EXPERIENTIA

Volume 39/No. 7 Pages 687-804 July 15, 1983

Reviews

Genetic engineering in the adaptation of plants to evolving human needs

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Genetic engineering is a technique for isolating individual genes from plants and for multiplying - cloning - these genes in bacteria and yeast. This makes it possible to determine the structure and the function of individual genes. It is hoped that procedures can be worked out to incorporate into the genomes of crop plants useful or even improved genes and thereby aid in the continuous adaptation of agricultural crops to human needs. Before describing the present state of gene technology as related to plant breeding, I would like to briefly emphasize that genetic engineering is a technique which is added to our arsenal of existing breeding techniques and will be complementing but not replacing the conventional breeding procedures.

Our major crop plants, rice, wheat, maize, barley, soybeans but also tobacco, sugar beets and rape have been bred by man - some more than 4000 years ago; some a few hundred years ago. At first, plant breeding was exercised by selecting bigger grain, non-shattering ears and diesease resistant plants from the variations produced in nature by crosses and mutation. Records of artificial fertilization of the date palm in Assyria date back to centuries before the time of Christ, but we have no record whether the genomes from *Triticum monococcum, Triticum searsii* and *Triticum tauschii* were combined into the 42 chromosomic bread wheat by artificial pollination. Perhaps artificial pollination also accounts for the amphidiploid tobacco, which may have been produced in South America in pre-Colombian times by hand pollination of *Nicotiana sylvestris* with pollen from *Nicotiana tomentosiformis* and accidental chromosome doubling in the hybrid. To this day plant breeding is mostly carried out empirically by crossing promising lines with each other and by selecting plants with improved yield, disease resistance and better climatic adaptation.

Especially since the end of the last century our knowledge of genetics and biochemistry has improved steadily and thereby given us increased opportunities to apply this knowledge to plant breeding in a rational manner[29]. We can cite a few examples to illustrate this. Hybrid vigor was discovered by Joseph Gottlieb Kölreuter in Germany in 1760 and was also recommended by him as a means of increasing yield. In 1908, after Carlsberg Laboratory alumnus Wilhelm Johannsen had clarified the theoretical basis for the distinction of pure genetic lines versus populations and coined the word gene for Mendel's units, George H. Shull in the United States produced by inbreeding pure lines of maize and established that single and double hybrids of these lines exhibit superiority in yield as a consequence of gene heterozygosity. Research at universities and breeding work at seed producing industries has led to the first commercial hybrid corn varieties in the early 1930's. In 1935 an average of 1.5 tons of maize was harvested per hectare and less than 1% of the area in the United States of America was planted with the new hybrid varieties. In 1968, 5.1 tons per hectare were harvested and 90% of the area was planted with hybrid maize. This enormous increase in yield is due to the combined results of plant breeding and improved agricultural practice. Further increases have been achieved over the last 15 years.

While the success of maize breeding examplifies the importance of suitable new combinations of genes, the triploid monogerm sugar beet introduced in the 1960's illustrates the use of a morphological mutation and polyploidy to adapt crop plants to modern society[18]. Originally, seeds of the sugar beet were produced in clusters on the flowering stem and when sown each cluster gave rise to several seedlings, which had to be singled by hand. In the monogerm lines, only 1 seedling is produced from each cluster and the labor effort to cultivate 1 hectare could be reduced from 290 man-hours to 13. In his thesis from the Carlsberg Laboratory, Øjvind Winge proposed in

Updated version of lectures presented at the 10th Anniversary of the Friedrich Miescher-Institut, Basel, March 21, 1980, and at the Symposium 'Biologie et avenir humain', Institut de la Vie, Lausanne, October 11, 1980.

1917 that species hybridization followed by chromosome doubling explains polyploid evolution. In the decades to follow, experimental work proved this to be correct and also that this is especially prevalent among our crop plants, e.g. tobacco, wheat, rape. The triploid sugar beet containing 3 sets of the chromosome complement instead of the normal 2 illustrates that increased yield can be obtained by increasing the number of genes in a balanced fashion and by providing in polyploids additional possibilities for heterozygosity at individual gene loci.

Oilseed rape *(Brassica napus)* with 38 chromosomes, is a crop plant which has arisen as a species hybrid of wild cabbage *(Brassica oleracea)* containing 18 chromosomes in its somatic cells and turnip *(Brassica campestris)* containing 20 chromosomes in the vegetative cells. Resynthesis of this amphidiploid oilseed rape by crossing the parent species and subsequent chromosome doubling or by first doubling the chromosomes of the parent species and thereafter adding them together in a fertile hybrid has been used extensively over the last 45 years to improve disease resistance, yield, and quality in oilseed rape. This crop plant also provides us with an example of the use of biochemical mutations in the improvement of the crop. After the discovery in the early 1960's by Canadian scientists that erucic acid can be eliminated by 2 recessive gene mutants, this fatty acid which negatively affects the technical quality of the oil and constitutes an unhealthy component of margarine has been eliminated from oilseed rape[1].

Chromosomal engineering was first successfully practised in wheat in the 1950's by using single chromosome addition or substitution lines. By crossing wheat with the distantly related weed, twitch *(Agropyron elongatum)*, and by appropriate selection, Ernest Sears was able to transfer resistance towards leaf rust fungi from the weed to bread wheat[53]. A majority of today's cultivated bread wheats contain genes from two twitch chromosomes which provide excellent disease resistance to the crop.

The tools of genetic engineering[2,17,30,33]

Figure 1 presents the principle of complementarity by which the genes govern the synthesis of proteins. The principle is built on the base pairing rule. The genetic information of the deoxyribonucleic acid (DNA) double helix is written with the 4 bases A, T, G, C (adenine, thymine, guanine, cytosin) in such a way that A in one strand always pairs by hydrogen bonding with T in the other (A–T) and G always pairs with C (G–C). Three bases form a codon containing the information for specifying 1 amino acid in a protein. When the DNA molecule is replicated into 2 identical daughter double helices, its 2 strands separate locally and 2 new strands are synthesized from nucleotides by the enzyme DNA polymerase. The sequence of the nucleotides in the new strand is conserved by the base pairing rule with the old strand serving as template.

When the genetic code is converted into a polypeptide chain, the enzyme RNA polymerase transcribes with the aid of the base pairing rule the coding strand of the DNA double helix into a complementary messenger RNA molecule (ribonucleic acid molecule). Instead of T, however, the base U (uracil) is incorporated in this case. The translation on the ribosomes again

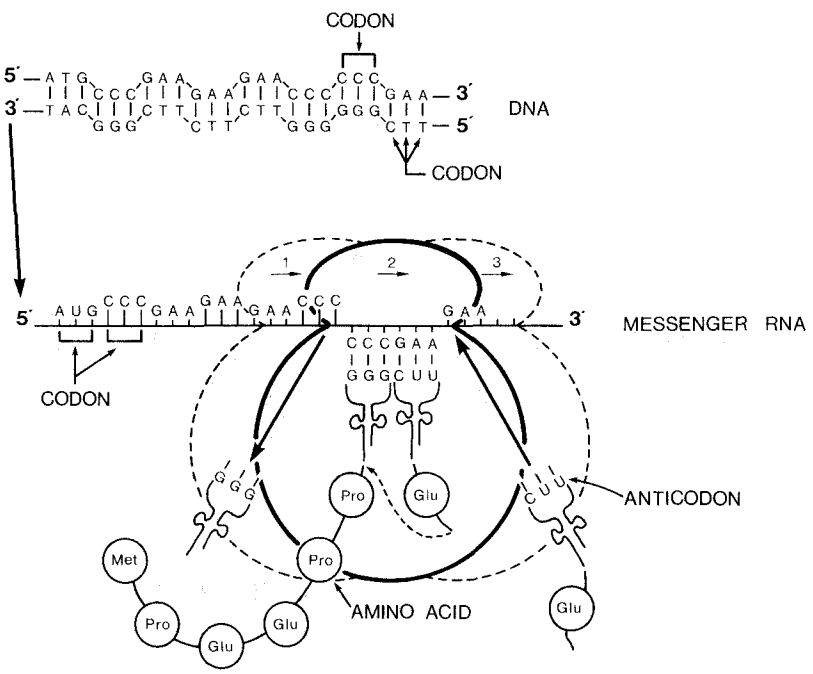


Figure 1. The principle of complementarity and the base pairing rule of nucleic acids govern the synthesis of proteins.

takes place according to the base pairing rule. To every codon in the messenger RNA fits an adaptor molecule, a clover leaf shaped transfer RNA molecule, which at one end displays the anticodon and at the opposite end carries the amino acid belonging to the anticodon in question. With 4 bases and a triplet code there are 64 possible codons. These correspond to 61 anticodons carried by transfer RNA molecules, while 3 codons are stop codons telling the ribosome that no further amino acids should be added.

The synthesis of all polypeptide (protein) chains starts with the amino acid methionine, which is coded in DNA by TAC, transcribed to AUG and recognized by the anticodon UAC. The most frequent of the 20 amino acids in the storage protein hordein of the barley grain, to be discussed below, is glutamic acid (35%) and proline (23%). Proline can be coded for by GGG in DNA, which is transcribed into CCC in the messenger RNA and recognized by the anticodon GGG. Glutamic acid can be coded by CTT, which is transcribed into GAA and recognized by transfer RNA molecules with the anticodon CUU. There is room in the ribosome for 2 transfer RNA molecules side by side. The growing polypeptide chain is attached to the left transfer RNA molecule, the amino acid to be added next at the right one. When this amino acid is bonded to the growing chain the ribosome moves simultaneously 1 codon towards the right. The left transfer RNA molecule slips out and the position on the right hand side becomes vacant to be filled with the appropriate transfer RNA molecule carrying the next amino acid to be added. As the ribosome moves along the messenger RNA, amino acids are added step by step with a speed of about 15 amino acids per second.

Moving from the molecular to the submicroscopic level in figure 2, we can sketch the biosynthesis of proteins in a plant cell as follows: The single DNA molecule spanning a chromosome from end to end is wound around the histone proteins forming little beads called nucleosomes. The lined up nucleosomes make up a fiber which is further folded or coiled into the chromosome visible in the light microscope. Portions of the DNA double helix in the chromosome are transcribed into messenger RNA molecules which then are transported from the nucleus into the cytoplasm for translation on ribosomes. Many messenger RNA molecules are translated on ribosomes which become attached to the membranes of the endoplasmatic reticulum as drawn in profile in figure 2. Simultaneously with the translation, the growing polypeptide chain is transferred across the membrane and discharged into the lumen of the membrane labyrinth and thereby separated, compartmentalized away from the groundsubstance of the cytoplasm. At first the 2 subunits of the ribosome bind to the 5' end of the messenger RNA and the N-terminal part of the polypeptide, a signal polypeptide is made. This signal peptide recognizes certain receptors in the membrane and attaches the ribosome to the membrane. As the polypeptide grows and is fed into the lumen of the endoplasmatic reticulum, a proteolytic enzyme cuts

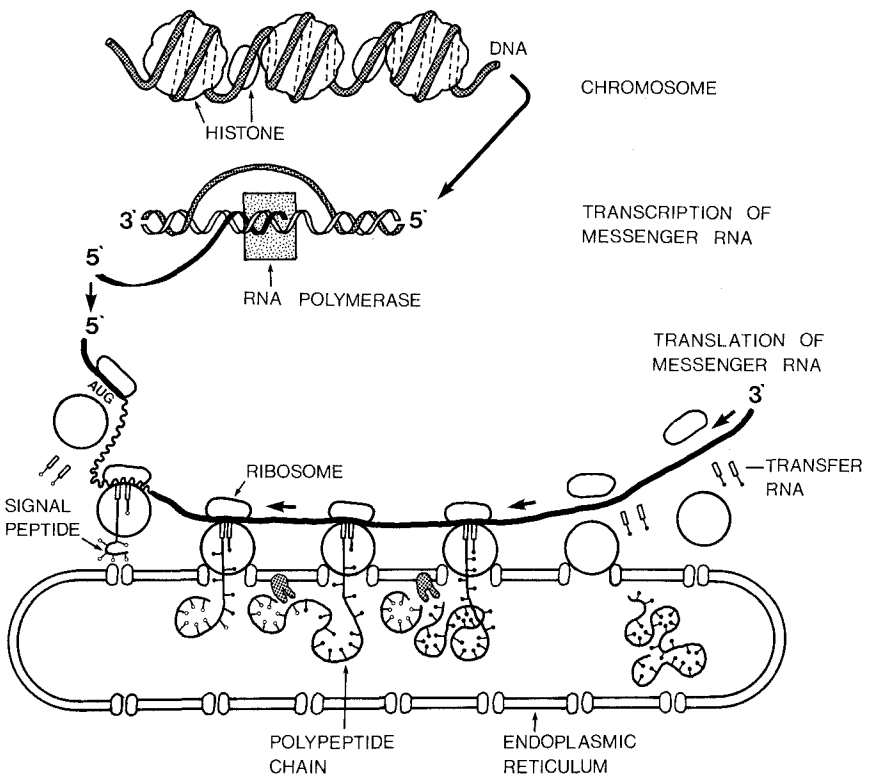


Figure 2. Diagram of the biosynthesis of proteins in a plant cell.

off the signal peptide as this has done its duty and is no longer needed for further transport or function of the polypeptide made.

The processes described with the aid of figures 1 and 2 can all be achieved in the test tube with components isolated from bacteria, plant cells or animal cells. Thus, pieces of DNA can be replicated into identical daughter molecules with DNA polymerase. If radioactive nucleotides are present in the medium, radioactively labeled daughter molecules can be made and traced in further experiments. Also messenger RNA can be transcribed from an appropriate piece of DNA in the test tube and the messenger RNA can be translated on isolated ribosomes with transfer RNA molecules into polypeptide chains. Using radioactive nucleotides we can label the messenger RNA and with radioactive amino acids we can tag the newly synthesized protein chains so that they are easily traced in new experiments.

The base pairing rule can be exploited to study like and unlike nucleic acid molecules: double stranded DNA can be separated into single strands by heating or treatment with alkali. Renaturation of the complementary strands into the double helix or 'hybridization' - that is, association of single strands originating from different cells, organisms, species - are easily achieved by cooling or acidification. A piece of single stranded DNA affixed to a solid support can catch and select a complementary strand from other preparations passed over it under renaturing conditions. Likewise one can collect messenger RNA molecules with the aid of the complementary strand of its gene. Finally, an enzyme produced by a chicken tumor virus was discovered which can reverse the transcrip-

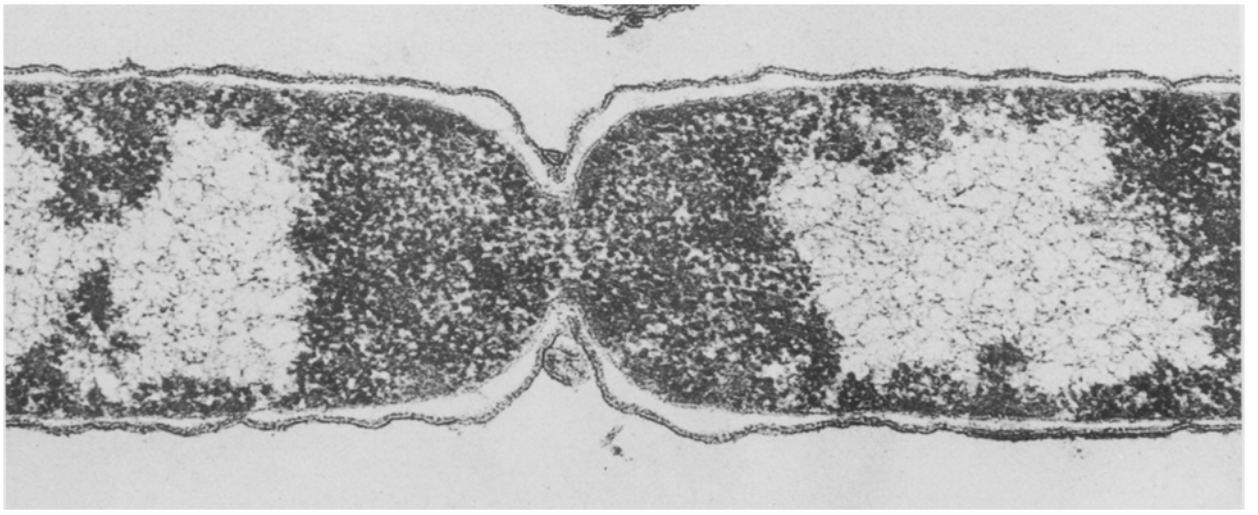


Figure 3. Thin section through a bacterium *(Salmonella typhimurium)* in division as viewed in the electron microscope. The threads visible in the light areas are portions of the large DNA double helix of the circular bacterial chromosome. The numerous small dark spheres are the ribosomes. × 80,000.

