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The Science Behind Bt Crops

BACKGROUND INFORMATION

The Nature of BT[1]

"Bt" is short for *Bacillus thuringiensis,* a soil bacterium whose spores contain a crystalline (Cry) protein. In the insect gut, the protein breaks down to release a toxin, known as delta-endotoxin. This toxin binds to the intestinal lining and creates pores, resulting in ion imbalance, paralysis of the digestive system, and after a few days, insect death.

Why Bt crops resist certain insects

Different versions of the Cry genes, also known as "Bt genes," have been identified. They are effective against different orders of insects, or affect the insect gut in slightly different ways. A few examples are shown in the table below.

The use of Bt to control insect pests is not new. Insecticides containing Bt and its toxins (e.g., Dipel, Thuricide, Vectobac) have been sold for many years. Bt-based insecticides are considered safe for mammals and birds, and safer for non-target insects than conventional insecticides. What is new in Bt crops is that a modified version of the bacterial Cry gene has been incorporated into the plant's own DNA, so that the plant's cellular machinery produces the protein. When the insect chomps on a leaf or bores into a stem of a Bt-containing plant, it ingests the toxin and will die within a few days.

Proteins and events

Different versions of the Bt (Cry) genes produce slightly different crystalline proteins. When one of these Bt genes is successfully inserted into the DNA of a crop, the process is called "transformation" and the result is called an "event." Some events cause the Cry protein to be produced only in certain parts of the plant. For example, one type of Bt corn produces the Cry protein only in the green tissue and pollen and not in the kernel, while another event produces it throughout the corn plant.

Each crop event is evaluated in the laboratory and in the field for yield and other agronomic traits that indicate how well it will grow in farmers' fields. When companies request regulatory approval to market a Bt crop, they request approval for the specific Cry gene insertion event that was used to make the crop insect resistant.

A more detailed explanation of the genetic engineering process and how transformation fits into it is in the next two sections.

Overview of genetic engineering[2]

Genetic engineering includes the directed addition of a foreign gene or genes to the genome of an organism. A gene holds information that will give the organism a trait.

Cry gene designation	Toxic to these insect orders
CryIA(a), CryIA(b), CryIA(c)	Lepidoptera moths, like European corn borers, and butterflies
Cry1B, Cry1C, Cry1D	Lepidoptera
CryII	Lepidoptera Diptera flies, including mosquitoes
CryIII	Coleoptera beetles, like Colorado potato beetle and corn rootworm
CryIV	Diptera
CryV	Lepidoptera, Coleoptera

[1]Table 1

Genetic engineering is one type of genetic modification. Traditional plant breeding also modifies the genetic makeup of plants. Plant breeding, which involves crossing plants and selecting new superior gene combinations, has been going on for hundreds of years. Every time people cross two plants to improve their traits, they are conducting genetic modification.

Plant breeding only can be done between two plants that can sexually mate with each other.

This limits the new traits that can be added to those that already exist in that species.

Genetic engineering is a tool for adding new traits to a crop. It physically removes the DNA from one organism and transfers the gene(s) for one or a few traits into another organism. Since crossing is not necessary, the sexual barrier between species is overcome. Therefore, genes from any living organism can be transferred into a plant.

Step 1: DNA extraction

The process of genetic engineering requires the successful completion of a series of five steps. To better understand each of these, the development of Bt corn will be used as an example. However, this same process applies to other genes besides the Bt gene.

Before the genetic engineering process can begin, a living organism that exhibits the desired trait must be identified. The resistance to European corn borer used in Bt corn was discovered about 100 years ago. Silk worm farmers in the Orient had noticed that populations of silk worms were dying. Scientists discovered that a naturally occurring soil bacteria was causing the silk worm deaths. The soil bacteria, called *Bacillus thuringiensis*, or Bt for short, produced a protein that was toxic to silk worms, the Bt protein.

Although the scientists didn't know it, they had made a discovery necessary in the process of making Bt corn. The same Bt protein found to be toxic to silk worms is also toxic to European corn borer because both insects belong to the taxonomic order Lepidoptera. The production of the Bt protein in the Bt soil bacteria is controlled by the soil bacteria's DNA.

To be able to work with the DNA of the Bt gene, scientists have to extract it from the soil bacteria. DNA extraction is the first step in the genetic engineering process. This is accomplished by taking a sample of bacteria containing the gene of interest and taking it through a series of steps that separate the DNA from other parts of a cell.

Step 2: Gene cloning

The second step of genetic engineering is isolating the DNA of a gene of interest and making copies of it, a process referred to as gene cloning. During DNA extraction, all of the DNA from the organism is extracted at once. This means the sample of DNA extracted from the *Bacillus thuringiensis* bacteria will contain the gene for the Bt protein, but also all of the bacterium's other genes. Scientists separate the single gene of interest from the rest of the genes extracted from the bacteria.

The next stages of genetic engineering will involve study and experimentation with this gene. To do that, a scientist needs to make thousands of exact copies of it by cloning.

Step 3: Gene design

Once a gene has been cloned, genetic engineers begin the third step, designing the gene to work when it is inside a different organism. This is done in a test tube by using enzymes to replace certain parts of the gene with other DNA that is needed to make the gene work properly in a specific crop.

The first Bt gene released was designed to produce a level of Bt protein lethal to European corn borer and to only produce the Bt protein in green tissues of the corn plant (stems, leaves). Later, Bt genes were designed to produce the lethal level of protein in all tissues of a corn plant (leaves, stems, tassel, ear, roots).

Step 4: Transformation

The genetically engineered gene, called a transgene, is used for the fourth step in the process, transformation or gene insertion.

Since plants have millions of cells, it would be impossible to insert a copy of the transgene into every cell. Therefore, tissue culture is used to propagate masses of undifferentiated plant cells called callus. These are the cells into which the transgene will be added.

The transgene is inserted into some of the cells using various techniques. Some of the more common methods include the gene gun, *Agrobacterium*, microfibers, and electroporation. The main goal of each of these methods is to deliver the transgene into the nucleus of a cell without killing the cell. For example, the gene gun method of transformation shoots microscopic gold or tungsten particles coated with DNA of the transgene at plant cells. Transformed plant cells containing DNA of the transgene are regenerated into plants.

The DNA of the transgene may or may not be successfully inserted into a chromosome. The cells that receive the transgene are called transgenic and are selected from those that are not transgenic. Single plant cells can develop into an entire plant. Therefore, individual transgenic cells can develop into an entire plant that has the transgene in every cell. The transgenic plants are grown to maturity in greenhouses and the seed they

produce is collected. The genetic engineer's job is now complete. He/she will hand the transgenic seeds over to a plant breeder who is responsible for the final step.

Step 5: Variety development

The fifth and final step in producing a genetically engineered crop is variety development. Transgenic plants are crossed with elite varieties using traditional plant breeding methods to combine the transgene with the desirable genes of the elite varieties. The goal is to develop a crop variety with the transgene that can be grown commercially.

The genetic engineering process is similar for any plant. The length of time required to complete the five steps from start to finish varies depending upon the transgene, crop, and available resources. It can take from 6 to more than 15 years before a new transgenic variety is ready to be grown in farm fields.

Tissue culture and transformation[2]

Genetic engineers must accomplish all of the following before they are successful in producing a genetically engineered crop plant.

1. The transgene must be delivered into the nucleus of a cell and inserted into a chromosome.
2. The cells that receive the transgene must stay alive.
3. The cells that contain the transgene must be identified.
4. The transformed cell must divide and give rise to a mature plant that produces seed.
5. The location where the transgene inserts into the chromosome must not interfere with the expression of the transgene.
6. The transgene must not insert into an existing gene in the chromosome that influences survival of the plant cell or productivity of the mature plant.

Geneticists have overcome these barriers by developing special techniques. The first technique is tissue culture.

Tissue culture is an important component of transforming plants with new genes. During tissue culture, plant cells are removed from various parts of a plant and placed on media in petri plates. The media does not contain the growth hormones normally present in a plant that tell the cells which tissue to become. As a result, the plant cells form a mass of undifferentiated cells called a callus.

After the transgene has been inserted into callus cells, growth hormones can be added to the media triggering the development of roots, shoots, and eventually entire plants.

Obtaining callus cells from a plant and regenerating them into new plants does not necessarily work with all crop lines. Some crop lines well suited for tissue culture are agronomically inferior to modern high yielding varieties and are not grown commercially.

The nutrient and environmental requirements of callus can differ among crop lines. Time must be invested to determine the optimal conditions for growing a particular callus line. Genetic engineers make their best progress by developing methods that work reliably on a small group of lines and continually working with them over several years. The better the agronomic traits of those lines, the shorter the time between developing a transgenic plant and developing a genetically engineered variety that can be grown commercially.

Alternative to tissue culture

Some genetic engineers bypass the tissue culture process by inserting the transgene directly into plant tissue, such as an immature seed embryo. This means that they introduce the new gene into some but not all of the cells in a young plant.

To be successful, some of the cells in the young plant that are transformed must develop into the pollen or egg producing tissues in the plant so that its seed will contain the transgene in every cell.

Selectable marker genes

After callus cells have gone through the transformation process, it takes weeks of recovery and growth in a petri dish before they can develop into plants. Thousands of cells are growing on a single petri dish, but only a few may have received the transgene. It would be very cumbersome to grow a plant from every cell to test for presence of the transgene. It is much more efficient for genetic engineers to select only those cells that contain the transgene and grow them into entire plants. Genetic engineers need a way to distinguish transgenic cells from non-transgenic cells.

Selecting out transgenic cells is done by connecting the transgene to a piece of DNA called a selectable marker gene. The two most commonly used selectable marker genes provide resistance to either a herbicide or an antibiotic.

To select the transgenic cells, cells are grown on media containing the herbicide or antibiotic. Only those cells containing the selectable marker gene can survive. The

surviving cells also contain the desired transgene. These cells are grown into entire plants. (See illustration on p. 32 or 43.)

An example of a selectable marker is the one used in the development of some Bt corn lines. The selectable marker gene that confers resistance to the herbicide called Liberty® was attached to the desired Bt gene, and the combination was transformed into corn cells. After transformation, the cells were grown on a medium that contained the Liberty herbicide. Cells that survived contained the Liberty resistance gene and the Bt gene. The surviving cells were grown into plants. When the selectable marker gene confers resistance to a herbicide, the transgenic Bt plant has two new traits that may be an advantage to the producer, insect resistance and herbicide resistance. Putting transgenes for two different traits into the same plant is referred to as "stacking."

Transformation

Transformation is used to genetically change a living organism. A genetically engineered plant has been transformed and is referred to as a transgenic plant. To make and identify a transgenic plant that will pass the new gene on to its offspring, the following must occur.

Transformation goals

1. Copies of the transgene must be inserted into the nucleus of the plant cell without killing the cell.
2. At least one copy of the transgene must insert into the chromosome of the plant cell.
3. The transformed cell must replicate its chromosomes, including the new gene, and produce new cells. The division process must be repeated until the transformed cell develops into a transgenic plant.

