Equivalent in the Southern Hemisphere 463

4 South American Upper Quaternary Vegetation History 465

5 Upper Quaternary Palaeoecology of Antarctica 469

6 Palaeoecology of the East Asian Monsoon Region 470

7 Upper Quaternary Palaeoecology of Australia and of the Pacific Islands 473

References 476

Biodiversity experiments – artificial constructions

or heuristic tools? 486

Carl Beierkuhnlein and Carsten Nesshöver

1 Introduction 486

2 Some Philosophical and Basic Aspects of Ecology and Diversity Research 490

3 Hypotheses and Concepts Addressing Biodiversity and Ecosystem Functioning 494

4 Experiments in Community Ecology and Biodiversity Research ... 497

4.1 The Role and the Value of Experiments 497

4.2 Historical Experiments 498

4.3 Modern Experiments 499

5 Advances and Frontiers – Insights into Mechanisms and Processes 503

5.1 The Biodiversity–Productivity Relationship 503

5.2 Plant Species Diversity, Invasibility and Community Dynamics 507

5.3 Influences of Species Diversity on Element Cycling 510

6 Developments in Methods and Approaches 512

6.1 Functional Types, Traits and Attributes 512

6.2 Individuality, Assembly Rules and Non-Stochastic Extinction 515

6.3 The Importance of Temporal and Spatial Scales 516

6.4 Separation of External Factors and Intrinsic Factors – Multi-Site Experiments and the Connection Between Experimental and Observational Studies 518

7 Outlook 520

References 524

Resource allocation in clonal plants	536
Markus Lötscher	
1 Introduction	536
2 Foraging Behaviour	537
2.1 Vertical versus Horizontal Growth	537
2.2 Lateral Spread	538
2.3 Branching	539
3 Clonal Integration	540
3.1 Extended Support of New Ramets	541
3.2 Cost/Benefit	542
3.3 Division of Labour	543
3.4 Resource Transport and Sectoriality	545
3.4.1 The Model Plants <i>Glechoma hederacea</i> and <i>Trifolium repens</i>	545
3.4.2 Complexity of the Clone	547
3.4.3 Genetic Variation	548
4 Importance of Storage Pools	549
5 Simulation Models	551
6 Conclusions	552
References	553
Subject Index	562

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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

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 CO₂-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.