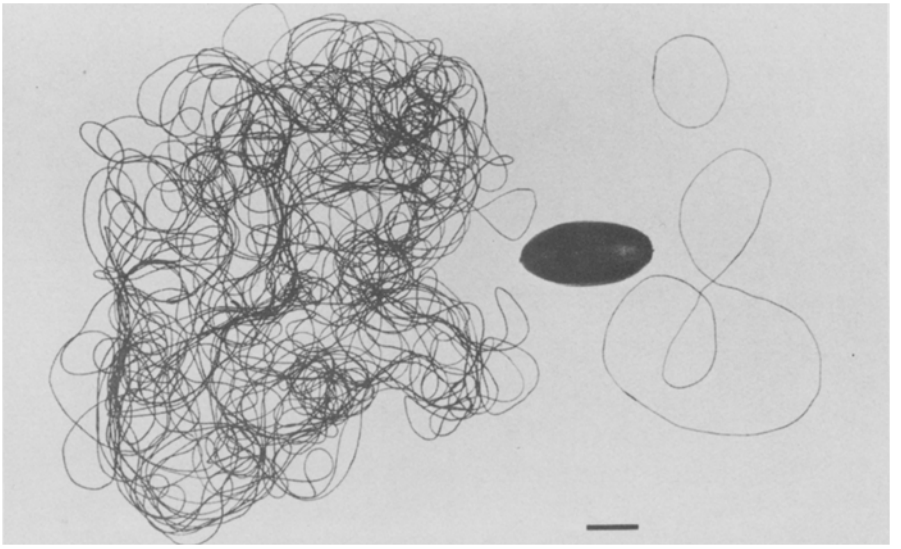


Figure 4. Model of the genome of an *Escherichia coli* cell at a scale of 10.000:1 (according to Arber[2]). If a bacterial cell is magnified 10.000 times it will have the size of an acorn 2 cm long and 1 cm in diameter. Its DNA chromosome will then be a 14 m long circularly closed thread with a diameter of 0.02 mm. The model illustrates that the chromosome has to be tightly folded to fit into the bacterial cell. Bacteria frequently shelter additional smaller or larger DNA circles, plasmids of the size depicted above the acorn. In contrast to the chromosomes they can occur in large copy numbers per cell. Bar = 1 cm.

tion process depicted in figure 2. From a single stranded primer, base-paired to the 3' end of the messenger RNA, this reverse transcriptase synthesizes a single DNA strand complementary to the messenger RNA molecule. After removal of the messenger RNA molecule by alkali treatment, the single DNA strand can be made into a double helix with DNA polymerase. In that way a gene can be synthesized from its messenger RNA.

Bacteria store their genetic information in circular chromosomes consisting of double helix DNA without nucleosome structure. Their genome is illustrated in figures 3 and 4. The 1.4 mm long *Escherichia coli* chromosome consists of about 4×10^6 base pairs. An average sized protein has something like 333 amino acids which would require 999, i.e. roughly 1000 base pairs for its gene. The *E. coli* chromosome can be estimated in this way to contain in the order of 4000 genes. We can see from figure 4 that the DNA plasmids found in the bacteria in addition to the chromosomes are much smaller with a coding capacity for a few genes only.

A yeast cell contains 3 types of DNA molecules: a large linear molecule with nucleosome structure in each of its 17 chromosomes in the cell nucleus; the 25-μm-long circular and naked DNA molecules of the mitochondria; and plasmids such as the 2-μm-long circular plasmid (figure 5) found in about 100 copies in addition to the chromosomes in the nucleus. The chromosome set of yeast contains almost 4 times more base pairs (15×10^6) than the single *E. coli* chromosome thus giving room for about 15000 genes. In the 75000 base pairs of the mitochondrial DNA about 30 genes are known and in the about 6000 base pairs of the 2-μm plasmid a few genes have been identified.

A higher plant cell, of barley for instance, contains 4 types of DNA molecules: The giant linear molecules of the 7 chromosomes in the nucleus contain 5×10^9 base pairs corresponding to some million genes. The naked DNA of the chloroplast is a closed circular molecule with a size of about 134000 base pairs. There are several of these molecules in each of the 20–100 chloroplasts or plastids of the cell. Also mitochondrial DNA in higher plants consists of circular molecules and then there is an as yet unknown number of plasmids in the nucleus and the cytoplasmic organelles.

Genetic transformation is the uptake of isolated DNA by a cell and the permanent incorporation of such DNA into the genome of the cell. A bacterium like the one depicted in figure 3 can be readily transformed with plasmids of the type depicted in figure 4, if the relatively thin bacterial cell membrane is made permeable for DNA molecules with high concentrations of calcium chloride and if the presence in the cell of major nucleic acid degrading enzymes has been eliminated by mutations. The identification and selection of the transformed cells is carried out with genetic markers. Thus the recipient cell typically requires an amino acid for growth and is sensitive towards an antibioticum. The donor DNA then contains the wild type gene which, after incorporation and expression in the recipient cell, permits it to grow without the amino acid supplement. The gene can remain on the plasmid or it can be transferred into the chromosome by recombination. Retained sensitivity to the antibioticum in the transformed cells serves as a control against contamination. Alternatively the donor DNA contains a gene supplying resistance towards the antibioticum and the resistance is used to select for genetic transformants among the recipient cells.

Yeast cells are surrounded by relatively thick walls (fig. 6). These have to be digested away with wall degrading enzymes to yield more or less naked proto-

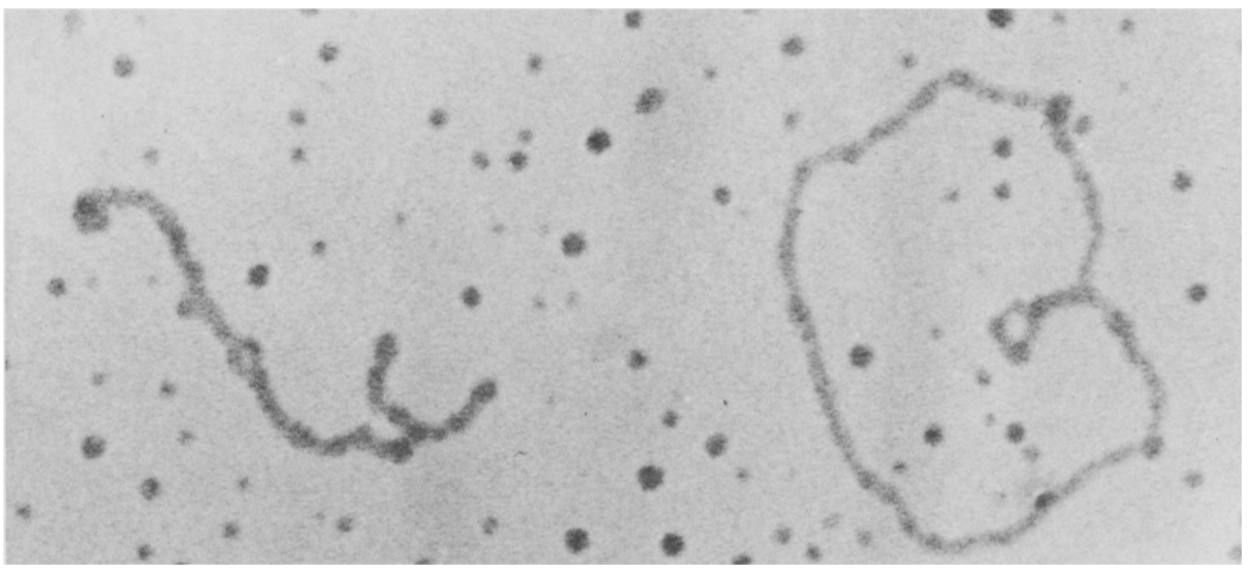


Figure 5. Electron microscopic picture of 2-μm DNA plasmids of yeast in open circular and super-coiled (below) form[59]. × 150,000.

plasts (fig. 7) which can be induced with polyethylene glycol and calcium chloride to take up DNA molecules like the plasmids depicted in figure 5. In appropriate growth media the protoplasts can subsequently regenerate a new cell wall and then be screened for genetic transformants.

The thick cellulose walls of higher plant cells (fig. 8) can also be removed by wall degrading enzymes and in a number of cases regeneration of the produced protoplasts into cells and whole plants is possible. So far, protoplast regeneration in our major crop plants such as wheat or soybeans has not been achievable. Likewise, genetic transformation of higher plant protoplasts with naked DNA has to be worked out. On the other hand, *Agrobacterium tumefaciens* causes crown gall tumors in plants such as tobacco or petunias by transfer of a plasmid and its partial incorporation into the host chromosomes. This is a typical case of genetic transformation of higher plant cells. By deletion from the plasmid of the genes producing the hormones which give rise to the undifferentiated tumor growth of the cells and by exploiting the crown gall bacterium as a vehicle to introduce this modified plasmid into the plant cell, healthy tobacco plants with the plasmid in one of its chromosomes have been produced[43]. These plants synthesize

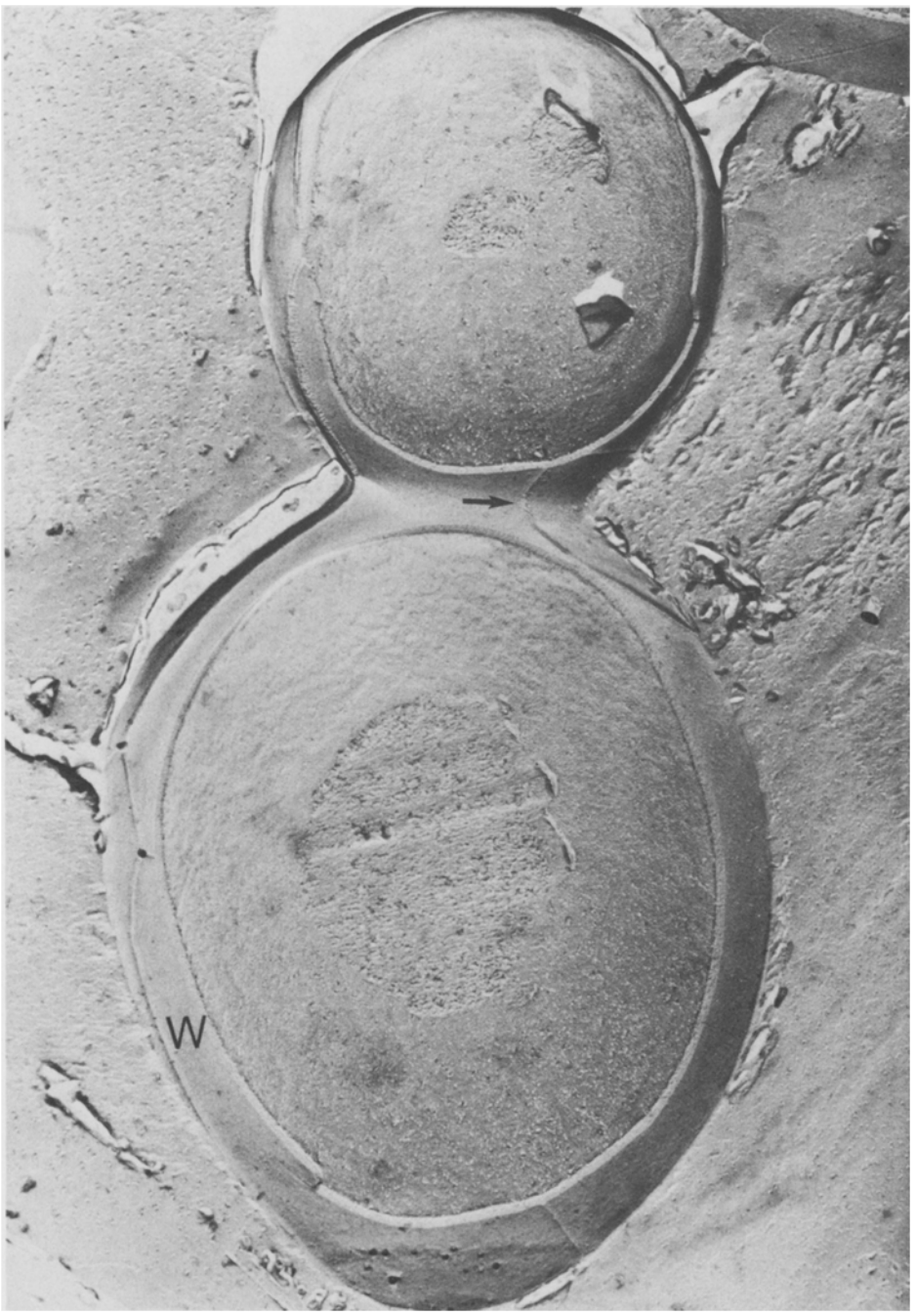


Figure 6. Picture of a yeast cell in division under the electron microscope[59]. The cell has been fractured in the frozen state and the three-dimensional impression of the fracture faces is obtained by evaporation of the ice along the surface of the cell. The fracture face has followed the plasma membrane over a large area. W = wall; arrow = bud scar. × 18,500.

