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The analysis of plant genes and chromosomes by using DNA cloned in bacteria

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Some examples are described in which the molecular cloning of plant DNA and the associated techniques of molecular biology have already contributed to plant genetics and plant breeding research. They include (*a*) the isolation and determination of the complete sequence of some nuclear genes, (*b*) the detection of genetic variation in gene structure and copy number, (*c*) the physical mapping of the chloroplast genome, (*d*) the physical mapping and identification of nuclear chromosomes by *in situ* hybridization, (*e*) a molecular description of highly repeated sequences in heterochromatin, (*f*) a rapid method for classifying cytoplasmic variants of maize and (*g*) characterization of mitochondrial DNA sequences necessary for normal pollen development in maize. It is emphasized that the mapping of genomes by using molecular methods does not require genetic variation or expression of the genes in the phenotype. Where a nucleic acid probe is available for a gene, then recessive allelic variants or inactive genes can be detected that do not contribute to the phenotype.

Introduction

The introduction of the molecular cloning of DNA in bacteria and the associated techniques of molecular biology into plant genetics has greatly enhanced the exploration of genetic variation at the molecular level. Consequently, the application of the techniques of molecular biology to plant chromosomes may be seen as a milestone in the history of plant genetics and in the exploitation of variation in plant breeding. It is my aim here to describe briefly some examples where the application of molecular biology has already contributed new knowledge on the structure of plant genes and on the molecular nature of genetic variation. I also wish to emphasize that chromosomes and very small segments of chromosomes can be mapped by using molecular biochemistry without a requirement for phenotypically expressed variation or estimates of recombination frequencies. The approach is therefore different from classical genetic analysis. Molecular biological approaches sometimes lead to new, more rapid methods of detecting genetic variation. To illustrate this, examples of the analysis of DNA sequences relevant to the control of endosperm development in triticale and to pollen development in maize are described.

Molecular cloning has brought new opportunities for studies in plant genetics for two principal reasons. The first is that the insertion of a small fragment of plant DNA into a bacterial plasmid *in vitro*, and the transfer of this recombinant plasmid into a bacterium, isolates the plant DNA fragment from all the others of the plant cell because only one chimaeric plasmid is taken up by each bacterium. The second reason is that the subsequent growth of the recipient bacterium to form a clonal population of cells, from which the plant DNA can be reisolated, provides large quantities of the pure plant DNA sequence. With large quantities of a pure sequence, extensive studies, including nucleotide sequencing, can be carried out.

The limiting step in the isolation of genes by bacterial cloning is the detection of the bacterium

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carrying the desired plant gene. Detection is relatively straightforward when a nucleic acid sequence homologous to the gene is available, and this is why most of the genes isolated to date are those whose RNA products are in large enough concentration, in at least some plant tissues, to be easily purified or highly enriched. Such genes include those specifying the ribosomal RNAs, storage proteins and leghaemoglobin. Studies on the ribosomal RNA genes of wheat purified by molecular cloning, which have led to new knowledge on gene structure and variation, are described below.

Variation in gene structure and copy number uncovered by molecular analysis

The genes specifying the ribosomal 25*S*, 18*S* and 5*S* RNA molecules are highly repeated in higher plant genomes. Hybridization of purified 5*S* RNA to wheat DNA digested with a restriction endonuclease and fractionated by electrophoresis showed that the 5*S* RNA genes reside in two different classes of repeating unit, one about 410 base pairs long and the other about 500 base pairs long (Appels *et al.* 1980). Gerlach & Dyer (1980) have purified examples of each size of repeating unit by molecular cloning and determined their complete nucleotide sequence. Because the 5*S* RNA sequence had been determined previously (Payne & Dyer 1976; Barber & Nichols 1978), it was possible to recognize the 120 base pairs in each repeating unit that specify the 5*S* RNA. The remaining DNA in each repeating unit is 'spacer' DNA.

A particular feature of the sequenced member of the 500 base pair size class of repeating unit is that it contained a 15 base pair duplication within the coding sequence. It is not known how many copies of this mutant gene are in the wheat variety Chinese Spring, but approximately 15–20% of the repeating units in the larger size class appeared to contain an additional 10–20 base pairs (Gerlach & Dyer 1980). Thus the 5*S* genes with the duplication may be common in this wheat variety. This is of particular significance because in *Xenopus* 5*S* RNA genes, the region homologous to that carrying the duplication in some wheat genes is very important in regulating transcription of the gene (Sakonju *et al.* 1980; Bogenhagen *et al.* 1980). No RNA transcript 135 (120 + 15) bases long was detected in wheat by Gerlach & Dyer (1980), so it appears that the duplication with the gene prevents transcription – an explanation consistent with the region carrying the duplication being important for gene expression also in wheat – or the RNA product is destroyed. Another possibility is that the genes with the internal duplication were silent in the tissues studied, owing to developmental control (not all 5*S* RNA genes are used at any one time).

