BACKGROUND INFORMATION

PART 1
Genetic Engineering

For most of history, farmers had to wait several plant generations before crops had the traits they most desired. The farmers used selective breeding, the process of choosing parent plants with the best traits over many generations. Selective breeding resulted in dramatic genetic changes to the species. While earlier farmers had no concept of the science of genetics, selective breeding based on observable traits allowed them to use plants' DNA to solve agricultural challenges and to improve the food supply. This approach to selecting specific traits is exemplified by the apple activity in Module 1.

Although selective breeding is still widely used, there are more modern processes available to alter the genetics of microorganisms, plants, and animals. More modern techniques to alter an organism's genetics includes mutation breeding, molecular marker-assisted breeding, genetic engineering, and genome editing.

Genetic engineering (GE) refers to deliberately modifying the characteristics of an organism by altering its genetic material. GE techniques include particle bombardment, Agrobacterium-mediated transformation, and targeted genome editing (the most recent additions to the genetic engineer's toolbox). Using GE technology, scientists can bring us improved agricultural products and practices faster than in the past.

Why genetically engineer plants?

Plants are genetically engineered for many of the same reasons that selective breeding is used: Better nutrition, higher **crop yield** (output), greater resistance to insect damage, and immunity to plant diseases.

Selective breeding techniques involve repeatedly cross-breeding plants until the breeder identifies offspring that have inherited the genes responsible for the desired combination of traits. However, this method may also result in the inheritance of unwanted genes responsible for unwanted traits (called **linkage drag**), and it can result in the loss of desired traits.

GE techniques can be used to isolate a gene or genes for the desired trait, add a gene from another organism or edit chromosomal DNA in a single plant cell, and generate a

Key biotechnology events related to food agriculture

1901	Japanese biologist Shigetane Ishiwatari discovered *Bacillus thuringiensis* (Bt), which makes a natural pesticide, found in soil worldwide and used by farmers since the 1920s.
1919	Karoly Ereky introduced the new term *biotechnology* (i.e., using biological systems to create products).
1971	Paul Berg completed a landmark gene splicing experiment.
1973	Stanley Cohen and Herbert Boyer created the first modified organism using recombinant DNA (rDNA) technology.
1974	Rudolf Jaenisch and Beatrice Mintz created the first transgenic animal (a mouse).
1978	Herbert Boyer starts a new company, Genentec and produces recombinant insulin.
1983	Mary-Dell Chilton inserted an antibiotic-resistant gene into a tobacco plant creating the first GE plant.
1987	Calgene creates the FlavrSavr® tomato.
1989	Chymosin from GE microorganisms authorized as a food processing aid by FDA.
1994	FDA concludes the FlavrSavr® tomato is as safe as comparable non-GE tomatoes.
1995	EPA approves the use of a Bt toxin as a plant-incorporated pesticide in a GE crop.
1998	GE virus-resistant papaya was grown commercially in Hawaii.
2012	CRISPR-Cas9 is used as a programmable RNA-guided DNA cutting tool.
2015	Genetically modified salmon is the first GE animal approved for food use in the United States.
2017	GE apples are available for sale in the United States.
2019	FDA completes consultation of high oleic soybean oil, first food from a genome edited plant.

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new plant with the trait from that cell. By adding one desired gene from the donor organism or by editing the gene in the chromosomal DNA of the single cell, the unwanted traits from the donor's other genes can be excluded. GE is used in conjunction with selective breeding to produce GE plant varieties that are on the market today.

Development of GE Tools in Bacteria

Throughout the past 100 years, several developments have led to current GE methods. After early geneticists were able to identify the gene locus for specific plant traits, various methods were used to try to transfer the specific DNA sequence from one plant to another. One method was injecting the DNA from the donor plant directly into the recipient plant cell to see if it would integrate into the recipient cell's genome. Unfortunately, the DNA was degraded, and the method was unsuccessful. It was like trying to send an envelope through the mail with only a zip code; the postal service wouldn't know where to deliver it. Scientists eventually used bacteria to transfer new DNA to the recipient plant cell.