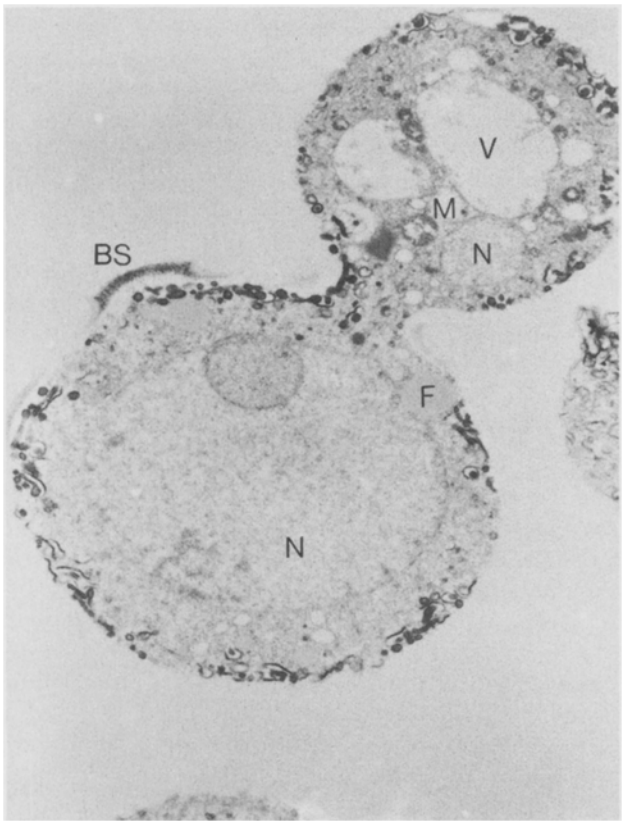


Figure 7. Section through yeast protoplast from budding cell after 60 min digestion with wall degrading enzymes[59]. Bud scars (BS) and wall remnants adhere to the protoplast. N = cell nucleus; V = vacuole; F = fat; M = mitochondrion. × 15,700

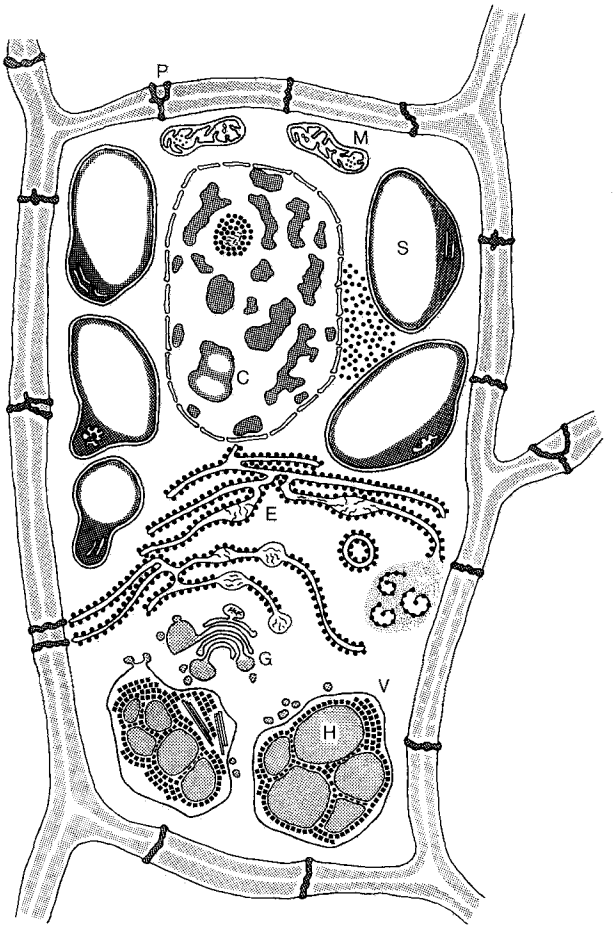
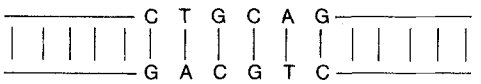


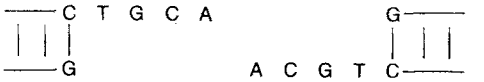
Figure 8. Endosperm cell of barley grain[59]. C = chromosomes in nucleus; E = endoplasmic reticulum with attached ribosomes; G = Golgi apparatus; H = hordein; M = mitochondrion; P = plasmodesmata traversing cell wall; S = starch in plastid; V = vacuole.

the unusual amino acid octopine under the direction of a gene on the plasmid and this property is passed on to the progeny. In nature, octopine is used by the crown gall bacterium as a nutrient source. Experiments are in progress to substitute the octopine gene with genes of interest to the plant breeder. This would then constitute a useful system to transfer individual genes for plant breeding purposes.

Bacteria have a sophisticated defense system to degrade, i.e. to restrict, foreign DNA. This is accomplished by the restriction endonucleases which recognize a specific base pair sequence and then cut the DNA at or near the recognition site. Each bacterial species has a different set of such restriction endonucleases and it marks its own DNA by methylating certain bases of the recognition sequence. The endonuclease cannot cut DNA which carries the base pair modifications, and the cell's own DNA is thus protected while foreign DNA will be recognized by the absence of the methylation and digested. The restriction endonuclease PstI from the bacterium *Providentia stuartii* cuts the double helix DNA at all places containing the following symmetric sequence of 6 unmodified base pairs.



The cleavage takes place in an oblique manner producing linear double stranded DNA molecules with short single stranded ends. These are called sticky ends, because each of these ends can be spliced together again by base pairing:



About 200 different enzymes have been isolated from various bacterial species and their specific recognition sequence determined. The enzymes provide unique tools to analyze the large complexity of DNA presented above: the recognition sites are used as physical markers of the DNA; they allow detailed mapping of any piece of DNA. The sticky ends are used to splice together the 2 ends of a restriction fragment to produce a circular molecule or the ends of different molecules to produce new linear as well as circular molecules. The enzyme DNA ligase can establish continuity of the strands in the spliced molecules by closing the gap between the 2 ends with a phosphate-diester bond.

The sizing of DNA molecules and the principle of mapping of restriction sites is illustrated in figure 9. Circular plasmids or linear DNA molecules are studied by intercalating the red fluorescing dye ethidium bromide between the 2 strands of the double helix and separating the molecules with an electric current in an agarose gel. Larger molecules will move slower than smaller ones. In lane a) of figure 9 the slow moving open circles of the 2-µm yeast plasmid (fig. 5) have been separated from the faster moving supercoiled molecules. In lane c) the plasmid has been cut with the restriction endonuclease PstI. A single band with a size of about 6000 base pairs is obtained, revealing that this plasmid contains only a single PstI recognition sequence.

The DNA of the bacterial virus lambda is cut with the restriction enzyme EcoRI at 5 sites resulting in 6 different fragments separated in lane d. Their size is very precisely known and they can therefore be used as a measuring stick to determine the size of unknown fragments. By the use of several restriction endonucleases individually and in pairs on the DNA segment to be analyzed, one can deduce from the patterns obtained the linear order of restriction fragments in the double helix and thereby prepare a map of the sites, which are cut by the restriction endonucleases. One is greatly helped in this puzzle by the possibility of extracting a given restriction fragment from the gel, whereafter it is digested with a new restriction endonuclease and the new fragments obtained are analyzed on another gel.

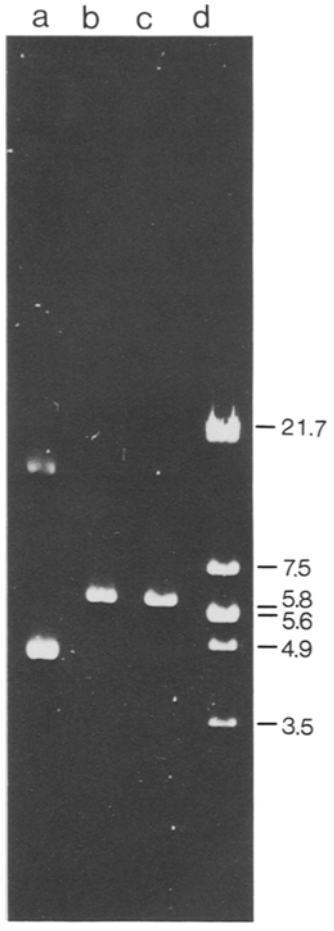


Figure 9. Gel electrophoresis of 2-µm DNA plasmid of yeast[59]. The molecules are made visible by UV-fluorescence of intercalated ethium bromide. *a* The upper faint band is the open circular form and the lower band is the supercoiled form of the plasmid, both depicted in figure 5. *b* and *c* show linear 2-µm plasmid molecules obtained by treatment with restriction endonucleases EcoRI (from *Escherichia coli)* and PstI (from *Providentia stuartii),* respectively. *d* Molecular weight standards in kilobase pair (= 1000 base pairs) for sizing of linear DNA molecules consistin of EcoRI restriction fragments of bacteriophage lambda DNA.

It has been mentioned above that the 2-µm yeast plasmid is cleaved at a single site by the restriction endonuclease PstI. This leads to linear molecules of the plasmid as shown in figure 10. The DNA of the 17 chromosomes of yeast can be cut with PstI several thousand times, giving rise to several thousand different linear DNA molecules. If these are mixed with a sufficient number of the opened 2-µm DNA molecules, the sticky ends will base pair and circles will be formed (cf. fig. 10) consisting of 2-µm DNA and a piece of chromosomal DNA and among these there will be circles which contain the histidine 4 gene, a gene required for the synthesis of histidine. Protoplasts are now produced from a yeast strain which carries a deletion mutant for this histidine gene and thus requires histidine for growth. The protoplasts are mixed with the constructed plasmids containing the large variety of chromosome fragments. After regeneration of cell walls one selects for cells that can grow without addition of histidine in the medium. These histidine independent transformants can arise only by having acquired from the DNA preparation a histidine 4 gene, which now allows them to produce histidine. By propagating the histidine independent cell, i.e. by making a clone we obtain many cells containing the plasmids. All the multiplied plasmids have the histidine 4 gene thus providing us with a unlimited supply of this gene. Occasionally the normal histidine 4 gene of the plasmid will become integrated by the natural mechanism of crossing over into chromosome III and substituting for the defective gene. In this way a sick gene can be exchanged with a healthy one. As described here for yeast, genes can be cloned in bacteria.

Once the plasmids containing the desired gene have been multiplied and isolated, the genes can be obtained in pure form by cutting them out again with the appropriate restriction enzyme. In the last few years 2 chemical techniques have been developed to determine the precise sequence of bases in a gene[34,48]. In the first method devised by Maxam and Gilbert, a strand of DNA is labelled at the 5′ end enzymatically with radioactive phosphorus. Then the preparation is divided into 4 aliquots and each subjected to a

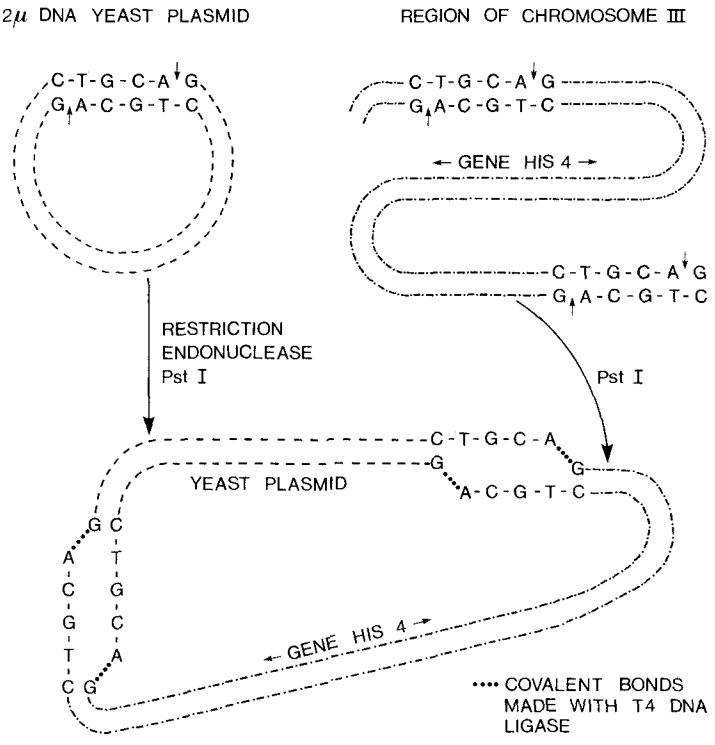


Figure 10. Example of the construction of a yeast plasmid containing the wild type gene of histidine 4 for genetic transformation of strains requiring histidine for growth.

different chemical reaction. One will randomly break the strand at G, the 2nd at A, the 3rd at C and the 4th at C as well as at T. The fragments obtained are then separated according to size on a gel side by side in 4 lanes and an autoradiograph of the gel prepared on X-ray film. The 4 lanes will contain bands each corresponding to a labeled fragment of certain size. The sequence of bases can be read directly from the comparison of the 4 lanes. The 2nd method devised by Sanger and Coulson is similar except that the fragments are produced by DNA replication using 4 different nucleotide analogues which terminate the synthesis of the new DNA strand when encountered by the DNA polymerase.

Prospects for improving the nutritional quality of cereal protein

Since the beginning of this century it has been known – especially from the work of Mendel and Osborne – that barley and maize are nutritionally poor as food for animals and humans. In both cereals the essential amino acid lysine is wanting. Therefore, imported proteins from soybeans have to be added if pigs are to be fed with barley grown in Denmark or elsewhere. Also soybeans alone form a poor diet; they are lacking the sulfur-containing amino acids.

Twenty years ago, Oliver Nelson and Edwin Mertz at Purdue University discovered that certain mutants of maize contain a considerably increased amount of lysine[37,42]. The increase is sufficient to turn these maize kernels of the mutant stocks into a balanced diet for animals and man. In barley, Lars Munck and his colleagues in Sweden found the first high-lysine line among a number of land varieties cultivated in Ethiopia[39,41]. This ancient variety and modern recombinant lines produced from it permit a reduction in the amount of soybean protein which has to be added to pig feed. A more drastic elevation in lysine content was obtained through artificial mutation by John Ingversen, Bertil Køje and Hans Doll giving rise to the mutant Risø 1508[24]. In a large scale trial it was shown that pigs fed with the mutant were ready for slaughtering after 100 days while the animals fed with present day barley attained the proper size only after 200 days[38]. Also, the pork chops and ham obtained from the latter was so poor in quality that they would not be bought by the consumer. A world-wide effort to use these mutants in breeding barley and maize of high nutritional quality has so far failed: it was not possible to overcome the severe yield depression suffered by the high lysine mutants. Molecular analysis of storage protein synthesis in barley and corn during the grain filling period has taught us why we have failed so far and in what manner we may achieve the coveted goal.

We studied first what happens when a barley grain is filled with starch and protein during the month of July[5,12,40,59]. The ripe grain consists of a small embryo, which will grow into a new plant, and the bulk of the kernel which represents 3 types of storage tissues. Of these, the so-called inner endosperm has gained its present large and dominating size by several thousand years of plant breeding. If we ana-

lyze such an endosperm cell in thin sections in the electron microscope during the time of active starch and protein synthesis, we obtain the picture presented in figure 8. The cell is surrounded by a cell wall, which is traversed by channels (plasmodesmata) providing communication between neighbouring cells. Towards the top is located the nucleus with the chromosomes that govern the cell's activities. The starch grains are produced in the plastids. Towards the bottom are located 2 of the cell's vacuoles containing the storage protein bodies. Biochemical studies in conjunction with the ultrastructural ones have revealed the biosynthetic pathway of the storage proteins[7,9–11,13]. The genes in the chromosomes contain the information as to their precise composition. This information is sent as messenger RNA to the ribosomes of the endoplasmic reticulum shown in the center of figure 8. The newly synthesized protein chains are discharged into the lumen of the labyrinth of the endoplasmic reticulum and they are thereafter packaged into small membrane vesicles originating from the reticulum. The protein chains in the vesicles are transported to the Golgi apparatus for further processing and from there to the vacuoles, where final deposition and concentration takes place. An actual electron micrograph of the vacuoles with the protein bodies, the profiles of the endoplasmic reticulum with attached ribosomes and plastids with starch grains is reproduced in figure 11.

The dense components marked H in figures 8 and 11 have been shown to be very rich in hordein polypeptides, the major storage protein of barley *(Hordeum vulgare)*. This protein contains very little lysine and belongs to the general class of cereal storage proteins called prolamines. In contrast to other proteins of the kernel, they are soluble in alcohol. If one grinds barley kernels and extracts the flour with alcohol these proteins will go into solution. By subsequently adding water to the extract they precipitate as a white powder. From flour of the lysine rich mutant Risø 1508 practically no hordein polypeptides are precipitated and we can thus state that the good nutritional quality of the mutant is due to the elimination of the lysine-poor hordein and a compensation in part by an elevated formation of lysine-rich endosperm components.

