

Transgenic Plants: How are they produced? How do they protein synthesis to make new protein? How can they silence genes?



Introduction

Gene technology has been used to create recombinant DNA for many years. The process of inserting genes from one organism into another was first discovered in bacteria, but has also been used successfully to produce plants and animals with new characteristics. These plants and animals are called transgenic. The genes inserted into transgenic plants have produced improved varieties that give higher yield, grow better under adverse conditions, resist pesticides, or produce their own insecticide. The original work in this area involved inserting a new gene into the plant's DNA that would produce a new protein, using the process of protein synthesis inside the cell. Now scientists have developed processes for actually turning off the protein synthesis process so that a gene coding for an undesirable characteristic does not produce its protein. This technique is called gene silencing. Gene silencing has saved the papaya from a viral disease and has been useful in producing a variety of soybeans that contain healthier, more durable oil.



Grades: 10-12

Time Needed: Six, 55-min class periods, or seven days if Internet research is done in class

Learning Objectives:

After completing this lesson, students will be able to:

1. Describe the processes of transcription and translation
2. Sequence the steps involved in protein synthesis after having modeled the process in class
3. Explain how one type of gene silencing turns off the protein synthesis process after transcription
4. Compare and contrast the process of protein synthesis with and without gene silencing
5. Research ways in which gene silencing has produced plants or animals with improved characteristics and predict how this technique could benefit humans in the future
6. Predict possible future uses for gene silencing through transgenic techniques in plants and humans

Next Generation Science Standards (NGSS)

As a result of activities in grades 10-12, all students should develop:

Topics

- **LS1:** Structure & Function
- **LS3:** Inheritance & Variation
- **LS4:** Natural Selection

Performance Expectations

- **HS-LS1-1:** Construct an explanation based on evidence for how the structure of DNA determine the structure of proteins which carry out the essential functions of life through systems of specialized cells
- **HS-LS4-2:** Construct an explanation based on evidence that the process of evolution primarily results from four factors: (1) the potential for a species to increase in number, (2) the heritable genetic variation of individuals in a species due to mutation and sexual reproduction, (3) competition for limited resources, and (4) the proliferation of those organisms that are better able to survive and reproduce in the environment
- **HS-LS3-2:** Make and defend a claim based on evidence that inheritable genetic variations may result from: (1) new genetic combinations through meiosis, (2) viable errors occurring during replication, and/or (3) mutations caused by environmental factors
- **HS-LS3-1:** Ask questions to clarify relationships about the role of DNA and chromosomes in coding the instructions for characteristic traits passed from parents to offspring.



Materials:

- Protein synthesis class model activity and follow-up matching, sequencing and quiz
- Transgenic plants Power Point, research sheet, and pre and post quiz
- Protein synthesis with gene silencing model activity and post quiz
- Papaya reading and soybean reading with discussion questions

Instructional Process

Day 1

1. Model of protein synthesis full class activity
2. Matching activity as a follow-up check for understanding
3. Protein synthesis sequencing activity using card sorting

Day 2

1. Quiz on protein synthesis.
2. Give pretest on transgenic plant production. Ask students to write down what they know before lesson - they can also use this sheet to take notes on what they don't know during. Power Point.
3. Show Power Point "How transgenic plants are produced."
4. HOMEWORK: Assign research student activity "Describe one transgenic plant." If this is to be done in class, allow one additional day.

Day 4

1. Retake transgenic plant quiz.
2. Share what students have found out about transgenic plants – display their work around the classroom.
3. Discuss concept of gene silencing and why we may want to do this (potato, tomato).
4. Show Power Point on gene silencing.
5. Show animation on RNAi gene silencing.

Day 5

1. Conduct classroom model of protein synthesis with gene silencing.
2. Give short quiz to check for understanding.
3. Discuss how gene technology could be used to silence a gene whose product is detrimental to the plant.

Day 6

1. Read about how gene technology initiated gene silencing and saved the papaya and has produced soybeans with a healthier kind of oil.
2. Discuss possible uses of this technique in the future for plants and for humans.