Transformation is the changing of the cell's genetic makeup through the addition of new DNA. The DNA can come from the environment surrounding the cell via "**horizontal gene transfer**" or be added in a laboratory through GE methods. The laboratory method developed to combine genetic sequences that would not otherwise be found in the genome is called **recombinant DNA (rDNA)** technology.

In 1973, Herbert Boyer and Stanley Cohen produced the first successful GE organism. Boyer had expertise using **restriction endonucleases** (enzymes that cut DNA at specific nucleotide sequences), and Cohen studied **plasmids** (small rings of DNA) in bacteria. They were able to use a restriction enzyme to cut open a plasmid loop from one bacterial species, insert a gene from a different bacterial species, and close the plasmid, which combined the genes from different bacteria into one rDNA molecule. An enzyme called ligase was used to help join the cut DNA strand. Then they transformed this rDNA plasmid into the bacterium *Escherichia coli (E. coli)* and showed that the bacteria could utilize the rDNA. In Boyer and Cohen's experiment, one gene coded for tetracycline resistance and the other for kanamycin resistance. Tetracycline and kanamycin are antibiotics that kill bacteria that do not have resistance genes. It was possible to see which of the *E. coli* in their experiment had successfully acquired the new genes by culturing them in the presence of the antibiotics, where only the successfully transformed bacteria could grow. These experiments showed that bacterial transformation could be used to deliver the desired DNA to a useful site, just as the postal service delivers mail to the correct address.

Restriction enzymes are like scissors that cut DNA at specific sequences. Some restriction enzymes leave blunt DNA ends while others leave short, single-stranded overhangs called sticky ends.

Ligase enzymes are like the glue or tape for connecting DNA sequences in GE, or molecular biology, procedures.

Bacterial transformation still serves as the basis for a number of DNA technologies. Bacteria are used extensively in the laboratory for rDNA research. There are even some species of bacteria that go through the transformation process naturally, but most bacteria needs manipulation to become **competent** (able to take up the plasmid). Using the techniques from bacterial transformation, scientists have learned how to change the genome of plants, including plants that we use for food.

Scientists worldwide continue to use the Boyer and Cohen techniques to improve GE tools that develop, modify, and improve consumer products, including many of the food products we eat.

Nature's Own Genetic Engineer

A widely used method of transferring a transgene to a plant is to use the soil bacterium *Agrobacterium tumefaciens* (*A. tumefaciens*). This bacterium has a natural ability to enter a plant cell and insert its own DNA into a plant's genome. A plasmid is constructed to include *A. tumefaciens* genes needed for transferring DNA into the recipient plant cell, the transgene of interest, and a selectible marker, such as a gene conferring antibiotic-resistance or herbicide tolerance. Scientists now use the bacterium's natural behavior to insert the transgene into a plant's genome.

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Simplified Steps of Plasmid Development

Application of GE Tools in Plants

Plants can be genetically engineered to be resistant to pests and herbicides, to increase crop yield, or to tolerate adverse weather conditions using a process similar to bacterial transformation. Plants can also be engineered to produce fruits and vegetables that have longer and more stable shelf-lives in the grocery store. These GE uses have potential trickle-down benefits from the farmer to consumers, animals, and the environment. Because plants are eukaryotic and contain a nucleus, a slightly different method than the one used for bacterial transformation is used to insert the gene of interest.

For example, if scientists find a gene for enhanced drought resistance in a plant, and they want to use the gene to make another plant more drought resistant, an advantage of GE over selective breeding is that less time is required and linkage drag is avoided. The desired gene to be transferred and added to the genome of the recipient plant is often referred to as a **transgene**.