The goal of any transformation technique is to transport the transgene and a selectable marker gene into the nucleus of a cell without destroying it. After that, the genetic engineer has no control as to how or even if the transgene will insert into a chromosome. Successful genetic engineers play the numbers game. Because they cannot control all of the steps, they repeat the process as many times as necessary to produce one genetically engineered plant.

Many methods of delivering the DNA of a transgene into the nucleus of plant cells have been tried and several have been successfully used to produce a transgenic plant. The most common methods will be described in the following sections.

Gene gun

The gene gun also is called particle acceleration or microprojectile bombardment. This method is one of the most commonly used to make genetically engineered crop plants.

The gene gun can be used on either tissue culture cells or young plant tissue. As the name implies, this method works by shooting DNA into the plant cells. Microscopic gold or tungsten particles are coated with hundreds of copies of the DNA of the transgene to be introduced.

In earlier versions of the gene gun, DNA-coated particles were shot into plant cells with a 22-caliber blank cartridge. Current versions place cells in a vacuum chamber and propel the DNA-coated particles with high pressure gas that is released in a sudden burst, much like a popped balloon.

Agrobacterium

Agrobacterium tumefaciens is a soil bacteria that works as a natural genetic engineer. *Agrobacterium* can invade plants through wounds in the stem or root. These bacteria have a piece of DNA called the Ti plasmid. Once inside the plant, a portion of the Ti plasmid will leave the bacteria, enter the plant cell, and insert itself into the plant's chromosomes. Upon insertion, the genes on the Ti plasmid are turned on. These genes cause plant cells to grow prolifically and produce enzymes that force the plant cell to make nutrients the bacteria need for growth. *Agrobacterium* genetically engineers the plant, forcing it to become a good source of food so the bacteria can multiply until the plant dies.

Genetic engineers have taken advantage of *Agrobacterium's* natural abilities. They have removed the genes from the Ti plasmid that make the plant become its source of food. In their place, they have incorporated into the Ti plasmid the desired transgene. The bacteria with their altered plasmids are used to infect plant cells growing in tissue culture. The transgene is inserted in the plant's chromosome to produce a transgenic cell.

Microfibers – "Whiskers"

Microfibers, also known as whiskers, have been used to transform cells. These microscopic fibers look like tiny needles with sharp ends. The whiskers are coated with hundreds of copies of the DNA of the transgene. The cells and whiskers are suspended together in a tube of solution and shaken vigorously. The tiny fibers stab the plant cells and deliver the DNA into the nucleus without killing it.

Electroporation

Electroporation uses a quick pulse of electricity to open tiny pores in the walls of plant cells. DNA is mixed in a solution with the cells. The DNA molecules are small enough to pass into the nucleus of the cell through the holes resulting from the electric pulse.

Events

Every successful transformation is called an event. For example, MON810 is an event used by the Monsanto Company to produce Bt corn. See Table 3 on p. 56 for other examples of events. Once an event is produced in a single cell, it is perpetuated from cell to cell when the chromosome replicates as the cells divide to produce a complete plant.

The event is passed from the plant to its seed. Eventually, selected events are incorporated into crop varieties through plant breeding. The three factors that differentiate events are:

1. Which transgene was inserted
2. Where on a chromosome it inserted
3. How many transgene copies were inserted in the plant's chromosomes

The position of insertion of the transgene DNA into the chromosome is not under the control of the genetic engineer.

Desirable events used by the plant breeder are those that have the transgene inserted at a location in the chromosome that does not disrupt other genes in the plant. A large percentage of the events produced by the genetic engineer never leave the lab or greenhouse because they disrupt the plant's growth. Others are rejected by the plant breeder because they interfere with plant productivity, leaving relatively few that are useful for producing a commercial transgenic variety.

For example, all the Bt corn varieties originated from only a few of the hundreds of events that were produced by transformation.

When a transgene is inserted into the DNA of a chromosome, it does so in a fashion similar to people cutting in line at the supermarket. The chromosome's DNA breaks and the transgene inserts. One copy or many copies of the transgene may insert into this site.

A problem can occur if a transgene inserts itself in the middle of an important plant gene. In this case, the DNA of the plant gene will be disrupted and the outcome is undesirable. The process can be compared to adding extra letters in the middle of a word so it will no longer make sense. If the disrupted plant gene is critical for growth or development, the plant will die. If the gene is important for a trait, such as grain yield, the undesirable event will not be noticed until the plant is tested in the field. The plant breeder evaluates many events to find those that are useful.

Table 2 on the next page summarizes examples of possible events that are rejected by genetic engineers.

Gene silencing

When scientists began to work with transgenic plants, they found that just because the transgene is present in seed does not necessarily mean that the plant grown from the seed will have the desired transgenic trait. This can happen because of a phenomenon called gene silencing.

Plants have a natural ability to silence their genes so that they will not be turned on, except when needed. This natural ability allows the plant to control extra genes that may occur in its chromosomes because of mistakes in replication, invading viruses, or some other poorly understood processes. For example, scientists know that certain genes that control the colors of corn kernels are silenced in the plant on some occasions.

When silencing occurs in transgenes, the plant cell somehow recognizes the transgenes and chemically modifies the DNA so it does not function properly. It is believed that silencing depends upon where the transgene inserts and how many copies of the transgene insert at that position of the chromosome. The insertion of more gene copies can result in a higher likelihood of silencing. Therefore, certain events will be more prone to gene silencing than others.

Transformation technology

Some plants are inherently easier to transform than others. Tobacco is one of the easiest species to transform and soybean is one of the most difficult. All transformation procedures involve a mix of art and science. The same procedures may not work equally well in the hands of all scientists and are labor intensive and expensive.

The goal of a genetic engineer is to reliably generate as many transgenic plants as possible. Most genetic engineering laboratories try to become efficient at using one method of transformation and stick with it. Sometimes their decision of which method to use is dictated by patent rights associated with the different methods. University and USDA genetic engineers have

the primary mission of studying how the transformation process works. They are also more likely to commit their efforts to crops that industry dedicates less time to, such as vegetable crops.

All transformation procedures are most successfully used when the law of averages is incorporated into the process. The insertion of DNA into a plant chromosome is a random process. The scientists can only control the amount of DNA of the transgene they use. As knowledge of the process improves, the procedure will become more predictable.

Credit Notes

[1]"The Nature of Bt" section is adapted from *Transgenic Crops: An Introduction and Resource Guide*. ©Center for Life Sciences and Department of Soil and Crop Sciences at Colorado State University, 1999-2001. All rights reserved. http://www.colostate.edu/programs/lifesciences/TransgenicCrops/. Used with permission.

[2]The "Overview of Genetic Engineering" and "Tissue Culture and Transformation" sections are adapted from *Crop Genetics: Crop Genetic Engineering*. Don Lee and Patty Hain, University of Nebraska. ©University of Nebraska, 2000. http://croptechnology.unl.edu/. Used with permission.

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Type of Event	Effect of Event	Outcome
1) The transgene is inserted into a part of the chromosome that contains a plant gene that is critical for cell survival or development.	The critical plant gene is knocked out and the cell dies or cannot divide.	The genetic engineer rejects the event during the selection process imposed on cells in tissue culture.
2) The transgene inserts into a part of the chromosome that does not allow for any expression of the transgene.	Plant derived from this event may contain the transgene, but the transgene cannot be detected.	The genetic engineer rejects the event during the selection process imposed on plants coming out of tissue culture.
3) The transgene inserts into a plant gene that is necessary for reproduction.	The transgenic plant does not produce seed.	The genetic engineer rejects the event because no seed is produced.
4) The transgene inserts into the chromosome and is present in the seed, but is silenced in reproduction.	The offspring of transformed plants do not have the trait expected from the transgene.	The plant breeder rejects the event in the early stages of variety development.
5) The transgene inserts into a part of the chromosome where it cannot function properly, or has an alteration in the sequence of its DNA.	The trait controlled by the transgene is not at an acceptable level.	The plant breeder rejects the event during variety development.
6) The transgene inserts into a plant gene that controls plant development in a more subtle fashion. This eventually influences agronomic and seed traits. The influence may be observed in some but not all environments and varieties.	The plant with this event may have all the desired characteristics, but some varieties that contain the transgene are not desirable enough to be grown commercially.	The plant breeder selects those varieties containing the event that can be grown commercially.

[2]Table 2

The Science Behind Bt Crops

TEACHING RESOURCES

Laboratory Lesson Plan: Fruit Cup DNA Extraction

DNA is present in the cells of all living organisms. This procedure uses household equipment and store supplies to extract DNA from kiwi or banana in sufficient quantity to be seen and spooled.

Science Content

- To use physical and chemical means to extract DNA from plant cells
- To observe and analyze the physical characteristics of DNA

Science Education Standards

Life Science, Content Standard C

– The cell (p. 184)
– The molecular basis of heredity (p. 185)
– Matter, energy, and organization in living systems (p. 186)

Source: *National Science Education Standards*, ©National Academy of Sciences, 1996. Used with permission. Page numbers refer to the seventh printing, November 1999 – also available on the Internet at http://books.nap.edu/html/nses/pdf/index.html.

Science Process Skills

- Observing
- Comparing
- Organizing
- Relating
- Inferring

Life Skills

- Learning
- Communicating
- Science processing

Time

Preparation: Time needed to buy materials at a grocery store plus 20 minutes classroom setup

Activity: 40 minutes

Materials

For each group of students, you will need:

- Two 5-oz plastic cups
- 1 set of measuring spoons
- plastic knife for cutting fruit
- plastic spoon for mixing and mashing fruit
- #2 cone coffee filter
- 30 ml of distilled water
- 1 teaspoon clear-colored shampoo, such as Suave Daily Clarifying Shampoo
- 1/2 of a kiwi fruit or 1/3 of a banana
- 2 pinches table salt, either iodized or non-iodized
- plastic transfer pipette or medicine dropper
- sealed test tube containing 95% ethanol (grain alcohol) or 91% rubbing alcohol

Optional: Photocopy student handout *See For Yourself – DNA Extraction* on p. 17 so each student has a copy.

Procedure

Divide the class into groups of four or a different group size, depending on how many students are the class. Tell the students:

"The process of extracting DNA from a cell is the first step for many laboratory procedures in biotechnology. The scientist must be able to separate DNA from the unwanted substances of the cell gently enough so that the DNA does not denature (break up).

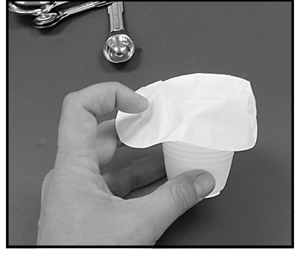
"You will prepare a solution of kiwi or banana treated with shampoo, distilled water, and salt. The detergent breaks down the cell membrane by dissolving the lipids (fatty molecules) and proteins of the cell, disrupting the bonds that hold the cell membrane together and releasing the DNA into the solution. The detergent forms complexes with the lipids and proteins, causing them to precipitate out of solution. The salt allows the DNA to precipitate out of a cold alcohol solution."