The effect of this mutation is also visible if we compare the protein bodies of the mother variety Bomi and the high-lysine mutant Risø 1508 in the electron microscope. Hordein is necessary to obtain

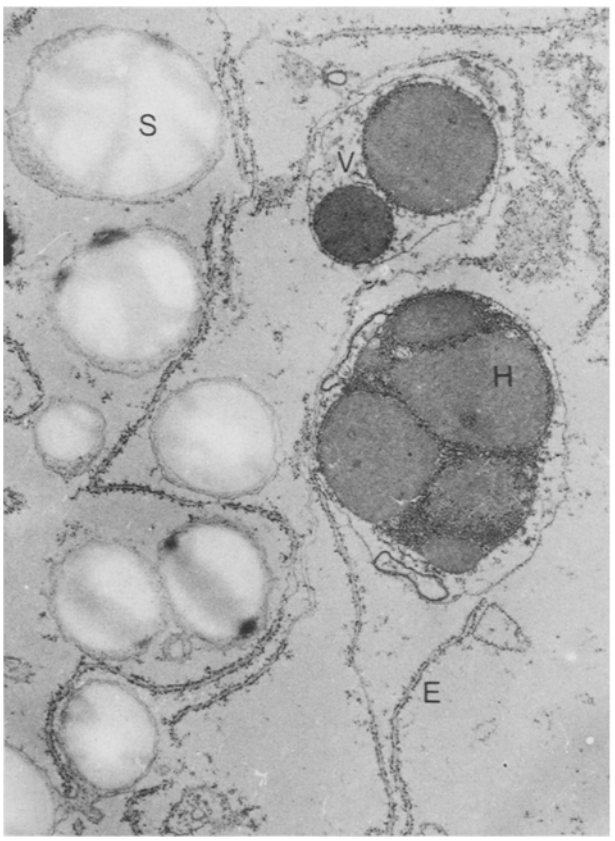


Figure 11. The storage proteins are deposited in the vacuoles (V) of the endosperm cell and the starch grains (S) are deposited in the plastid compartment. E = profiles of the endoplasmic reticulum; H = hordein. × 11,350

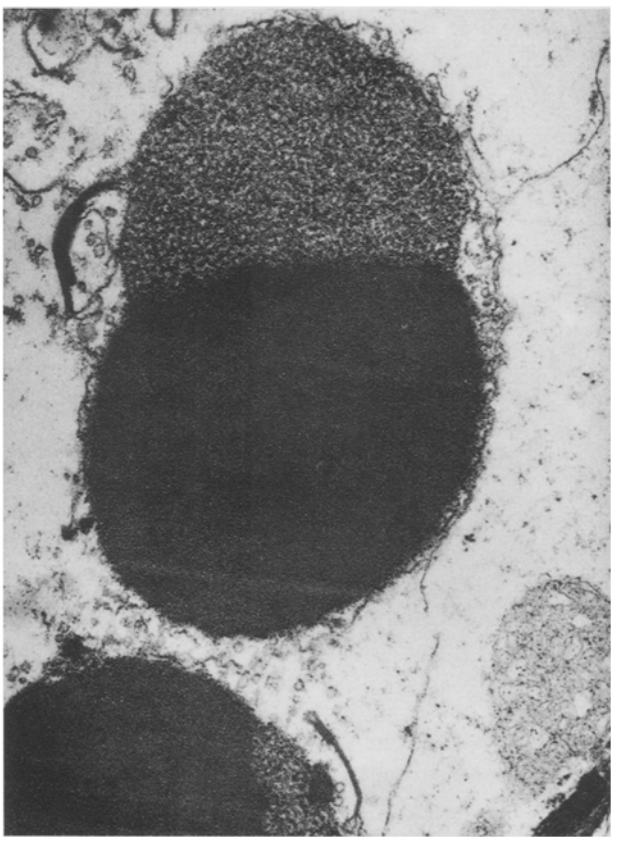


Figure 12. Developing protein bodies in the wild type Bomi barley consist of a large amount of opaque homogeneous material rich in hordein polypeptides and a fibrillar matrix. × 21,450

the compaction of the reserve proteins into the dense mass visible in figure 12, while this condensation is retarded in the mutant protein bodies of figure 13.

The isolated hordein polypeptides can be separated on a polyacrylamide gel by electrophoresis into at least 9 components with apparent molecular weights ranging from 27,000 to 67,000 (figure 14). They are grouped into the low-molecular-weight B hordein polypeptides (27,000–38,000) and the C hordein polypeptides (48,000–67,000). An additional larger D hordein polypeptide, not visible in figure 14, is also found[3]. Exploiting genetic polymorphisms (cf. figure 14, Carlsberg II and Bomi), it has been established that these 3 groups of polypeptides are coded for by 3 widely separate regions in barley chromosome 5 comprising 3 different mendelian gene loci designated *hor*2, *hor*1, and *hor*3, respectively[15,31]. Studies of the primary structure of the B and C families of hordein polypeptides by A.A. Holder, J.M. Schmitt, I. Svendsen and P.R. Shewry have shown that each polypeptide band has a distinct amino acid sequence composition although the sequences within a family reveal extensive homology[22,50–52,54]. We therefore expected one or several messenger RNA species to code for each of the polypeptide bands recognizable. In figure 14 are also presented the polypeptide patterns of mutant Risø 56 which is a mutation in or close to the *hor*2 gene resulting in a drastic reduction if not total elimination of the B hordein polypeptides[14]. Mutant Risø 1508 lacks practically all hordein polypeptides; it is a mutation in a gene in chromosome 7, thus not in the regions for the genes coding for hordein polypeptides.

We then proceeded to analyze the structure of the genes. By isolating portions of the endoplasmic reticulum (so-called microsomes) from the endosperm cells (fig. 15) and using these to translate in the test tube the messenger RNA of the cells A. Brandt, J. Ingversen and Verena Cameron-Mills established that all hordein polypeptides are translated on the ribosomes of the endoplasmic reticulum and discharged into the

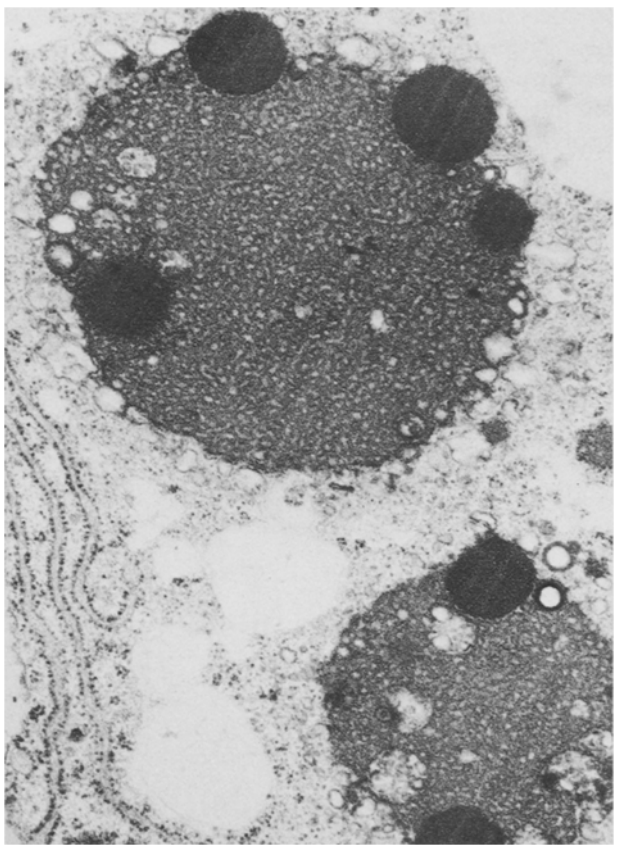


Figure 13. Developing protein bodies in the high-lysine mutant 1508 consist of the fibrillar matrix. The hordein dependent packaging into homogenous protein grains is retarded in the mutant. The dense spheres are a component also found in the wild type grain. Profiles of the ribosome studded endoplasmic reticulum at left. × 21,450

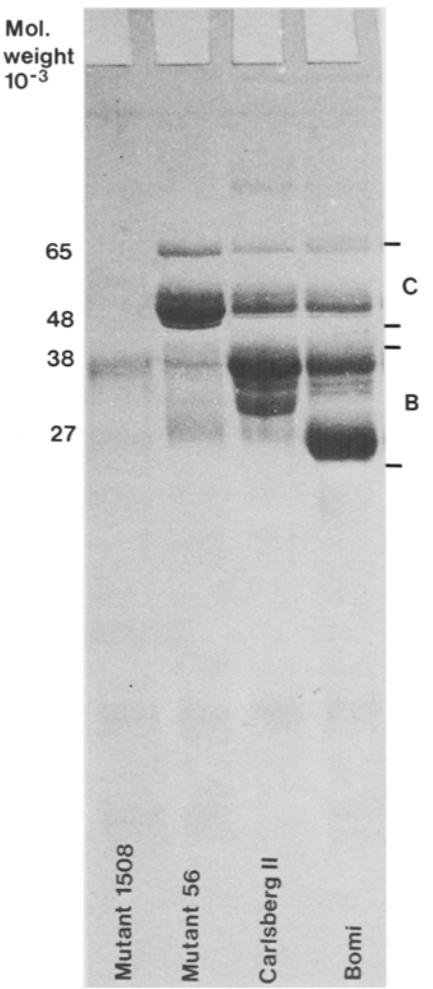


Figure 14. The alcohol soluble storage protein hordein can be separated by electrophoresis into 2 families of polypeptides, B and C comprising 4 and 5 polypeptide bands, respectively. The varieties Bomi and Carlsberg II differ genetically in the B family but are identical in the C family. Mutant 56 from Carlsberg II does not produce B hordein polypeptides, Mutant 1508 from Bomi barley lacks all hordein polypeptides.

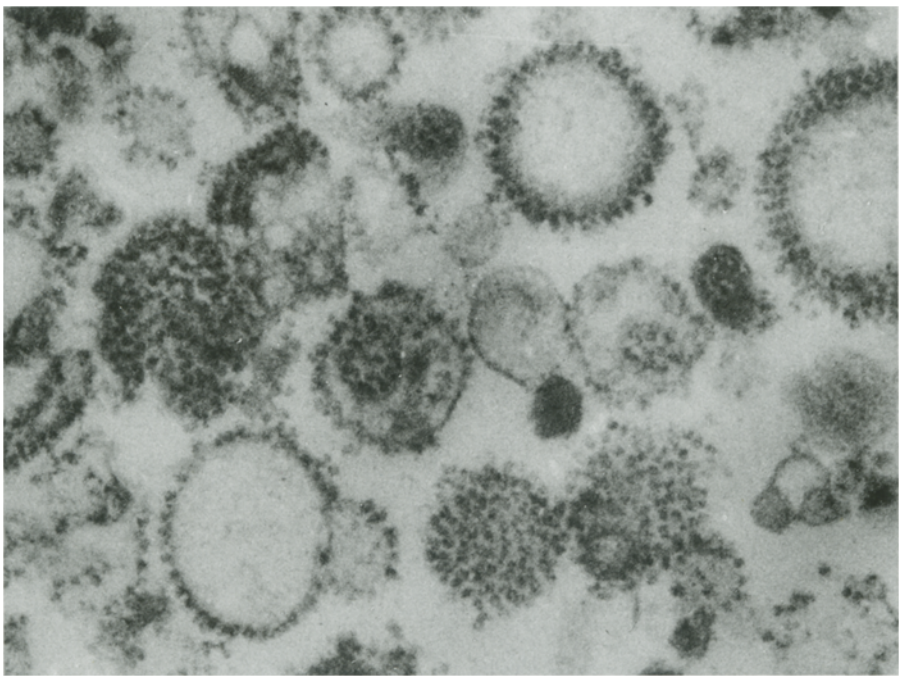


Figure 15. Isolated fragments of the endoplasmic reticulum. Such fragments are active in the synthesis of storage proteins and serve as source for messenger RNA. × 62,000.

lumen of this membrane system. This can be shown by adding proteolytic enzymes to the microsome preparation after translation is completed. The newly synthesized radioactively labeled polypeptides are protected by the membrane against digestion through the enzymes. Using messenger RNA from mutant Risø 1508 no hordein polypeptides were obtained and mutant Risø 56 produced only C hordein polypeptides. It was also shown that the primary translation products have an addition of about 20 amino acids at their N-terminal end, corresponding to the signal peptide cleaved off after transfer into the lumen of the endoplasmic reticulum.

Applying reverse transcriptase and cloning in a bac-

Figure 16. Portion of the 3′ end of the messenger RNA for hordein polypeptide B_1^8. The nucleotide sequence comprising 283 nucleotides has been obtained from a reverse transcribed complementary DNA sequence which was cloned in a bacterial plasmid. Below, the nucleotide sequence is given, in green, the C-terminal and an internal amino acid sequence determined for hordein B_1; above, the amino acid sequence deduced from the genetic code. The stop codons are marked in red and the polyadenylation signals in blue.

terial plasmid, A. Brandt, E. Hopp and S.K. Rasmussen obtained complementary DNA clones to the messenger RNA for B_1 hordein polypeptides[6,8]. These clones are being nucleotide sequenced and a portion of one of them is depicted in figure 16. The nucleotide sequence for the 3′ end of the messenger RNA including the carboxy-terminal end of one hordein polypeptide B_1 is presented as it would appear in the DNA strand which is complementary to that transcribed into messenger RNA. This gives the same sequence as that present in the messenger RNA except that T stands instead of U. Above the nucleotide sequence is presented the amino acid sequence deducible according to the genetic code and it can be compared to the amino acid sequences which have been determined directly on the polypeptide (given in green). Very good agreement is revealed. At the place where the reading frame ends, that is, next to the codon for the carboxy-terminal amino acid valine, are 3 stop codons (red) followed by 205 non-coding nucleotides to the very end of the molecule. As is the case with all messenger RNA translated on the endoplasmatic reticulum, a series of 34 adenine bases is found close to the end of the RNA. The 6 nucleotides AATAAA framed in blue are the so-called polyadenylation signals, a sequence which is present whenever a polyadenylated tail is to be added to the messenger RNA. In fact there are 3 such signals in the sequence shown, which also contains 2 additional stop codons in the untranslatable portion of the molecule. As more of the clones are sequenced it emerges that there are several closely related messenger RNA molecules even for the B1 hordein polypeptides. Thus we have to conclude that each of the hordein polypeptide bands seen in figure 14 is composed of a number of different polypeptides each made from its own messenger RNA corresponding to a separate gene.

Besides establishing the base sequence of the messenger RNA and the amino acid sequence of a hordein B_1 polypeptide, Anders Brandt and Esteban Hopp used the artificially synthesized gene as a probe to study the type of messenger RNA present in the wild type endosperm cells and in the high-lysine mutants mentioned above. This is illustrated in figure 17. The messenger RNAs isolated from Bomi and Carlsberg II barley as well as those of the mutants Risø 1508 and Risø 56 were separated on an agarose gel. The RNA molecules were then blotted from the gel into a filter paper, whereafter the filter sheet was soaked in a solution containing the artificial genes. These had been labeled with radioactive nucleotides and separated into single strands to allow hybridization with the messenger RNA molecules according to the base pairing rule. After the filter sheet had been dried, it was placed back to back with an X-ray film for autoradiography. In studying the autoradiogram, we can see for Bomi and Carlsberg II hybridization to messenger RNA molecules ranging in size from 1200 to 1400 nucleotides. Assuming 400 bases for non-translatable portions of the messenger RNA this would provide 260–330 amino acids which is the range expected for the family of B polypeptides. The artificial B_1 gene did not hybridize to any messenger RNA of mutant Risø 56. Obviously this mutant is unable to produce messenger for B hordein polypeptides. There was a weak hybridization to 2 RNA molecules in mutant Risø 1508 indicating the presence of a small amount of messenger RNA in this mutant. Under the conditions employed, the probe did not hybridize to messenger RNA molecules coding for the family of C hordein polypeptides known to be present in mutant Risø 56 and with expect sizes of about 2000 nucleotides.