Next Generation Science Standards (NGSS)

Dimension

Practices:

- Developing and using models
- Obtaining, evaluating & communicating information

Disciplinary Core Ideas:

- LS3.A Inheritance of Traits
- LS3.B Variation of Traits
- LS4.C Adaptation
- LS1.A Structure & Function

Cross-Cutting Concepts:

- Structure & Function
- Cause & Effect



Sources

SITE YOUR SOURCES – websites, images, resources, books, etc

Sources for transgenic plants:

- <http://www.scq.ubc.ca/your-guide-to-plant-cell-culture/>
- <http://www.scq.ubc.ca/transgenic-crops-how-genetics-is-providing-new-ways-to-envision-agriculture/>
- <http://passel.unl.edu/pages/informationmodule.php?idinformationmodule=958077244&topicorder=3&maxto=7>
- <http://cls.casa.colostate.edu/transgeniccrops/how.html>
- <http://filebox.vt.edu/cals/cses/chagedor/crops.html>

Sources for gene silencing

- http://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=5&ved=0CGAQFjAE&url=http%3A%2F%2Fusers.rcn.com%2Fjkimball.ma.ultranet%2FBiologyPages%2FA%2FAntisenseRNA.html&ei=X60eUM-zFuroiwLEt4GACw&usg=AFQjCNFKNs_eqwqwsX8iEyygHVdX0HRzCg
- <http://learn.genetics.utah.edu/content/tech/genetherapy/gtapproaches/>
- http://www.odec.ca/projects/2004/mcgo4s0/public_html/t3/rna.html#RNA

RNAi animation:

- <http://blogs.nature.com/news/2011/12/video-animation-rna-interference.html>

Image and graphics citations are included in the notes section of Power Points.

Gene silencing and the papaya

- <http://www2.hawaii.edu/~doisteph/Papaya/rainbow.html>
- http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-41582005000400004

Protein Synthesis Roll-Play and Race



Introduction

In this activity members of a typical science class (at least 28 students) will be used to model the process of protein synthesis. Members of the class will role-play the activity of the various molecules involved in the processes of transcription and translation. They will construct a mini-protein made of about 30 amino acids and the process will be timed. Then a second gene will be used and the class will have an opportunity to improve their protein synthesis time to produce a second mini-protein.

Materials:

- Stopwatch
- Sealed envelopes containing DNA sequence for gene one and two
- 20 small jars labeled with the name of amino acids
- 20 sets of different colored, shaped, designed beads to represent different amino acids
- 1 jar labeled Stop Codon containing small cards that say “terminate” protein synthesis
- Large signs to designate areas of the room nucleus, cytoplasm, ribosome
- Index cards for transcribing DNA into codons of RNA (must be hole punched on each end)
- Index cards for anticodons
- String for stringing amino acid beads into mini-protein
- Smaller signs with sting attached for students representing molecules DNA, mRNA, RNA polymerase, ribosome, 20 tRNAs
- Twist-ties to hold codons of mRNA together into a single stranded chain
- Chart showing how tRNA anticodon translates into protein

**** Templates for signs to be worn by role players AND labels for classroom areas are attached separately****



Procedure:

Teacher's role

1. Assign students to roles
2. Distribute signs and materials to students
 - a. DNA needs sign and gene envelopes
 - b. mRNA needs sign, pen and pile of double punched index cards
 - c. RNA polymerase needs sign, and pile of twist ties
 - d. Ribosome and Ribosomal RNA need signs and string
 - e. tRNAs need sign and individual anticodon card and a copy of the tRNA to amino acid chart
3. Label areas of classroom (nucleus, cytoplasm, ribosome)
4. Set out jars containing beads and labeled with amino acid name around the classroom
5. Make sure each student understands his/her role
6. Tell class when to begin and make sure all tRNAs have their amino acid bead and mRNA and RNA polymerase have completed their single strand of chain before ribosome team can begin translation.