Ask the students to predict how quickly they think the DNA will begin to appear after the fruit solution is added to the alcohol and whether they think every group will have the same amount of DNA appear in the test tube. If using the optional handout *See For Yourself – DNA Extraction* on p. 17, provide each student with a

copy. An alternative, if available, is to project the PowerPoint® presentation described on p. 15 as a guide for students as they are doing the procedure.

1. In one of the 5 oz cups, make a solution consisting of 1 teaspoon of shampoo and two pinches of table salt. Add distilled water to make a final volume of 30 ml or approximately 1/3 the volume of the cup. Dissolve the salt and shampoo by stirring slowly to avoid foaming.

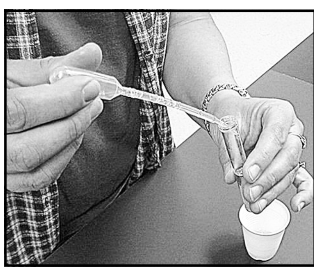
2. Using the plastic knife, peel and cut 1/2 of a kiwi (or 1/3 of a banana) into small pieces and add it to the solution from step 1. Mash the fruit against the side of the cup with the back of the spoon for 10 minutes. *(Mashing helps break open the cell walls and membranes so the cellular contents are exposed to the shampoo, salt, and water. The detergent dissolves the lipids that hold the cell membranes together, which releases the DNA into the solution. The detergent causes lipids and proteins to precipitate out of the solution, leaving the DNA. The salt enables the DNA strands to come together.)*



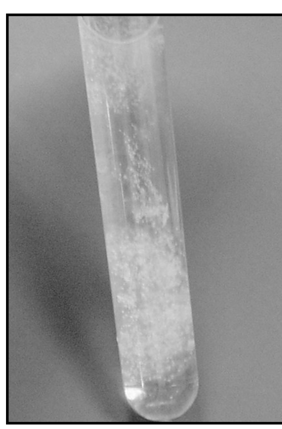
3. While one member of the group mashes the kiwi (or banana), another member will place a #2 cone coffee filter inside the second 5 oz plastic cup. Fold the coffee filter's edge around the cup so that the filter does not touch the bottom of the cup.

4. Filter the mixture by pouring it into the filter and letting the solution drain for several minutes until there is approximately 5 ml of filtrate to test (covers the bottom of the cup). The filter collects the lipid/protein complexes and allows DNA solution to filter through.

5. Obtain a test tube of cold alcohol. For best results, the alcohol should be as cold as possible.



6. Fill the plastic pipette with fruit solution and add it to the alcohol. *(DNA is not soluble in alcohol. When alcohol is added to the mixture, the components of the mixture, except for DNA, stay in solution while the DNA precipitates out into the alcohol layer.)*



7. Let the solution sit for 2 to 3 minutes without disturbing it. It is important not to shake the test tube. You can watch the white DNA precipitate out into the alcohol layer. When good results are obtained, there will be enough DNA to spool on to a glass rod. Using a Pasteur pipette that has been heated at the tip to form a hook, you can retrieve some of the DNA. DNA has the appearance of white, stringy mucus.

Reflect and Apply

1. Why were you able to see the DNA in the test tube only after alcohol was added to the mixture?

   DNA is not soluble in alcohol like it is in water because DNA is a sugar. When alcohol was added to the test tube, the DNA precipitated out into the alcohol layer and became visible.

2. It is more difficult to extract DNA from some fruits and vegetables than it is others. What factors do you think might account for the differences?

   Students might mention the thickness of the cell wall or the amount of DNA contained in the cells.

3. All living organisms contain DNA, including plants, animals, and humans. Do you think scientists are able to use the same methods of extracting DNA for all living things? Why?

   Students may point out that plant cells have a cell wall and animal cells do not, which might require

different procedures for plant and animal cells.

4. Suppose you extracted DNA from a banana and DNA from the monkey eating the banana. If you showed the two test tubes of DNA to a scientist, how could the scientist tell by looking at the extracted DNA which DNA came from the banana and which from the monkey? Why?

   The scientist could not tell. All DNA looks the same.

5. DNA fingerprinting is a technique used by forensic scientists to place a suspect at the scene of a crime by identifying the DNA he/she left behind. Lawbreakers sometimes wear gloves to avoid leaving traditional fingerprints at a crime scene. Would wearing gloves work for lawbreakers who do not want to leave DNA fingerprints? Why?

   No, because DNA fingerprints could be done using bodily fluids like blood or saliva, a strand of hair, or skin cell that a suspect left at the scene of a crime.

PowerPoint® Tutorial

A PowerPoint® tutorial of this laboratory lesson is available for downloading on the World Wide Web at http://www.biotech.iastate.edu/publications/ppt_presentations/default.html. There is also an html (web page) version of the tutorial at this site.

The tutorial has been placed on a compact disc (CD) that can be opened on either PC or Macintosh computers with CD or DVD drives. The CD of educational resources from Iowa State University's Office of Biotechnology is provided free to Iowa teachers. To order the CD, phone 515-294-9818, toll-free in Iowa 800-643-9504, or e-mail biotech@iastate.edu.

Internet Ideas

Crop Genetics: Crop Genetic Engineering
http://croptechnology.unl.edu/
©University of Nebraska, 2000.

Transgenic Crops: An Introduction and Resource Guide
http://www.colostate.edu/programs/lifesciences/TransgenicCrops
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See for yourself . . .

DNA Extraction

Fruit Cup DNA Extraction

The process of extracting DNA from a cell is the first step for many laboratory procedures in biotechnology. The scientist must be able to separate DNA from the unwanted substances of the cell gently enough so that the DNA does not denature (break up).

You will prepare a solution of kiwi or banana treated with shampoo, distilled water, and salt. The detergent breaks down the cell membrane by dissolving the lipids (fatty molecules) and proteins of the cell, disrupting the bonds that hold the cell membrane together and releasing the DNA into the solution. The detergent forms complexes with the lipids and proteins, causing them to precipitate out of solution. The salt allows the DNA to precipitate out into cold alcohol.

Materials

For each group of students, you will need:

- Two 5-oz plastic cups
- 1 set of measuring spoons
- plastic knife for cutting fruit
- plastic spoon for mixing and mashing fruit
- #2 cone coffee filter
- 30 ml of distilled water
- 1 teaspoon clear-colored shampoo, such as Suave Daily Clarifying Shampoo
- 1/2 of a kiwi fruit or 1/3 of a banana
- 2 pinches table salt, either iodized or non-iodized
- plastic transfer pipette or medicine dropper
- sealed test tube containing 95% ethanol (grain alcohol) or 91% rubbing alcohol

Predictions

1. How quickly do you think the DNA will begin to appear after the fruit solution is added to the alcohol?

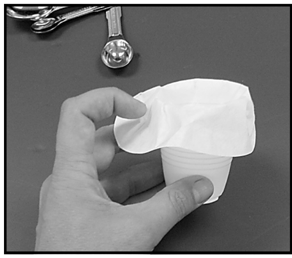
2. Do you think that every group will have the same amount of DNA appear in the test tube? Why or Why not?

Procedure

1. In one of the 5 oz cups, make a solution consisting of 1 teaspoon of shampoo and two pinches of table salt. Add distilled water to make a final volume of 30 ml or approximately 1/3 the volume of the cup. Dissolve the salt and shampoo by stirring slowly to avoid foaming.

2. Using the plastic knife, peel and cut 1/2 of a kiwi (or 1/3 of a banana) into small pieces and add it to the solution from step 1. Mash the fruit against the side of the cup with the back of the spoon for 10 minutes. *(Mashing helps break open the cell walls and membranes so the cellular contents are exposed to the shampoo, salt, and water. The detergent dissolves the lipids that hold the cell membranes together, which releases the DNA into the solution. The detergent causes lipids and proteins to precipitate out of the solution, leaving the DNA. The salt enables the DNA strands to come together.)*

3. While one member of the group mashes the kiwi (or banana), another member will place a #2 cone coffee filter inside the second 5 oz plastic cup. Fold the coffee filter's edge around the cup so that the filter does not touch the bottom of the cup.



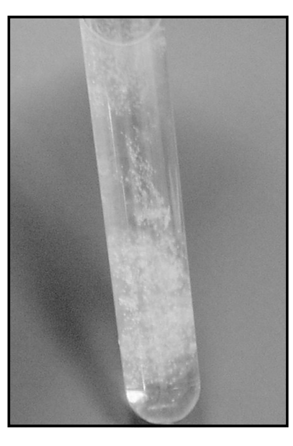
4. Filter the mixture by pouring it into the filter and letting the solution drain for several minutes until there is approximately 5 ml of filtrate to test (covers the bottom of the cup). The filter

collects the lipid/protein complexes and allows DNA solution to filter through.

5. Obtain a test tube of cold alcohol. For best results, the alcohol should be as cold as possible.

6. Fill the plastic pipette with fruit solution and add it to the alcohol. *(DNA is not soluble in alcohol. When alcohol is added to the mixture, the components of the mixture, except for DNA, stay in solution while the DNA precipitates out into the alcohol layer.)*

7. Let the solution sit for 2 to 3 minutes without disturbing it. It is important not to shake the test tube. You can watch the white DNA precipitate out into the alcohol layer. When good results are obtained, there will be enough DNA to spool on to a glass rod. Using a Pasteur pipette that has been heated at the tip to form a hook, you can retrieve some of the DNA. DNA has the appearance of white, stringy mucus.



Reflect and Apply

1. Why were you able to see the DNA in the test tube only after alcohol was added to the mixture?

2. It is more difficult to extract DNA from some fruits and vegetables than it is others. What factors do you think might account for the differences?

3. All living organisms contain DNA, including plants, animals, and humans. Do you think scientists are able to use the same methods of extracting DNA for all living things? Why?

4. Suppose you extracted DNA from a banana and DNA from the monkey eating the banana. If you showed the two test tubes of DNA to a scientist, how could the scientist tell by looking at the extracted DNA which DNA came from the banana and which from the monkey? Why?

5. DNA fingerprinting is a technique used by forensic scientists to place a suspect at the scene of a crime by identifying the DNA he/she left behind. Law-breakers sometimes wear gloves to avoid leaving traditional fingerprints at a crime scene. Would wearing gloves work for lawbreakers who do not want to leave DNA fingerprints? Why?

Credit Note

This activity was developed by Mike Zeller for the Office of Biotechnology, Iowa State University, and is used with permission.

… and justice for all
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TEACHING RESOURCES

Laboratory Lesson Plan: DNA Transformation of Bacteria – Red Colony

This experiment illustrates how a gene of interest can be inserted into bacteria. The transformed bacteria can be used as living factories to produce large numbers of copies of the gene for insertion into plants.