In each repeat unit there is an array of A–T base pairs immediately following the sequence specifying the 5*S* RNA. This array dictates, in part, the termination of transcription. The 70 base pairs before the beginning of the coding sequence are very similar in the cloned sequences from both the 410 and 500 base pair size classes. Most of the other spacer DNA has diverged considerably between repeating unit classes. Thus the 70 base pairs before the start of the coding sequence are probably conserved to determine the correct initiation of transcription of 5*S* RNA.

This study on 5*S* RNA genes illustrates the value of molecular analysis to genetics in many ways. It has revealed (1) details of gene structure relevant to the control of gene expression, (2) variation in spacer DNA length and sequence between genes, (3) a small duplication within a gene, which may make the gene permanently silent thus preventing its presence ever being

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detected in studies of the phenotype. Also comparison of the nucleotide sequences of the 120 base pair regions coding for the 5*S* RNA in four clones has revealed single base pair differences (mutations) at eight different sites in different copies of the gene (Gerlach & Dyer 1980). The biological significance of these mutations is as yet unknown.

The copy number of repeated genes, such as the ribosomal RNA genes, commonly varies between individuals of a species. This is easily detected by using cloned sequences as probes. Variation in the copy number of 25*S* + 18*S* ribosomal RNA genes which are tandemly arrayed at homologous loci in wheat and rye has been detected by *in situ* hybridization of cloned rDNA to metaphase chromosomes (Miller *et al.* 1980). Heterozygosity for the number of copies of rRNA genes has also been detected in diploid rye by similar methods. These rapid ways of detecting variation, including heterozygosity, without carrying out a breeding experiment, clearly illustrate the value of the techniques of molecular biology to genetics.

The physical mapping of chromosomes

The mapping of plant chromosomes by molecular techniques not based upon detecting variation in gene expression is best developed for the chloroplast genome (Bedbrook & Kolodner 1979). The progress obtained by this route is important not only because of the importance of the chloroplast genes to photosynthesis and other key areas of plant metabolism, but also because in many crop plants, recombination either does not occur between maternal and paternal chloroplast genomes or, if it does take place, it happens at a frequency below that necessary to enable the genes to be mapped by conventional genetic techniques. Segments of chloroplast DNA, generated by digesting purified DNA with restriction endonucleases, have been ordered on circular maps in a number of species including maize, pea, spinach (reviewed in Bedbrook & Kolodner 1979) and wheat (Bowman *et al.* 1981). Every segment has been cloned in bacteria, and consequently large quantities are available to map and sequence any region of the circular chromosome. The genes for chloroplast ribosomal RNAs and many transfer RNAs have been positioned on the genomes by hybridization of the RNAs to specific DNA fragments. The position of other genes, including that of the large subunit of ribulose bisphosphate carboxylase (LS) has been determined by finding the cloned DNA segment that, when mixed with appropriate extracts from *E. coli*, produces an mRNA that translates into LS (reviewed in Bedbrook & Kolodner 1979). The faithful transcription and translation of chloroplast genes in *E. coli* extracts implies that *E. coli* contains the information to recognize the start and stop signals for gene expression on chloroplast DNA. This has been recently proved by the demonstration that LS is synthesized *in vivo* by *E. coli* cells carrying the chloroplast LS gene (Gatenby *et al.* 1981). As expected from this result, the segment of DNA that occurs just before the coding region of the LS gene that has been cloned from maize possesses features similar to those that bacteria use to control the initiation of transcription and translation (McIntosh *et al.* 1980; Gatenby *et al.* 1981). From these examples it may be seen that the mapping of genes on chloroplast DNA is proceeding relatively rapidly, and detailed sequencing and other studies are providing details of the signals involved in gene expression. Such studies could scarcely be contemplated without resorting to molecular cloning.

