



Figure 1. Chloroplast structure

Chloroplasts are bounded by two membranes—the outer membrane and the inner membrane. They also contain an internal membrane system, composed of the **thylakoid membranes**, which carries chlorophyll and is the site of light absorption. The region of the chloroplast between the inner membrane and the thylakoid membranes is called the stroma. It is the site of chloroplast DNA and most chloroplast biosynthetic pathways, including the fixation of carbon during photosynthesis, and the synthesis of oil and starch.

Chloroplasts evolved from photosynthetic bacteria living inside the primitive ancestors of plant cells. Reflecting their origin, chloroplasts still resemble bacteria: they carry their own DNA and possess some of their original bacterial genes. Researchers have found, however, that most 'chloroplast' genes are no longer inside chloroplasts - during evolution, thousands of genes moved from chloroplast DNA to DNA in the cell nucleus. Now biologists are piecing together how and why this natural genetic engineering occurred.

## INTRODUCTION

Chloroplasts (Figure 1) have both the power and the raw materials to be sophisticated biological factories. They are rich in energy collected from light, in sugar produced by photosynthesis, and in minerals imported from the plant cell. From this starting point, they synthesise starch, lipids, pigments, key vitamins and amino acids, proteins, RNA, DNA, and a variety of other complex molecules.

The DNA that chloroplasts make is their own and carries natural chloroplast genes. Its presence betrays something fundamental about how plants evolved: it shows that the ancestors of modern plants didn't invent chloroplasts, they ate them! Chloroplasts evolved from photosynthetic bacteria living inside primitive animal-like cells (Figure 2). The huge range of biochemical reactions that chloroplasts perform reflects this history: as bacteria, chloroplasts' ancestors synthesised all the organic molecules necessary for life.

By studying DNA in photosynthetic bacteria, in chloroplasts and in plant cell nuclei, biologists have shown that a key feature of the evolution of chloroplasts was the transfer of most of their original bacterial genes into the plant cell nucleus. Now researchers are piecing together how and why this transfer occurred.

[www](http://www.saps.plantsci.cam.ac.uk/worksheets/ssheets/ssheet20.html)

## PRACTICAL INVESTIGATION

### *Can leaf-discs make starch in the dark?*

[[www-saps.plantsci.cam.ac.uk/worksheets/ssheets/ssheet20.html](http://www-saps.plantsci.cam.ac.uk/worksheets/ssheets/ssheet20.html)]

### QUESTION 1

Given that chloroplasts evolved from free-living organisms, what characteristics (in addition to biosynthesis) would you expect them to display?

## THE DISCOVERY OF CHLOROPLAST GENES

The discovery of chloroplast genes is closely tied to the history of genetics itself. Although Gregor Mendel set out the principles of genetics in 1865 (from his research on peas), for many years his theories were almost completely ignored. The science of genetics lay discovered but unknown until the spring of 1900 - when three botanists Correns (Germany), de Vries (Holland) and Tschermak (Austria) independently published re-discoveries of Mendel's principles. Nine years later, Correns helped show that not only cells, but also chloroplasts possess genes.

Correns was investigating **variegated** plants, whose shoots contained a mixture of green cells (carrying normal chloroplasts) and white cells (carrying colourless chloroplasts). Working with the four-o'clock plant *Mirabilis jalapa*, he found that fertilising a flower on a green section with pollen from a white section produced green seedlings. In contrast, fertilising a flower in a white section with pollen from a green section produced pure white seedlings (which soon died). In other words, seedlings always inherited their colour from the tissue producing the egg cells.

This pattern went against the genetic principles that Correns had helped re-discover. Instead of offspring inheriting genes from both parents, they appeared only to inherit them from the mother; a phenomenon called **maternal inheritance**. To explain his results, Correns concluded that whether a chloroplast was green or white depended on genes inside the chloroplast, and that chloroplasts were only inherited from the egg cell.

### QUESTION 2

Although many cultivated plants have green and white variegation (for example many garden varieties of ivy, holly and privet), very few wild plants have this characteristic. Suggest two reasons for this observation?

## WHY DO CHLOROPLASTS HAVE GENES?

By the time Correns published his research, the reason chloroplasts might carry their own genes was already suspected. As long ago as the 1840s, biologists had noticed that chloroplasts were about the same size and colour as **cyanobacteria** (known as the 'blue-green algae' at the time). In 1905, the Russian biologist Konstantin Merezhkovsky had proposed that chloroplasts evolved from blue-green algae - if so, it would not be surprising if chloroplasts possessed ancestral bacterial genes.

