Chapter 17

Biotechnology



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SUMMARY

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BIOTECHNOLOGY: *RNA Gives Plants the Silent Treatment*

KEY CONCEPTS

1. DNA can be purified. Purified DNA can be broken into pieces that contain individual genes, and different pieces can be recombined to form new combinations of genes.

2. Recombined DNA can be inserted into plant cells. The plant cells may express the genes contained by the inserted DNA.

3. The insertion of specific genes into plants can enable them to form useful industrial products.

4. The genetic manipulation of plants can produce useful agricultural and horticultural varieties with traits that would have been extremely difficult or impossible to select by traditional breeding methods.

17.1 USEFUL MATERIALS FROM LIVING ORGANISMS

Biotechnology, a word that has been in the news only since about 1985, could mean many things. Because *bio*- refers to life, and *technology* is the application of science to the creation of products for human use, processes, and services, biotechnology could refer to almost any branch of agriculture, to the fermentation and food-processing industries, and to many industries and services supporting health providers. However, new and spectacular discoveries about the structure and function of genes and enzymes have tended to overshadow more traditional fields, and the word *biotechnology* now generally implies the genetic manipulation of organisms to give them new capabilities or improved characteristics. This chapter describes some of the techniques that allow genetic engineers to modify the genes of plants and some of the advantages that can be expected from the new organisms.

17.2 STEPS TOWARD GENETIC ENGINEERING

DNA Can Be Purified, Recombined, and Cloned

Years of basic research and many technologic breakthroughs were needed before scientists learned to manipulate genes in ways that were truly useful.

PLASMIDS The technology for the chemical manipulation of DNA began in the 1960s with the discovery of **plasmids** in bacteria. Plasmids are small pieces of DNA--separate, autonomous, circular minichromosomes--generally not required for the survival of the bacterial cell. Some plasmids carry genes that help the cell survive in unusual environments, some make the host bacterium resistant to antibiotics or to certain viruses, some carry genes for enzymes that metabolize rare compounds, some provide unusual biochemical pathways. Plasmids have the ability to be replicated in a cell, just like the main chromosome. An example is a plasmid called pBR322, which was devised for genetic engineering in the gut bacterium *Escherichia coli*. It contains two genes--one that

provides resistance to ampicillin, and one that provides resistance to tetracycline--and a site where DNA replication can start.

Plasmids are useful because they are easy to purify and work with. The main chromosome of the bacterium *E. coli* has 4.7 million base pairs and thousands of genes, but a plasmid might have only 2,000 base pairs and 2 genes (see Fig. 19.3). This makes the plasmid more stable in a test tube and easier to analyze. Furthermore, bacterial cells can be induced to take up circular plasmids from the surrounding solution, a process known as **transformation**. By this process, a recipient cell obtains new genetic information.

RECOMBINANT DNA Also in the 1960s, microbiologists discovered that bacteria contain enzymes capable of cutting DNA at specific base sequences. These enzymes are called **restriction endonucleases** or **restriction enzymes**, because their function is to protect the cell by restricting invasion by foreign DNA. Cells modify their own DNA so that it is not cleaved by the restriction enzymes. Different restriction enzymes (from different species or even strains of bacteria) recognize different sequences of bases in DNA.

When isolated and purified, restriction enzymes were found to be extremely useful because they allowed scientists to cut purified plasmid DNA in specific, reproducible places (Fig. 17.1). Furthermore, the cuts can be reversed. Many restriction enzymes make cuts with *sticky ends*, at which there are overlapping regions of complementary DNA strands. At lower temperatures these ends stick together and the DNA can be covalently connected (*ligated*) using another enzyme, DNA ligase.



Figure 17.1. How restriction enzymes work. A restriction enzyme--here *EcoR1*, one of several restriction enzymes produced by *Escherichia coli*--cuts DNA at particular base sequences. The sticky ends from any two DNAs cut by the same enzyme can form hydrogen bonds and be connected covalently by DNA ligase. Pieces of DNA from different sources can be combined because the sticky ends formed by a particular restriction enzyme all have the same base sequence, no matter what their source. These pieces will stick together and can be ligated, forming a **recombinant DNA** molecule (Fig. 17.2). If the recombination process inserts a new gene into a plasmid, and if the DNA becomes circular (and is not too large), the new gene can be taken up with the plasmid by a receptive bacterium. In this case, the plasmid is called a **vector** for the transfer of the new gene.

When a researcher adds to a test tube a mixture of pieces of DNA (plasmids and isolated copies of a gene, for instance), all cut with the same restriction enzyme, and allows them to recombine, the result is a random mixture of DNA molecules of various sizes. Some pieces of DNA stick their own ends together to form small circles; two plasmids (or two genes) may combine together just as well as one plasmid with one gene; finally, three, four, or more pieces may join end to end to form really large circles. Cutting the DNA with two restriction enzymes simplifies the situation. Because each molecule has sticky ends with different base sequences, single pieces cannot reform circles. Still, much of the art in genetic engineering is in selecting the desired combination of ligated pieces of DNA--through a procedure known as cloning.



Figure 17.2. The formation of recombinant DNA. Restriction enzymes (such as *EcoR1*) can be used to insert a piece of DNA into a plasmid. If the plasmid has only one site recognized by the restriction enzyme, the foreign DNA can only be inserted there.

CLONING A **clone** is a colony or group of cells or organisms, all of which have the same genes. Cloning is replication of the cells in the colony. In the context of DNA biotechnology, cloning is a simple method for separating and eventually characterizing individual molecules of DNA. It works because an individual DNA molecule inserted into a single bacterial cell can be replicated many times as the cell divides, until the colony contain hundreds of thousands of copies of the same molecule.