DNA role (one student):

1. Maintain envelopes containing genes
2. Open envelope one containing DNA sequence for first gene
3. Give sequence to mRNA for transcription onto index cards

mRNA role (one student):

1. Transcribe DNA code into codons and writes the codon onto an index card
2. Hands codon to RNA polymerase one at a time
3. Bring completed single strand or mRNA codons to ribosome

RNA polymerase role (one student):

1. Assemble a single stranded chain of RNA codons
2. Use twist ties to connect RNA codons together

Ribosome and ribosomal RNA roles (two students):

1. Detach codons one at a time from single stranded mRNA
2. Make a knot in the string when the start codon (methionine) is detached
3. Detach and holds up each succeeding mRNA codon so tRNAs can see it
4. Wait until tRNA brings amino acid
5. Check to see that anticodon card held by tRNA matches with codon
6. Add bead received from tRNA with matching codon to string of forming protein
7. Make a knot at the end of the string when the Stop Codon is received



Role of all tRNAs (21 students)

1. Use tRNA to amino acid code chart to figure out which amino acid to carry.
2. Obtain one bead representing appropriate amino acid from labeled jar and holds it.
3. Watch ribosome until codon that matches anticodon card is held up by ribosome.
4. Deliver appropriate amino acid bead to ribosomal RNA and show anticodon card to prove it matches codon card held by ribosomal RNA.
5. Immediately get another appropriate amino acid bead from jar and again watches ribosome for matching codon.

Role of timers (2 students)

1. One student uses stopwatch to time process
2. Other student says “start” and “stop” at beginning and end of process, and announces or writes on the board the time intervals as process proceeds.

GENES TO USE FOR SYNTHESIS (PLACE EACH GENE INSIDE A SEALED ENVELOPE)

Gene one:

TAC CTA TCT TTT TAT TGA ACG GGA ATA CGT AGC GAG AAG GAG ATA CAG CTT
GTG CGT TAT TGA AAG CTA ATA AGC TTG TTT ACC TGA CAG GTC ATT

Gene two:

TAC TAT TGA ACG CGT TTT GAG TGA CTA TCT AGC CAG GTC TTT GTG GTC TAT
TTG TTT GGA ATA GAG AAG GAG CTA ACC AGC CUU CAG CGT AAG ATT

Gene three:

TAC CGT ATA AGC CGT TGA AAG TTT GTC TAT ATA AGC TT T GAG TGA GAG
AAG ACG CGT TAT TGA CTA GAG TTT ATA CAG CGT CAG GTC CTA TCT ATT

Gene four:

TAC GGA AGC GTC ACC GAG ATA TTG AAG CGT TTT CTT GTG CTA ACG CAG TAT
TGA TCT CCT TTT GGA CGT TAT CTA ATA CAG TGA TTG AAG ATT



tRNA Anticodon to Amino Acid Chart

(Use this chart to determine what amino acid a tRNA molecule will carry with it for protein synthesis)

First Position ↓	Second Position U	C	A	G	Third Position ↓
U	lys lys asn asn	arg arg arg arg	ileu met ileu ileu	thr thr thr thr	U C A G
C	glu glu asp asp	gly gly gly gly	val val val val	ala ala ala ala	U C A G
A	Stop Stop tyr tyr	Stop trp cys cys	leu leu phe phe	ser ser ser ser	U C A G
G	gln gln hist hist	arg arg arg arg	leu leu leu leu	pro pro pro pro	U C A G

*** Note this is not the same chart that has been used to go from mRNA to the amino acid that is found in many texts and on the Internet. ***

List of 20 tRNA anticodons (Write these on index cards and hand them out to 21 role-players.)

- | | | | |
|------------|------------|------------|------------|
| UAC | GGA | AUA | GUG |
| CUA | AGC | UUG | ACG |
| UCU | GUC | AAG | CAG |
| UUU | ACC | CGU | UGA |
| UAU | GAG | CUU | CCU |

You will also need the tRNA anticodon for the Stop **AUU**



tRNA role players will use these anticodons and the preceding and following chart to figure out what amino acid they should be carrying. They should also use these anticodons to figure out what codon to watch for the ribosome to hold up.

Amino Acid Abbreviations Table

Alanine	ala
Arginine	arg
Asparagine	asn
Aspartic Acid	asp
Cysteine	ays
Glutamine	gln
Glutamic Acid	glu
Glycine	gly
Histidine	hist
Isoleucine	ileu
Leucine	leu
Lysine	lys
Methionine	met
Phenylalanine	phe
Proline	pro
Serine	ser
Threonine	thr
Tryptophan	trp
Tyrosine	tyr
Valine	val

Follow-up for Protein Synthesis Model



Matching Exercise

Demonstrate your understanding of protein synthesis by matching the events that occurred during the class protein synthesis model activity with the events that occur inside the cell during protein synthesis.