Later research on chloroplast biochemistry and genetics confirmed this theory. Like chloroplasts, cyanobacteria can photosynthesise. 'They use the same series of reactions,' says Peter Nixon at the Imperial College of Science, Technology and Medicine in

London. For example, in both chloroplasts and cyanobacteria, photosynthesis is powered by light energy captured by chlorophyll (see Figure 3). Researchers discovered that chloroplasts contain DNA in 1962 and in the early 1970s they showed that chloroplast DNA is circular - like that of most bacteria. More recently, biologists have shown that the genes on chloroplast DNA are closely related to genes in cyanobacteria. These findings indicate that chloroplasts and cyanobacteria are related.

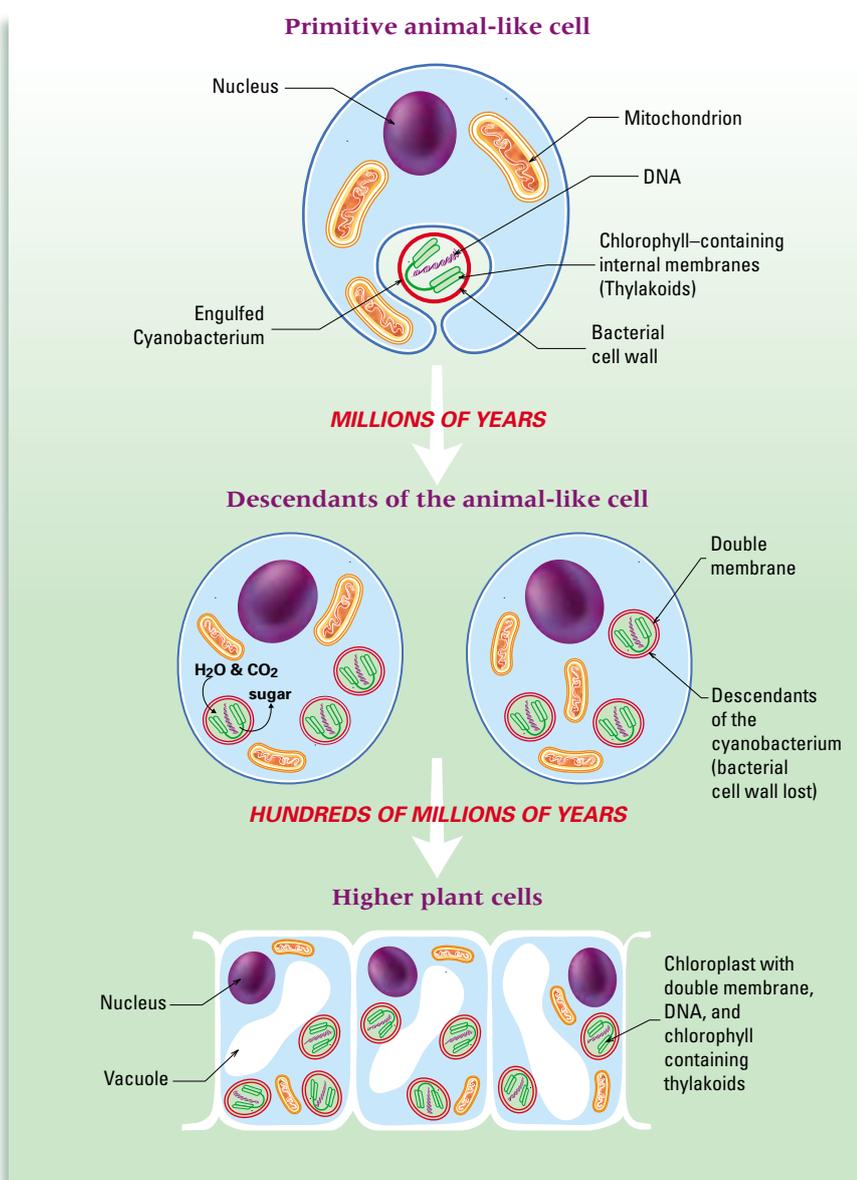
Chloroplast evolution is explained by the theory of **endosymbiosis** (Figure 2). Sometime before 1.2 billion years ago (the date of the earliest, uncontroversial fossilized plants), an organism a little like a single-celled animal engulfed a cyanobacterium but failed to digest it. Instead, the cyanobacterium survived and continued to divide: its descendants living on inside the descendants of its host. Over many generations, the lives of the internal cyanobacteria and of their host cells became completely entwined. The host provided water, carbon dioxide and nutrients, and the cyanobacteria - now chloroplasts - repaid with sugars produced by photosynthesis.