Small segments of nuclear chromosomes, which, of course, contain much more DNA than the chloroplast genome, are being mapped by similar techniques. Furthermore, plant nuclear chromosomes contain many families of repeated sequences (Flavell 1980), and when members

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of the same family are highly clustered or in tandem, their position can be determined by *in situ* hybridization (Pardue & Gall 1970). In this technique, a particular piece of DNA purified by molecular cloning is tritium-labelled and hybridized to denatured metaphase chromosomes spread on a glass slide. The sites of hybridization are recognized by radioautography. The locations of the multicopy $25S+18S$ cytosolic ribosomal RNA genes in wheat and rye have been revealed this way (Gerlach & Bedbrook 1979; Miller *et al.* 1980). This technique provides a way not only of recognizing the chromosomal sites of such purified genes, or other repeated sequences, but also of distinguishing the chromosomes containing them from those that do not. The physical mapping of chromosomes by *in situ* hybridization with the use of highly purified repeated sequence DNA is best developed in wheat and rye (Gerlach *et al.* 1978, 1980; Gerlach & Peacock 1980; Dennis *et al.* 1980; Jones & Flavell 1981 *a, b*; Bedbrook *et al.* 1980; Hutchinson *et al.* 1980) and further details for rye are described below.

A molecular analysis of highly repeated sequences and heterochromatin in rye

The project to purify and study the chromosomal location of specific families of repeated sequences in rye was undertaken because (*a*) the heterochromatic segments at the telomeres of cultivated rye appear to be responsible in triticale for aberrations in meiosis (Merker 1976), endosperm development and grain shrivelling (reviewed in Thomas *et al.* 1980), and (*b*) heterochromatin contains mostly repeated sequence DNA (reviewed by John & Miklos 1979). The role of rye heterochromatin in causing aberrant endosperm development with consequent grain-shrivelling in triticale is described in the contribution to this symposium by Bennett.

The purification by molecular cloning of members of families of highly repeated sequences likely to reside in rye telomeric heterochromatin (Appels *et al.* 1978) is described in full elsewhere (Bedbrook *et al.* 1980). The organization of the families in the genome was discovered by carrying out *in situ* hybridization of the radioactively labelled cloned DNAs to metaphase chromosomes. Four families were found to account for 45–65 % of the DNA of heterochromatic C-bands (Bedbrook *et al.* 1980; Jones & Flavell 1981 *a, b*). In figure 1 the mapping of the four families of repeats in chromosomes of the rye variety King II is summarized. The C-bands, which are shown on the right hand member of each chromosome pair, are taken from the results of Singh & Röbbelin (1975, 1976) for King II.

The telomeric heterochromatin on the short arm of each chromosome includes tandem arrays of each of the four families. Chromosomes 1R, 4/7R, 5R and 6R contain interstitial heterochromatin bands that contain only one of the families, while chromosome 6R also contains interstitial arrays of another family. Where telomeric blocks of heterochromatin occur on the long arms, they include arrays of sequences from one, two or four of the families studied. Thus different blocks of heterochromatin can be distinguished by hybridization with different cloned sequences, enabling certain chromosomes to be distinguished one from another. However, each family of repeats is present on all chromosomes. Further structural details on the repeated sequences in these families are described elsewhere (Bedbrook *et al.* 1980). These details, together with the results summarized here (see also Jones & Flavell 1981 *a, b*), provide a molecular description of those segments of the genome that appear to be major contributors to grain-shrivelling in triticale, a phenomenon first observed by plant breeders.

It is interesting to note that a genetic analysis of grain-shrivelling in triticale would show

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that this phenomenon is controlled by many loci, some of larger effect than others. The inheritance would therefore be of the polygenic, continuous kind. However, the analysis of heterochromatin by molecular analysis shows that in this particular instance the polygenes are long arrays of repeated sequences that are probably not transcribed. It is possible that the arrays exert their phenotypic effect in triticale by being replicated later than most of the wheat and