To see how it works, assume that we prepare a mixture of recombinant DNA molecules formed from a plasmid and a gene of particular interest to us. We use a special plasmid, pUC19, which has the gene for resistance to the antibiotic ampicillin and a gene

that makes the enzyme ß-galactosidase. We treat the plasmid with a restriction enzyme that makes one cut in the middle of the ß-galactosidase gene, then add the new gene, which has been cut with the same enzyme, and ligate. We combine the mixture of DNA molecules with a suspension of bacterial cells in such a way that each cell will, on the average, take up only one DNA molecule. Only circular molecules will be taken up. Next the bacteria are spread out on a Petri dish containing nutrient agar and ampicillin (Fig. 17.3). The bacteria are diluted enough so that each cell is separated from every other cell. Those bacteria that did not take up plasmids will be prevented from growing by the ampicillin. Those bacteria that do contain plasmids will survive and grow into individual colonies (clones). We know that each clone must contain a plasmid. In the nutrient agar is a colorless chemical that turns blue in the presence of ß-galactosidase. Those colonies of bacteria that contain intact ßgalactosidase genes will synthesize ßgalactosidase and turn blue. Colonies that contain plasmids in which the gene



Figure 17.3. Colonies (or clones) of *Escherichia coli* on an agar plate. The cells in the blue colonies (most visible at the left) are making ß-galactosidase, which liberates the blue dye from its colorless complex with the sugar galactose. The white colonies (most visible at the right) do not make ß-galactosidase, because their gene for that enzyme has been interrupted by the insertion of a foreign piece of DNA.

of interest has been inserted into the middle of the ß-galactosidase gene will be unable to make ß-galactosidase and will remain white. We check the bacteria from a white colony to verify that they contain the gene we want. By growing cells from the selected colony in nutrient medium, we can have an inexhaustible supply of that particular gene.

We can also determine the exact base sequence of the DNA we are interested in, although this step is not necessary for use to reproduce the DNA, transfer it to other bacteria, or recombine it with other pieces of DNA. The basic strategy is to synthesize DNA, starting at a fixed point and stopping the synthesis at particular bases, so that the size of the fragments relates to the positions of the bases (see sidebar "IN DEPTH: Determining the Base Sequence of DNA"). It is possible to separate DNA fragments of different sizes by electrophoresis, a technique that moves DNA molecules through an electric field at a rate proportional to their molecular weights (or lengths), resolving fragments that differ by just one base pair. This allows us to see directly the relative positions of different bases--the base sequence.

There Are Many Ways to Isolate a Useful Gene

To use cloning to produce multiple copies of a gene, it is first necessary to identify and isolate an initial sample of that gene. There are several strategies to do this; the choice depends on the information available. One set of methods starts with a purified protein, whereas another set starts with the bases sequence of related genes.

REVERSE TRANSCRIPTASE AND CDNAS Imagine that you have discovered a fungus that produces a protein that is toxic to insect pests. You want to clone the gene for this protein. You know that the base sequence of the gene is reflected in a messenger RNA (mRNA) that serves as a template for the synthesis of the protein. That mRNA is one among the many mRNAs in the fungus.

The first step is to extract the mRNAs and reproduce their base sequences in DNA molecules. An enzyme called **reverse transcriptase** can produce DNA using an RNA template. Starting with the mRNAs, a "primer," which is a small piece of DNA complementary in base sequence to the mRNAs, and substrates (nucleoside triphosphates, see Chapter 2), that enzyme adds nucleotides to the primer to form single strands of DNA with base sequences complementary to the mRNA templates. The primer often is a line of T bases, "oligo(dT)," which is complementary to the poly(A) tail of most eukaryotic mRNAs. The result is a mixture of "complementary" or "copy" DNAs, abbreviated cDNAs.

POLYMERASE CHAIN REACTION The next step is to isolate the cDNA for the protein of interest from among the cDNAs made with reverse transcriptase. Again, there are several strategies. One is useful if you know the base sequences at the ends of the cDNA. Even though you do not know the base sequence of the whole gene, you may be able to guess the base sequence of one end. There are chemical ways of determining the amino acid sequence of the toxic protein, starting with one end (the "N-terminal" end). Given the sequence of about seven amino acids, you can infer mRNA codons that produced that amino acid sequence and synthesize a DNA oligonucleotide with the same base sequence as the codons. That oligonucleotide, together with poly(dT), defines the ends of the cDNA you want.

A DNA **polymerase chain reaction** (PCR, Fig. 17.4) will produce multiple copies of the desired gene. The reaction combines the cDNAs with the oligonucleotides (now serving as *primers*), nucleoside triphosphates, and a special DNA polymerase, the enzyme that synthesizes DNA. The special DNA polymerase is not destroyed by high temperature, and, in fact, it needs a fairly high temperature to function. Each reaction cycle includes three steps at different temperatures. (1) The reaction solution is heated, almost to boiling, to separate any complementary strands of DNA, making each strand a potential template. (2) The reaction solution is cooled; this allow primers to bind to the ends of any DNA with complementary base sequences. (3) Finally, the reaction solution is heated to the optimum temperature for the DNA polymerase, allowing the synthesis of new DNA by the addition of nucleotides to the primers. Each cycle produces a new DNA for every template-primer complex. If two primers are specific for the gene of interest, the concentration of that gene doubles with each cycle, and after about 30 cycles, almost all the DNA in the reaction solution consists of that gene.



Figure 17.4. The polymerase chain reaction (PCR).

PCR is a flexible technique. It is possible, for instance, to use it to detect traces of animal or plant genes in criminal investigations. It also is possible to use it to synthesize a gene with added restriction sites at the ends. This allows the gene to be inserted into a plasmid and cloned in bacteria, a useful preparation for transforming plants.

GENOMICS In the last few years, scientists have deciphered the base sequences of the entire genetic material of many viruses, bacteria, and even human cells, as well as many species of plants. The first plants to have their entire DNA sequences determined were Arabidopsis thaliana (mouse-eared cress, a relative of mustard and a favorite experimental material) and Oryza sativa (rice). By 2014, the full sequences of over one hundred plants were known or close to that state. The full assemblage of genetic material of a cell is called the genome: therefore the study of genome structure, function, and evolution is called genomics.