Genetic Engineering

- Allows the direct transfer of one or just a few genes between either closely or distantly related organisms
- Achieves crop improvement in a shorter time compared to conventional breeding
- Allows plants to be modified by adding, removing, or switching off particular genes

Adapted from: Agricultural Biotechnology (A Lot More than Just GM Crops).
www.isaaa.org/resources/publications/agricultural_biotechnology/download/Agricultural_Biotechnology.pdf

Advanced Content

The technologies used to clone or synthesize genes are changing and evolving. The three major methods currently used are:

- Traditional cloning – isolating DNA directly from the genome of the donor organism and inserting it into a plasmid for later use
- Subcloning the gene of interest – copying the gene from an existing collection of DNA clones ("DNA library")
- *De novo* gene synthesis – building a gene from scratch, using single nucleotides or short oligonucleotide strands without the need for a physical template

The techniques used by scientists to assemble and insert DNA pieces into the plasmid are also evolving along with the complexity of multi-gene DNA constructs. While simple restriction enzyme protocols can be used to create a single gene insert, multi-gene constructs such as those required for complex plant traits require more complex assembly strategies.

What is a DNA Library?

A DNA library is a collection of cloned DNA fragments that are stored in plasmids, which in turn are maintained and propagated in bacterial or yeast cells. The type of library is classified by the source of the DNA and the plasmid – referred to as a cloning vector – used to construct the library. Sources of DNA may be a single cell, a tissue, an organism, or an environmental sample containing multiple organisms. The DNA may be obtained from genomic sequences or from isolated mRNA and converted to complementary DNA (cDNA). Scientists use DNA libraries to find and study DNA encoding proteins or other functions of interest.

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General Plasmid Preparation

Bacterial plasmids are used to store a ready supply of the gene of interest. In the case of Agrobacterium-mediated plant transformation, the plasmids are used to transfer the gene of interest to the genome of the recipient plant. To receive the gene of interest, the bacterial plasmids are treated with a restriction enzyme that is compatible with the gene. This way, the plasmid DNA will have the same sticky ends as the gene, so they will combine more easily. The gene and plasmid DNA preparations are mixed with DNA ligase to seal the sticky ends of the DNA molecules together.

Scientists may also modify the bacterial plasmid using a similar process to insert one or more **selectable marker** genes. The selectable marker genes will be important later in the GE process when bacteria or plant cells with the gene of interest are being isolated. There are many selectable markers used to screen for bacterial, as well as plant transformants.

Selectable markers include:

- Auxotrophy (selects for the ability to grow on certain carbon sources)
- Antibiotic resistance (selects for ability to grow in the presence of a specific antibiotic)
- Herbicide tolerance (selects for ability to grow in the presence of a specific herbicide)

This new bacterial plasmid is called a **transformation plasmid** and has the gene of interest as well as the selectable marker gene. The transformation plasmid is added to bacteria using a bacterial transformation method. Finally, the bacteria are plated onto a medium containing the selection factor that will inhibit the growth of bacteria that did not take up the plasmid. The Petri plates are incubated to encourage bacterial growth, and only the bacteria that have taken up the transformation plasmid with the selectable marker gene will grow. Bacteria without it will not grow, resulting in millions of bacteria with the gene of interest in their DNA.

The next step is to transfer the gene to the plant cells. Currently, the most frequently used technique is Agrobacterium-mediated transformation. Bombardment with a gene gun is less common and typically used in cases where Agrobacterium-mediated methods don't work. Agrobacterium is a plant pathogen that has the natural ability to transfer DNA to plant cells. GE methods use a version of the Agrobacterium plasmid that has been "disarmed": the modified plasmid still has the ability to transfer DNA into the plant's genome, but it's disease-causing genes have been removed. Agrobacterium that have been transformed with the plasmid carrying the gene of interest and selectable marker are mixed with the plant cells. The Agrobacterium enters the plant cells and inserts a segment of the plasmid DNA (containing the gene and selectable marker gene) into the plant's genome. Once the Agrobacterium has had time to transform the plant cells, the cells are placed on medium containing: (1) An antibiotic that kills the Agrobacterium, (2) the selection factor that will inhibit growth of plant cells that did not take up the plasmid DNA, and (3) plant hormones that encourage the transformed cells to grow into new plants.