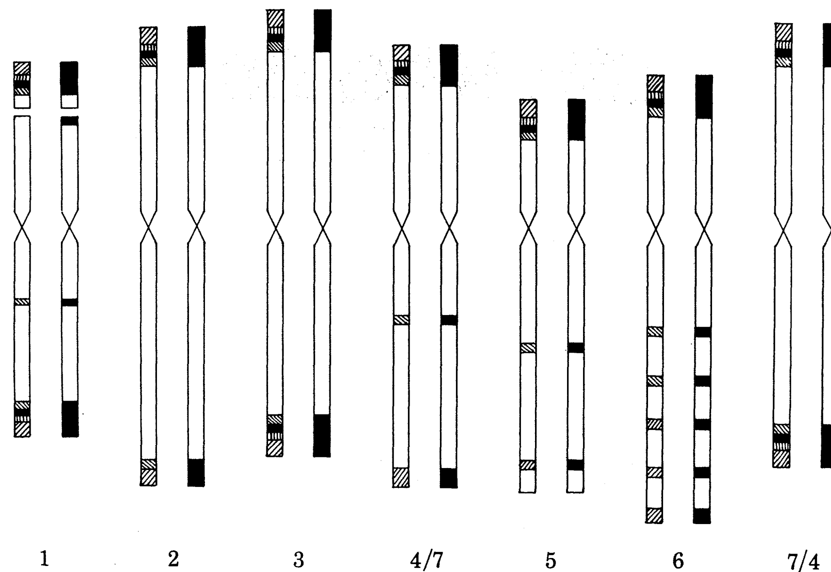

FIGURE 1. The composition of heterochromatic C-bands in rye, variety King II. The right-hand member of each chromosome pair illustrates the position of C-bands (Singh & Röbbelin 1975, 1976) and unpublished results of M. D. Bennett and colleagues). The left-hand member illustrates the arrays of repeated sequences found in the heterochromatin by *in situ* hybridization (Bedbrook *et al.* 1980; Jones & Flavell 1981 *a*). Molecular descriptions of each of the four kinds of array are given in Bedbrook *et al.* (1980). Each kind of repeating sequence has a different shading: ▨, ▥, ■, and ▧.

remaining rye DNA, with the consequence that rye anaphase bridges are formed but not resolved (Bennett 1977; Thomas *et al.* 1980). That is, the presence of the repeats affects the phenotype through their influence on chromosome behaviour in a specific tissue. This example shows that heterochromatin and highly repeated sequences are not inert when considered in the context of the total biology of an organism.

The cloned repeated sequences described here are, of course, useful probes for uncovering chromosomal variation within and between individuals. Many chromosomes have been found in *Secale* species with *in situ* hybridization patterns different from those illustrated in figure 1 (Jones & Flavell 1981 *a*, *b*). The probes are also useful together with C-banding for characterizing triticale lines in breeding programmes that have lost telomeric heterochromatin and are therefore likely to have less shrivelled grains (Bennett, this symposium).

The loss of telomeric highly repeated DNA from rye chromosomes maintained as disomic additions in wheat has already been demonstrated by the cloned probe DNAs (Jones & Flavell 1981 *a*).

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Variation in mitochondrial DNA and cytoplasmically inherited male sterility in maize

Techniques of molecular biology including molecular cloning have recently made useful contributions to our understanding and exploitation of cytoplasmic mutations that cause the failure of pollen development. These mutations are used extensively in hybrid crop breeding and hybrid seed production. For example, in maize during the 1960s almost all the hybrid