### QUESTION 3

Biologists believe that mitochondria also evolved from engulfed bacteria (of a different type, called the 'purple bacteria'). Considering the structure of plant and animal cells, is it more likely that the chloroplasts evolved before mitochondria, or vice versa? Give reasons for your answer.

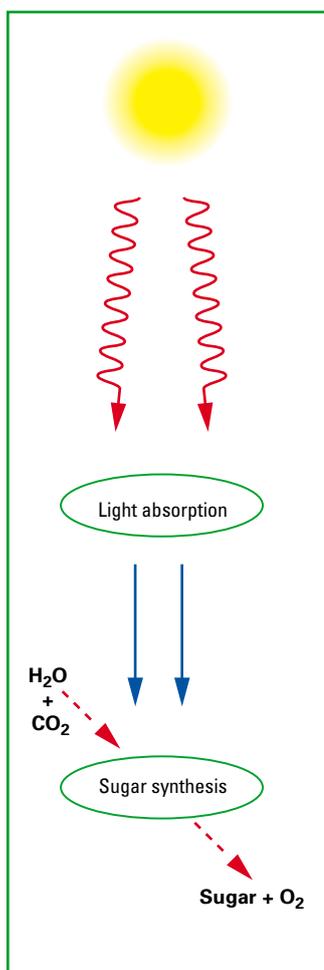
During plant evolution, the biochemistry of plant cells and chloroplasts integrated so completely that although chloroplasts are present only in photosynthetic tissues, all plant cells carry chloroplast-like organelles - collectively called **plastids**. According to John Gray at the University of Cambridge, they can't afford not to: in addition to photosynthesis, plastids are the only parts of the cell able to perform a variety of vital biochemical reactions. 'It's a fairly long list of important things,' says Gray. 'For example, the only place that fatty acids are made is in plastids, and a large number of amino acids are also made only in plastids.'

Showing their common ancestry, all plastids contain identical DNA. However, in different parts of the plant, plastids specialise to perform different tasks. Hence just as chloroplasts specialise in photosynthesis (see Figure 3), **amyloplasts** specialise in synthesising starch (for example in potatoes), and **chromoplasts** in synthesising



**Figure 2. How chloroplasts evolved**

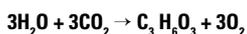
According to the theory of endosymbiosis, chloroplasts evolved from a cyanobacterium that was engulfed by a primitive, animal-like cell. The descendants of the engulfed cyanobacterium and those of the original host integrated their metabolism and development, eventually becoming chloroplasts and plant cells respectively.



**Figure 3. Photosynthesis**

Photosynthesis takes atoms from low energy molecules- water and carbon dioxide- to produce high-energy molecules - sugars. This requires an energy input that is provided by light captured by chlorophyll.

The immediate products of photosynthesis are 3-carbon sugars (**trioses**) which have the formula C<sub>3</sub>H<sub>6</sub>O<sub>3</sub>. A very crude summary of the chemical reaction in photosynthesis is therefore:



The excess oxygen produced by photosynthesis is the only reason you have anything to breathe. It comes from the oxygen atoms in the water; oxygen atoms in the carbon dioxide are incorporated into the sugar.

pigments (for example in colourful flowers and fruits). Underlining their need for plastids, some cells contain colourless plastids with no specialised function - called **leucoplasts**. In growing parts of the plant, cells carry small **proplastids** that can develop into the entire range of mature plastids. Mature plastids can also change types: as a tomato ripens, for example, green chloroplasts change to red chromoplasts.

### WWW PRACTICAL INVESTIGATION

- Green DNA. Simple isolation, restriction and electrophoresis of chloroplast DNA. Bioscience Explained, 1(2) available at <http://www.bioscience-explained.org/EN1.2/PDF/ctDNAEN.pdf>

### THE GENE DRAIN

Despite the many similarities between chloroplasts and cyanobacteria, there are some important differences. For example, the DNA in cyanobacteria holds over 3000 genes, corresponding to the number of different proteins the bacteria produce. When the complete DNA sequence of tobacco chloroplasts was published in 1986, however, it showed that they only contain 76 protein-coding genes. What happened to the rest?

Chloroplasts still contain about the same number of proteins as cyanobacteria, so these genes haven't simply been lost. Instead, in the hundreds of millions of years that chloroplasts have been inside plant cells, most of the original chloroplast genes have moved to the cell nucleus.

Looking for an evolutionary explanation for this phenomenon, biologists point out that whereas genes in the nucleus are transferred from generation to generation by sexual reproduction, genes in chloroplasts are inherited asexually by chloroplast division. They suggest that the advantages conferred by sexual reproduction may have been the driving force behind the transfer of genes from the chloroplast to the nucleus.