In the next step, isolation of the natural gene or genes for the hordein B1 polypeptide was attempted, again using the reverse transcribed gene as a probe. A

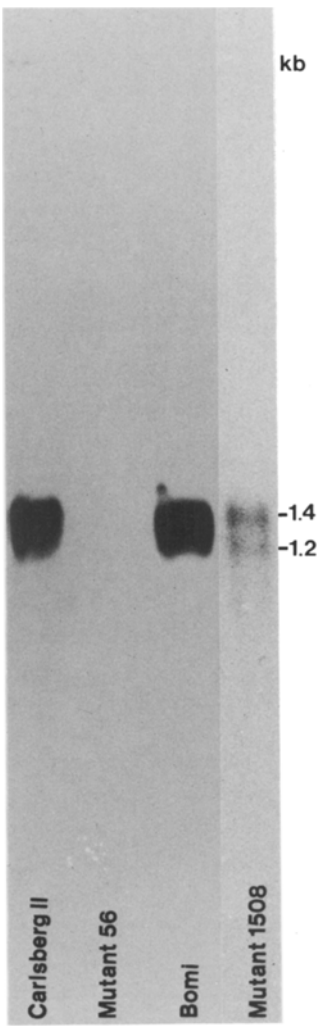


Figure 17. Hybridization of the gene reverse transcribed from the messenger of a B_1 hordein polypeptide to endosperm messenger RNA of the barley varieties Carlsberg II and Bomi as well as the high-lysine mutants Risø 56 and Risø 1508[8].

library of barley genes was made by splicing EcoRI restriction endonuclease fragments of total endosperm barley DNA into bacteriophage lambda DNA. The DNA was packaged into complete virus particles and appropriate bacteria infected. After propagation of the bacteriophage the bacteria were lysed and those phage particles selected which can hybridize to the reverse transcribed gene. One of the obtained lambda clones is analyzed in figure 18. In the 2 lanes designated 'undigested', the bacteriophage lambda chromosome with the inserted segment of the barley chromosome containing hordein genes is visible after gel electrophoresis as an ethidium bromide stained band and by autoradiography after hybridization to the radioactive labeled reversed transcribed gene. By cutting the clone with the EcoRI restriction endonuclease, 4 ethidium bromide stained fragments are obtained. The 2 larger ones (21,700 and 18,000 base pairs) are the left and right chromosome segments of the bacteriophage which flank the 2 barley DNA EcoRI restriction endonuclease fragments (5200 and 3000 base pairs) inserted into the lambda vector, when the gene library was made. Only the 5200 base pair barley DNA fragment contains nucleotide sequences hybridizing to the reverse transcribed B_I hordein gene, as only it reveals a black band on the X-ray film after autoradiography (right most lane).

The artificially made hordein gene was further used as a probe to study the chromosomes of the barley varieties Bomi and Carlsberg II as well as the high lysine mutants Risø 1508 and 56. The DNA of the chromosomes was cut with the restriction endonu-

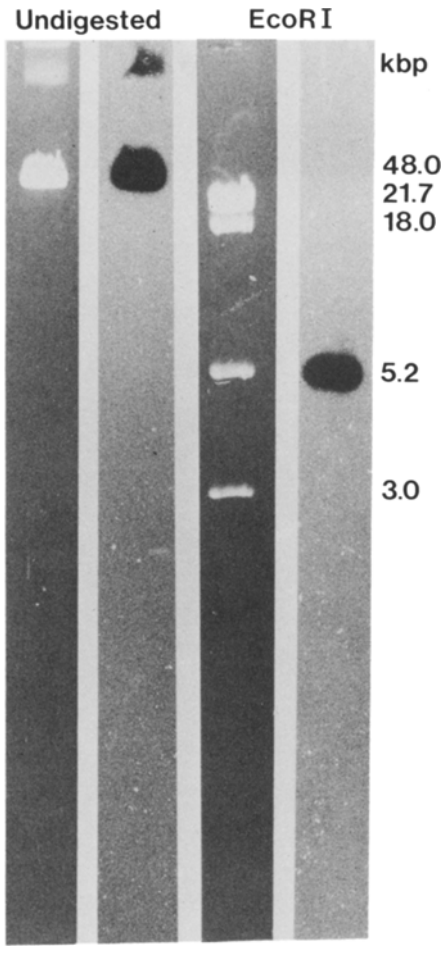


Figure 18. Analysis of a bacteriophage lambda clone containing a barley chromosome segment with hordein genes by gel electrophoresis[8]. In the 2 lanes labeled 'undigested' the phage DNA with the insert has been made visible by ethidium bromide staining at left and by autoradiography after hybridization to the radioactive gene reverse transcribed from the messenger RNA for a B_I hordein polypeptide. The size is 48 kilobase pairs (kbp). In the 2 lanes labeled EcoRI this restriction endonuclease was used to cut the DNA clone. With ethidium bromide staining 4 restriction fragments are made visible of which only the 5.2 kilobase pair fragment hybridizes to the radioactive artificial hordein gene.

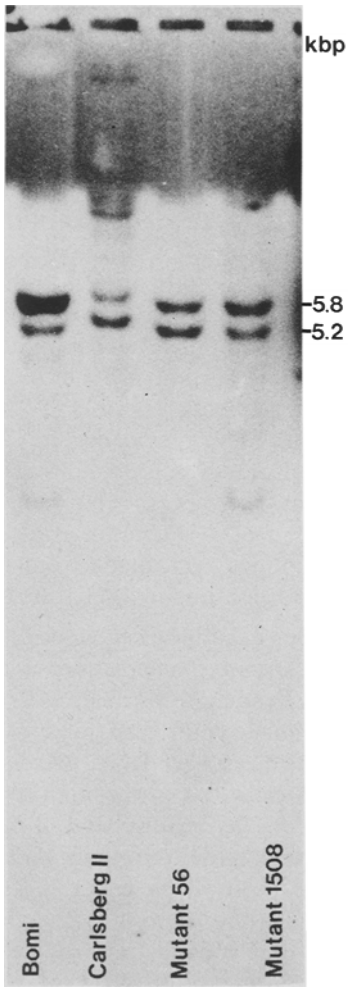


Figure 19. Analysis of the endosperm DNA of barley varieties Bomi and Carlsberg II as well as the high-lysine mutants Risø 1508 and 56 for the presence of the genes coding for the B hordein polypeptides[8]. The DNA of the 4 lines was restricted with EcoRI endonuclease. The resulting fragments were separated by electrophoresis, denatured and transferred to a filter sheet, which was then soaked in a solution containing the radioactive denatured artificial hordein B_I gene. After hybridization by base pairing an autoradiograph was prepared. The bands visible and marked are the DNA fragments of the chromosomes containing genes coding for hordein polypeptides.

clease EcoRI and the resulting many thousand fragments separated in the 4 parallel lanes illustrated in figure 19. The fragments were then blotted onto a filtersheet, hybridized to the radioactive probe and an autoradiogram prepared. It can be seen that the probe hybridizes to a 5200 base pair EcoRI fragment corresponding to the one isolated and cloned in the previous experiment. But the probe also reveals a 5800 base pair large fragment to carry genes for hordein B polypeptides as well as 3 smaller fragments which hybridize less extensively, either because of a smaller degree of homology or because of their presence in smaller numbers. It is clear that the chromosomes of all 4 lines contain the genes recognized by the probe. This is expected for mutant Risø 1508 but reveals for mutant 56 that its failure to produce messenger RNA for hordein B polypeptides is due to a point mutation rather than a large deletion in the chromosome.

The intensity of the black bands in figure 19 is proportional to the amount of radioactive probe hybridized. By comparing the intensity of hybridization exhibited by the 5200 base pair band obtained from the restricted barley chromosome with that obtained from a dilution series of a known amount of the natural, genomic lambda clone, one can estimate if the particular restriction fragment is present in a single copy or in multiple copies in the barley chromosome 5. It turns out that there are in the order of 50 copies of the 5200 base pair EcoRI fragment and also in the order of 50 copies of the 5800 base pair EcoRI fragment present in a single chromosome. Each of the 2 fragments could encode several B hordein polypeptides. We thus arrive at the following picture of the B hordein gene family in barley: there are in the order of 100 to several hundred genes for these hordein polypeptides. The messenger RNA molecules and the polypeptides analyzed reveal that a considerable number of genes have different primary structures. Sequence analysis of genomic clones is required to determine to what extent the 100 or more genes are moderately or highly polymorphic. We expect a similarly complex situation for the C hordein polypeptides which are equally poor in nutritional quality.

The 3 high-lysine mutants we have available in barley today are clearly too small a sample to provide suitable germ plasm for a successful breeding program. Mutant Risø 1508 prevents the formation of sufficient or stable messenger RNA not only for all hordein polypeptides, but also for proteins with a suitable nutritional composition. Mutant Risø 56 prevents the transcription of the 100 or so B hordein genes in an as yet unknown manner. Like in the mutant 1508 this drastic cut-off in storage protein synthesis additionally causes premature cessation of starch synthesis leading to low yield. The high-lysine line from Ethiopia, Hiproly, owes its nutritional quality not to a reduced amount of hordein but to an increased amount of water soluble albumin polypeptides. One of these, the chymotrypsin inhibitor 2, is prominent and has been purified and sequenced by Ib Jonassen, Ib Svendsen and Brian Martin[25–27,58]. It was found to be very rich in the amino acid lysine containing 8 lysine residues among a total of 83 residues, i.e. about 10%. The lysine content of the present day barley grain is about 3.4% of its crude protein. We know that the storage protein bodies depicted in figures 11–13 contain other proteins than hordein but we do not know yet if the lysine rich albumins are found there. The latter are translated on the endoplasmic reticulum[28] and could thus be co-transported with the hordein polypeptides into the storage vacuoles.

Eventually it will be possible to tailor the hordein genes into genes specifying polypeptides with increased amounts of lysine. First, however, ways to transform barley cells genetically and to regenerate transformed cells into fertile plants will have to be discovered. Until then we will have to screen for mutants with diminished hordein production and others with elevated levels of lysine rich storage proteins. The precise knowledge of the number and types of proteins stored as well as of the organization of their structural genes is a necessary fundament for devising screening procedures to obtain large numbers of mutants. Mutation breeding has taught that only one out of several hundred induced or spontaneous mutants will decrease hordein without depressing yield and, likewise, that only one out of several hundred mutants will increase the lysine rich albumin polypeptides without infringing on yield potential.

The prolamin which is, the alcohol soluble storage protein of maize *(Zea mays)* is called zein. In electrophoretic patterns zein gives only 2 protein bands; still, it exhibits the same diversity in the primary structure of its constituent polypeptide chains. Cloning and nucleotide sequence analyses of 3 from messenger RNA reverse transcribed zein genes and 2 natural zein genes by Daniel Geraghty, Joachim Messing, Irwin Rubenstein, Karl Pedersen, Brian Larkins and their coworkers have revealed that also zein is the product of a highly diversified multigene family[16,23,44]. Indeed, the primary structure of all 5 genes analyzed was different. In figure 20 is presented the nucleotide sequence of one of the genes obtained by reverse transcription and the amino acid sequence derived from it. The green N-terminal portion is part of the signal sequence removed after transfer of the polypeptide across the membrane of the endoplasmic reticulum. The glutamin residues are framed red and the proline residues blue. Indeed there is not a single lysine residue among the 213

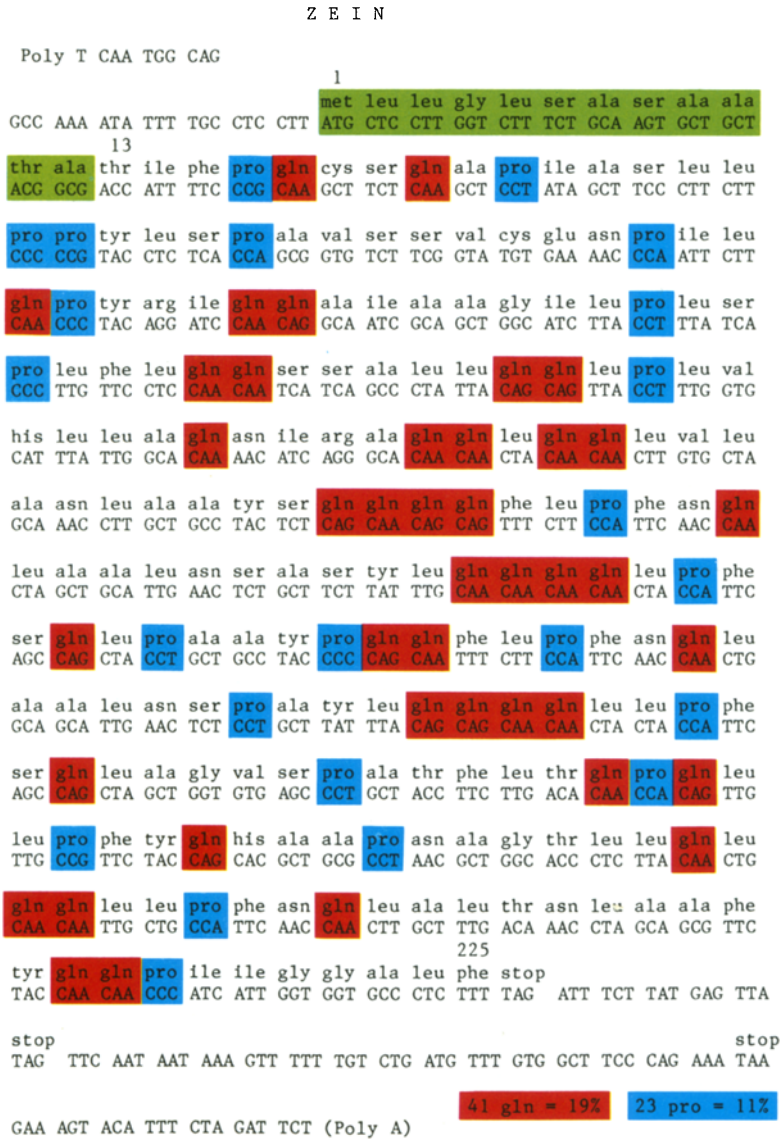


Figure 20. Nucleotide sequence of gene reverse transcribed from messenger RNA for a zein storage polypeptide of maize. The codons have been translated into the amino acid sequence. The signal sequence is outlined in green, glutamin residues in red and proline residues in blue. According to the data of Geraghty, Messing and Rubenstein[16].

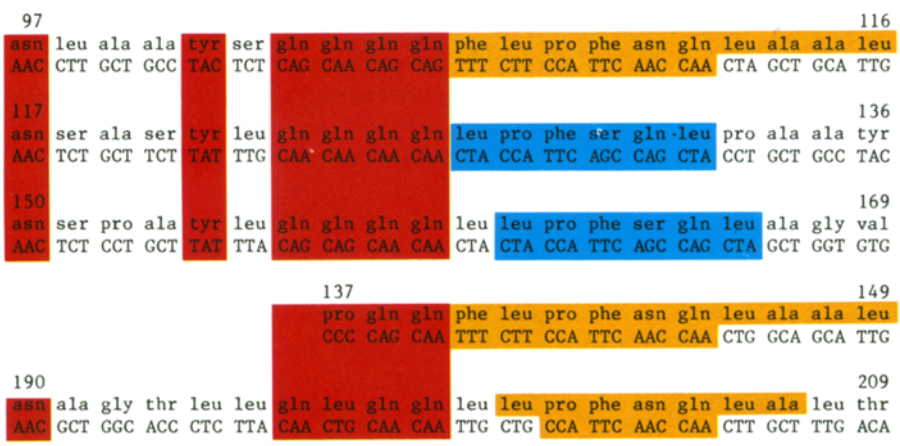


Figure 21. Internal duplications in the zein polypeptide. Striking homologies of amino acid sequences are outlined in red. Conservation at both the codon and amino acid level are outlined in blue and yellow. Numbering of the amino acids is from the N-terminal end.

amino acids of this zein polypeptide. Remarkable internal duplication of portions of the polypeptide and of portions of the gene are observed. This is illustrated in figure 21: if the glutamin tetrapeptides (red) occurring at 3 different positions of the polypeptide chain are lined up, strict conservation of a tyrosine residue 2 positions upstream and of an asparagine 6 positions upstream is observed. The functional significance of the conservation of the amino acids is stressed by the fact that some of their codons have mutationally changed during evolution by single nucleotide substitutions. On the other hand the nearby blue and yellow outlined sequences have been evolutionarily highly conserved both at the nucleotide and amino acid sequence level. All 5 analyzed zein genes show close homology in these particular regions of the polypeptides and their genes, while other regions exhibit extensive polymorphisms. The latter regions are those where incorporation of lysine residues by mutations can be envisioned.