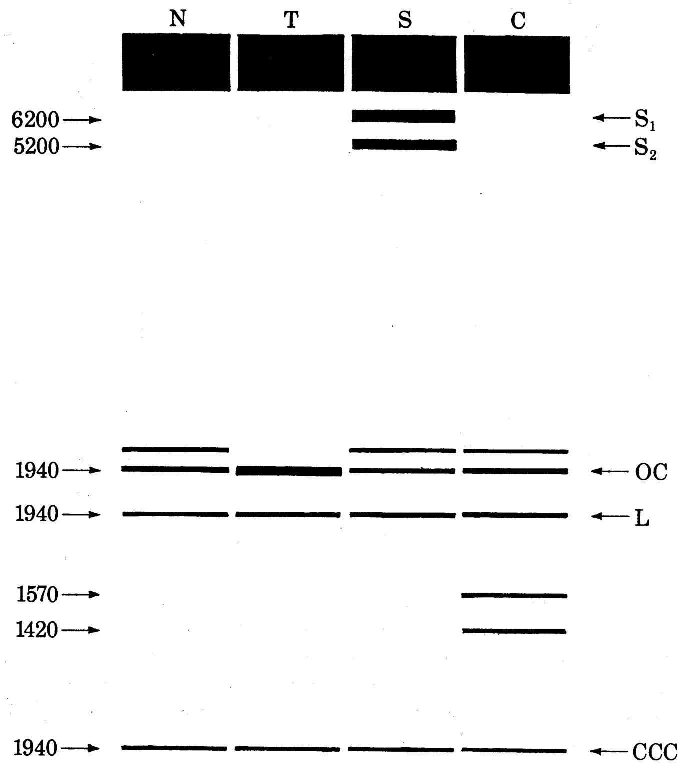

Figure 2. The classification of maize cytoplasms by electrophoresis of mitochondrial DNA. Mitochondrial DNA from each of the four types of cytoplasm (N, T, S and C) is fractionated by electrophoresis on an agarose gel. The linear and circular low molecular mass DNAs provide a unique pattern for each cytoplasm (results schematically redrawn from Kemble *et al.* 1980) and Kemble & Bedbrook 1980)). Electrophoresis was from the top to the bottom of the diagram. The major block of DNA at the top of the gel is high molecular mass DNA in the principal mitochondrial genome. S_1 and S_2 are linear pieces of DNA found only in S cytoplasm. OC, L and CCC are the open circular, linear and covalently closed circular forms respectively of the same nucleotide sequence. The numbers on the left-hand side are the lengths of the DNA pieces in nucleotide pairs.

maize seed production in the U.S.A. was achieved by the use of the 'Texas' cytoplasm, which causes severe pollen failure in certain nuclear genetic backgrounds under agricultural conditions (Duvick 1965). The pollen failure ensures that plants with the Texas cytoplasm are fertilized by pollen from another plant and therefore that only hybrid seed is formed. Male sterility due to cytoplasmically inherited mutations can be suppressed by specific nuclear genes (fertility restorer genes). This has enabled geneticists to distinguish three different types of maize cytoplasm (T, C and S) that confer male sterility. Each is suppressed by different restorer genes (Beckett 1971). To determine to which group an unknown cytoplasm belongs, it was

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formerly necessary to transfer it by backcrossing into the nuclear background of the tester stocks with different fertility restorer genes and then to score fertility. Cytoplasm classification can now be carried out much more rapidly by studying DNA components of mitochondria or the proteins synthesized in isolated mitochondria (Kemble *et al.* 1980; Forde *et al.* 1980).

Mitochondria of maize contain small pieces of DNA, in addition to their principal genomes. Some are linear, some are circular (Kemble & Bedbrook 1980). These small pieces are readily seen when mitochondrial DNA is fractionated by electrophoresis on an agarose gel and 'stained' with ethidium bromide. The complement of small DNA molecules is unique for each of the N, C, T and S cytoplasms, as illustrated in figure 2. This was concluded from a survey of 31 lines carrying different cytoplasms previously classified by their interaction with specific restorer genes (Kemble *et al.* 1980). The classification of maize cytoplasms by this method requires etiolated shoots from only 20–30 seeds, takes only 1 day after harvesting the plant material, many samples can be processed simultaneously and the characteristic gel banding pattern is independent of the nuclear genetic background, i.e. the cytoplasm can be classified into the appropriate group whether there is a nuclear restorer gene present or not. The method is therefore of considerable use to breeders for analysing sources of cytoplasmically inherited male sterility or other cytoplasmically inherited traits.

Progess in understanding the molecular basis of the variation causing infertility in plants with T, C or S cytoplasms has come from cloning DNA sequences in the two small DNA pieces, S_1 and S_2, found only in mitochondria from S cytoplasm (figure 2). Restriction endonuclease maps of these fragments, first recognized by Pring *et al.* (1977), have been established by research groups in Florida, North Carolina and Cambridge, U.K. Cloned fragments were selected that specifically hybridized only to S_1 or S_2 (Thompson *et al.* 1980) and used as hybridization probes to show that S_1 and S_2 sequences are present in the principal genome (high molecular mass mitochondrial DNA) in the normal cytoplasm but not in the male sterile cytoplasms S, T or C (Thompson *et al.* 1980). Thus male sterility is correlated with the deletion of S_1 and S_2 sequences from the mitochondrial genome. That the loss of S_2 sequences from the principal mitochondrial genome is the *cause* of cytoplasmic male sterility is strongly supported by the finding that phenotypic revertants of S cytoplasm, i.e. plants with normal pollen development, have lost the linear S_1 and S_2 molecules and have S_2 sequences integrated into high molecular mass mitochondrial DNA (Levings *et al.* 1980).