In this activity, a plasmid with the DNA of a gene for resistance to the antibiotic ampicillin and the lacZ gene will be transferred into a susceptible strain of *Escherichia coli* (*E. coli*) bacteria. The cells that take up this plasmid will show resistance to the antibiotic and produce a color change as the lacZ gene converts lactose in the media.

Science Content

- To have students understand how transformation occurs
- To give students experience in changing the genetics of an organism
- To help students understand the role of selective markers

Science Education Standards

Science as Inquiry, Content Standard A, Abilities Necessary to do Scientific Inquiry

- – Identify questions and concepts that guide scientific investigations (p. 175)
- – Design and conduct scientific investigations (p. 175)
- – Formulate and revise scientific explanations and models using logic and evidence (p. 175)
- – Recognize and analyze alternative explanations and models (p. 175)

Life Science, Content Standard C

- – The cell (p. 184)
- – The molecular basis of heredity (p. 185)
- – Matter, energy, and organization in living systems (p. 186)

Source: *National Science Education Standards*, ©National Academy of Sciences, 1996. Used with permission. Page numbers refer to the seventh printing, November 1999 – also available on the Internet at http://books.nap.edu/html/nses/pdf/index.html.

Science Process Skills

- Observing
- Comparing/measuring
- Relating
- Applying

Life Skills

- Communication
- Science processing
- Disease prevention
- Problem solving

Time

Preparation: Estimated 6 hours, beginning at least 24 hours in advance

Activity: 40 minutes

Materials

Iowa teachers can order the following supplies without charge from the ISU Office of Biotechnology by phoning toll-free 800-643-9504.

- *E. coli*
- Plasmid
- Media
- Ampicillin
- Calcium chloride
- Petri dishes
- Sterile pipettes
- Innoculating loops
- Capped glass test tubes

Optional: Make copies of student handouts 1-b, -c, and -d on p. 27-37 and the overhead transparency masters 1-a through 1-h on p. 39-53.

Day 1

Students should work in groups of three students. Each group of students will need:

- 2 capped microcentrifuge tubes (1.5 ml) containing 2 drops of sterile $CaCl_2$ and labeled "$CaCl_2$."
- Ice container, such as a styrofoam cup
- 1 petri dish containing colonies of *E. coli* (DH5 alpha strain)
- 1 aluminum foil packet containing 4 sterile toothpicks
- 1 container for used toothpicks
- 1 Sharpie marking pen

Day 2

Each group of three students will need:

- 2 capped microcentrifuge tubes from Day 1
- 4 sterile plastic pipettes
- 1 aluminum foil packet containing 4 sterile paper clips that are large and smooth. The clips should be opened into a 90º angle and the small end bent to close it.
- 1 Sharpie marking pen
- 1 glass test tube with a cap containing 2 ml of sterile nutrient broth and labeled “Broth”
- 2 petri dishes containing only nutrient agar and labeled “No Amp” on the bottom
- 2 petri dishes containing nutrient agar and the antibiotic ampicillin. The dishes should be labeled “Amp” on the bottom.
- 3 copies of the laboratory instructions, 1-d on p. 35-37, one for each student
- 1 microcentrifuge tube (1.5 ml), labeled “P,” containing 4 drops of plasmid DNA that is placed on ice to keep cold until used. The tube should be labeled “DNA.”
- 1 container for used toothpicks
- 1 ice container, such as a styrofoam cup, for placing tubes on ice after DNA has been added.
- 1 container, such as a styrofoam cup, for the 42º C water bath.

The teacher should have available for the entire class:

1 incubator for the petri dishes set at 37° C or less. It is difficult to maintain the temperature precisely unless a research incubator is used. Prolonged temperatures above 40° C will kill the bacteria. Temperatures lower than 37° C will result in slower growth of the bacteria, but will not kill them.

Educator Background Information

Genes control the traits that living organisms possess. Bacteria, such as *E. coli*, have genes on their chromosomes and on a small circular piece of DNA called a plasmid. Genes on the plasmid can be transferred from one bacteria to another by a process known as transformation.

In this experiment, a plasmid with the DNA of a gene for resistance to the antibiotic ampicillin and the lacZ gene will be transfered into a susceptible strain of the bacteria. The cells that take up this plasmid will show resistance to the antibiotic and produce a color change (dark red) as the lacZ gene converts lactose in the media.

The same technique is used to transfer the DNA of genes for production of insulin, growth hormones, and other proteins into bacteria. The transformed bacteria are used in fermentation to produce commercial quantities of the protein for treating diseases or for other uses. See p. 26 for information about a step-by-step PowerPoint® tutorial for this activity.

Lesson Plan

Pre-Lab Preparation and Storage of Materials by Teachers

STERILE SUPPLIES

1. **Packets of toothpicks and large paper clips bent as spreaders.** Wrap each item in aluminum foil, label the contents with a marking pen, and
   (a) bake them in an oven at 350º F for 15 minutes, or
   (b) put them in a pressure cooker at 15 pounds for 15 minutes, or
   (c) place them in an autoclave for 15 minutes.

   The pressure cooker and autoclave should be at the desired pressure for the 15-minute period. After the packets have cooled, they should be stored unopened at room temperature. The students should be instructed when opening the packets to touch only that part of the object that will not come in contact with the solutions or petri dishes.

2. **1.5 ml microcentrifuge tubes.** Wrap in aluminum foil all the tubes needed by the teacher to prepare the supplies for the students and
   (a) bake them at 250º F (they melt at 350º F) for 30 minutes, or
   (b) put them in a pressure cooker at 15 pounds for 15 minutes, or
   (c) place them in an autoclave for 15 minutes.

3. **Calcium chloride**. Dissolve 0.75 g of $CaCl_2$ into 50 ml of distilled water in a labeled 100-ml glass bottle with a cap. Keep the cap loose and place the glass bottle in
   (a) boiling water for 30 minutes, or
   (b) a pressure cooker at 15 pounds for 15 minutes, or
   (c) an autoclave for 15 minutes.

   Allow the bottle to cool until it is comfortable to hold, cap it tightly, and store in a refrigerator until it is used.

4. **Ampicillin solution**. For each 1,000 ml of Amp agar to be prepared, dissolve 50 mg of ampicillin sodium salt in 1 ml of cool sterile distilled water. The water can be sterilized before adding the ampicillin by placing it in a glass bottle that is not more than half full, putting the cap on loosely, and placing the glass bottle in
   (a) boiling water for 30 minutes, or
   (b) a pressure cooker at 15 pounds for 15 minutes, or
   (c) an autoclave for 15 minutes.

   The sterile water should be stored in the refrigerator until it is used to make the ampicillin solution. The ampicillin solution should not be prepared and stored in advance for an extended period. The solution should be prepared and put in the refrigerator immediately before the nutrient broth solution (Item 5, Sterile Supplies) and the agar plate solution (Item 2, Other Supplies) are prepared.

5. **Nutrient broth solution.** Calculate the amount of nutrient broth that is to be supplied to the students and add extra for spillage and other factors. Weigh 25 mg of LB premix per ml of distilled water into a bottle and label it. Add the appropriate volume of distilled water to the bottle. The bottle should not be more than half full so that it does not boil over during sterilization. With the cap of the bottle loose, place the bottle in
   (a) boiling water for 30 minutes, or
   (b) a pressure cooker at 15 pounds for 15 minutes, or
   (c) an autoclave for 15 minutes.

   After the LB has cooled and is comfortable to hold, cap it tightly and store in a refrigerator until it is dispensed to the class.

   Before the class, put 2 ml of the LB into glass test tubes, leave the caps loose, and place them in an appropriate rack in boiling water for 30 minutes to sterilize them. After the 30 minute-period, remove the tube rack from the boiling water, let the tubes cool, then tighten the caps. Unused broth can be reboiled and stored in the refrigerator for future use.

OTHER SUPPLIES

1. **Plasmid DNA solution.** The plasmid DNA used in the laboratory has a gene for ampicillin resistance. The plasmid DNA is obtained from the supplier in a concentrated solution, which has to be diluted to 0.005 µg/µl for the DNA transformation experiment. The DNA should be distributed to the students in tubes kept on ice. Any unused 0.005 µg/µl DNA can be stored in the freezer for future use. In a self-defrosting freezer, the DNA should be put on ice in an insulated container, such as a Thermos jar.

2. **MacConkey agar plates.** Two types of agar plates should prepared: without ampicillin "No Amp" and with ampicillin "Amp." Prepare separate solutions for the "No Amp" and the "Amp" plates. For each type of plate, 25 ml of agar solution will be required per plate. Label the plates on the underside, not the lid, before they are poured.

   "No Amp" plates:
   Prepare three "No Amp" plates for each group of three students. The student group will use one of the "No Amp" plates to prepare the starter culture and the other two "No Amp" plates for transformation. It is best to prepare about five extra "No Amp" plates for the entire class in case contamination occurs in one or more of the plates.

   Place the required volume of distilled water in one or more glass bottles with caps. The bottle should not be more than half full. Add 50 mg of lactose MacConkey medium per ml of distilled water. With the cap loose, sterilize the agar solution by
   (a) boiling water for 30 minutes, or
   (b) a pressure cooker at 15 pounds for 15 minutes, or
   (c) an autoclave for 15 minutes.

   Cool the bottle of agar to 55º C by method A or B as described in the note on the next page and pour the "No Amp" plates. Allow the plates to harden for about 30 minutes or until the agar has a milky or opaque appearance, then turn the plates upside down (lid down, agar up). If the plates are to be kept for more than two days, store them upside down in a refrigerator. The plates can be kept refrigerated for a month.

   "Amp" plates:
   Prepare two "Amp" plates for each group of three students. Follow the same procedure as for the "No Amp" plates until the agar has cooled to 55º C. Add 1 ml of the ampicillin solution (Item 4, Sterile Supplies) per liter (1,000 ml) of solution, swirl to mix, and pour immediately the plates labeled

"Amp." If the agar solidifies, it cannot be reheated because the ampicillin will be destroyed above 60º C.

Allow the "Amp" plates to harden for about 30 minutes or until the agar has a milky or opaque appearance, then turn the plates upside down (lid down, agar up). If they are to be kept for more than two days, store them upside down in a refrigerator. The plates can be kept refrigerated for a month.

Note:
People differ in their sensitivity to temperature and a teacher may prefer to measure the temperature of the agar to determine when 55º C is reached, particularly for the solution to which ampicillin is added. It is not possible to put a thermometer into the heated agar solution because it will become contaminated. There are two alternatives:

(A) The bottle of agar can be put into a container with the same volume of cool tap water as the volume of the medium inside the bottle. When the temperature of the tap water reaches 55º C, the contents inside the bottle should be at a similar temperature.

(B) The bottle of agar can be put into a hot water bath at 55º C and allowed to stand for 30 minutes.