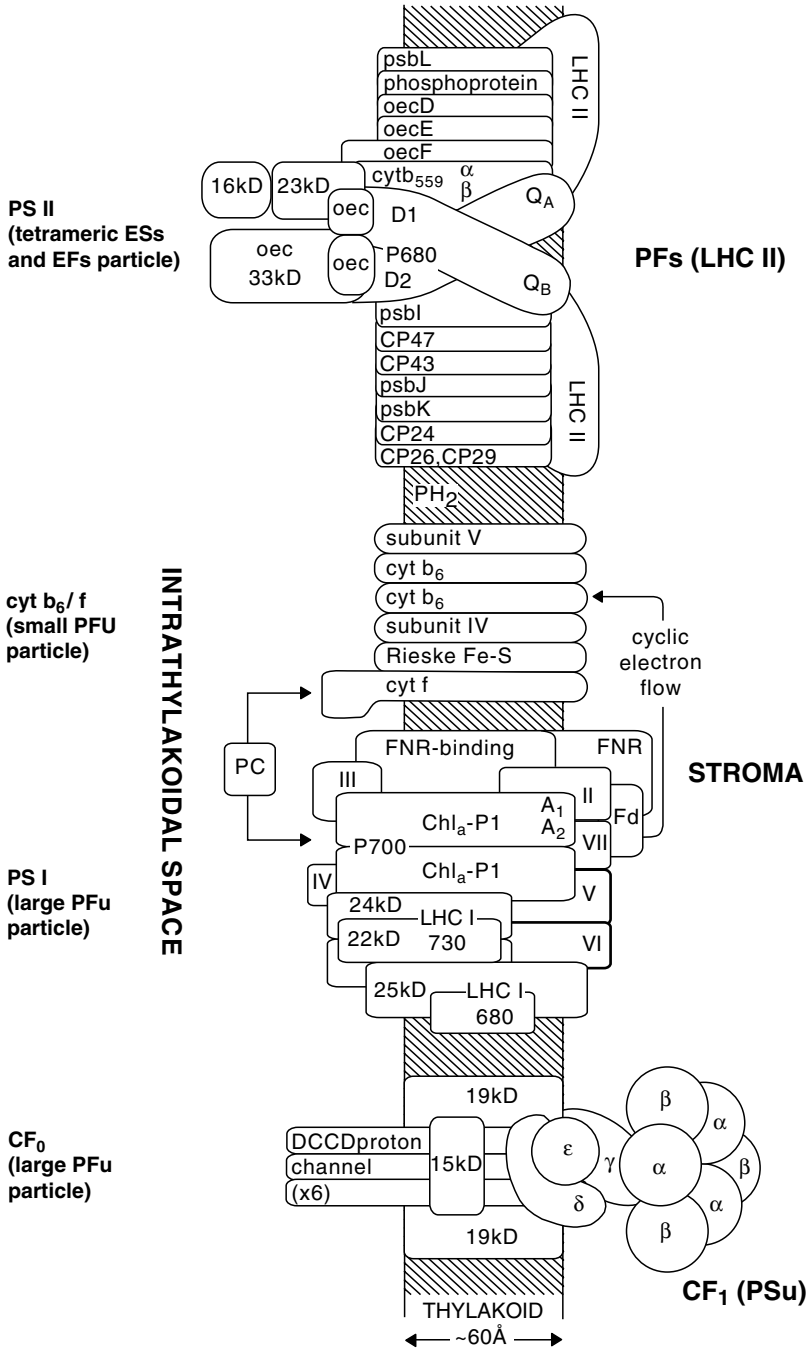
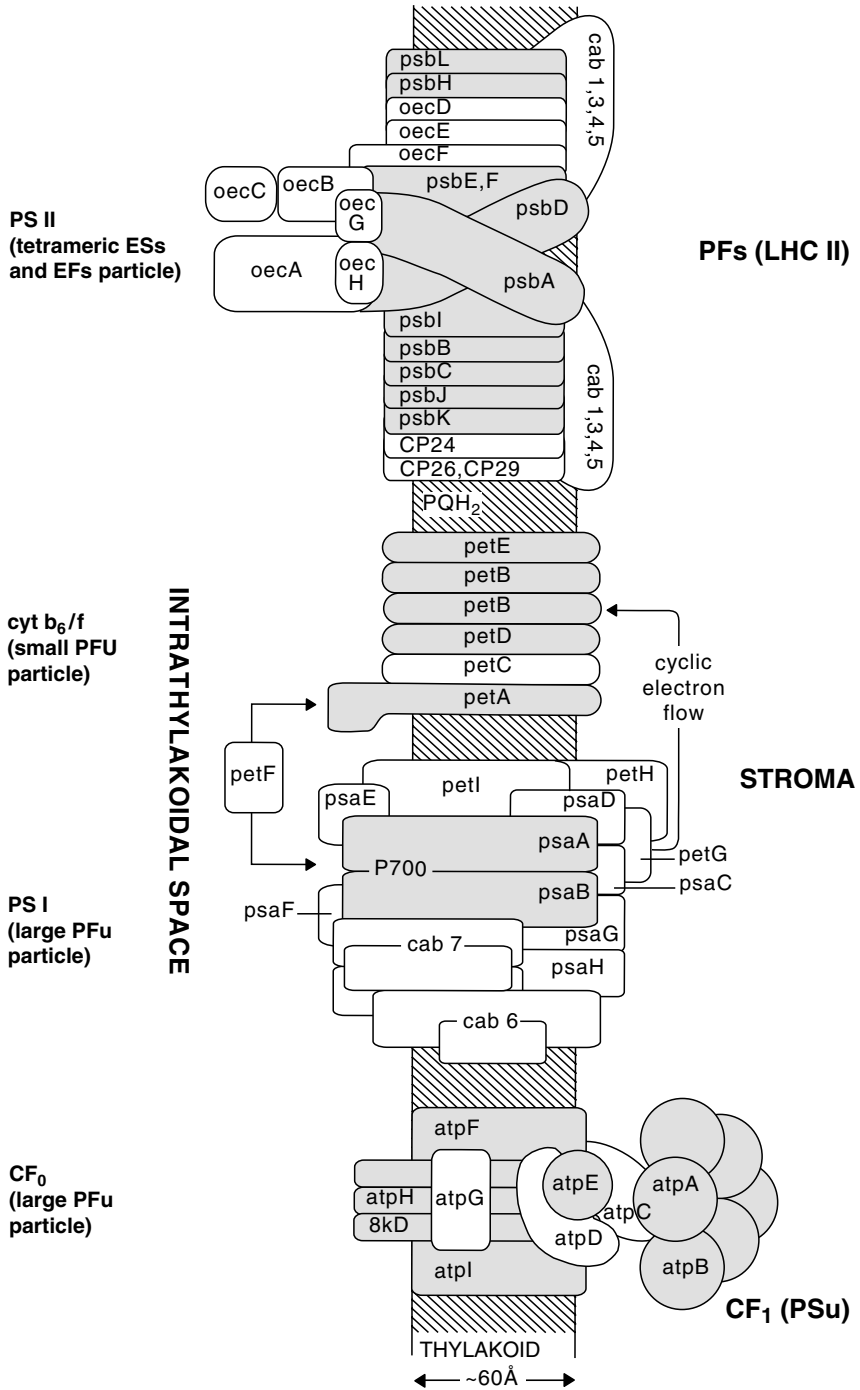