The information from genomics is useful in identifying genes of interest. This is because genes with similar functions have similar base sequences. Computer programs can compare base sequences, identifying similarities and differences. Thus, it is possible to find a gene in the rice genome for a particular enzyme by its similarity to a corresponding gene in humans. Once the rice gene base sequence is known, a scientist can design primers and use PCR to produce many copies of the gene or to isolate the same gene form a different plant. Also, by comparing the sequences of a gene in plants, animals, and fungi, it is possible to identify the base sequences of the genes (and thus the amino acid sequences of their proteins) that are

essential for the proteins' function and get ideas about how to improve the function. Perhaps most important, the information from genomics is teaching us how networks of genes are regulated. By identifying genes and proteins that control the expression of other genes, we may learn to influence the development of plants in complex ways.

Scientists Use a Special Bacterium to Insert DNA into Plants

The techniques described in the previous sections were developed using DNA form bacteria, fungi, and animals. DNA in plants has the same chemical structure as DNA in other organisms, and it can be extracted from plant cells and manipulated in a test tube using many of the same techniques, including reverse transcription, PCR, and cloning. This means that we can select and purify plant genes. It does not, however, automatically mean that we can put the genes back into plant cells in a way that allows the cells to survive and to use the new genetic information.

In searching for a way to insert genes into plant cells, scientists focused on a condition called crown gall disease. This disease, caused by the soil bacterium Agrobacterium tumefaciens, results in tumors (galls) growing in places where a plant has been wounded (Fig 17.5). The disease is called crown gall because plants often are wounded at the crown, the junction between shoot and root. A. *tumefaciens* cells attach to the walls of plant cells exposed in the wound and cause the plant cells to start dividing. That the plant cells continue to divide even when the bacteria are killed with antibiotics shows that the bacteria *transform* the plant cells, apparently by turning off their normal mechanisms for limiting cell division. The result is rather like an animal cancer, and in fact, much of the early research on crown gall disease was financed by the National Cancer Institute.

What mechanism do the bacteria use to transform the plant cells? For many years, biologists suspected that a transfer of genes was responsible, and in the mid-1970s, the DNA that contained those genes was identified. Infectious strains of *A. tumefaciens* have a large plasmid, called the *Ti (tumor-inducing)*



Figure 17.5. Crown galls are formed when *Agrobacterium tumefaciens* infects wounded plant tissue. The wounds often occur around the crown (area between stem and root), but can also be higher on the stem, like the gall on this walnut (*Juglans* sp.) tree. The gall tissue grows actively in the laboratory.

plasmid (Fig. 17.6), part of which the bacteria inject into plant cells (Fig. 17.7). The region that is injected (the T-DNA) contains three main genes that cause the cells to divide and grow. Two of the genes code for enzymes that make the plant hormone auxin (indoleacetic acid); one of the genes codes for an enzyme that makes another plant hormone, a cytokinin (isopentenyl adenine). It has been known for years that an excess of auxin and cytokinin will cause plant cells, especially the parenchyma cells at the surface of a wound, to divide and grow.



Figure 17.6. The Ti plasmid from a strain of *Agrobacterium tumefaciens*, showing the original T-DNA that is transferred to plant cells and stimulates growth of galls and a typical replacement T-DNA used by genetic engineers to add a genetic function to a plant. The promoter controls when and in what tissue the gene of interest is expressed. The "reporter" gene allows the selection of transgenic plants: it might provide resistance to an antibiotic or a herbicide, allow the plant to grow on an exotic nutrient source, or make the cells fluorescent or luminescent (Fig. 17.8).

Another T-DNA gene is for an enzyme that synthesizes an unusual type of amino acid called an opine. Opines generally are combinations of two normal amino acids (there can be different combinations, depending on the enzyme). Once synthesized in the plant cells, opines leak out into the intercellular spaces. The bacteria growing in the intercellular spaces of the tumor also make enzymes that allow them to take up and metabolize opines, using them as a source of carbon, nitrogen, and energy. Thus, the plant cells transformed by the bacteria--that is, injected with new bacterial genes--nourish their bacterial parasites. In short, *A. tumefaciens* has evolved a natural mechanism for conducting genetic engineering.

For human genetic engineers, this system is valuable because the T-DNA of the Ti plasmid can be modified to serve as a vector to carry genes into plant cells (Fig. 17.7). In





Figure 17.8. Plants can be transformed when infection by a genetically engineered *Agrobacterium tumefaciens* Ti plasmid introduces new genetic information. This tobacco (*Nicotiana tabacum*) plant glows in the dark because the new gene that was inserted, which came from a firefly, produces the enzyme luciferase.

practice, researchers start with T-DNA that has lost the genes for auxin and cytokinin synthesis, so that it does not form tumors. Into the T-DNA, they insert the gene of interest, controlled by a promoter that regulates when and in what tissues it is turned on, together with a "reporter" gene that allows them to select for cells that have stably incorporated the T-DNA (for instance, a gene coding for the enzyme luciferase: Fig. 17.8). This recombinant T-DNA, generally in the form of a miniplasmid, is transferred to an *A. tumefaciens* cell that has a Ti plasmid lacking its own T-DNA. Spread on the cut surface of a piece of leaf, the bacteria transfer the recombinant T-DNA to the plant cells. The leaf is transferred to a medium containing antibiotics that kill the bacterial cells, and the engineers select those plant cells that have incorporated the reporter gene in the T-DNA. After these steps, the plant cells can be coaxed to regenerate new plants by using standard techniques of tissue culture (see Chapter 15). The plants, with new genetic information, are called *transgenic*.