### QUESTION 4

**What are the advantages of sexual reproduction, and therefore of having chloroplast genes in the nucleus?**

Moving a gene from chloroplasts to the nucleus would take several steps. The first might be the accidental destruction of a chloroplast. This would release chloroplast DNA into the cell, allowing a chloroplast gene to enter DNA inside the nucleus. To discover how often this might occur, researchers in Australia inserted a foreign gene into tobacco

chloroplasts (see Plastid biotechnology). In about 1 in 16,000 offspring from the modified plants, a copy of the gene had moved to nuclear DNA.

Of course, a gene transferred from a destroyed chloroplast will still be present inside surviving chloroplasts - which is just as well because at first the transferred gene would not be expressed.

### QUESTION 5

**A gene is expressed if the protein it encodes is produced by the cell. What are the two main processes required for gene expression?**

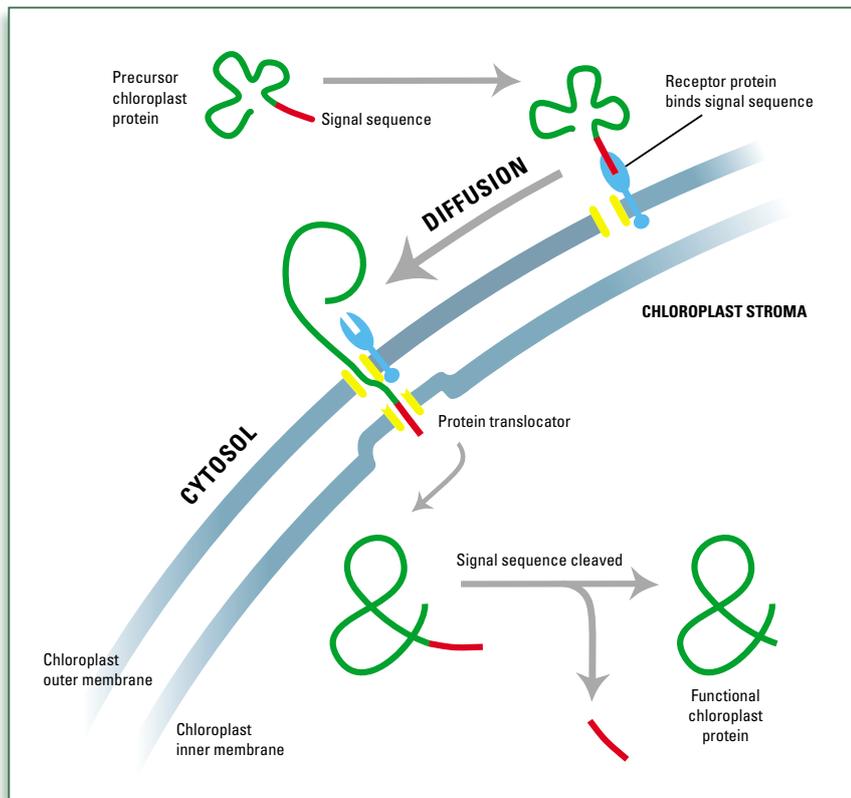
As anyone who has tried swapping files between different brands of computer knows, a program that works in one system is often not compatible with another. Biologists have found inactive chloroplast genes in the nuclei of several plants. Some of these still carry the regulatory DNA sequences that directed their transcription in chloroplasts but the genes are not transcribed in the nucleus because these sequences are not 'recognised' by nuclear proteins. Therefore, before a transferred chloroplast gene is expressed, it must acquire the correct 'nuclear' regulatory DNA.

Even this, however, is not enough to allow the plant to lose the original version of the gene in its chloroplasts. Genes in the nucleus are used to produce proteins in the cytoplasm and such proteins can only enter chloroplasts if they carry the correct 'address label' - a short string of amino acids known as a **signal sequence** (Figure 4). Therefore, a chloroplast gene transferred to the nucleus also needs the chance addition of the small section of DNA that encodes the signal sequence. Until then, the protein that the gene encodes is trapped in the cytoplasm.