After a gene has been successfully inserted into the plant's genome, the modified plant must be able to grow and reproduce with its newly modified genome. First, the genotype of the plant must be studied so that the scientists only grow plants in which the genome has been modified correctly. When this is done, the GE plants will be grown under controlled conditions in a greenhouse and then in field trials to make sure that the new plants possess the desired new trait and show no new undesired characteristics.

Food from GE Plants

The first GE plant evaluated by the FDA for human consumption was the FlavrSavr® tomato. FDA concluded that the FlavrSavr® tomato was as safe as comparable non-GE tomatoes. It was brought to market in 1994, but it was not sufficiently profitable to continue production. Although there are currently no GE tomatoes on the market, other GE food crops are commercially available. Most of these GE plants were engineered to increase resistance to disease or pests, or tolerance to specific herbicides.

As of 2019, there were 10 GE food crops available in the U.S. Of these, only a few GE crops in the grocery store are available as whole produce. Whole produce could include certain cultivars of apple, potato, papaya, sweet corn, and squash. Ingredients derived from GE corn, soybeans, sugar beets, and canola (such as flour, oil, starch, and sugar) are used in a wide variety of foods including cereal, corn chips, veggie burgers, and more.

> The 10 GE crops today are: Alfalfa, apples, canola, corn (field and sweet), cotton, papaya, potatoes, soybeans, squash, and sugar beets.

Animal food: In the United States, more than 95 percent of food-producing animals consume food containing ingredients from GE crops. GE plants can also be found in food for non-food producing animals, such as cats and dogs.

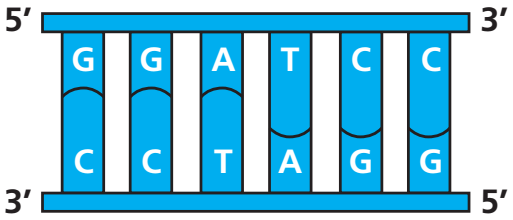
PART 2
Targeted Genome Editing

While original rDNA techniques would often result in random integration of the desired gene(s), newer **genome editing** techniques use tools to target the desired gene or the "edit" to a precise locus in the genome. One genome editing technique currently used by plant scientists is the CRISPR-Cas system. It's part of a natural bacterial defense system that scientists are using to cut and modify DNA more precisely than any previous GE method.

What is CRISPR and how is it used by bacteria?

CRISPR stands for **Clustered Regularly Interspaced Short Palindromic Repeats**. CRISPRs are sequences of nucleotides in the bacterial genome where bacteria keep a record of previous infections by a virus and later use it to identify and fight subsequent attacks by the same virus. When a bacterial cell is infected by a virus, the cell incorporates pieces of the viral DNA into the CRISPR sequence, which then produces small, non-coding RNAs that act like virus detectors. This is a form of **adaptive immunity**.

Sample Palindromic Sequence



The sequence read in one direction on one strand matches the sequence read in the opposite direction on the complementary strand.

Acronym Alert

Early genetic engineering (GE) began about half a century ago, while genome editing is a more recent technique. Although both two-word phrases begin with a G and an E, in this curriculum, genome editing will always be spelled out, and GE refers to the broader category of genetic engineering techniques.

Close to the CRISPRs are **CRISPR-associated (Cas)** genes that encode for Cas proteins. In bacteria, Cas proteins are part of the adaptive immune system. Some Cas proteins help the bacterial cell to capture small pieces of invading viral DNA for insertion into the CRISPR sequences during the initial infection; others silence the attacking virus' DNA during subsequent infections to protect the bacteria. For example, the small RNAs made from the CRISPR sequence containing the previously captured pieces of viral DNA (from the first infection) bind to the Cas9 endonuclease enzyme and target it to cut the viral DNA of repeat invaders.