3. ***E. coli* Starter Plate.** One petri dish containing live *E. coli* is needed for each group of three students. A strain of *E. coli* should be used that does not have resistance to ampicillin.

   Use a sterilized transfer loop, a paper clip bent into a loop and sterilized, or a sterilized toothpick. Gently touch the device to a colony of bacteria from a petri dish or test tube. Spread the bacteria on the starter plates in a zig-zag pattern to obtain individual colonies as the concentration of bacteria on the transfer device becomes less. Incubate the plates at 37º C for 24-36 hours. Colonies should grow to the size of this 0 for the lab procedure.

Clean Up After the Laboratory
Sterilize used toothpicks and 1.5 ml microcentrifuge tubes before placing them in the regular trash. Sterilize the used pipettes before washing them.

Sterilization can be achieved by placing the items in
(a) boiling water for 30 minutes, or
(b) a pressure cooker at 15 pounds for 15 minutes, or
(c) an autoclave for 15 minutes, or
(d) a 10% liquid chlorine bleach solution.

Wash glass bottles, pipettes, and paper clips for future use.

Doing the Laboratory

Day 1

Materials:

For each group of three students:

- 2 capped microcentrifuge tubes (1.5 ml) containing 2 drops of sterile $CaCl_2$ and labeled "$CaCl_2$"
- Ice container, such as a styrofoam cup
- 1 petri dish containing colonies of *E. coli* (DH5 alpha strain)
- 1 aluminum foil packet containing 4 sterile toothpicks
- 1 container for used toothpicks
- 1 Sharpie marking pen

Procedure:

Use optional student handouts on p. 27-33, optional overhead transparency masters on p. 39-53, or other methods to provide background on transformation to students. Ask students to predict what they will see on the Amp/B1, Amp/B2, No Amp/B1, and No Amp/B2 plates at the end of the experiment. Ask students to record their predictions on student handout 1-d on p. 35 or elsewhere. Depending on the time available, the background information and student predictions may need to be discussed prior to the Day 1 laboratory. Divide class into groups of three students each and ask students to complete the following two steps.

1. Use two separate sterile toothpicks to transfer a colony of *E. coli* about the size of this 0 into each of two tubes of calcium chloride that have been kept on ice. Use the toothpicks to stir the cells vigorously and thoroughly into the solution. The solution should appear milky. Close the caps of both tubes and discard the toothpicks into the container provided for that purpose. One person in the group should use the marker to label one of the tubes "B1." Another person should label the other tube "B2."

2. Place the tubes back in the ice and place the container of ice with tubes back in the refrigerator. (DO NOT FREEZE.) The cold calcium chloride in the tubes conditions the surface of the bacteria for DNA uptake the following day.

Day 2

Materials

For each group of three students:

- 2 capped microcentrifuge tubes "B1" and "B2" on ice from Day 1
- 4 sterile plastic pipettes
- 1 aluminum foil packet containing 4 sterile paper clips that are large and smooth. Each clip should be opened into a 90º angle and the small end bent to close it.
- 1 Sharpie marking pen
- 1 glass test tube with a cap containing 2 ml of sterile nutrient broth and labeled "Broth"
- 2 petri dishes containing only nutrient agar and labeled "No Amp" on the bottom
- 2 petri dishes containing nutrient agar and the antibiotic ampicillin. The dishes should be labeled "Amp" on the bottom.
- 3 copies of the laboratory instructions on p. 35-37, one for each student
- 1 microcentrifuge tube (1.5 ml), labeled "P," containing 4 drops of plasmid DNA that is placed on ice to keep cold until used. The tube should be labeled "DNA."
- 1 container for used toothpicks
- 1 ice container, such as a styrofoam cup, for placing tubes on ice after DNA has been added.
- 1 container, such as a styrofoam cup, for the 42º C water bath.

The teacher should have available for the entire class:

1 incubator for the petri dishes set at 37º C or less. It is difficult to maintain the temperature precisely unless a research incubator is used. Prolonged temperatures above 40º C will kill the bacteria. Temperatures lower than 37º C will result in slower growth of the bacteria, but will not kill them.

Procedure

1. Finger flick the "B1" and "B2" tubes to resuspend cells.

2. Open the tube labeled "B1" and with a sterile pipette add one drop of solution from the "P" tube. Close the "B1" tube. Do not add anything to the tube labeled "B2." (*The plasmid DNA from the "P" tube has a gene for resistance to ampicillin and the lacZ gene.*)

3. Place the "B1" tube on ice for 15 minutes. (*The cells are kept cold to prevent them from growing while the plasmids are being absorbed.*)

4. Remove the "B1" tube from the ice and immediately hold it in a 42º C water bath for 90 seconds. After 90 seconds, immediately place the tube back on ice for at least 1 minute. (*The marked temperature change causes the cells to readily absorb the plasmid DNA*).

5. Use a sterile pipette to add 5 drops of sterile nutrient broth to the "B1" tube. Close the tube. Mix by tipping the tube and inverting it gently (*The bacteria are provided nutrients to help them recover from the calcium chloride and heatshock treatments. For better results, allow the cells to recover at 37º C for a few minutes, preferably 20 minutes, before proceeding.*)

6. Label the underside of the four petri dishes with your group's names. Print "B1" on one "Amp" plate and "B2" on the other "Amp" plate. On one "No Amp" plate, print "B1" and on the other "No Amp" plate, print "B2."

7. Use a fresh sterile pipette to place three drops of cell suspension from the tube labeled "B1" onto the center of the petri dish labeled "Amp/B1" and three drops to the center of the dish labeled "No Amp/ B1."

   Use another fresh sterile pipette to place three drops of cell suspension from the tube labeled "B2" onto the center of the dish labeled "Amp/B2" and three drops to the center of the dish labeled "No Amp/B2."

   Use a fresh sterile large paper clip to spread the liquid evenly across the surface of each plate. Do not touch the part of the paper clip that comes in contact with the agar.

8. Incubate the plates upside down for 24 hours at 37º C.

9. Analyze the results of the transformation by placing the two plates labeled "Amp" and the two plates labeled "No Amp" side-by-side. (*The plate labeled "Amp/B2" should not have bacterial growth because the bacteria are killed because they did not have resistance to the antibiotic ampicillin. Bacterial growth on the "Amp/B1" plate is from cells that took up plasmids added in step 2 and that became resistant to ampicillin and became dark red. There is extensive bacterial growth on both of the "No Amp" plates*

because the antibiotic was not present and both resistant and nonresistant bacteria could grow. Antibiotic resistance is often used as a selective marker to identify cells that have been transformed. See illustration on p. 32 or 43.)

Reflect and Apply

These questions appear on optional student handout 1-d on p. 35-37.

1. Compare the predictions you made for each plate to your actual results. Were your predictions correct? Why or why not?

   Answers will vary.

2. How do you account for the different appearances of the Amp/B1 and Amp/B2 plates?

   The dark red bacterial growth on the Amp/B1 plate is from cells that took up plasmids added in step 2 to make them resistant to ampicillin. The Amp/B2 plate should not have bacterial growth because the bacteria were killed by the ampicillin.

3. How do you account for the similar appearances of the No Amp/B1 and No Amp/B2 plates?

   There was no ampicillin on either of these plates, so bacteria could grow unhindered.

4. Suppose that you are a scientist trying to insert a transgene into corn. How could you use ampicillin to help you determine which corn cells have taken up the transgene?

   You could attach the DNA of the transgene to the DNA of an antibiotic resistance gene, insert the DNA of the attached genes into corn cells, and grow the corn cells in the presence of the antibiotic. Only the corn cells that incorporated the antibiotic resistance gene, with the transgene attached, will grow.

PowerPoint® Tutorial

A four-part PowerPoint® tutorial of this laboratory lesson is available for downloading on the World Wide Web at http://www.biotech.iastate.edu/publications/ppt_presentations/default.html. There is also an html (web page) version of the tutorial at this site.

In addition, the tutorial has been placed on a compact disc (CD) that can be opened on either PC or Macintosh computers with CD or DVD drives. The CD of educational resources from Iowa State University's Office of Biotechnology is provided free to Iowa teachers. To order the CD, phone 515-294-9818, toll-free in Iowa 800-643-9504, or e-mail biotech@iastate.edu.

The four parts of the tutorial and their PowerPoint® file sizes are:

1. Transformation: Student Instructions – 1.9 Mb
2. Transformation: Materials Lists – 2.1 Mb
3. Transformation: Media Preparation – 2.5 Mb
4. Transformation: Teacher Preparation-Sterile Materials – 2.6 Mb

Internet Ideas

Bt Corn & European Corn Borer: Long-Term Success Through Resistance Management
http://www.extension.umn.edu/distribution/cropsystems/DC7055.html
©1997 Regents of the University of Minnesota. All rights reserved.

Crop Genetics: Crop Genetic Engineering
http://croptechnology.unl.edu/
©University of Nebraska, 2000.

DNA Transformation Demonstration
http://project.bio.iastate.edu/Courses/Gen308-SS99/Laboratories/DNA_Transformation/files/maina.html
Project BIO, Iowa State University.

Practical Protocols
http://www.ncbe.reading.ac.uk/NCBE/PROTOCOLS/menu.html
National Centre for Biotechnology Education, The University of Reading, United Kingdom.

Transgenic Crops: An Introduction and Resource Guide
http://www.colostate.edu/programs/lifesciences/TransgenicCrops
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Credit Notes

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Learning more about . . .
Bacillus thuringiensis

The Nature of BT[1]

"Bt" is short for ***Bacillus thuringiensis***, a soil bacterium whose spores contain a crystalline (Cry) protein. In the insect gut, the protein breaks down to release a toxin, known as delta-endotoxin. This toxin binds to the intestinal lining and creates pores, resulting in ion imbalance, paralysis of the digestive system, and after a few days, insect death.

Why Bt crops resist certain pests

Different versions of the Cry genes, also known as "Bt genes," have been identified. They are effective against different orders of insects, or affect the insect gut in slightly different ways. A few examples are shown in the table below.

The use of Bt to control insect pests is not new. Insecticides containing Bt and its toxins (e.g., Dipel, Thuricide, Vectobac) have been sold for many years. Bt-based insecticides are considered safe for mammals and birds, and safer for non-target insects than conventional insecticides. What is new in Bt crops is that a modified version of the bacterial Cry gene has been incorporated into the plant's own DNA, so that the plant's cellular machinery produces the protein. When the insect chomps on a leaf or bores into a stem of a Bt-containing plant, it ingests the protein and dies within a few days.

Proteins and events

Different versions of the Bt (Cry) genes produce slightly different crystalline proteins. When one of these Bt genes is successfully inserted into the DNA of a crop, the process is called "**transformation**" and the result is called an "**event**." Some events cause the Cry protein to be produced only in certain parts of the plant. For example, one type of Bt corn produces the Cry protein only in the green tissue and pollen, while another event produces it throughout the corn plant.