There Are Other Methods for Injecting DNA into Plants

Although the *A. tumefaciens* system is the favorite method of transforming plant cells, there are a few other ways to add new genetic material to these cells. One is called biolistics, a play on the word ballistics. The apparatus also is called a gene gun. DNA containing the gene of interest is adsorbed onto the surface of small (subcellular-sized) particles of gold or tungsten. These particles are pressed onto the front of a bullet. The bullet is then loaded into a gun, and the gun is fired at the plant tissue. Originally, a common .22 caliber gunpowder-powered bullet was used, but a pellet of a high-pressure air (or helium) pistol also is effective and avoids the toxic gases produced by the gunpowder. A special metal plate with a hole that is smaller than the cross section of the bullet abruptly stops the flight of the bullet before it reaches the tissue, but the gold or tungsten particles keep going with enough energy to penetrate the cells. They are so small that they appear not to leave holes in the cell membrane after they pass through; they also appear not to harm the cells in any other way. After the particles come to rest inside the cells, the adsorbed DNA dissolves into the cytoplasm.

A new method, based on the same principle, uses thin silicon carbide fibers coated with DNA. Mixed with a suspension of plant cells and agitated briskly, some fibers penetrate the cells and release their DNA. In at least some cells, the DNA reaches a place where it can be used as a template for RNA synthesis, and its genetic information is expressed.

Another method for getting DNA into a plant cell is electroporation. This is based on the discovery that a short, high-voltage charge of electricity can produce temporary holes in a plasma membrane without permanently harming the cell. For this technique, it is necessary to make protoplasts--cells without walls--from the recipient plant cells. The cell walls can be removed by treatment with polysaccharide-hydrolyzing enzymes (cellulase and polygalacturonase) while the cells float in an osmotically balanced medium to prevent water form entering and breaking them open. The protoplasts are then placed between two electrodes in an ice-cold solution that contains the DNA. A few pulses of electricity, each less than 0.1 second in duration, produce the membrane holes. These close up in seconds or minutes, but during this time some DNA can enter the cells. Cultured under the proper conditions, protoplasts regenerate their cell walls, begin dividing, and even regenerate whole plants--plants that may express the genes of the DNA that entered the protoplasts.

Viruses also can be used to inject genes into plants. Plant viruses (subcellular particles of nucleic acid and protein) infect plant cells, multiply, and spread throughout the plant (see Chapter 19). The genes of most plant viruses are usually made of RNA rather than DNA, but genetic engineers can produce DNA copies of the viral genes and connect them to genes of interest. In the plant, the introduced genes are expressed in the cytoplasm of cells wherever the virus spreads. This method does not produce a permanently transformed plant, because the viral and introduced genes are not incorporated into the plant's nuclear DNA, and they are not passed to seed formed by the infected plant. However, proteins made by the infected plant in response to the introduced genes can be extremely useful.

17.3 APPLICATION OF BIOTECHNOLOGY

Because bacteria were the first organisms to be transformed with new genes, they were the first to be used to produce new commercial products. The most valuable have been proteins. For instance, patients with diabetes who require daily injections of insulin currently receive human insulin produced by bacterial cells transformed with the human gene for insulin. Previously, they received pig insulin, purified from the pancreases of slaughtered pigs; this procedure was costly, and the insulin was not as satisfactory. Other proteins having medical uses that are currently produced by genetic engineering include somatotropin, erythropoietin, clotting factors, and interferon. Genetically engineered bacteria (or yeasts) also produce the enzymes added to laundry detergents. And, of course, many of the restriction enzymes and DNA polymerases on which genetic engineering depends are produced in large quantities by genetically engineered bacteria.

Plants Can Serve as a Source of Biochemicals for Medicine and Industry

Although bacteria can grow rapidly, nothing can beat plants for the economical production of large quantities of protein. Through photosynthesis, plants acquire carbon and harness solar energy for protein synthesis, whereas bacteria require a supply of growth medium. It is possible to harness the metabolic capacity of transgenic plants to produce large quantities of a protein valuable for industrial uses.

Plants are being engineered to produce vaccines that are cheaper and more acceptable to people. A vaccine contains an inactivated or weakened bacterial or viral pathogen or a part of a pathogen. Injected into a person's body, it stimulates the immune system to produce antibodies or immune cells that will kill an active pathogen if it infects the person at a later time. Some vaccines, for instance, the Sabin polio vaccine, can be taken orally. With this in mind, scientists are designing and testing food plants that contain genes for proteins from important pathogens. Two intriguing examples are a banana (*Musa sapientum*) that makes a protein from the hepatitis B virus and an alfalfa (*Medicago sativa*) sprout that contains a part of the cholera toxin. In the future, for the cost of a few seeds and some careful farming, even an impoverished region may be able to protect its population from serious lethal disease through such vaccines.

In addition to proteins, plants synthesize oils as storage materials for their seeds. By changing the enzymes of the synthetic pathway, it is possible to design plants that produce specialized oils or waxes. These materials could be useful in detergents, cosmetics, pharmaceuticals, and lubricants. Most biological oils are biodegradable. Previously, this has been seen as undesirable in an industrial product such as motor oil, because it means that the product has an uncertain (and possible short) lifetime. The risks of polluting the environment and the cost of disposing of non-biodegradable wastes, however, have pointed out the advantages of easy degradability.

Gene Manipulations Can Produce Many Types of Useful New Plants

NEW HORTICULTURAL VARIETIES Transforming plants with new or altered genes may yield plants with new traits that are useful or attractive. Much is known about the genes for the enzymes that make anthocyanins--the red, blue, and purple pigments found in plant flowers, as well as in other organs. Plant molecular biologists have produced plants that have additional genes for enzymes in the anthocyanin pathway and that, as a result, have flowers with unusual colors or patterns (Fig. 17.9). There is hope of producing a blue rose, something not found in searches among wild varieties and random mutants.