Developing CRISPR-Cas as a New GE Tool

In 2012-2013, several scientific teams tested whether they could adapt the bacterial CRISPR-Cas immune system for use as a genome editing tool. First, they determined which specific components of the system were needed: The Cas9 enzyme and a guiding RNA. Next, they showed that they could target the Cas9 enzyme to cut a specific locus of their choosing simply by changing part of the guiding RNA sequence to match the targeted genome sequence. Collectively, multiple scientific teams showed CRISPR-Cas9 could be used as a programmable RNA-guided DNA cutting tool in bacteria, plant, mouse, and human cells.

This discovery was important because it meant that scientists could now cut and "edit" genomic DNA at a specific location of their choice. When the cell tries to repair the broken DNA strand by joining the pieces back together, scientists could take advantage of this process to add or remove specific DNA sequences. They could also include a repair template (with a mutation or a new gene entirely) to guide a specific repair by the cell's own mechanisms. In agriculture, genome editing using CRISPR-Cas, or one of several other available DNA targeting and cutting tools, can be used to create plants that produce higher yields, are more nutritious, and have characteristics that will help them endure extreme weather conditions.

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Here's the CRISPR-Cas9 process:

1. The scientist first identifies the precise location for the desired edit in the plant's genome.
2. A small piece of guide RNA is designed to target the DNA sequence at that location.
3. The guide RNA and Cas9 can be introduced into the plant cell as either DNA, RNA, or an RNA-protein complex called a ribonucleoprotein.
4. The guide RNA locates and binds to the targeted plant genomic DNA sequence. Its associated Cas9 enzyme then cuts the DNA at the targeted location.
5. The plant cell's own repair machinery re-attaches the cut DNA ends. During the process, nucleotides may be removed from or added onto the cut DNA ends. This can result in the loss of an undesirable trait or the expression of a new desired trait.
6. The cells are grown into mature plants with edited DNA.
7. The edited DNA is now heritable and can be passed on to the offspring.

Note: Depending on the method by which the guide RNA and Cas9 were introduced, they may not be present in the mature plant.

If the scientist includes a repair template during the plant transformation process (step 3), the repair template will direct the repair of the genomic DNA at the cut site (step 5).

CRISPR-Cas Delivery

There are several possible CRISPR-Cas delivery methods. The choice of delivery method depends on several factors, including which method is most efficient for the type of plant being edited and whether the scientist's goal is transient or stable expression of the CRISPR-Cas components.

Advanced Content

Plasmid-mediated delivery transforms the cell with a plasmid or plasmids carrying the genes for the guide RNA and Cas protein, similar to rDNA technology. Alternatively, direct delivery of the Cas9 protein with guide RNA into plant cells can be used.

In 2013, scientists discovered how to use the CRISPR-Cas system to edit a plant's genome. Since this discovery, many scientists throughout the world have been working to improve our food supply through genome editing using CRISPR-Cas as well as other targeted DNA cutting systems like TALEN and Zinc Finger Nucleases. These genome editing tools are being used to improve:

- a plant's yield performance
- nutritional value
- tolerance to biotic stress such as viral, fungal, and bacterial diseases
- tolerance to abiotic stress such as environmental conditions, including changes in water availability, temperature, and soil chemistry

The most studied crops are rice, corn, tomato, potato, barley, and wheat. Specific examples of researchers and their projects include scientists at Pennsylvania State University who used genome editing to extend the shelf-life of white mushrooms by disabling an enzyme that causes the mushrooms to brown, and scientists in Spain who used genome editing to modify the genome of wheat strains to be significantly lower in gluten.

The first food produced from a genome-edited crop became commercially available in 2019: High oleic soybean oil is lower in unhealthy fats than original soybean oil. Scientists are continually testing the potential of genome editing techniques to solve a range of food-related problems, such as:

- producing bananas that are resistant to a fungal disease that destroys the crop
- providing a solution to the citrus greening disease that is threatening U.S. orange trees
- protecting the world's chocolate supply by improving the cacao plant's ability to fight a virus that is destroying the crop in West Africa

CRISPR-Cas9