Improvement of plant productivity by knowing about photosynthesis?

Plant photosynthesis converts solar energy into chemical energy. This process produces directly or indirectly all the food we eat, all the oxygen we breathe, and much of the energy we use. The plant cover of our planet and the algae in the oceans produce every year 10 times as much chemical energy as is used by mankind. One has also calculated that the total energy contained in the presently known oil reserves represents only 100 years of photosynthesis. Our other geological energy source, coal, is entirely of plant origin and can satisfy our energy demands for the next 300 years. The oxygen of the atmosphere originates apparently from plant photosynthesis and its complete renewal corresponds to 2000 years of photosynthesis, a short period considering the age of this planet.

Sugar and starch are the early products of photosynthesis, and the chlorophyll carrying organelles, the chloroplasts (fig. 22), are the photosynthetic power stations of the cell. The overall process can be described by the following formula:

$$6\,CO_2 + 6\,H_2O \xrightarrow[\text{light}]{\text{chloroplast}} C_6H_{12}O_6 + 6\,O_2$$

$$(G = 48 \times 10^4\,J = 114\,kcal)$$

In order to reduce 1 g molecule carbon dioxide (CO_2) to glucose ($C_6H_{12}O_6$), 48×10^4 Joule or 114 kilocalories of solar energy are required, 1 g molecule photons (light quanta) at the red wave-length of 680 nm contains 17.6×10^4 J or 42 kcal. Thus, at least 3 light quanta of solar energy are necessary to fix 1 molecule carbon dioxide from the air. The oxygen produced by plant photosynthesis originates from water (H_2O) in the membrane system of the organelle, while the fixation of carbon dioxide and the movement of carbon through the chloroplasts' intermediary metabolism to sugar, starch and many other chemical compounds takes place in the stroma, the soluble part of the chloroplast (fig. 22). The chloroplast uses for the reductions of every molecule CO_2 to sugar:

2 molecules reduced nicotinamide-adenine-dinucleotide phosphate ($NADPH_2$) and 3 molecules adenosine triphosphate (ATP).

These are the energy-rich chemical compounds used by the cell to synthesize and degrade substances. The photosynthetic formation of a single sugar molecule requires $6 \times 3 = 18$ ATP molecules.

Figure 23 presents a section through the chloroplast's

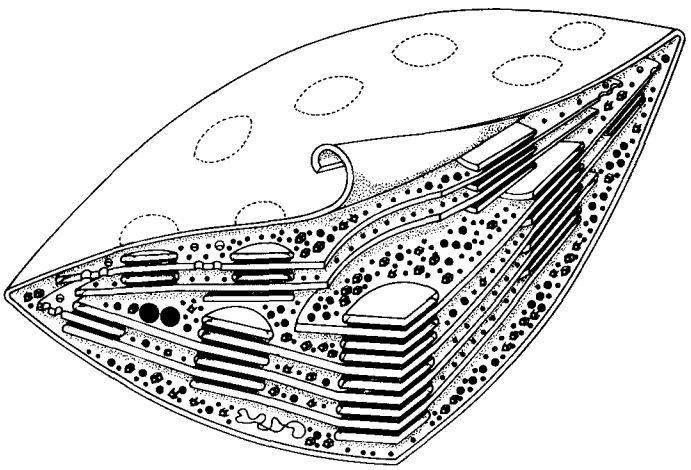


Figure 22. Model of chloroplast. Between the photosynthetic membranes is located the stroma which contains ribosomes, the CO_2 fixing enzyme ribulose bisphosphate carboxylase, many other enzymes and circular DNA molecules, the organelle's own genetic information.

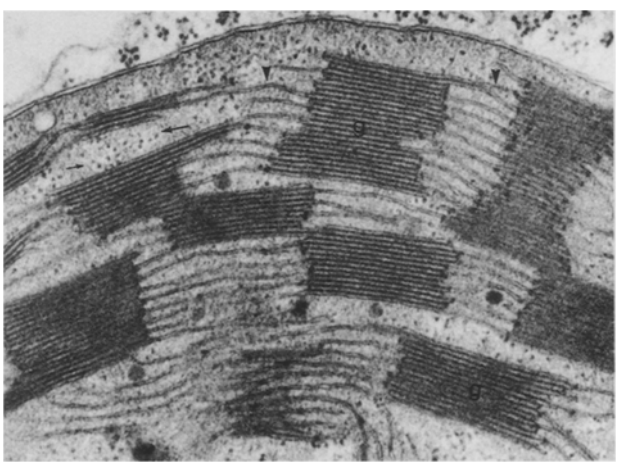


Figure 23. Electron micrograph of a section through a chloroplast in a barley leaf[55]. The photosynthetic membranes, thylakoid membranes, are organized into grana stacks (g) and unstacked regions (arrow heads). Also visible are the protein synthesizing ribosomes in the chloroplast stroma (arrows) and ribosomes in the cell's cytoplasm located outside of the chloroplast envelope (e). × 64,000.

membrane system which consists of flattened sacks (thylakoids). The cavity inside the sack is electron optically empty, and the photosynthetic membrane separates this space from the chloroplast stroma containing hundreds of enzymes in addition to the protein synthesizing chloroplastic ribosomes. In the grana stacks there are twice as many thylakoids as in the other parts of the membrane system and the membranes of the adjoining thylakoids are closely appressed across their entire surface. Many thylakoids in the grana can be viewed as disks with approximately the same diameter, yet it is to be noticed that the disk's cavity is continuous with the cavity of the thylakoids outside the grana, i.e. in the stroma region. It is now known that the splitting of water into protons (hydrogen ions) and oxygen occurs mainly – if not exclusively – in the grana stacks, while the production of the high-energy compounds $NADPH_2$ and ATP takes place at the outer surface of the unstacked thylakoids.

Let us explain, with the aid of figure 24, how we perceive today the formation of $NADPH_2$ and ATP by the photosynthetic membrane[21,32,55,60]. The light quanta are absorbed primarily by the chlorophyll of the light harvesting yellow-green proteins, whereby 1 of the atoms in the chlorophyll molecule is excited, i.e. 1 of the atom's electrons is lifted in the course of 10^{-15} sec into a wider, energy-rich orbit. Frequently, the electron falls back from this singlet state in the course of 10^{-9} sec to the ground state, called the doublet state (double spectral lines), and re-emits thereby the light quantum in the form of red fluorescence. The electron can also transit into a different high-energy state, the long-lived triplet state in which the paired electrons have a spin of the same direction. The transition to the ground state in this case takes place in the form of phosphorescence over a period of msec (10^{-3} sec) unless the energy in the meantime has been stored in chemical form. Triplet states are attractive since many biochemical reactions proceed with msec velocity. By resonance energy transfer the high-energy singlet or triplet states migrate from chlorophyll molecule to chlorophyll molecule, preferentially from the light harvesting chlorophyll-proteins (Figure 24, LHC) via the light focussing chlorophyll *a*-protein 2 to the chlorophyll *a*-protein 3 that contains the reaction center for photosystem II, a special chlorophyll molecule with absorption at 680 nm (P680). Resonance energy transfer can only take place over distances of a few Å (10^{-7} mm) and it is thus the primary task of the chlorophyll-proteins to hold the many chlorophyll molecules in a fixed position and at a distance which allows the energy transfer to occur. In the reaction center the high-energy electron is moved in an unknown way to the primary electron acceptor Q (possibly a complex of pheophytin and quinone) which thereby is reduced. The chlorophyll *a*-protein 3 is expected to span the membrane to allow charge separation between the 2 sides of the membrane as it takes place when the electron moves to Q at the stroma side and the 'hole' to the opposite side. In order to fill the 'hole' an electron is donated from the 3 components Z_1, Z_2, M, which thereby become oxidized, and which are known functionally to effect the splitting of water into protons and molecular oxygen. Lately, several of the proteins participating in the water splitting have been isolated and we hope therefore that this white area soon can be filled with color[19,20]. Cytochrome b_{559} belongs to photosystem II, but its function is unknown.

From the primary electron acceptor Q the electron is moved to R. This protein binds several well-known herbicides and the binding blocks the electron transport. From R the electron is transferred to plastoquinone which is found in larger quantities and shuttles in the membrane to transport electrons from photosystem II to photosystem I. The transport must take place over considerable distances if photosystem II is located in the grana and photosystem I in the unstacked thylakoids.

Many years ago Robert Emerson demonstrated that more photosynthesis is obtained upon illumination of an alga with both long- and shortwave red light than with 1 wavelength alone. The enhancement is also obtained if 1 photosystem is illuminated first and, after a dark period, then the other one. The energy can thus be stored and electron transport experiments have shown that the 2 photosystems are serially linked. The electron is transported from plastoquinone via the Rieske iron-sulphur protein, cytochrome f, the copper containing plastocyanin to the primary donor for photosystem I. At this point new energy has to be conveyed to the electron transport chain. This takes place by absorption of light quanta in the chlorophyll *a*-protein 1 and energy transfer to the reaction center for photosystem I with an absorption at 700 nm (P700). This chlorophyll-protein is likewise expected to span the membrane to effect a charge separation across the membrane. From the primary electron acceptor A_1 the electron is transferred via the iron-sulphur proteins A_2, A or B to ferredoxin, and the enzyme NADP-ferredoxinreductase (yellow in figure 24) uses it to produce the high-energy compound $NADPH_2$.

When plastoquinone during the electron transport receives 2 electrons, it has also to take up two protons (H_2) in order to become completely reduced. These 2 hydrogen ions come from the stroma side. When the reduced plastoquinone donates the electrons to the next carrier it has to release the 2 hydrogen ions again, but they move now into the cavity of the thylakoids. Thus, the hydrogen ion concentration in the cavity of the thylakoids increases during illumination, i.e. the pH value decreases and the cavity

becomes more acid. Additional hydrogen ions are produced by the water-splitting reaction. The proton gradient thus established represents an energy potential, which is used by the 'coupling factor' (CF_0 and CF_1 in figure 24) for the formation of ATP, the other high energy substance produced in photosynthesis. The protons are conducted from the thylakoid's cavity through a channel consisting of protein molecules to the ATP synthesizing enzyme at the stroma-side of the photosynthetic membrane. The catalyzer for the conversion of adenosine diphosphate to adenosine triphosphate consists of the protein molecules designated with α and β in figure 24. The coupling factor has its name from the observation that ATP production is strictly coupled in the photosynthetic membrane with electron transport and oxygen evolution. We understand now why the photosynthetic membrane is organized into closed, flattened sacks or disks. The membrane must not allow protons to leak through and the cavity in the grana disks has to be continuous with the cavity of the unstacked regions in order to permit the protons produced in the grana to be utilized by the ATP synthesizing enzymes on the unstacked membrane regions.

I mentioned in the beginning that in theory at least 3 red light quanta are required to produce 2 $NADPH_2$ and 3 ATP molecules for the reduction of 1 molecule of CO_2 to the level of glucose. Experiments with light flashes revealed 1 molecule of oxygen (O_2) to be released when 4 electrons were transferred from the reaction center of photosystem II to plastoquinone. Likewise, the transfer of 4 electrons through the entire chain is necessary to reduce 1 molecule of CO_2. Since a separate light quantum has to be used for each electron sent off from the reaction center, 4 light quanta are required in photosystem II and 4 in photosystem I or 8 quanta in all to reduce 1 molecule of CO_2 and to liberate 1 molecule of O_2. This is indeed the quantum efficiency determined in the best experimental measurements. $NADPH_2$ and ATP in photosynthesis are thus produced with a unique efficiency of $\frac{3}{8}$ or 37%. Measuring the efficiency on plant growth or the total production of biomass though yields rarely more than 1–5%. Plants therefore consume a considerable amount of the chemical energy attained in photosynthesis in their metabolism. The primary task of plant breeding is to improve the efficiency by which $NADPH_2$ and ATP are used for the desired product such as storage of starch, oil or nutritionally valuable protein.

When the beech trees every spring flush with countless new green leaves, the photosynthetic membranes in the leaves' chloroplasts are built from scratch. In the course of the summer the membranes are maintained and damaged portions repaired. All this takes place by an intimate co-operation between the genes in the nucleus and those in the chloroplast DNA[4,60]. The proteins that are coded in the chromosomes of the nucleus are transcribed into messenger RNA, which is moved to the ribosomes of the cytoplasm where the code is translated into the sequence of amino acids in the proteins. Thereafter the latter are moved through the chloroplast envelope and built into the photosynthetic membrane. As we have discussed for the proteins transported across the membrane of the endoplasmic reticulum, the movement across the 2 membranes of the chloroplast envelope is accomplished with the aid of an N-terminal signal peptide, which is cleaved off once the protein is on the other side of the envelope. Proteins colored yellow in figure 25 are coded in the nucleus, synthesized in the cytoplasm and transported into the chloroplast. Among these are, for example, the light harvesting chlorophyll *a*/*b*-protein 2. Proteins that are coded in the chloroplast DNA and synthesized on the chloroplasts' own ribosomes are colored purple in figure 25. To these belong for instance the chlorophyll *a*-protein 3 with the reaction center for photosystem II. The figure shows that the interplay between the genes in the nucleus and those in the chloroplast must be closely correlated in order to procure the membranes' intricate mosaic.

The location and the primary structure of the genes in the nuclear DNA and those in the chloroplast DNA bringing about this mosaic is being explored. The presence of small multigene families for the genes located in the nucleus becomes progressively apparent, while the genes in the chloroplast DNA are highly conserved in spite of the presence of 150–200 copies of chloroplast DNA molecules per cell.