### QUESTION 6

**The genetic code is the way in which the sequence of bases (As, Gs, Ts & Cs) on the DNA in a gene encodes the sequence of amino acids in a protein. What does the fact that chloroplast genes can transfer to the nucleus imply about the code in prokaryotes and eukaryotes?**

Of the genes that remain in chloroplasts, many encode proteins involved in photosynthesis, but others are needed for different roles. In particular, chloroplast genes frequently encode proteins that are required to express chloroplast genes! These proteins form vital parts of the molecular machinery



**Figure 4.**  
**Importing proteins**  
**into chloroplasts**

Chloroplast proteins encoded by nuclear genes are synthesised in the cytosol as precursor proteins. These carry a signal sequence that is recognised first by a receptor on the chloroplast outer membrane, and then by a translocator which transports the precursor protein into the chloroplast. Once inside, the signal sequence is cleaved and the protein folds into its functional form.

that transcribes chloroplast DNA to produce messenger RNA, and translates the messenger RNA to synthesise protein.

Although the chloroplasts in most species of higher plants possess very similar sets of genes, comparing chloroplast DNA across the whole plant kingdom shows much more variation. Researchers looked at plastid genes from 16 different species, ranging from tobacco to single-celled algae. They found that between them, the plastids in the 16 species carried 274 different protein-coding genes. Out of these 274, however, 230 had successfully transferred to the nucleus in at least one of the species investigated - i.e. most genes were present in the plastids of some species but in the nucleus of others.

These results suggest that whether or not an original chloroplast gene has transferred to the nucleus is largely a matter of chance. So there is no reason to suppose that the flow of genes out of chloroplasts has stopped: if you could fast-forward a few hundred million years, chloroplasts would probably possess even fewer genes. 'I believe that the time will come when they will all be gone,' says Gray. 'We are just at a point in time in evolution. You can't imagine that where we are now will be the end of it.'

## PLANT BIOTECHNOLOGY- FUTURE HOPES

The ability of plastids to synthesise complex biological molecules makes them an attractive target for biotechnology. For example, plastids manufacture several vitamins, suggesting that manipulating plastid biochemistry in food crops could improve the nutritional quality of food. Probably the best known example of this approach is the creation of genetically modified rice that is enriched in  $\beta$ -carotene, the precursor of vitamin A.

$\beta$ -carotene is synthesised in chloroplasts and other plastids and is usually obtained by eating fresh fruits and vegetables. Unfortunately, although the chloroplasts in rice leaves synthesise  $\beta$ -carotene, the amyloplasts in rice seeds do not. This causes serious health problems in regions of Asia, Africa and South America where rice forms the majority the diet. Worldwide, it is estimated that vitamin A deficiency causes between 1 and 2 million deaths each year in children under 4.

In an attempt to alleviate this problem, biologists in Switzerland and Germany have created  $\beta$ -carotene-enriched rice (called 'golden rice' because the carotene is orange). They inserting three genes encoding  $\beta$ -carotene-synthesizing enzymes into the rice nucleus, attaching the genes to regulatory sequences specific to the seed to ensure that the enzymes were produced in the right place. This research was published in 2000 and the inserted genes are now being crossed into local varieties of rice to grow in the affected countries.



### QUESTION 7

To synthesise carotene, the enzymes encoded by the three genes had to be imported into plastids. Two of the genes were isolated from daffodils and the enzymes they encoded were imported into rice plastids without the researchers needing to make modifications. However, the third gene was taken from a bacterium. Look at Figure 4 and then suggest how the researchers ensured that the enzyme encoded by this gene was imported into the plastids of the rice grain?

Although the genes used to create golden rice were inserted into the cell nucleus, the presence of DNA inside plastids has allowed researchers to introduce foreign genes directly into chloroplasts (Figure 5) By doing this, biologists hope to answer one of the main criticisms of genetically modified crops - that introduced genes may spread into the environment when pollen from GM plants fertilises the flowers of related wild species. In most plants, plastids are only inherited from the egg cell (see **The discovery of chloroplast genes**) and so genes on plastid DNA are not transferred in pollen.

Researchers are investigating this approach as a means of making crops pest- or herbicide-resistant (like current GM crops: see question 8, below), or of improving the nutritional quality of foods (as in golden rice). However, many of the genes inserted into plastids so far have had a different purpose - to synthesise pharmaceuticals.

In March 2000, scientists in St. Louis, Missouri unveiled tobacco plants whose chloroplasts make a human growth hormone. The researchers had inserted the growth hormone gene into chloroplast DNA. The chloroplasts now use that gene to synthesise the hormone, a protein called **somatotropin**. Somatotropin is used to treat some forms of dwarfism and to combat excessive weight loss such as that associated with AIDS.

Since 2000, researchers have genetically modified chloroplasts in tobacco plants to produce several other medicinally important proteins, ranging from antibodies that could combat tooth decay, to proteins that could form edible vaccines against tetanus and cholera. According to Nixon, the motivation behind the research is simple - GM plants may offer a way of producing much cheaper drugs. 'At the end of the day, what's driving it is a commercial argument' he says.