MORE PEST RESISTANCE Predation by insects and damage caused by viral, bacterial, and fungal diseases are among the major factors that limit the productive harvest from food crops. Classical plant breeding traditionally has selected varieties that resist these pests, and with genetic mating techniques it has been possible to combine resistance genes with genes for high-quality vegetables, cereal grains, and fruit. But classical genetic techniques





Figure 17.9. Petunia plants that are transformed with additional flower color genes produce unusual patterns, include sectors that have no pigment at all.

are inefficient, requiring many cycles of back crossing and selection. As we identify the genes for resistance, we can transfer them quickly and efficiently using molecular techniques (Fig. 17.10). For instance, seeds of common beans produce a protein that blocks the digestion of starch by two insect pests, cowpea weevil and Azuki-bean weevil. The gene for this protein has now been transferred to the garden pea. In tests, it protected stored pea seeds for infestation by these insects, killing or slowing the development of the larvae.

Furthermore, we can identify methods of resistance that plants never evolved. *Bacillus thuringiensis* is a bacterium that makes a protein toxin that kills insects. Scientists have already inserted the gene for *B. thuringiensis* toxin into important crop plants, including potato, tomato, corn, and cotton. These plants synthesize the toxin and kill many of the insects that graze on them. There are other antibacterial proteins that might also be useful. For instance, an apple sapling containing the insect gene for the attacin E protein is less susceptible to fire blight, a disease caused by the bacterium *Erwinia amylovora*.

Figure 17.10. Plants can be transformed with genes that provide resistance to insects and viruses, as well as bacteria and fungi. (a) Caterpillars dine on a cotton (*Gossypium hirsutum*) plant, but (b) not if it is genetically modified to resist attack. (c) A normal potato (*Solanum tuberosum*) plant is susceptible to viral attack, but (d) a genetically modified strain is resistant.

Tobacco mosaic virus infects crops in the plant family Solanaceae (tomato, potato, eggplant, green pepper). Inserting the gene for the viral coat protein into the plant genome for unknown reasons makes the plant resistant to infection by the virus. Scientists in Mexico have used the same technique to produce potato varieties immune to potato viruses X and Y, and in Hawaii, a genetically engineered papaya resistant to a ringspot virus saved the Hawaiian papaya industry from impending disaster (Fig. 17.11). Protecting agricultural plants against viral infection increases the yields of fruits or other useful crops that can be harvested from those plants.

Protecting plants against competition from weeds takes a different strategy. Some of the first plants to be engineered were plants resistant to herbicides. In extensive use are varieties of soybeans, cotton, sugar beet, corn, and canola that are resistant to glyphosate (trade name Roundup). Many of the same crop species, as well as others, such as radicchio and rice, have been engineered for tolerance to the herbicides glyfosinate, sulfonylurea, and bromoxynil. A resistant crop allows farmers to use herbicides to kill weeds in the middle of a field of crop plants. Properly applied, the crop allows a more discriminating use of safer herbicides.

IMPROVED QUALITY OF FRUIT AFTER HARVEST In many countries, a large proportion of harvested crops never reaches consumers, because it spoils en route to the market. In America, a great deal of the energy and cost associated with food production results from the complications of getting food to market in edible and attractive form. Controlling the atmosphere (limiting the amounts of oxygen and ethylene) and reducing the temperature are two expensive techniques for preventing certain fruits from spoiling. Many fruits are

Figure 17.11. A strand of papaya trees in Hawaii. The trees on the left were infected with papaya ringspot virus and show serious symptoms of decline. The trees on the right contain the gene for the virus coat protein and are resistant to infection by the virus.

picked green, stored, and then artificially ripened with an ethylene treatment. How much more effective and efficient it would be if we could insert genes that slowed the rate of senescence (aging), thus slowing spoilage. One of the enzymes needed for the softening of tomatoes as they rot is polygalacturonase. The first bioengineered food crop approved in the United States was the "FlavrSavr" tomato, which contained a gene that blocked the synthesis of polygalacturonase, thus delaying the senescence of the fruit. A more recent strategy to slow the over-ripening of tomatoes, also applied to cantaloupe, has been to insert genes that reduce the synthesis of the ripening hormone ethylene.

IMPROVED NUTRITION Some crops that are dietary staples are not necessarily the most nutritious. A well-known example is corn, which is low in lysine and tryptophan. Both lysine and tryptophan are essential amino acids in the diets of human and non-ruminant animals. High-lysine corn varieties have been developed by classical genetic techniques. But vitamin and mineral deficiencies are still responsible for much suffering in underdeveloped countries, suffering that could be alleviated by more nutritious plant products.

A deficiency in vitamin A results in blindness. It is estimated that 124 million children worldwide are vitamin A-deficient; and in southeast Asia, 250,000 children go blind each year because of this deficiency. Scientists in Switzerland have designed a variety of rice that produces seed with endosperm rich in ß-carotene. ß-carotene is the precursor for vitamin A. The new rice has enough ß-carotene to color the seeds, leading to its name, "golden rice." Another project has produced a rice variety that is rich in ferritin, a protein that binds iron. Iron deficiency, a problem for 1 to 2 billion people worldwide, results in anemia, increased susceptibility to infection, and impaired learning ability in children.

A variety of canola (*Brassica napus*, or oilseed rape) has been given the gene for a fungal enzyme, phytase. Phytic acid is a storage form of phosphate in plants. By releasing the phosphate from phytic acid in pig and chicken feed, phytase improves the nutrition of these animals--they grow faster and stronger. This is partly because of the

improved availability of phosphate, and partly because of the destruction of phytic acid, which inhibits the uptake of iron in the gut. Not incidentally, the phytase also reduces the phosphate excreted in manure, thus reducing environmental pollution of farm effluents.

For many decades, margarines made from plant oils have served as butter substitutes. The fatty acids in plant oils are generally unsaturated (have double bonds), in contrast to fatty acids in butter fat, which are saturated. Humans require some unsaturated oils in their diet, and saturated fatty acids are considered a health risk. Yet polyunsaturated fatty acids (two or more double bonds) are generally liquid at room temperature--inconvenient for a bread spread--and they oxidize to form chemically reactive free radicals, leading to a lower shelf life and possible toxic effects. The ideal fatty acid might be oleic acid, a 12-carbon fatty acid with one double bond. DuPont scientists have produced a soybean with a gene that causes it to accumulate high amounts of oleic acid.