In figure 26, the restriction endonuclease site maps for the chloroplast DNA molecules of maize, wheat and barley are compared[46]. Very similar restriction endonuclease fragment sizes and locations are indeed observed in barley (HvC-DNA) and wheat (TaC-DNA) and similarities are recognizable when these 2 agricultural plants are compared with maize. The total sizes are 134, 137 and 135 kilobase pairs (= 1000 base pairs), respectively. All 3 are characterized by an inverted repeat of 20–25 kilobase pairs. (In the legumes no such inverted repeat is found, while in the alga *Euglena* a triple tandem repeat is observed). In figure 27 are presented the genes that have been located by Carsten Poulsen on the restriction endonuclease site map of barley. Like in maize and wheat, the genes for the ribosomal RNA molecules (rrs, rrl and rrf) are located in the inverted repeat. These RNA molecules provide the skeleton upon which the 34 proteins of the large ribosomal subunit and the 25 proteins of the small ribosomal subunit are assembled. The ribosomes of the chloroplast are similar in general construction to those of the cytoplasm, but still very different in the structural details of the individual components. Known is also the location of

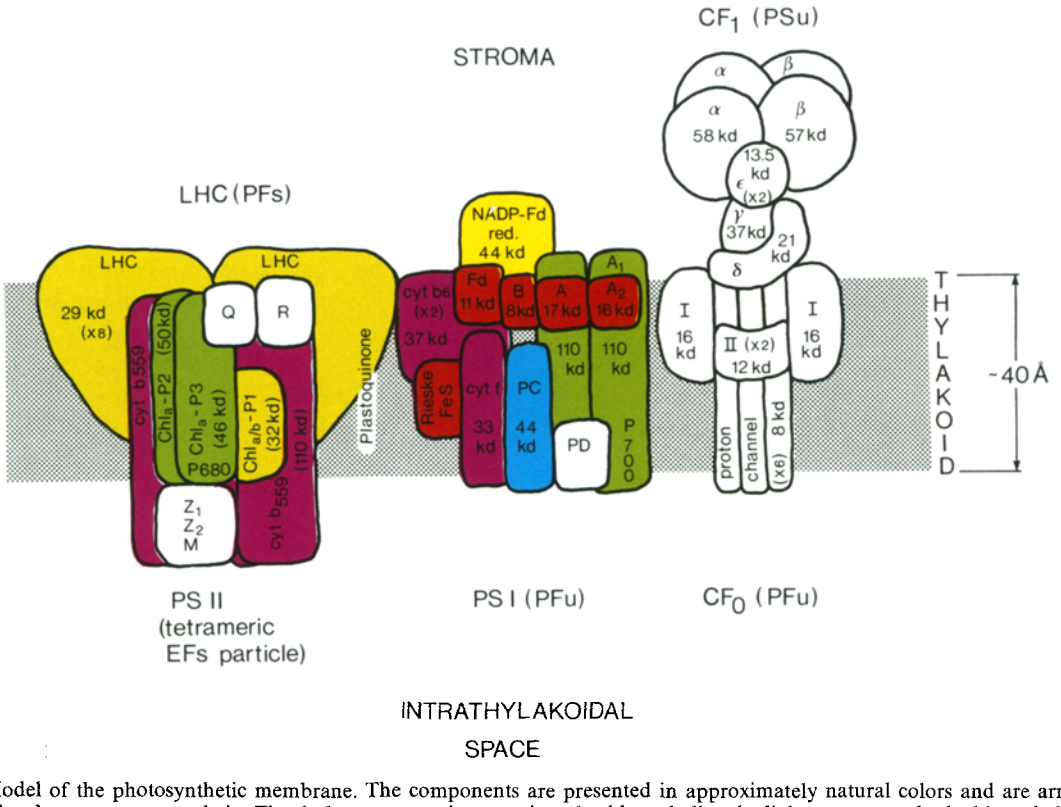


Figure 24. Model of the photosynthetic membrane. The components are presented in approximately natural colors and are arranged as required by the electron transport chain. The dark-green proteins contain only chlorophyll *a*, the light-green ones both chlorophyll *a* and *b*, the brick-red ones iron and sulphur, the purple ones iron in haem and the blue one copper.

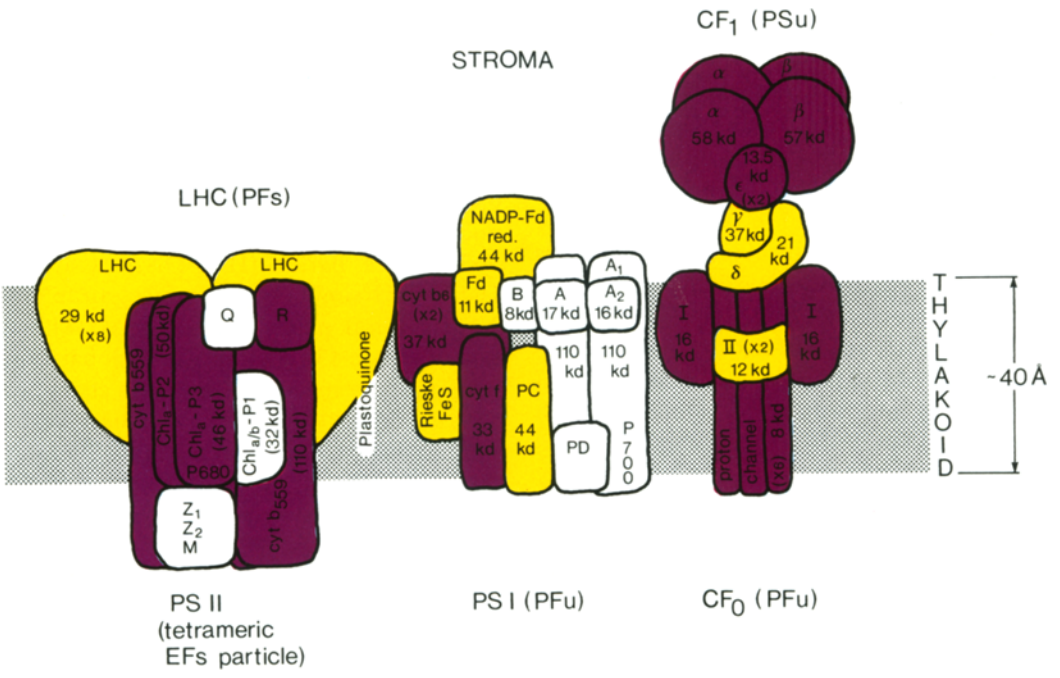


Figure 25. Model of the photosynthetic membrane. Proteins encoded in genes of the cell nucleus are colored yellow. These proteins are synthesized on ribosomes in the cytoplasm and transported across the chloroplast envelope, whereafter they are built into the photosynthetic membrane. Proteins encoded in genes of the chloroplast's own DNA and synthesized on chloroplast ribosomes are colored purple. For the white proteins the site of synthesis and the genes' localization is not yet known.

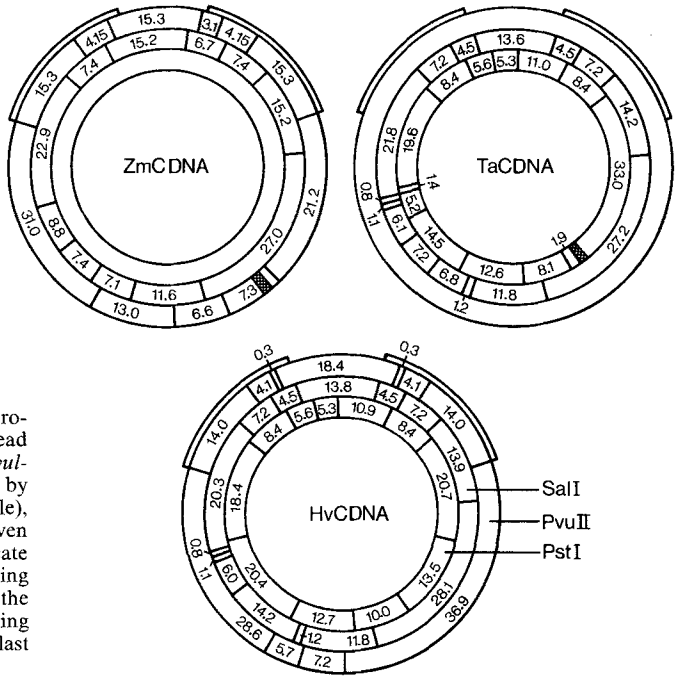


Figure 26. Restriction endonuclease maps of the circular chloroplast DNA molecules of maize *(Zea mays,* ZmC-DNA), bread wheat *(Triticum aestivum,* TaC-DNA) and barley *(Hordeum vulgare,* HvC-DNA)[46]. The circles give the fragments obtained by digestion with the restriction endonucleases PvuII (outer circle), SalI (middle circle) and PstI (inner circle). Fragment sizes are given in kilobase pairs (1000 base pairs). The cross hatched areas indicate the location of the gene for the large subunit of the CO_2 fixing enzyme ribulose bisphosphate carboxylase. The 2 arcs covering the outer circle indicate the inverted repeats of the molecule containing inter alia the genes for the RNA components of the chloroplast ribosomes.

the gene for the herbicide binding protein R and of the genes coding for the β and ε subunit of the ATP synthesizing enzyme (atpB, atpE). The latter two lie adjacent to each other on the DNA molecule and are transcribed into a common single messenger RNA molecule. Also shown in figure 27 are 11 other regions which are transcribed into messenger RNA molecules. The initiation of transcription of these particular genes requires light. In all, more than 70 regions of the chloroplast DNA molecule transcribed into messenger RNA have been identified by Carsten Poulsen[46].

I have tried to give an impression of our progressing knowledge of the interaction between the nucleus, the cytoplasm and the chloroplast at the levels of functioning, coding and synthesis. Exciting and close insights are being obtained on how a plant cell in general and photosynthesis in particular works. It will be sometime before the plant breeder can directly apply this knowledge to improving our crop plants in a rational manner.

However, there is one example in which the molecular biologist has provided a clear-cut answer to a problem posed by plant breeders: Can the efficiency of the CO_2 fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase be improved?

This most abundant enzyme in the chloroplast stroma participates both in the photosynthetic carbon reduction cycle and in the photorespiratory carbon oxidation cycle[45]. In the former, the enzyme catalyzes the only reaction known to give a net increase in the amount of carbon compounds by fixing 1 molecule of CO_2 to generate 2 moles of glycerate-3-phosphate. In the latter, the enzyme acts as an oxygenase converting ribulose-1,5-bisphosphate into glycerate-3-phosphate and phosphoglycolate. 2 moles of glycolate thus produced in the chloroplast are converted in the cytoplasm into glycerate-3-phosphate with the release of 1 molecule of CO_2. The glycerate-3-phosphate can reenter the carbon reduction cycle and the CO_2 is available for fixation by the carboxylase reaction. The flux of carbon through the linked photosynthetic reduction and the photorespiratory oxidation cycles is determined by the internal concentrations of CO_2 and O_2. If there is enough CO_2, the ribulose-bisphosphate

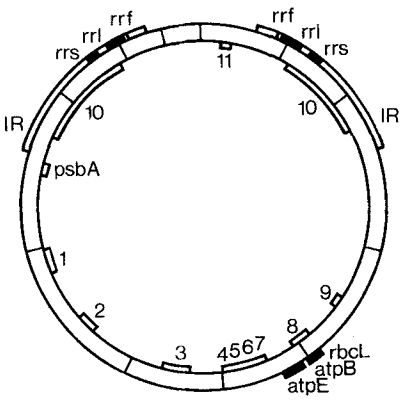


Figure 27. A map of the circular barley chloroplast DNA molecule with a coding capacity of 134 kilobase pairs[46]. IR = inverted repeats Indicated is the location of the following genes: atpB, atpE, coding for protein subunits β and ε of the adenosine triphosphate synthesizing enzyme on the photosynthetic membrane (cf. fig. 25); rbcL, coding for the large subunit of ribulose bisphosphate carboxylase; psbA, coding for the herbicide binding protein R (cf. fig. 25); rrs, rrl, rrf coding respectively for the RNA molecules of the small and large subunit of chloroplast ribosomes and the 5 S-RNA. The messenger RNA of the genes designated with numbers 1–11 and the psbA gene is transcribed only upon illumination of the barley seedlings.

carboxylase reduces CO_2 with the aid of the energy rich compounds provided by the photosynthetic membrane. If external CO_2 is limited in supply, the enzyme functions as an oxygenase and thereby protects the photosynthetic machinery from damage as it continues to operate in the sunlight. It does so by using the energy for respiration, i.e. for cooling. In barley and wheat this leads to the production of CO_2 and regeneration of the substrate to be used for the fixation of CO_2. In maize, which can operate at higher temperatures, the enzyme ribulose-bisphosphate carboxylase/oxygenase is only present in the bundle sheath cells of the leaves, while the large amount of surrounding mesophyll cells use photosynthesis to produce and supply CO_2 with the aid of a different set of enzyme reactions. In maize leaves then there is no photorespiration because of the guaranteed supply of CO_2 to the carboxylase. Could the 'waste' of energy for photorespiration in barley and wheat be prevented by changing the structure of ribulose-1,5-bisphosphate carboxylase, i.e. by abolishing the oxygenase activity?

As is diagramatically shown in figure 28, the enzyme consists of 2 subunits, one with a mol. wt of about 15,000 and the other of about 55,000. The analysis by Strøbaek, Gibbons, Haslett, Boulter and Wildman[57] of this enzyme in the amphidiploid tobacco and its parent species has revealed that *Nicotiana tabacum* with 48 chromosomes contains the large subunit passed on maternally by the chloroplast DNA from *Nicotiana sylvestris* (24 chromosomes). This can be shown by isoelectric focussing of the large subunits of

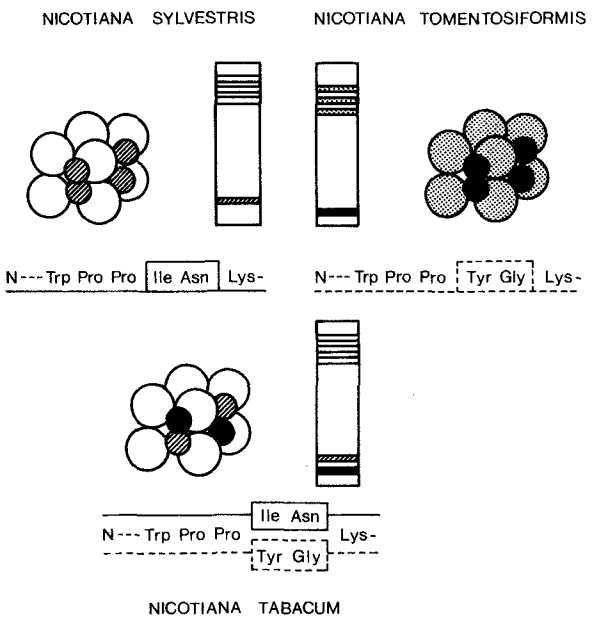


Figure 28. Molecular analysis of the inheritance of the large and small subunits of the CO_2 fixing enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase in the amphidiploid *Nicotiana tabacum* and its parent species[57].

the 3 species in gels as presented in figure 28. Such a procedure gives a 'finger print' of the different three-dimensional shapes a protein molecule can assume and is a very sensitive indication of the primary structure of the molecule. The large subunit actually contains the catalytic site for the CO_2 fixing reaction. Quite a different behavior is shown for the small subunit polypeptide chain. Its terminal amino acid sequence from the amphidiploid *Nicotiana tabacum* (48 chromosomes) is polymorphic for isoleucine/tyrosine at position 7 and for asparagine/glycine at position 8. Examination of the equivalent positions in the parent species revealed isoleucine-asparagine in *Nicotiana sylvestris* (24 chromosomes) and tyrosine-glycine in *Nicotiana tomentosiformis* (24 chromosomes), which allows the conclusion that the amphidiploid tabacum has inherited 2 alleles for the small subunit, 1 from each parent species. These alleles have been retained - conserved - for at least several hundred generations and this may indeed be one of the many reasons for the better climatic adaptability or productivity of the amphidiploid tobacco compared to its diploid parent species. The small subunit appears to have a regulatory function for the enzyme.

The gene of the large subunit of the enzyme from maize chloroplast DNA has been cloned and used to locate the corresponding genes in wheat and barley. A comparison of the maps in figures 26 and 27 shows precisely the same location in the 3 species. Lee McIntosh, Carsten Poulsen and Lawrence Bogorad have determined the sequence of the 1425 nucleotides of the gene from maize and deduced from it with the genetic code dictionary the sequence of the 475 amino acids as presented in the diagram of figure 5[35]. This has made it possible to determine which amino acids are important in catalysis of ribulose-1,5-bisphosphate to phosphoglyceric acid using the information provided by G.H. Lorimer, J.V. Schloss, C.D. Stringer and F.C. Hartman on peptide fragments binding the substrate and various effectors[45]. Radioactively labeled substrate analogues can be covalently bound to certain amino acid side chains and thereby reveal the location of the substrate binding sites. As shown in figure 29 the substrate analogue D-3-bromo-1,4-dihydroxybutanone-1,4-bisphosphate (BBBP), which is 1 carbon atom shorter than the 5-carbon sugar ribulose-1,5-bisphosphate, binds to lysine residues 175 and 334. Thus these 2 amino acids are located closely together in the pocket for the substrate formed by the folded polypeptide chain. Another analogue, N-bromoacetylethanolamine phosphate (NBA-EAP), having the same length as the natural substrate but otherwise different chemical groupings, binds to cysteins 172 and 458 showing these residues likewise to be in the pocket of the substrate binding site. The enzyme has to be activated for catalysis into a different shape by 2 effectors, namely magnesium and

CO_2. If NBA-EAP is provided after activation with magnesium it will bind to lysine 175 like BBP and no longer to the cystein residues.