At the moment, human proteins used in medicine are either isolated from donated blood or produced using cultured cells or genetically modified (transgenic) bacteria. There are problems with both methods. Only the most abundant blood proteins can be isolated in sufficient amounts from donated blood, while cultured cells and bacteria must be grown

in rigidly controlled conditions. Somatotropin, for example, is made commercially using transgenic *Escherichia coli* (a bacterium that lives naturally in the large intestine).

'For *E. coli*, you have to grow the bacteria at 37°C,' says Nixon. 'If you just have a hectare of tobacco plants, that is going to be much cheaper to grow and you already have the infrastructure to harvest and process the crop.'

### CONCLUSIONS

Charles Darwin proposed that evolution - driven by natural selection - proceeds by the gradual accumulation of minute changes over many generations. In time, he suggested, this would change the form of a species, for example altering the shape of a finch's beak to suit the local diet.

Overall, of course, Darwin was absolutely correct. However, just occasionally evolution depends not on the tiny and gradual, but on the spectacular, individual and highly improbable. You would not be reading this sentence if, 65 million years ago, the meteorite that struck the earth leading to the extinction of the dinosaurs had missed. With the land still occupied by giant reptiles, the planet would be closed to the evolution of modern mammals. Likewise, the dinosaurs might have had nothing to eat if, sometime well over a billion years ago, a particular cyanobacterium had not survived becoming lunch for a particular animal-like cell!

Having compared DNA from chloroplasts across the plant kingdom, researchers now believe that all chloroplasts trace their origin to a single, engulfed cyanobacterium, i.e. the endosymbiosis that created chloroplasts from cyanobacteria happened only once. The entire plant kingdom rested on this event - after which, evolution could proceed in its usual, gradual manner. Chloroplasts and other plastids evolved by the slow conversion of the ancestors of the engulfed cyanobacterium into organelles integrated into plant cells.

In modern plants, the growth, metabolism and development of cells and plastids are so deeply integrated that neither partner could survive alone. Part of this integration was the transfer of most of the original bacterial genes from the evolving chloroplasts into the cell nucleus. This has left plastids with only a small remnant of their original genes, and researchers believe that even these few may eventually shift to the nucleus.



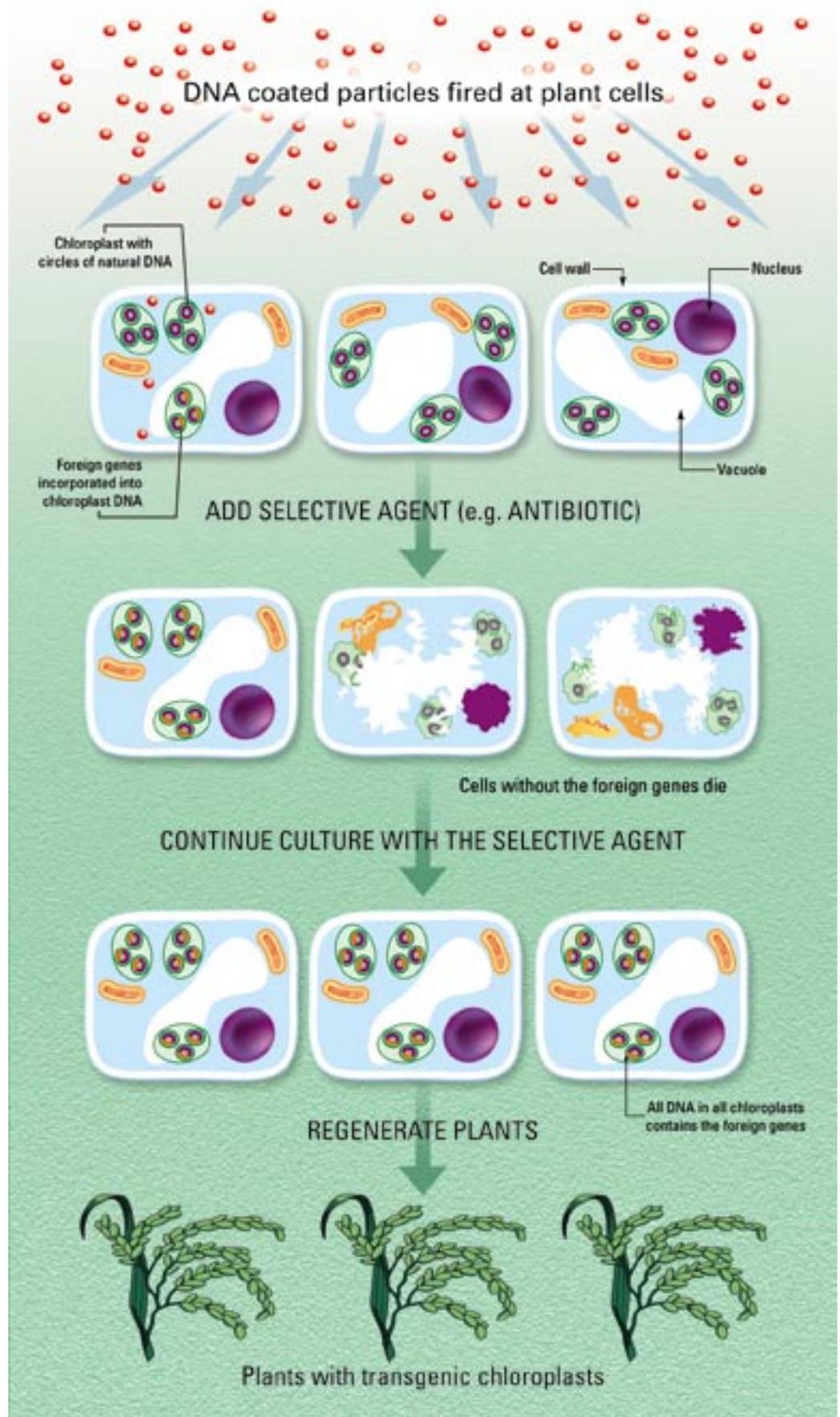
**Figure 5. Genetic modification of chloroplasts**