Model activities:

- ___ 1. Opening of the gene envelope
- ___ 2. Transfer code inside envelope to slightly different code
- ___ 3. Write the modified code on index card in groups of three letters
- ___ 4. Assemble cards into chain held together by twist ties
- ___ 5. Tie original knot in string
- ___ 6. Each tRNA receives a card with three letter anticodon
- ___ 7. Each tRNA gets a different color, shape or pattern bead
- ___ 8. A bead is delivered to the ribosome
- ___ 9. tRNA brings bead to ribosome and bead is accepted or rejected
- ___ 10. Beads are added to the string one by one
- ___ 11. The tRNA gets another bead after giving one to the ribosome
- ___ 12. A knot is tied on the end of the string
- ___ 13. The completed bead string is brought up to the teacher

Actual cellular activities

- A. Amino acids attached together one at a time to assemble a protein
- B. The DNA is unzipped and uncoiled before it can be read
- C. Each different tRNA molecule holds one and only one specific amino acid
- D. A start codon (coding for methionine) is fed into the ribosome to begin the protein synthesis process
- E. The anticodon and codon must match for the ribosome to accept the amino acid carried by the tRNA
- F. RNA polymerase brings complimentary RNA nucleotides to assemble a single strand of mRNA
- G. The DNA code is transcribed into mRNA code
- H. Three mRNA bases constitute a codon
- I. The completed protein is exported from the cell
- J. Since tRNA can only hold one amino acid when it gives one to the ribosome, it must replace it with amino acids found in cell cytoplasm
- K. mRNA is fed into ribosome one codon at a time
- L. A stop codon fed into ribosome stops the synthesis process.
- M. Each different tRNA molecule contains specific group of 3 bases called an anticodon

Key for Matching Answers;

1. B 2. G 3. H 4. F 5. D 6. M 7. C 8. K 9. E 10. A 11. J 12. L 13. I



Quiz on Protein Synthesis

1. The instructions for making your proteins are stored in a huge twisted molecule called _____. This molecule must always remain inside of the _____. This molecule contains about 3 billion pairs of _____ used to store information. It must first _____ and _____ so its information can be read.
2. Sections of bases long enough to code for a protein are called _____. Each of these sections can actually code for as many as _____ proteins.
3. Another nucleic acid is needed to facilitate the manufacture of these proteins. The other nucleic acid goes by the three letter name of _____. There are actually _____ different forms of this nucleic acid: _____, _____ and _____. All these molecules are _____ stranded. They are made of sub-units called _____. These sub-units contain the sugar _____, and the base _____ instead of _____.
4. The first step in protein synthesis is called _____. During this step the code is transferred from the bases of the molecule inside the nucleus to the bases of _____. After this molecule is completed it leaves the _____ and journeys out into the _____ of the cell.
5. When it reaches the protein manufacturing center, a tiny _____, it feeds its bases into the manufacturing center _____ bases at a time. This section of 3 bases is called a _____.
6. Then the second step in protein synthesis begins. This step is called _____. During this step small nucleic acid molecules, called _____, found in the cytoplasm of your cells are pulled into action. These molecules are made of only three _____. The sequence of bases here is called an _____. Each of these molecules carries with it an _____.
7. If the 3 bases of this molecule match the 3 bases held up at the manufacturing center, a temporary bond is created between the _____ molecule and the _____ molecule. When this happens an _____ is dropped off and is hooked to a chain of similar molecules. Eventually the chain is long enough to be called a polypeptide or a _____. The manufacturing is over when a _____ codon is reached. The long chain drops off and coils up into its appropriate structure.

Word bank (may be used more than once)

DNA, anti-codon, genes, stop, mRNA, thymine, amino acid, protein, tRNA, nucleotides, uncoiled, transcription, single, nucleus, three, four, ribosome, rRNA, uracil, bases, RNA, untwisted, translation, cytoplasm, ribose



Cards to be used for Protein Synthesis Sequencing Activity

Student groups should cut out the cards, arrange them in order and then number the back of the card