IMPROVED TOLERANCE TO ENVIRONMENTAL STRESS One of the most difficult challenges is to produce plants that grow better under stressful conditions. We know that certain plant varieties gave genetic information that allows them to survive saline conditions, toxic metals, droughts, high light intensities, or chilling temperatures. In some cases, we even know how the resistance works, in a general way. But seldom do we understand what genes or proteins are involved.

One strategy used by plants to survive and even thrive in saline soils is to transport salt into their cells' vacuoles, where it does not interfere with cellular processes. A specific protein, called a Na^+/H^+ antiporter, serves as the channel between cytoplasm and vacuole. Researchers have inserted into a tomato plant a gene that synthesizes high levels of this channel (Fig. 17.12). The plant grows, flowers, and bears fruit when irrigated with 200 mM NaCl, a concentration 50 times the normal limit for tomato. The salt accumulates in the vacuoles of the leaves, but not the fruit; therefore, the fruits are edible, and harvesting the leaves can help clear a field of salt.

Figure 17.12. Salt tolerance of wild-type tomato (Solanum *lycopersicum*) plants and transgenic salt-tolerant plants. (a) Fruits from wildtype plants grown normally (left) and transgenic plants grown in the presence of 200 mM NaCl (right). (b) Wild-type plants and (c) transgenic plants growing in the NaClcontaining medium. The transgenic plants synthesize high levels of a sodium carrier, which allows the leaf cells to sequester NaCl in their vacuoles.

The same strategy can be used to produce plants that will take up toxic heavy metals, such as lead, cadmium, and uranium from contaminated soil and water, a process known as *phytoremediation*.

A number of companies are working on finding genes that will confer drought tolerance on maize plants. In 2012, Monsanto announced the successful testing and commercial availability of their "DroughtGard" hybrid maize variety, which has a bacterial gene for cold shock resistance. The gene produces a protein that binds to and unfolds RNA molecules that have been tangled and inactivated under water stress conditions.

It is thought that resistance to some stresses depends on several genes, which complicates efforts to increase it. Much current research is directed toward identifying the genes that differ between stress-tolerant and stress-sensitive varieties. The objective is to produce varieties of plants for a wider range of climate and soil conditions.

17.4 IS BIOTECHNOLOGY SAFE?

In 2002, a survey conducted by the International Service for the Acquisition of Agri-biotech Applications found that genetically modified crops were planted on 59 million hectares (148 millions acres) of farmland. These crops included more than 20% of the world's soybeans, corn, cotton, and canola, and 75% of soybeans in the United States. By 2013, the total was 170 million hectares (426 million acres). The use of genetically modified crops, which is expanding rapidly, has not been without controversy.

In 1992, a group of 1,000 chefs signed a pact promising not to use any genetically engineered foods in their cooking. In 1998, the European Union instituted a moratorium on the planting and use of genetically modified crops. What are people worried about? In part, the worry reflects recent experience that new technologies may cause problems. If the technologies are widely and carelessly applied, these problems can become serious. The problem of disposing of radioactive and toxic chemical wastes is one example. In part, the worry reflects general antitechnology feelings. Much of the controversy about genetically engineered plants reflects the public perception that little is known about genes, especially about the genes that are "unnaturally" introduced into organisms.

One counterargument points out that crops have been genetically manipulated for centuries--by breeding and selection. Familiarity and our positive experiences with the resulting new plant varieties have made their use acceptable. Few people realize how little is known about the genetic composition of new crop varieties that have been developed through random mutagenesis (production of mutants) or crossbreeding with wild types. We may be able to say that we know more about changes in the genes of a crop modified by genetic engineering than about changes in the genes of one developed through classical breeding.

In the early days of genetic engineering, when little was known about engineered organisms, scientists themselves placed regulations and limits on different types of experiments involving recombinant DNA. Different types of experiments required different levels of care. For instance, experiments with cancer viruses and pathogens that infect mammals required (and still require) stringent containment procedures, whereas simple experiments cloning genes in a genetically weakened and well-understood strain of bacteria could be performed in a regular laboratory with simple aseptic procedures. The marketing of engineered plants has more risks, but these have been examined, and ways for eliminating uncertainties have been identified. The scientific issues to be evaluated in the approval of a genetically engineered food are: (1) Does the product contain any new allergenic material that might affect especially sensitive groups? (2) Are new toxic compounds introduced into the food supply, or are existing toxins increased to unacceptable levels? (3) Are nutrient levels adversely affected? (4) Will the use of genes for antibiotic resistance (used to indicate when a plant has been stably transformed) compromise the use of important therapeutic drugs? If the answers to these questions are all negative, the food is considered to be safe.

A second type of problem involves environmental effects--for instance, the impact of new plants on wildlife or the possibility that new genes from the desired recipient crop species could be transferred to a related wild, weedy species. This is a concern when the new gene confers protection against natural pests or chemical herbicides. A 1999 report that pollen from corn carrying the *B. thuringiensis* toxin gene could kill Monarch butterfly larvae caused consternation among ecologists and biotechnologists alike. Glyphosate resistance has been found in weedy *Brassica* species near canola fields in Canada. Understanding how serious these findings are will require long-term ecological research; this is one reason why some groups urge governments to delay the widespread release of transgenic plants.

The problems involved with plant biotechnology should be balanced against the advantages. Biotechnology provides an excellent example of information-intensive solutions replacing energy-intensive solutions to problems. Planting crops that resist pests takes much less energy (in terms of fuel, chemicals, and labor) than applying insecticides. Designing crops to avoid spoilage reduces the waste of energy (fertilizers, labor, and fuel for both farm operations and distribution) that occurs when spoiled crops are discarded. For this reason, it is not surprising that biotechnologists sometimes display frustration when the products of their work are not quickly accepted. In this growing field, research is the key. It is certain that the more we understand about plant and animal physiology and ecology, the more safely and effectively we can use biotechnology to improve our lives.