Each crop event is evaluated in the laboratory and in the field for yield and other agronomic traits that indicate how well it will grow in farmers' fields. When companies request regulatory approval to market a Bt crop, they request approval for the specific Cry gene insertion event that was used to make the crop insect-resistant.

Cry gene designation	Toxic to these insect orders
CryIA(a), CryIA(b), CryIA(c)	Lepidoptera moths, like European corn borers, and butterflies
Cry1B, Cry1C, Cry1D	Lepidoptera
CryII	Lepidoptera Diptera flies, including mosquitoes
CryIII	Coleoptera beetles, like Colorado potato beetle and corn rootworm
CryIV	Diptera
CryV	Lepidoptera, Coleoptera

[1]Table 1

Overview of genetic engineering[2]

Genetic engineering is the directed addition of the DNA of a gene or genes into the chromosomes of an organism. A gene holds information that will give the transformed organism a new trait.

Step 1: DNA extraction

The process of genetic engineering requires the successful completion of a series of five steps. To better understand each of these,

the development of Bt corn will be used as an example.

Before the genetic engineering process can begin, a living organism that exhibits the desired trait must be identified. The resistance to European corn borer used in Bt corn was discovered about 100 years ago. Silk worm farmers in the Orient noticed that populations of silk worms were dying. Scientists discovered that a naturally occurring soil bacteria was causing the silk worm deaths. The soil bacteria, called *Bacillus thuringiensis*, or Bt for short, produced a protein toxic to silk worms, the Bt protein.

The same Bt protein found to be toxic to silk worms is also toxic to European corn borer because both insects belong to the Lepidopteran order. The production of the Bt protein in the Bt soil bacteria is controlled by the DNA of a gene.

To be able to work with the DNA of the Bt gene, scientists have to extract it from the soil bacteria. **DNA extraction** is the first step in the genetic engineering process. This is accomplished by taking a sample of bacteria containing the Bt protein gene and taking it through a series of steps that separate the DNA from other parts of a cell.

Step 2: Gene cloning

The second step of genetic engineering is isolating the DNA of a gene of interest and making copies of it, a process referred to as **gene cloning.** During DNA extraction, all of the DNA from the organism is extracted at once. This means the sample of DNA extracted from the bacteria will contain the Bt protein gene and all of the bacterium's other genes. Scientists separate the Bt protein gene from the rest of the genes extracted from the bacteria.

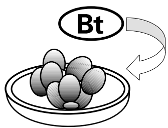
The next stages of genetic engineering involve study and experimentation with the Bt protein gene. In order to do that, a scientist needs to make thousands of exact copies of it by cloning.

Step 3: Gene design

Once the Bt gene has been cloned, genetic engineers begin the third step, which is designing the gene to work when it is inside a different organism. This is done in a test tube by using enzymes to replace certain parts of the gene with other DNA that is needed to make the gene work properly in a specific crop.

The first Bt gene released was designed to produce a level of Bt protein lethal to European corn borer and to only produce the Bt protein in green tissues of the corn plant (such as stems and leaves). Later, Bt genes were designed to produce the lethal level of protein in all tissues of a corn plant (leaves, stems, tassel, ear, roots).

Step 4: Transformation



The modified gene is used for the fourth step in the process, transformation or gene insertion.

Since plants have millions of cells, it would be impossible to insert a copy of the Bt gene into every cell. Instead, tissue culture is used to grow masses of undifferentiated plant cells called callus. Callus is one kind of plant tissue used for transformation. Callus are the cells to which the new Bt gene will be added.

The Bt gene can be inserted into cells using various techniques. Some of the more common methods include the gene gun, *Agrobacterium*, microfibers, and electroporation. The main goal of each of these methods is to deliver the Bt gene into the nucleus of a cell without killing the cell.

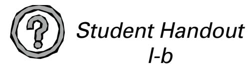
After the Bt gene reaches the nucleus of a plant cell, it may or may not be successfully inserted into a chromosome. The cells that do incorporate the Bt gene into their chromosomes are called **transgenic** and must be selected from those cells that are not transgenic. The selected transgenic cells develop into plants that have the Bt gene in every cell. The transgenic plants are grown to maturity in greenhouses, and the seeds they produce are collected. The transgenic seeds are used by a plant breeder for the final step.

Step 5: Variety development

The fifth and final part of producing a genetically engineered crop is development of a productive variety that can be grown commercially by farmers. Transgenic plants are crossed with elite varieties using traditional plant breeding methods to combine the transgene with the desirable genes of the elite varieties.

The Process of Plant Genetic Engineering – Start to Finish

The genetic engineering process is similar for any crop. The time required to complete the five steps from start to finish varies depending upon the Bt gene, crop, and available resources. It can take from 6 to more than 15 years before a new transgenic variety is ready to be grown in farm fields.



Learn the Language

Bacillus thuringiensis
A soil bacterium whose spores contain a crystalline (Cry) protein

DNA extraction
The process of separating DNA from the other components of a cell

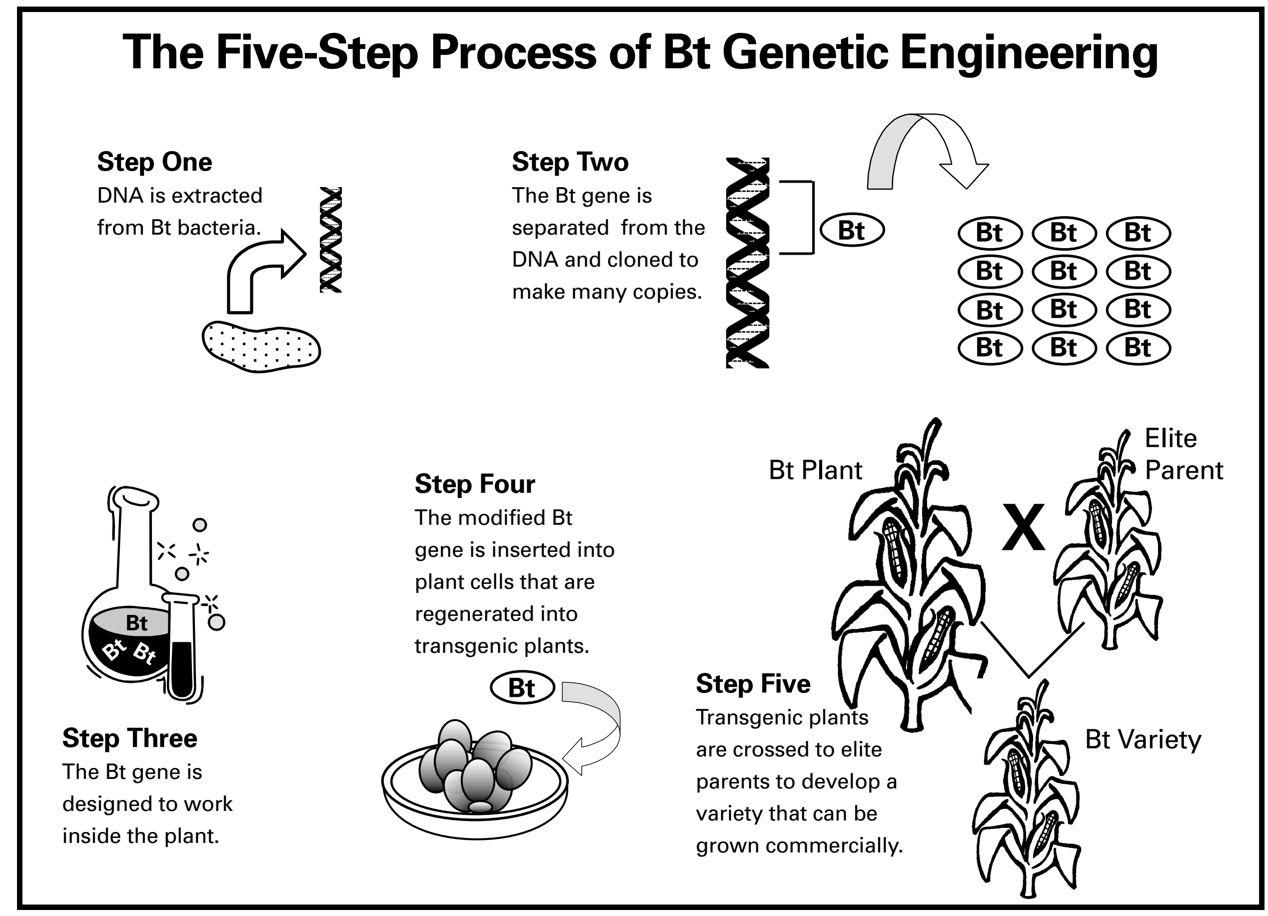
Event
The successful insertion of the DNA of a gene into the chromosomes of plant cells that can grow into mature plants and produce seeds

Genetic engineering
The addition of the DNA of a gene into the chromosomes of an organism by use of laboratory methods

Gene cloning
Separating the DNA of a gene of interest from the DNA of other genes and making exact copies

Transformation
The process of inserting the DNA of a gene into the chromosome of an organism

Transgenic organism
A plant or other organism into which DNA of a gene has been inserted by transformation



Credit Notes

[1]"The Nature of Bt" section is adapted from *Transgenic Crops: An Introduction and Resource Guide*. ©Center for Life Sciences and Department of Soil and Crop Sciences at Colorado State University, 1999-2001. All Rights Reserved. http://www.colostate.edu/programs/lifesciences/TransgenicCrops/. Used with permission.

[2]The "Overview of Genetic Engineering" section is adapted from *Crop Genetics: Crop Genetic Engineering*. Don Lee and Patty Hain, University of Nebraska. ©University of Nebraska, 2000. http://croptechnology.unl.edu/. Used with permission.

"Five-Step Process" chart designed by Glenda Webber, Iowa State University.

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Learning more about . . .
Tissue culture

Tissue culture and transformation[1]

The transformation step of producing a genetically engineered crop plant with a Bt gene must accomplish all of the following before it is successful.

1. The Bt gene must be delivered into the nucleus of a plant cell and inserted into a chromosome.
2. The cells that receive the Bt gene must stay alive.
3. The cells that contain the Bt gene must be identified.
4. The transformed cell must divide and give rise to a mature plant that produces seed.
5. The location where the Bt gene inserts into the chromosome must not interfere with the ability of the gene to produce the Bt protein.
6. The Bt gene must not insert into an existing gene that influences survival of the plant cell or productivity of the mature plant.

Genetic engineers have overcome these barriers by developing special techniques. One technique is **tissue culture.** In one type of tissue culture, clusters of undifferentiated plant cells are grown on petri plates.

Another technique is **transformation** in which genetic engineers introduce the Bt gene into cells using one of several possible methods including:

- Gene gun (particle bombardment)
- *Agrobacterium tumefaciens*
- Electroporation
- Microfibers

Tissue culture

The tissue culture process is used to produce plant cells appropriate for transformation. During this procedure, plant cells are removed from various parts of a plant and placed on media in petri plates. The media does not contain the growth hormones normally present in a plant that tell the cells which kind of tissue to become. As a result, the cells form a mass of undifferentiated cells called a callus.