Fig. 1a, b. Model of the photosynthetic membrane showing the polypeptide components of the major complexes. The site of coding is indicated by shading (chloroplast DNA) or is unshaded (nuclear gene). (Modified from Simpson and von Wettstein 1989)



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 glycine 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, Mg²⁺ and protoporphyrin. This opened the way to learn more about how the metal ion is incorporated into the porphyrin ring. The insertion of Mg²⁺ 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 Mg²⁺. 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 Mg²⁺ 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 Mg²⁺ 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 Mg²⁺ into protoporphyrin IX (Fodje et al. 2001). It provides the starting point for clarifying the mechanism by which Mg²⁺ 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 Mg²⁺ into protoporphyrin 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 Mg²⁺.

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

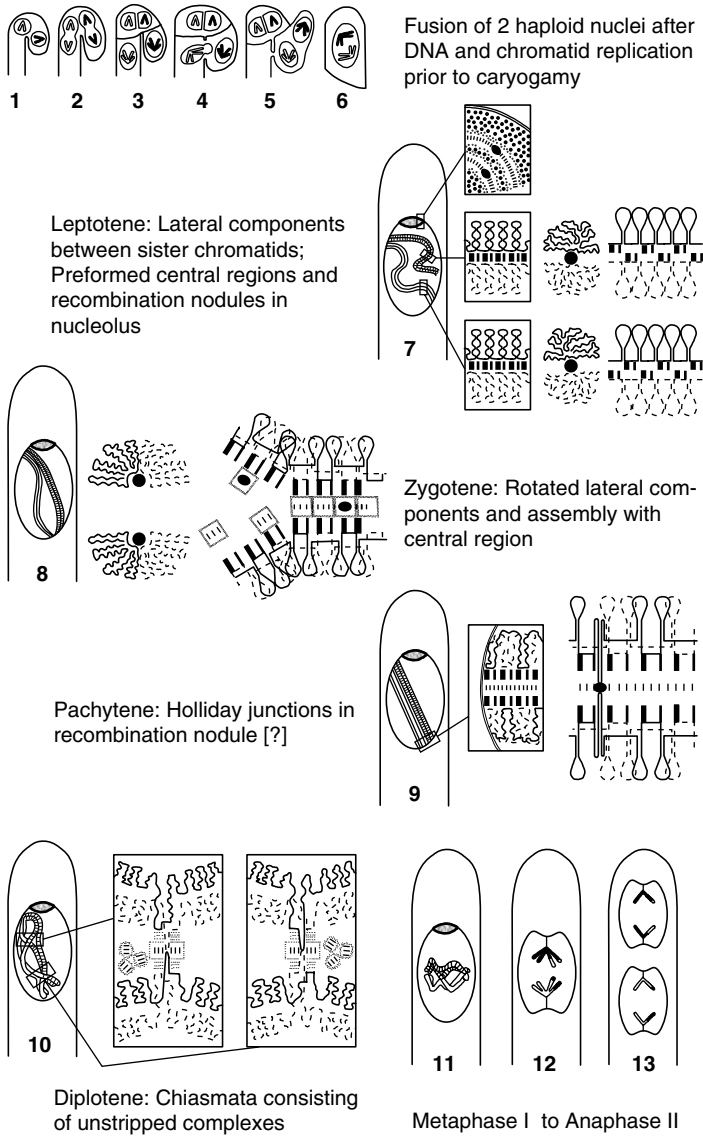


Fig. 2. Formation of the synaptonemal complex and chiasmata during meiosis in an ascomycete (*Neottiella*)

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