**A)** To insert a foreign gene into chloroplasts, researchers use the 'gene gun' technique. They coat tiny particles of gold or tungsten with DNA containing the gene. Using compressed gas, they fire the particles at cultured plant cells. Some of the particles enter chloroplasts, and some of the chloroplasts incorporate the foreign gene into their DNA. By selecting the DNA sequences that flank the foreign gene on the introduced DNA, researchers can target the gene to precise points in the chloroplast DNA.

**B)** Researchers also include a marker gene on the introduced DNA. This confers an ability that allows cells with modified chloroplasts to be selected. For example, the marker gene may give antibiotic resistance. The cultured cells are then treated with the antibiotic so that only those carrying the marker gene survive.

**C)** At this stage, usually only one chloroplast in each cell carries foreign DNA. However, repeated tissue culture and antibiotic treatment generates cells in which all chloroplasts contain the introduced genes in all DNA copies.

**D)** These cells are regenerated into plants. (Following fears that GM crops carrying antibiotic resistance genes might transfer the antibiotic resistance to harmful bacteria, other marker genes can now be used.)



### QUESTION 8

In 2003, more than 99% by area of GM crops grown worldwide produced either the bacterial B.t. toxin to kill pests, or an enzyme that made them resistant to the herbicide glyphosate (so that spraying glyphosate would kill weeds but leave the crop untouched), or both. It would be unwelcome if either of these traits spread to wild relatives of crops via pollen, but for different reasons. What are these reasons?

### QUESTION 9

Suggest some of the main day-to-day costs facing a factory culturing *E. coli* on a large scale that are not incurred by a farmer growing a hectare of tobacco plants.

### QUESTION 10

a) A high yielding crop such as maize gives a harvest of about 9260 kg/hectare. If you produced a pharmaceutical protein in the maize kernels that reached 1% of their total weight (this has already been achieved by genetically engineering the maize nucleus) and managed to extract 50% of that protein, how much protein could you sell per hectare?

b) Human antibodies for use in science and medicine are currently produced in cultured mammalian cells. It is estimated that the worldwide capacity to produce antibodies in this way is enough for about 2500 kg of antibodies per year. If your maize produced human antibodies, about how many hectares would you need to grow to match the current world capacity?

c) Currently, human antibodies cost about £190 per gram. Maize costs about £1540 per hectare to grow and harvest. If you assume a similar cost per hectare to extract your antibody from the maize, how much would it cost per gram to produce?