<p>The DNA molecule in the nucleus unwinds somewhere in the middle of the molecule.</p>	<p>The mRNA feeds into the ribosome three bases at a time. These three bases are called a codon.</p>	<p>When a stop codon is reached, the protein synthesis stops the polypeptide and the mRNA break away from the ribosome.</p>
<p>When the mRNA molecule is complete, it breaks away from the DNA and leaves the nucleus.</p>	<p>As the codon become visible a tRNA with the matching anti-codon attaches to the mRNA with its specific amino acid.</p>	<p>Messenger RNA nucleotides attach themselves to the exposed DNA bases, forming a half ladder.</p>
<p>When two tRNA molecules are attached to the mRNA inside the ribosome the amino acids they are joined together by a peptide bond.</p>	<p>The mRNA goes to the ribosome and attaches to it.</p>	<p>The DNA molecule unzips to expose a number of bases that comprises one gene.</p>
<p>The DNA molecule rejoins and coils back up inside the nucleus.</p>	<p>One of the two tRNA molecules in the ribosome detaches from the amino acid and the mRNA, and goes out to look for another amino acid.</p>	<p>The mRNA molecule may go to another ribosome and cause another protein to be produced. After a few times it breaks down and cannot make any more proteins.</p>



Transgenic plant research requirements:

1. Use the Internet to find out information about how plant scientists have been able to add genes into the DNA of a particular plant and produce a variety of that plant that has improved characteristics for improving yield, resisting pesticides, producing an insecticide, or growing in adverse conditions, such as a drought.
2. Make sure the sources you use for your research are credible and authoritative. (Use scientific journals, university sites, etc., not Wikipedia)
3. Be prepared to share the following information with the class:
 - a.) Name of Plant
 - b.) Improved characteristic after insertion of new gene
 - c.) When and where this work was done – is it complete
 - d.) Picture of plant
 - e.) Citations used for research
 - f.) Has food from this plant been approved for consumption in the U.S.?
4. Also be prepared to display your work for other classes to see.
5. Plant list could be used to assign plants to students so there would be no duplication of effort.

The list of transgenic plants should include:

corn, cotton, rice, potato, papaya, soybeans, squash, flax, chicory, tobacco, peas, sugar beets, tomatoes, rapeseed, peanut, alfalfa, eggplant, wheat, barley, Flowers: Gladiolus, Petunia, Chrysanthemum, sunflower, trees: Poplar, Spruce, Cottonwood, Sweetgum, grasses: bluegrass, creeping bent grass, Fruits and vegetables: sugarcane, apple, cranberry, grape, plum, raspberry, strawberry, watermelon, broccoli, carrot, lettuce, and pepper.

Transgenic plants Power Point included separately

How to make transgenic plant pre-test and final quiz



1. Describe the basic genetic principles involving DNA genes, and protein synthesis that makes genetic engineering possible.

2. The process for creating transgenic plants has 5 steps. Describe in your own words what happens at each step.

Step 1: DNA Extraction using restriction enzymes

Step 2: Gene Cloning using bacteria vectors

Step 3: Gene Design incorporating marking and regulation into transgene

Step 4: Transformation using gene guns and tissue culture

Step 5: Backcross Breeding to produce hardier varieties of GMs



Gene silencing pretest and post-test

1. Why would we want to turn off a gene?
2. Is RNA always found in the single stranded form?
3. What happens when an antisense strand of RNA comes in contact with the sense strand of RNA?
4. How does the antisense method stop a gene from being expressed?
5. What kind of molecules are Dicer and Argonaute?
6. Fill in the equations:
Double stranded RNA + _____ = siRNA
siRNA + Argonaute = _____
RISC + mRNA = no _____ synthesis
7. List three plants that have been modified by gene silencing techniques

Gene silencing Power Point including modeling instructions and link to interference animation found separately



Introduction to Webquest: How gene silencing saved the papaya

The papaya has been an economically important crop in Hawaii since its introduction there in 1910. However, by 1997, the papaya ring spot virus had almost destroyed the papaya industry in Hawaii.

Researchers at Cornell University had been working on genetically engineering a papaya that would resist this virus since 1984. The genetically modified (GM) papaya strain they were working on was called Rainbow Papaya. Their work was thoroughly reviewed by the USDA. In 1997 the government gave permission to 200 farmers in Hawaii to plant the GM Rainbow Papaya. The transgenic papaya grew successfully and resisted the virus. The papaya industry was saved.