KEY TERMS

clone genome genomics plasmids polymerase chain reaction recombinant dna restriction endonucleases restriction enzymes reverse transcriptase transformation vector

SUMMARY

1. Biotechnology is the application of biology to the creation of products for human use, including particularly the genetic manipulation of organisms to give them new capabilities or improved characteristics.

2. DNA can be manipulated through chemical and biochemical techniques. This allows a scientist to isolate, identify, and reproduce (through cloning) a gene.

3. Genes can be inserted into plant cells by using the Ti plasmid from Agrobacterium tumefaciens, a parasitic bacterium that naturally transfers DNA to plant cells, or by using physical techniques, including hiolistics and electroporation. In either case, if the inserted genes include one that is selectable (for instance, that provides resistance to a toxic antibiotic), it is possible to find cells that have stably incorporated the genes into their own chromosomes.

4. The techniques of molecular biology and genetic engineering are used to produce bacteria and fungi that synthesize new pharmaceutical drugs and industrial compounds.

5. New varieties of plants produced by genetic engineering can serve as a source of biochemicals--for instance, proteins or lipids useful in industrial processes.

6. Changing the genes of plants can produce new horticultural varieties with attractive and unusual flowers.

7. Genetic engineering may produce pest-resistant crops more efficiently than classical genetic methods. By creating plants resistant to herbicides, genetic engineering may allow chemical methods of weed control to be used more safely.

8. Certain genes may be altered by genetic engineering to produce fruit that maintains its quality longer after harvest.

9. Genetic engineers are working to identify genes that will give plants improved tolerance to environmental stresses such as salt, heat, cold, or drought.

10. The genetic engineering of plants, and especially the presence of genetically engineered foodstuffs on the market, has engendered concern among members of the public. Assuring the safety of new plant varieties is no more difficult for transgenic plants than for plants produced by classical breeding techniques. Understanding the ecological implications of transgenic plants requires long-term research.

Questions

1. A mutant *E. coli* bacterium without a plasmid cannot grow on a medium containing either ampicillin or streptomycin (or both). When the same strain of *E. coli* is infected with the pBR322 plasmid, it can grow on amplicillin or on tetracycline (or both). Inserting a new gene into the middle of the ampicillin-resistance or the tetracycline-resistance gene (in the pBR322 plasmid) inactivates that gene. Assume that Strain A of *E. coli* contains a pBR322 plasmid that has a piece of DNA inserted into the ampicillin gene and that Strain B contains a different pBR322 plasmid that has a piece of DNA inserted into the tetracycline gene. Complete the table below:

	Grows on ampicillin	Grows on tetracycline
Bacterium with pBR322 plasmid	No	No
Bacterium with normal pBR322 plasmid	Yes	Yes
Strain A	?	?
Strain B	?	?

If you had a suspension that contained a mixture of Strain A and Strain B, how could you select and grow a culture of Strain A cells?

2. Some investigators have found DNA associated with ancient plant fossils. They suggest that this DNA comes from the cells of the original plants. (Alternatively, the DNA might come from ancient bacteria or fungi or from a modern contaminant.) To analyze the DNA, they must have large quantities of individual DNA pieces, which can obtain by cloning the pieces. Describe the process by which they might obtain these quantities.

3. Match the following terms on the left with their corresponding definition on the right:

pieces of bacterial DNA independent of the chromosome
DNA used to carry a gene into a cell
enzyme that digests plant cell walls
enzyme that cuts DNA at specific sites
technique for making plasma membrane permeable to DNA
technique for isolating a single strain of bacterium or single
DNA sequence
colored compound in a plant cell
bacterial strain used to transform plant cells

4. Outline the arguments for and against the following projects:

a. Producing a bacterium that could live in the human gut and break down cellulose to the sugar glucose. Relate this to plant food in the human diet.

b. Producing a strain of bacterium that could fix nitrogen symbiotically with any species of plant (as *Rhizobium* does with legumes).

c. Producing a strain of wheat, the kernels of which produce a toxic compound that kills mildew fungi.

d. Producing a banana that makes a protein from the hepatitis B virus.

5. Suggest a trait that cold be added to an existing plant to improve it in some way; then outline arguments for and against your proposal.

6. New techniques allow genetic engineers to produce plants that lack genes and traits of their species--rather than gain new ones. Suggest useful plants that might be created using these techniques.

All the information in DNA is encoded in its sequence of bases. Determining the sequence is useful because it allows researchers to tell whether the DNA could code for a protein and, if so, what the amino acid sequence of the protein would be. It allows them to compare the DNA being studied with other known DNAs to detect mutations or potential evolutionary relationships. Sequencing DNA is currently a major activity of biotechnologists, and many millions of DNA sequences are stored in computer databases.

The procedure for determining DNA sequences works by making DNA fragments of sizes that correspond to the positions of particular bases. Originally devised by F. Sanger and his colleagues, it uses DNA synthesis to make the different-sized fragments. Assume that you have cloned a piece of DNA of unknown sequence in a vector. The cloned DNA is double-stranded; you heat the strands to separate them so that you can use one strand as a template for DNA synthesis. Then you add a short piece of DNA with a sequence complementary to the vector DNA that is next to the sequence to be determined. This short piece, the primer, binds to

The components are mixed in a test tube and the DNA polymerase is allowed to work

New DNA strands are assembled. At random times, a terminator nucleotide stops DNA synthesis. The color of fluorescence of the DNA indicates the base at that particular size of strand.

The mixture is placed in one lane of gel and subject to electrophoresis. The nucleotide sequence can be read from the sequence of bands on the gel. This picture shows six vertical lanes, representing six DNAs, from a real gel.