These studies therefore illustrate that a phenotypic characteristic important in plant breeding is probably the result of loss of DNA sequences or of the excision and maintenance of the sequences in a small, presumably non-functional fragment. The reversion of the phenotype upon reintegration of the sequences suggests that the location of the sequences is important for correct function. Furthermore, the S_1 and S_2 cloned DNA fragments are very useful probes for detecting plants with the deletion(s). Such plants may possess a cytoplasm likely to produce pollen failure in certain environments and nuclear genetic backgrounds. This assay, which would give a positive answer whether nuclear restorer alleles were present or not, again illustrates the value of molecular biological approaches for detecting recessive variation.

Prospects

Examples such as those described in this paper represent the beginning of the application of molecular cloning to plant genetics. A great deal of new information about genetic variation,

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gene expression, differentiation and development can be confidently expected to emerge over the next few years. If genetic problems important in plant breeding are studied, such as that described above relating to cytoplasmic male sterility, there is every chance that information and techniques helpful to plant breeders will emerge. But even more than this, when it becomes possible to insert DNA into plants, the genes isolated by molecular cloning techniques can be used to modify plants in novel directed ways (Cocking 1981; Flavell 1981). This achievment will herald another era in the history of plant genetics and breeding made possible by molecular cloning. There is indeed much to look forward to in plant genetics because of the results from the past 40 years of intensive research in bacterial genetics.

I am grateful to my colleagues Dr T. A. Dyer and Dr C. N. Law for help in the preparation of the manuscript and to other colleagues at the Plant Breeding Institute who provided much of the experimental evidence presented in this paper.

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Discussion

P. R. Day (*Plant Breeding Institute, Cambridge, U.K.*). Most plant breeders will be daunted by the detailed analysis necessary to identify and manipulate useful genes in plant transformation. In the meantime, what are the prospects of introducing useful variation by the cruder techniques of transforming with shotgun plant DNA clones?

R. B. Flavell. I cannot at present be particularly optimistic about 'shotgun' approaches to plant modification because they are so inefficient. Consider a plant with a haploid genome size about that of barley (5.3×10^9 nucleotide pairs). If a bank of cloned sequences were created, with the average piece of plant DNA being 20000 nucleotide pairs, then approximately 2.6×10^5 different clones would be required to provide a complete complement of the genome. Clearly in practice the number of clones required would be substantially in excess of this. If only one cloned DNA piece were taken up into each plant, any single copy gene would be present in only one out of 260000 plants. Because in practice the efficiencies would be *very much* less than this for technical as well as theoretical reasons, the number of plants necessary to be handled is too great to make the approach attractive. However, if techniques of specific gene enrichment are developed and/or important genes or regulatory sequences are represented many times in a plant genome, the shotgun approach becomes more favourable. Clearly, unless transformation frequencies approach 100% it is also essential to be able to select those plants containing inserted DNA.

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J. Heslop-Harrison, F.R.S. (*Welsh Plant Breeding Station, Aberystwyth, U.K.*). Although there is still some uncertainty about what cytoplasmic organelles, if any, are actually conveyed into the egg in the course of fertilization in angiosperms, some aspects are fairly clear. Notwithstanding some earlier suggestions to the contrary, it is now well established that the plastid and mitochondrial lineages are continuous through meiosis in the anther, although the organelles do undergo dedifferentiation followed by later redifferentiation during the meiotic prophase (H. G. Dickinson & J. Heslop-Harrison, *Phil. Trans. R. Soc. Lond.* B **277**, 327–342 (1977)). During the subsequent pollen mitosis, the organelles are partitioned between the vegetative and generative cells; mitochondria appear invariably to pass into the generative cell, but plastids may (e.g. in Gramineae) or may not (e.g. in Orchidaceae) be incorporated. Where plastids do not enter the generative cell, there seems no likelihood that any can reach the egg. There is still a further period when there could be an elimination of organelles, namely during the passage through the receptive synergid. At this time the male gametes appear to be thoroughly 'scrubbed' of pollen tube cytoplasm, so there is little likelihood of organelles from this source passing into the egg. Light microscopic images often appear to show naked male nuclei participating in the two fertilizations, and indeed some electron microscopic evidence (e.g. by D. D. Cass & W. A. Jensen, *Am. J. Bot.* **57**, 62–70 (1970)) might be taken to indicate that the gametes are themselves cleansed of organelles in passing through the synergid. However, this does not appear to be convincingly established yet for any species. The genetical evidence, of course, indicates that in at least some families, plastids must be transferred into the egg with the male gamete. I would expect there to be variation in this, just as there is in the earlier partition of organelles after pollen mitosis.

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