The transgenic papaya produced by the Cornell researchers had a gene from the ring spot virus implanted into its DNA. The implanted gene coded for the protein coat of the virus. Evidence suggests that the transgene conferred PRSV (papaya ring spot virus) resistance because it utilizes post-transcriptional gene silencing or PTGS. Researchers have actually found the siRNAs and dsRNA used in this process when they analyzed the genome of the transgenic papaya.



Papaya fruit with ring spot virus infection
freshplaza.com



Transgenic papaya without ring spot
bio349.biota.utoronto.ca

References:

<http://www2.hawaii.edu/~doisteph/Papaya/rainbow.html>

http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0100-41582005000400004

ddr.nal.usda.gov/bitstream/10113/32809/1/IND44045692.pdf

hortsci.ashspublications.org/content/40/7/2083.full.pdf



Webquest

Step One: View the first segment (1 of 12) and second segment (2 of 12) from Harvest of Fear on the Watch Know Learn website <http://watchknowlearn.org/Video.aspx?VideoID=38182&CategoryID=11628>

Answer the following questions:

First segment questions:

1. What are the symptoms of a ring spot virus infection on the papaya?
2. What methods were used (prior to genetic engineering) to stop the virus? Were they effective?
3. Who were the two scientists from Cornell University that used genetic engineering to save the papaya?
4. What was their original idea about how to protect the papaya from the virus?
5. What viral gene did they plan to incorporate into the papaya's DNA?
6. What is a gene gun and how does it work?

Second segment questions:

1. How did the researchers test the effectiveness of the transgene?
2. What were the results of these tests?
3. Give one example of some other genetic engineering work on plants being done at Cornell at this time.

Step Two: Read the information on the following website and answer the questions that follow:

<http://www.hawaiipapaya.com/rainbow.html>

1. What does it mean to say "rainbow papaya is an F 1 hybrid"?
2. When was rainbow papaya commercialized? What effect did planting this variety have on the papaya industry in Hawaii?
3. What percent of the papaya crop in Hawaii was rainbow papaya in 2009?
4. When was rainbow papaya scheduled to be exported to Japan?
5. What evidence is given to support the fact that rainbow papaya is environmentally safe?

Step Three: Read the information on the following website and answer the questions that follow:

<http://www.agbioforum.org/v7n12/v7n12a07-gonsalves.htm>

1. What can you infer from looking at the pictures in figures 1-5?
2. Look at the data in **Table 1**. Papaya production in Hawaii, 1992-2001. Then explain why papaya production declined from 1992 -1997 and why it rose from 1998 – 2001.
3. At the time this article was written, what other countries were interested in developing PRSV-resistant transgenic papaya for their countries?
4. Think about why they would need to develop a different transgenic papaya and could not just plant rainbow papaya.



Step Four: Read the information on the following website and answer the questions that follow:

<http://www.biofortified.org/2012/06/rainbow/>

1. This article says that the transgenic papaya contains “A gene from the ring spot virus (that) was inserted into the papaya, where it acts like a built-in vaccine against the virus.” How does this statement compare to what you know about the gene silencing technique used to give the transgenic papaya protection against the ring spot virus? Do you think the statement a simplification or an untruth?
2. The author of this article describes an interview with Dr. Gonsalves, the researcher who developed rainbow papaya. Give some information about Dr. Gonsalves background.
3. What did Dr. Gonsalves say about transgenic papayas eventually losing their immunity to ring spot virus?
4. What did he say about human consumption of the ring spot virus?
5. What did he say about the long term impact genetically modified papaya will have on humans and the environment?

Step Five: The following website contains a highly technical paper on the gene silencing effect of incorporating a gene for the protein coat of the ring spot virus into the papaya:

<http://www.ueb.cas.cz/en/content/post-transcriptional-gene-silencing-involved-resistance-transgenic-papayas-papaya-ringspot>

Download the PDF file and read the abstract and as much of the article as you feel you can understand.

1. What does the author say about the process that confers resistance to the ring spot virus in the transgenic papaya?
2. What proof does he put forth to support his claim?

Step Six: Read the news release from January of 2012 on the site below:

<http://www.usda.gov/wps/portal/usda/usdahome?contentid=2012/01/0005.xml&contentidonly=true>

1. What announcement is made in this news release?
2. Why is it good news for Hawaii and for genetic engineering?