After transformation, growth hormones can be added to the media triggering the callus cells to develop roots, shoots, and eventually mature plants.

Obtaining callus cells from a plant and regenerating them into new plants does not necessarily work with all crop lines. Some crop lines well suited for tissue culture are agronomically inferior to modern high yielding varieties and are not grown commercially.

The nutrient and environmental requirements of callus can differ among crop lines. Time must be invested to determine the optimal conditions for growing a particular callus line. Genetic engineers make their best progress by developing methods that work reliably on a small group of lines and continually working with them over several years. The better the agronomic traits of those lines, the shorter the time between developing a transgenic plant and developing a genetically engineered variety that can be grown commercially.

Alternative to tissue culture

Some genetic engineers bypass the tissue culture process by inserting the Bt gene directly into plant tissue, such as an immature seed embryo. This means that they introduce the Bt gene into some but not all of the cells in a young plant.

To be successful, some of the cells in the young plant that are transformed must develop into the pollen or egg producing tissues in the plant so that its seed will contain the Bt gene in every cell.

Selectable marker genes

After callus cells have gone through the transformation process, it takes weeks of recovery and growth in a petri dish before they can develop into plants. Thousands of cells are growing on a single petri dish, but only a few may have received the Bt gene. It would be very cumbersome to grow a plant from every cell to test for presence of the Bt gene. It is much more efficient for genetic engineers to select only those cells that contain the Bt gene and grow them into entire plants. Genetic engineers need a way to distinguish cells with the Bt gene from cells that do not have the Bt gene.

Selecting out cells with the Bt gene is done by connect-

ing the Bt gene to a piece of DNA called a selectable marker gene. The two most commonly used selectable marker genes provide resistance to either a herbicide or an antibiotic.

To select the cells with the Bt gene, all the cells are grown on media containing the herbicide or antibiotic. Only those cells containing the selectable marker gene can survive. The surviving cells also contain the Bt gene. These cells are grown into entire plants.

See the illustration below.

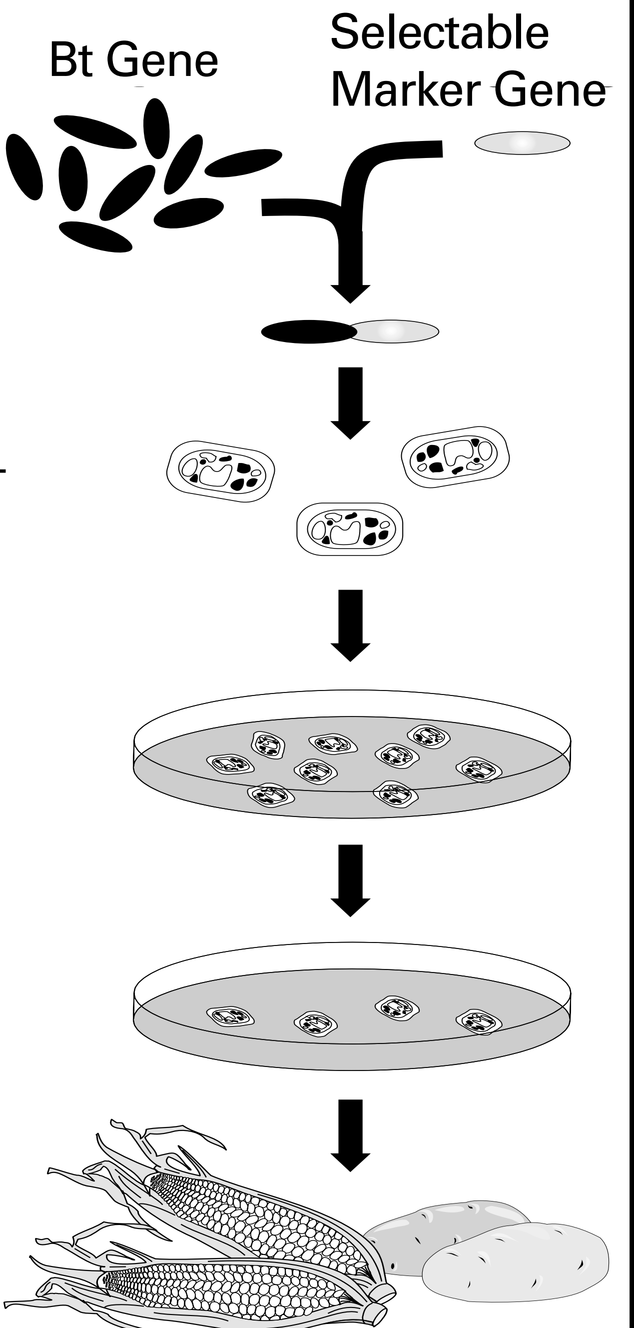
An example of a selectable marker is the one used in the development of some Bt corn varieties. The selectable marker gene that confers resistance to the herbicide called Liberty® was attached to the desired Bt gene and the combination was transformed into corn cells. After transformation, the cells were grown on a medium that contained the Liberty herbicide. Cells that survived contained the Liberty resistance gene and

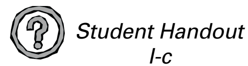
1. Bt gene that produces the desired lethal protein is joined to a selectable marker gene.

2. Selectable marker gene + Bt gene are inserted into plant cells during transformation.

3. Plant cells are grown in the presence of a chemical that selects for the selectable marker gene.

4. Cells that carry the selectable marker gene + Bt gene survive and are grown into plants.





the Bt gene. The surviving cells were grown into plants. When the selectable marker gene confers resistance to a herbicide, the transgenic Bt plant has two new traits that may be an advantage to the producer, insect resistance and herbicide resistance. Putting transgenes for two different traits into the same plant is referred to as "stacking."

Learn the Language

Selectable marker gene
A gene that controls a trait that is easily detectable, such as herbicide or antibiotic resistance, and that is attached to the desired transgene

Tissue culture
A process in which plant cells are grown on petri ` dishes in the absence of normal growth hormones, producing undifferentiated cells that can be genetically modified, then treated with hormones to grow into whole plants

Transformation
The process of inserting the DNA of a gene into the chromosome of an organism

Credit Notes

[1]"Tissue Culture and Transformation" is adapted from *Crop Genetics: Crop Genetic Engineering*. Don Lee and Patty Hain, University of Nebraska. ©University of Nebraska, 2000. http://croptechnology.unl.edu/. Used with permission.

"Tissue Culture" diagram designed by Glenda Webber, Iowa State University.

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See for yourself . . .

Bacterial transformation

DNA Transformation of Bacteria – Red Colony

This experiment shows how a gene can be inserted into bacteria. The transformed bacteria can be used as living factories for gene cloning to produce the large numbers of gene copies needed for insertion into plants during transformation.

In this activity, a plasmid with the DNA of a gene for resistance to the antibiotic ampicillin and the lacZ gene will be transfered into a susceptible strain of *Escherichia coli* (*E. coli*) bacteria. The cells that take up this plasmid will show resistance to the antibiotic and produce a color change as the lacZ gene converts lactose in the media.

Day 1

Materials

For each group of three students:

- 2 capped microcentrifuge tubes (1.5 ml) containing 2 drops of sterile $CaCl_2$ and labeled "$CaCl_2$"
- Ice container, such as a styrofoam cup
- 1 petri dish containing colonies of *E. coli* (DH5 alpha strain)
- 1 aluminum foil packet containing 4 sterile toothpicks
- 1 container for used toothpicks
- 1 Sharpie marking pen

Predictions

After listening to your teacher's explanation of this experiment, predict what you will see on each of the petri dishes at the end of this experiment. Draw what you think you will see on each dish below.

Procedure:

1. Use two separate sterile toothpicks to transfer a colony of *E. coli* about the size of this 0 into each of two tubes of calcium chloride that have been kept on ice. Use the toothpicks to stir the cells vigorously and thoroughly into the solution. The solution should appear milky. Close the caps of both tubes and discard the toothpicks into the container provided for that purpose. One person in the group should use the marker to label one of the tubes "B1." Another person should label the other tube "B2."

2. Place the tubes back in the ice and place the container of ice with tubes back in the refrigerator. (DO NOT FREEZE.) The cold calcium chloride, in the tubes, conditions the surface of the bacteria for DNA uptake the following day.

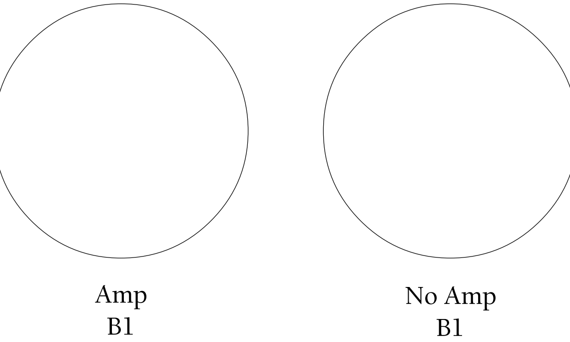
Day 2

Materials

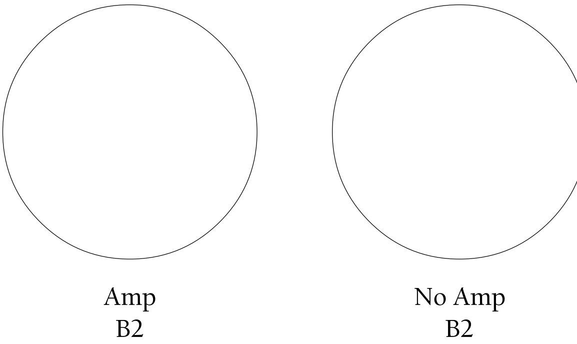
For each group of three students:

- 2 capped microcentrifuge tubes "B1" and "B2" on ice from Day 1
- 4 sterile plastic pipettes
- 1 aluminum foil packet containing 4 sterile paper clips that are large and smooth. Each clip should be opened into a 90º angle and the small end bent to close it.

(continued on next page)



Amp B1 | No Amp B1



Amp B2 | No Amp B2

- 1 Sharpie marking pen
- 1 glass test tube with a cap containing 2 ml of sterile nutrient broth and labeled “Broth”
- 2 petri dishes containing only nutrient agar and labeled “No Amp” on the bottom.
- 2 petri dishes containing nutrient agar and the antibiotic ampicillin. The dishes should be labeled “Amp” on the bottom.
- 3 copies of the laboratory instructions, one for each student
- 1 microcentrifuge tube (1.5 ml), labeled “P” containing 4 drops of plasmid DNA that is placed on ice to keep cold until used. The tube should be labeled “DNA.”
- 1 container for used toothpicks
- 1 ice container, such as a styrofoam cup, for placing tubes on ice after DNA has been added.
- 1 container, such as a styrofoam cup, for the 42º C water bath.