The binding site for the activating CO_2 molecule, distinguishable from the substrate CO_2 molecule involved in the carboxylation, has been identified as lysine 201. This has been done by activating the enzyme with radioactive CO_2 and filling the substrate pocket with the transition state analogue D-2-carboxyarabinitol-1,5-biphosphate (an analogue which mimics the carboxylated intermediate of ribulose-1,5-bisphosphate to the phosphoglyceric acid molecules, i.e. D-2-carboxy-3-keto-arabinitol-1,5-bisphosphate). This protects the substrate site from binding radioactive CO_2. Subsequently the bound radioactive CO_2 was covalently linked with diazomethane to the ε-amino group of lysine 201.

If the amino acid sequences of these regions pinpointed as important in catalysis are compared for maize, spinach and barley, exactly the same sequences are found while other regions are quite polymorphic. Apparently the pocket used for carboxylation and oxygenation of the substrate ribulose-bisphosphate is the same in evolutionarily very distantly related plant species, irrespective whether the plant exhibits photorespiration or not. Any reduction in the oxygenase activity will also affect the carboxylase activity. The proposal to breed away the oxygenase activity, i.e. photorespiration from wheat or barley by restructuring ribulose-1,5-bisphosphate carboxylase is incompatible with our present molecular knowledge of the enzyme.

Combining species which cannot be sexually hybridized

I mentioned in the introduction that a considerable number of our crop plants have arisen by species hybridization and chromosome doubling. The production of stable protoplasts by enzymatic digestion of the cell walls, subsequent fusion of protoplasts with the aid of polyethylene glycol in the presence of calcium chloride and regeneration of naked protoplasts into cells and whole plants has opened the way for somatic hybridization of species which cannot be crossed. These techniques have been especially developed for higher plants by Georg Melchers, W.A. Keller, K.N. Rao and H.R. Michayluk[36]. Somatic hybrids are expected to have a chromosome complement comprising the sum of the diploid chromosome numbers present in the fusing partners. They should be amphidiploid, fertile and true breeding. This is indeed the case with those somatic hybrids produced that also can be obtained by sexual hybridization e.g. *Nicotiana tabacum*.

The tomato and potato cannot be sexually hybridized. The plant breeder's dream in this case is to obtain somatic true breeding hybrids which produce potato tubers under ground and tomato fruits above ground. Well, the result could also be that the hybrid develops tomato roots and potato-like fruits. -

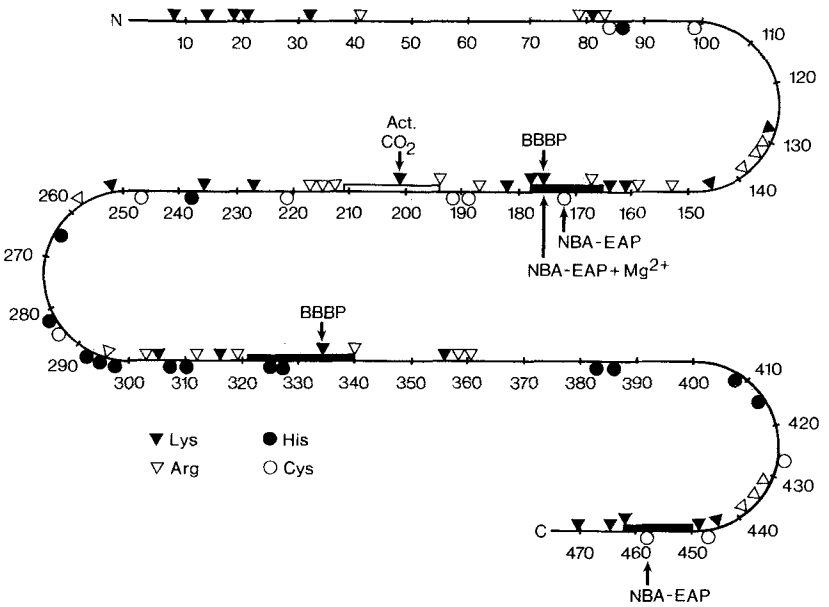


Figure 29. Relationship between the structure and function of the large subunit of ribulose-1,5-bisphosphate carboxylase[45]. From the sequence of 1425 nucleotides determined for the gene of maize, the primary structure of the 475 amino acids was deduced and the polypeptide backbone is presented here as a serpentine line with the distribution of certain amino acid side chains indicated (lysine, arginine, histidine and cystein). Using the radioactively labeled substrate analogue D-3-bromo-1,4-dihydroxybutanone-1,4-bisphosphate (BBBP) lysine 175 and lysine 334 can be identified as amino acids important for the binding of the substrate ribulose-1,5-bisphosphate in the process of CO_2 fixation. Similarly, the role of cystein 172 and cystein 458 in binding the substrate can be asserted by the use of NBA-EAP (N-bromoacetylethanolamin phosphate). Amino acid lysine 201 is important for activation of the enzyme by CO_2.

Georg Melchers set out to hybridize protoplasts from leaves of a yellow -green mutant of tomato *(Lycopersicon esculentum)* with 24 chromosomes and a dihaploid potato *(Solanum tuberosum)* with 24 chromosomes. The latter protoplasts were produced by cell wall degradation of submersed cultured callus cells which are white since they contain only proplastids. Such a fusion product can be recognized in figure 30. The cytoplasms of the fused protoplasts have not yet mixed and the tomato-potato fusions can therefore be recognized as protoplasts with a colorless part and a green part. Regeneration was accomplished on appropriate media and shoots selected that were normal green. A considerable number of putative hybrids were grown[36,47]. Two of these are shown in flower in figure 31, in comparison with the flowers of the potato partner and those of a typical tomato. The morphology of the flowers as well as the leaves of the plants were quite aberrant and differed from the potatoes and the tomatoes. The plants grow slowly and are continuously propagated by cuttings. They produce small tubers, which can sprout into new plants but they are completely sterile, producing no good pollen or ovules. Anthony Holder has studied in the hybrids the 2 subunits of ribulose bisphosphate carboxylase as illustrated in figure 32. The small subunits of the enzyme from potato and tomato give different isoelectric focusing patterns due to different primary structures of the small subunits in the 2 species, and have as a consequence thereof different conformational states. As discussed in the previous chapter the small

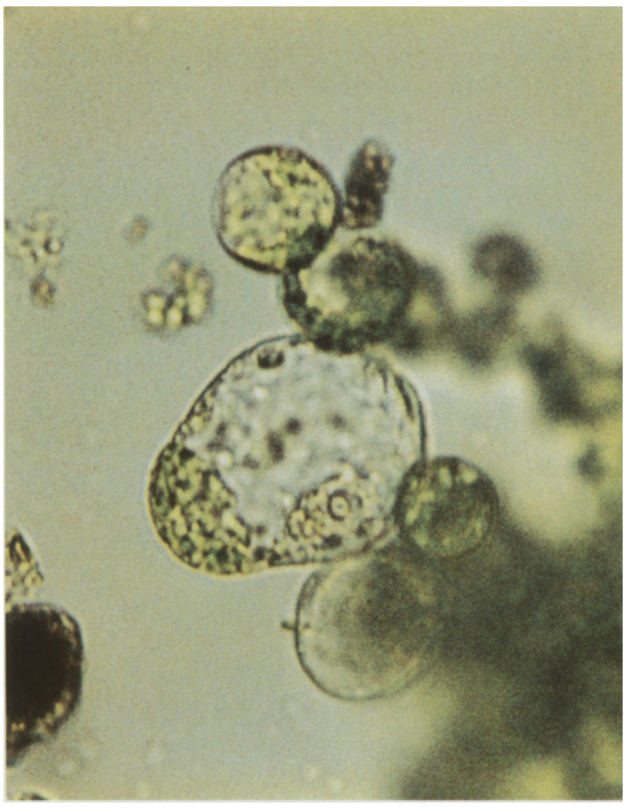


Figure 30. Hybrid protoplast created by fusion of a protoplast from tomato and a protoplast from potato[36]. The cytoplasm of the tomato is recognizable by the green chloroplast originating from the tomato mesophyll cells used while the colorless part of the cytoplasm can be traced to the other partner, a colorless potato tissue culture cell.

Figure 31. Comparison of flowers from potato (top), a somatic tomato-potato hybrid (6b, middle) and tomato (bottom). Inset: Flower of another tomato -potato hybrid (1b)[36].

subunit polypeptide is encoded by nuclear genes and we expect in somatic hybrids both the tomato and potato genes to be present and thus a mixture of the 2 polypeptide patterns. Indeed polypeptide bands from both the tomato and potato small subunits are found in the hybrids (fig. 32) revealing that they do contain genes from both species and that these are expressed. Additional peptide maps have substantiated these results[47]. The large subunit of the enzyme is encoded in a gene in chloroplast DNA. Fortunately, again the isoelectric focusing patterns are different for the 2 species (fig. 32). However, there is no summation of the patterns in the hybrids: 3 of them contain the polypeptide bands characteristic for the tomato and the 4th for that of the potato. This reveals that the hybrids regenerated from the fused protoplasts retain either the tomato chloroplast DNA or the potato chloroplast DNA while the organelle DNA of the other species is eliminated. If a large enough sample of hybrid plants is investigated, about half of them are topatoes (that is, hybrids with tomato chloroplasts) and half of them pomatoes, or hybrids with potato chloroplasts. This has been subsequently substantiated by restriction endonuclease patterns of the chloroplast DNA in the 2 species and the somatic hybrids[49]. Somatic segregation of chloroplast DNA is a well established phenomenon from classical genetic studies.

Why are these pomatoes and topatoes sterile, slow growing plants? Maria Sacristán who has studied their chromosome numbers finds that practically all hybrids have a chromosome number of more than the expected 48, mostly 50, some 56 and even 72. The last mentioned hybrid could be a triple fusion of 2 potato protoplasts and 1 tomato protoplast ($2 \times 24 + 24$ chromosomes). The extra chromosomes could originate from aneuploid cells with extra chromosomes present in the potato cell cultures. Such aneuploid plants of unbalanced chromosome constitution are known to be abnormal in growth and often sterile. It could, however, also be that the potato and tomato nuclear genomes cannot cooperate with each other or with the chloroplast genome of the foreign species.

The hybrids are more chill-resistant than the tomato parent, but not as resistant as the potato parent[56]. No doubt the importance of such exotic somatic hybrids lies in the possibility of bringing together genes from widely different species. If we can learn to regulate the pairing and crossing over of the homoeologous chromosomes in the hybrids and thereby transfer the desired genes for cold, heat or disease resistance from the chromosome of one species to that of another, this will be a helpful tool in plant breeding. It will, however, also require that we subsequently again diploidize the plants. We may also be optimistic and search for more successful somatic hybrids.

Outlook

To date, the success of plant breeding has primarily been based on selection of improved varieties from plant populations with a variability provided more or less by chance through crossing, polyploidy or mutation. A purposeful and rational plant breeding – especially of traits concerning quality and environmental adaptation – has been limited by the unavailability within the framework of classical genetics and biochemistry of methods to characterize the large number of genes involved. Genetic engineering has now provided, first and foremost, the tools to analyze the structure and function of any of the million genes present in the plant cell nucleus, the chloroplast and the mitochondrion. This helps us to understand the genetic control of the traits which we wish to improve. Increasingly, we hope to use individual, characterized genes in the rational improvement of the quality and environmental adaptation of crop plants by conventional breeding techniques in combination with the novel techniques of genetic transformation and somatic hybridization.

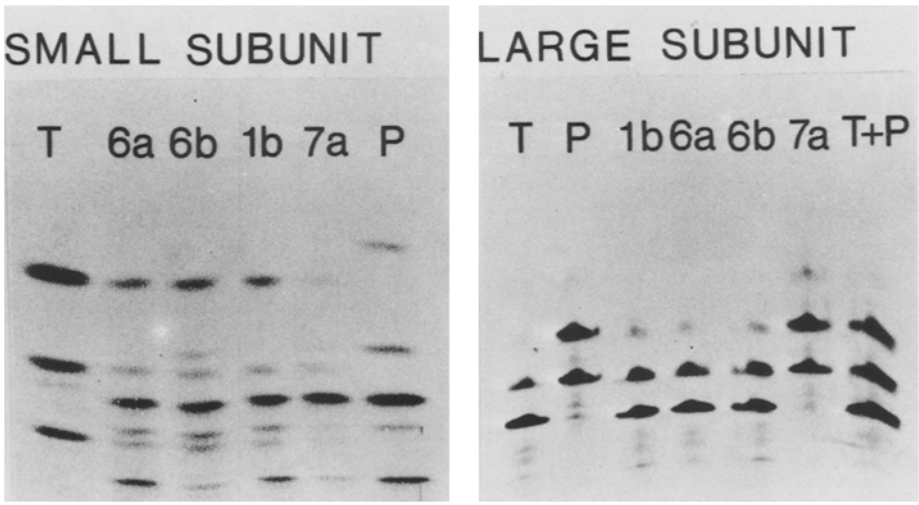


Figure 32. Finger prints of the conformational states of the small and large subunits of ribulose-bisphosphate carboxylase in the tomato (T), potato (P) and 4 somatic hybrids[36]. The small subunit patterns of the hybrids are an additive mixture of the two parents revealing the presence of nuclear genes of both parents in the hybrids. The large subunit patterns in 3 hybrids are like that of the tomato, in the 4th like that of the potato. The hybrids thus contain either tomato chloroplast DNA or potato chloroplast DNA but not both. (T + P, in vitro mixture of tomato and potato large subunits).

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0014-4754/83/070687-27$1.50 + 0.20/0

Full Papers

Intracellular topography of immunoreactive gastrin demonstrated using electron immunocytochemistry

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Summary. Gastrin (G)-producing cells from the mammalian gastric antrum have been investigated using computer-assisted morphometry and a novel double colloidal gold-labeled-immunoglobulin electron immunocytochemical procedure. Correlation analysis of human antral G-cells indicates ($p < 0.001$) that a single population of granules exists with small (160 nm) electron-dense and large (240 nm) electron-lucent forms representing the extremes. Non-crossreacting region-specific antisera have been used to visualize G-17 and G-34 (progastrin) to the small electron-dense granules and G-17 to the other intermediate forms. From the results we propose a topographic segregation of immunoreactive gastrins within 2 apparently distinct granule subclasses and suggest that this may represent the pathway of granule maturation.

Introduction

Post-translational cleavage of precursor to smaller molecular forms of bioactive peptides has been established in recent years[18,19,31]. The majority of endocrine cell types exhibit a range of secretory granule morphology[12] which could reflect storage of different molecular structures. One such example is the mammalian antral G-cell which has been claimed to contain 2 main granule populations, 1 large electron-lucent and 1 small dense-cored[9,13,32]. The observed variations in granule ratio has been suggested by different groups to represent the functional state of the secretory cycle[8,13], the effect of fixation[20,21], the co-existence of 2 unrelated products[16] or of 2 molecular forms of gastrin[13,36] within the same cell. Recent advances in electron immunocytochemistry particularly the introduction of region-specific antisera[17] and immunogold procedures[5,8,28–30,37] have allowed the ultrastructural topographic distribution of separate regions of peptide molecules to be visualized[26,33]. In this study we have combined newly developed electron immunocytochemical procedures with computer-assisted morphometry in order to characterize the granule population in mammalian antral G-cells.

Materials and methods

Fresh antral mucosa was obtained from adult human subjects ($n = 6$) at surgery, and from adult cats ($n = 10$) following sodium pentobarbitone anesthesia ('Euthatal'; 200 mg/ml, 1 ml/kg). The tissue was processed for conventional electron microscopy or for electron immunocytochemistry[37]. Electron micrographs of cells fulfilling the criteria acknowledged for the classification of G-cell granules[7] were analyzed