Figure 1. The sequence of a section of DNA can be determined by synthesizing pieces of DNA. Each band in the electropherogram (right) represents a piece of a particular length: the closer the band is to the top, the larger the piece of DNA. All bands start at the same bases, determined by the base sequence of the primer; all bands with the same color end at the same type of base (G,A,T, or C), determined by the bases of the terminator nucleotide. The relative positions of the bands than give the relative positions (sequence) of the different bases.

its complementary sequence in the vector DNA and serves as the starting point for DNA synthesis.

To this template-primer complex you add a DNA-synthesis reaction mixture. The mixture contains the DNA synthesizing enzyme DNA polymerase and activated nucleotides, which are substrates (reactants) for making the new DNA. The mixture also contains a small amount of special substrates that stop DNA synthesis at particular bases. On substrate stops synthesis at A bases, one at G bases, one at C bases; and one at T bases (Fig. 1a). Each substrate is linked to a dye that fluoresces a particular color.

When you run the DNA-synthesis reaction, you will be making four sets of DNA fragments. Each set is itself a mixture of DNAs of different lengths. The mixture with the A-terminating reagent, for instance, has a mixture of DNAs. All of them started at the primer, but some of them stopped at the first A of the synthesized DNA, some at the second, some at the third, and so forth. The relative sizes of these fragments indicate the relative positions of the A bases (and their complementary T bases in the template). All of these fragments glow green (Fig. 1b). Another set, with a G-terminating reagent will have sizes that indicate the relative position of G bases, and will glow yellow. A set with T-terminating reagent will glow red, and a set with C-terminating reagent will glow blue.

The newly synthesized DNA is separated by electrophoresis. The mixture is placed in an artificial plastic gel and subjected to an electric field. The DNA fragments all have negative ionic charges, so they move away from the negative and toward the positive pole. The holes between the gel molecules are small, so fragments move at different speeds, with short fragments moving through the gel more quickly than long ones. Even fragments that differ by only one base in length can be distinguished. At the end of the period of electrophoresis, the positions of the different fragments are visualized by their fluorescence. The presence of a band at a certain position on the gel that glows green means that there was an A at that position. The colors of the bands that are one base longer and shorter show the bases adjacent to that A. The sequence of the DNA can be read directly from the electropherogram.

BIOTECHNOLOGY: RNA Gives Plants the Silent Treatment

We know that RNA is involved in gene transcription and translation, but RNA can also turn off ("silence") gene expression. Rich Jorgensen from the University of Arizona first stumbled onto the co-suppression mechanism in 1990. He accidentally silenced genes in petunias, when trying to deepen the purple color through genetic engineering. He initially thought it was a bizarre attribute of the species. Hairpin gene silencing was first discovered and described in plants in the mid-1990s. Dr. Peter Waterhouse, a molecular geneticist from CSIRO (Commonwealth Scientific & Industrial Research Organization) Plant Industry in Australia, described the first successful gene silencing in tobacco in 1995, but there was a delay in filing a patent until 1998.

The term RNAi (*RNA interference* or hairpin gene silencing) was coined in 1998 by Andrew Fire (then at the Carnegie Institution of Washington) and Craig Mello (University of Massachusetts Medical School) in *Nature*. RNAi has been called the *third revolution in biotechnology*. (The first was recombinant DNA technology and the second was the PCR technique.)

RNAi is an evolutionarily ancient mechanism. It is believed that RNAi is part of a plant's natural defense mechanism against double-stranded (ds) RNA viruses and transposable elements. RNAi may also be involved in normal genetic control during plant development. Short strands of dsRNA called *siRNA* and microRNA (*miRNA*) exist naturally in the cells of many species, where they inactivate mRNAs with either complete or partial base-sequence similarity, respectively. siRNA mediates mRNA degradation, whereas miRNA is though to act predominately as a translation regulator.

The process of RNAi is straightforward, although incompletely understood. The dsRNA (complementary to the target mRNA sequence) is cleaved by the *Dicer* enzyme into many siRNA molecules (each about 21-23 base-pairs long). These siRNAs bind to the RISC (RNA-induced silencing complex) protein complex, which then attacks the target mRNA, leading to its degradation and post-transcriptional gene silencing (PTGS). In plants, the silent state can spread systemically, demonstrating that mobile silencing signals are present (Fig. 1).

The use of RNAi tools is revolutionizing the field of functional genomics. siRNA can be synthesized and used to silence genes quickly and easily. Scientists can see how genes are involved in cellular processes by observing the phenotypic effects. The laboratory of Abhaya Dandekar at the University of California at Davis is using this gene silencing technique in model plant systems to silence the crown gall oncogenes and prevent crown gall tumor formation induced by *Agrobacterium tumefaciens* (Fig. 2).

What is post-transcriptional gene silencing (PTGS)?

Figure 1. Mechanism of RNAi (RNA interference): double-stranded RNA (dsRNA), acted on by Dicer enzyme, provides the instructions (siRNA) for the RISC nuclease/helicase to locate and cleave any messenger RNA (mRNA) with the same base sequence as the dsRNA. This blocks the expression of the gene that produced the mRNA.

Figure 2. RNAi-induced silencing of crown gall oncogenes (tumor-inducing genes) in tomato. (Left) The tomato plant has been given a gene that produced a dsRNA complementary to one of the oncogenes that crown gall bacterium injected into the stem. Because of this, no tumor (gall) is formed. (Right) In contrast, the unprotected plant forms a gall.

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http://phys.org/news/2014-04-biotechnology-approach-sustainable-production-pharmaceutical.html

Fig 17.3. T.M. Murphy Fig 17.5. T.M. Murphy Fig 17.8. Keith V. Wood Fig 17.9. T.M. Murphy Fig 17.10. (a,b) Monsanto Co. (c,d) Calgene, Inc. Fig 17.11. T.M. Murphy Fig 17.12. Zhang, Blumwald. 2001. Nature Biotechnology 19:765 with permission