The teacher should have available for the entire class:

1 incubator for the petri dishes set at 37º C or less. It is difficult to maintain the temperature precisely unless a research incubator is used. Prolonged temperatures above 40º C will kill the bacteria. Temperatures lower than 37º C will result in slower growth of the bacteria, but will not kill them.

Procedure

1. Finger flick the “B1” and “B2” tubes to resuspend cells.

2. Open the tube labeled “B1” and with a sterile pipette add one drop of solution from the “P” tube. Close the “B1” tube. Do not add anything to the tube labeled “B2.” (*The plasmid DNA from the “P” tube has a gene for resistance to ampicillin and the lacZ gene.*)

3. Place the “B1” tube on ice for 15 minutes. (*The cells are kept cold to prevent them from growing while the plasmids are being absorbed.*)

4. Remove the “B1” tube from the ice and immediately hold it in a 42º C water bath for 90 seconds. After 90 seconds, immediately place the tube back on ice for at least 1 minute. (*The marked temperature change causes the cells to readily absorb the plasmid DNA*).

5. Use a sterile pipette to add 5 drops of sterile nutrient broth to the “B1” tube. Close the tube. Mix by tipping the tube and inverting it gently (*The bacteria are provided nutrients to help them recover from the calcium chloride and heatshock treatments. For better results, allow the cells to recover at 37º C for a few minutes, preferably 20 minutes, before proceeding.*)

6. Label the underside of the four petri dishes with your group’s names. Print “B1” on one “Amp” plate and “B2” on the other “Amp” plate. On one “No Amp” plate, print “B1” and on the other “No Amp” plate, print “B2.”

7. Use a fresh sterile pipette to place three drops of cell suspension from the tube labeled “B1” onto the center of the petri dish labeled “Amp/B1” and three drops to the center of the dish labeled “No Amp/ B1.”

   Use another fresh sterile pipette to place three drops of cell suspension from the tube labeled “B2” onto the center of the dish labeled “Amp/B2” and three drops to the center of the dish labeled “No Amp/B2.”

   Use a fresh sterile large paper clip to spread the liquid evenly across the surface of each plate. Do not touch the part of the paper clip that comes in contact with the agar.

8. Incubate the plates upside down for 24 hours at 37º C.

9. Analyze the results of the transformation by placing the two plates labeled “Amp” and the two plates labeled “No Amp” side-by-side.

Reflect and Apply

1. Compare the predictions you made for each plate to your actual results. Were your predictions correct? Why or why not?

2. How do you account for the different appearances of the Amp/B1 and Amp/B2 plates?

3. How do you account for the similar appearances of the No Amp/B1 and No Amp/B2 plates?

4. Suppose that you are a scientist trying to insert a transgene into corn. How could you use ampicillin to help you determine which corn cells have taken up the transgene?

Credit Note

This activity was developed by Mike Zeller for the Office of Biotechnology, Iowa State University, and is used with permission.

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What is Bt?

- Bt *Bacillus thuringiensis* is a rod-shaped soil bacterium.
- Bt Its spores contain a crystalline (Cry) protein.
- Bt In certain insects, the protein releases a toxin called delta-endotoxin.
- Bt Delta-endotoxin creates tiny holes in the insect's intestinal lining, which stops digestion.
- Bt This causes the death of the insect.

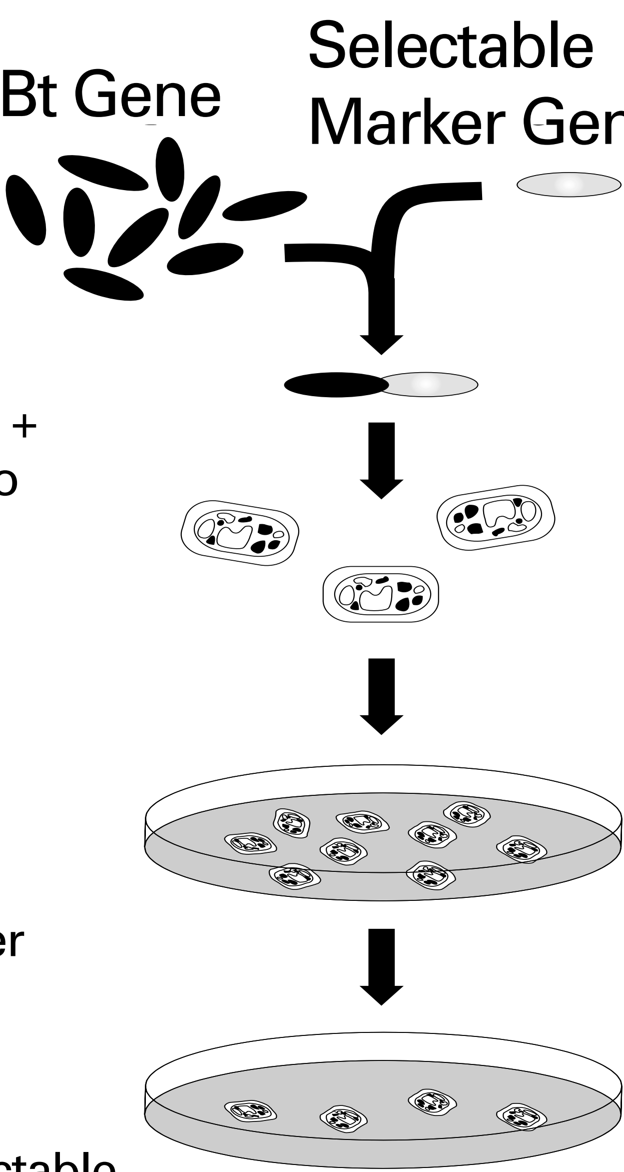
Cry Proteins

Different types of Cry proteins, also known as Bt genes, affect different insects.

Cry gene designation	Toxic to these insect orders
CryIA(a), CryIA(b), CryIA(c)	Lepidoptera moths, like European corn borers, and butterflies
Cry1B, Cry1C, Cry1D	Lepidoptera
CryII	Lepidoptera Diptera flies, including mosquitoes
CryIII	Coleoptera beetles, like Colorado potato beetle and corn rootworm
CryIV	Diptera
CryV	Lepidoptera, Coleoptera

[1] Chart text adapted from *Transgenic Crops: An Introduction and Resource Guide*. ©Center for Life Sciences and Department of Soil and Crop Sciences at Colorado State University, 1999-2001. All rights reserved. http://www.colostate.edu/programs/lifesciences/TransgenicCrops/. Used with permission.

Bt Technology



1. Bt gene that produces the desired lethal protein is joined to a selectable marker gene.

2. Selectable marker gene + Bt gene are inserted into plant cells during transformation.

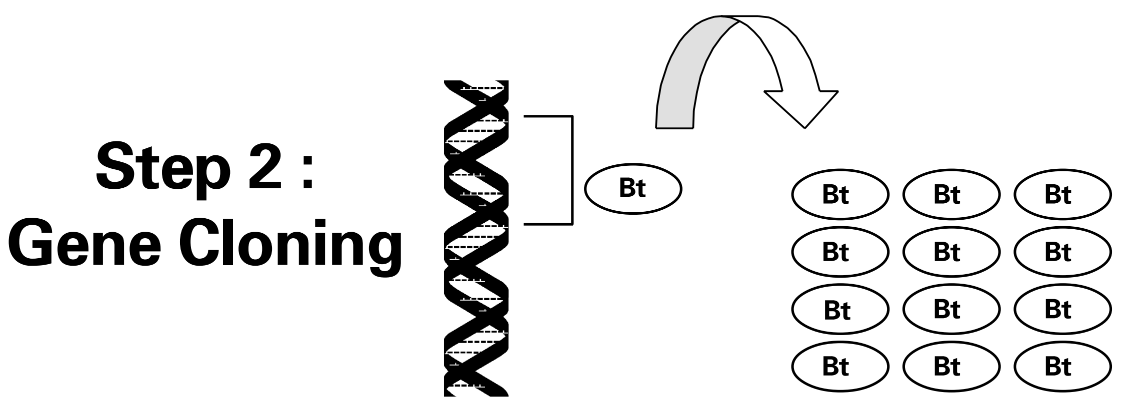
3. Plant cells are grown in the presence of a chemical that selects for the selectable marker gene.

4. Cells that carry the selectable marker gene + Bt gene survive and are grown into plants.

A Closer Look at Bt Genetic Engineering

All of the DNA from a sample of *Bacillus thuringiensis* bacteria is extracted at once, including the Bt gene.

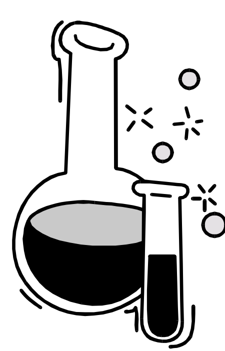
A Closer Look at Bt Genetic Engineering



- DNA of the Bt gene is separated from the DNA of all the other genes in the Bt bacterium.
- Thousands of exact copies of the Bt gene are produced by cloning.

A Closer Look at Bt Genetic Engineering

Step 3 : Gene Design

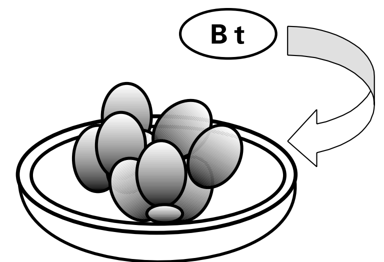


- In a test tube, enzymes are used to replace certain parts of the gene with other DNA that is needed to make the gene work properly in a specific crop.

- The DNA of a selectable marker gene for either antibiotic or herbicide resistance is fused to the DNA of a Bt gene.

A Closer Look at Bt Genetic Engineering

Step 4 : Transformation

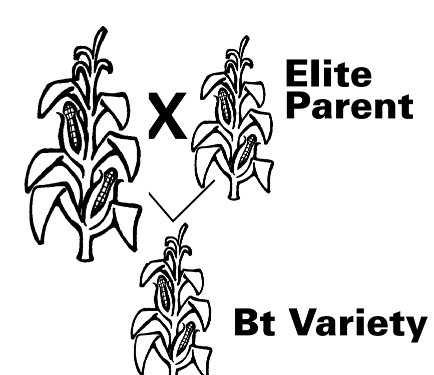


- Bt The modified Bt gene is inserted into plant cells.

- Bt The plant cells are grown in a medium containing an antibiotic or herbicide. Cells that survive have antibiotic or herbicide resistance controlled by the selectable marker gene and the attached Bt gene.

A Closer Look at Bt Genetic Engineering

Step 5 : Variety Development



- Cells with the Bt gene are grown into mature transgenic plants that produce seed.

- Plant breeders cross the transgenic plants with elite parents that have desired traits, such as high yield.

- Elite offspring from the cross that have the Bt gene and other desirable traits are selected as varieties for commercial production.