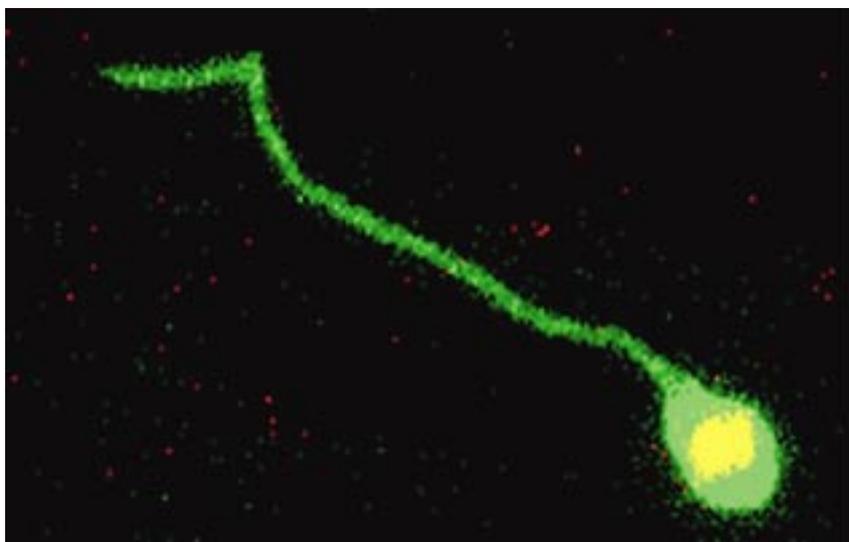


Figure 6. Stromule

#### BOX 1

### STROMULES

Figure 1 of this booklet shows the traditional image of a higher plant chloroplast: a lens shaped organelle, about 5 - 7  $\mu\text{m}$  long and 1 - 3  $\mu\text{m}$  in diameter. Although this is an accurate snapshot of many chloroplasts, it is missing a dynamic feature of chloroplast structure - **stromules**. Stromules are thin tubules that extend from chloroplasts and other plastids. They are about 0.8  $\mu\text{m}$  in diameter and reach up to 40  $\mu\text{m}$  long. However, they are difficult to see because they do not carry the chlorophyll-containing **thylakoid membranes**, only the colourless fluid of the stroma (see fig. 6). The name 'stromule' derives from 'stroma-filled tubule'.

Recently biologists have found that by genetically modifying plants to produce a fluorescent protein in the stroma, they can 'colour in' the stromules on living plastids. This shows that at any one time stromules may be visible on all, some or none of the plastids in a cell. Furthermore, individual stromules can be seen emerging, disappearing and even forming connections between plastids (pictures and movies of stromules are available on the web, see Web Resources in the Teacher's Guide).

Now researchers are trying to decide on the function of stromules. 'It's very much up in the air,' says Gray. 'But what you do see is that if you have a cell in which the plastids are small and a long way apart, then you have more stromules.' This suggests that stromules may help plastids interact with the rest of the cell. 'There is a general belief that somehow stromules are involved in the transfer of metabolites either into or out of plastids,' says Gray. 'They may also have a role in allowing the plastid to sense distant parts of the cell.'

By connecting plastids, stromules may also allow communication between them. Researchers have found that if the fluorescent protein in a plastid is bleached by very strong light, then fluorescence can pass through stromules from neighbouring plastids into the bleached plastid. Therefore, stromules can transfer proteins between plastids, perhaps helping to coordinate plastid activity.

## GLOSSARY

<b>Amyloplast</b>	a plastid specialised in starch synthesis.
<b>Chromoplast</b>	a plastid specialised in pigment synthesis.
<b>Cyanobacteria</b>	a large group of photosynthetic bacteria.
<b>Endosymbiosis</b>	a symbiosis in which one organism exists within the cells of another.
<b>Eukaryote</b>	a single-celled or multicellular organism in which cells contain a nucleus, including plants, animal and fungi.
<b>Marker gene</b>	a gene conferring a trait designed to identify cells that carry that gene.
<b>Maternal inheritance</b>	inheritance of a characteristic in which the phenotype of the offspring depends only on the phenotype of the mother.
<b>Plastid</b>	the generic name for chloroplasts and related organelles.
<b>Prokaryote</b>	a type of living cell that lack a nucleus, i.e. a bacterium
<b>Proplastid</b>	a small plastid present in cells in growing regions of the plant. Proplastids develop into other plastid types as tissues mature.
<b>Signal sequence</b>	a short string of amino acids that directs a protein to a particular location within the cell.
<b>Somatotropin</b>	a growth-promoting hormone secreted by the pituitary gland of the brain.
<b>Stroma</b>	the fluid-filled region of the chloroplast that lies between the inner chloroplast membrane and the thylakoid membranes (see fig. 1).
<b>Stromule</b>	a stroma-filled tubule projecting from a chloroplast or other plastid.
<b>Thylakoid membrane</b>	an internal chloroplast membrane that carries the photosynthetic pigments.
<b>Transgenic</b>	carrying a gene inserted using a biotechnological approach rather than by conventional breeding, now synonymous with 'genetically modified'.
<b>Triose</b>	a three-carbon sugar.
<b>Variegated</b>	composed of tissues of different colours.