

## Genetic Engineering Introduction

- Recombinant DNA Technology
  - Very basic, will cover only what I think is necessary for this course:
- Gene Cloning
  - Vectors, Enzymes
- Gene Structure
  - Coding sequence and control elements
- Transformation Procedures
- Germplasm Selection
- Regulatory Issues
- Breeding Issues
- Public Perception Issues

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## Gene Cloning

- Isolation and amplification of an individual gene sequence by insertion of that sequence into a bacterium where it can be replicated
- Involves the construction of novel DNA molecules by joining DNA from different sources
- Product is Recombinant DNA (rDNA)

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## Basic Events in Gene Cloning

- Isolation of gene of interest
- Incorporate gene into a vector (small replicating DNA molecule, usually circular)
- Introduce recombinant vector into host cell via transformation
- Select for the cells that have acquired the recombinant DNA molecule
- Multiply recombinant vector within host cell to produce a number of identical copies of the cloned gene

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## Components of Gene Cloning

- Vectors (cloning vehicles)
- Enzymes for cutting and joining the DNA fragments
- The DNA fragments (libraries)
- Selection process

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## Vector Features

- Must contain a replicon that enables it to replicate in host cells (region of DNA that is amplified, *i.e.*: has origin of replication)
- Several marker genes
- Unique cleavage site(s)
- For expression, must contain control elements, such as promoters, terminators, ribosome binding sites, etc...

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## Types of Vectors

- Plasmids
- Cosmids
- Phages
- Yeast Artificial Chromosomes (YACs)
- Transposons
- Bacterial Artificial Chromosomes (BACs)

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## Plasmids

- Double stranded, circular DNA which exist in bacteria, yeast and organelles
- May exist as single copy per cell or multi-copy per cell (10-20 genomes/cell), or even under relaxed replication control where up to 1000 copies/cell can be maintained
- Replication by enzymes encoded on host chromosomes
- Size of rDNA insertions limited to ~10kb

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## Cosmids

- Plasmid vectors that contain a bacteriophage lamda cos site
- The cos site results in efficient packaging of lamda DNA into virus particles
- So, with the cos site, larger DNA inserts are possible (up to ~40 kb)

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## Bacteriophage Vectors

- Viruses that attack bacteria
- Must first deactivate lysogenic growth component of phage (phage DNA inserts into host DNA, creating prophage)
- Allow lytic growth – cell death after infection and replication. Cell death revealed as plaques
- Insert rDNA into phage (usu. up to 25kb)
- Infect bacteria with phage
- Infected bacteria form plaques
- Advantage: Transformation, selection very easy

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## Yeast Artificial Chromosome

- Artificially produced mini chromosome
  - Centromere, two telomeres, origin of replication, plus marker genes
- Can accommodate very large inserts (~1,000 – 2,000 kb)

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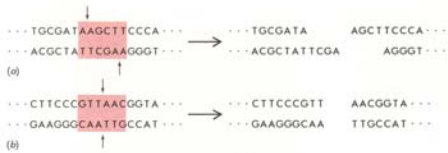
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## Restriction Enzymes

- Recognize a specific DNA sequence (4 to 6 bp) and cut the DNA
- Will make either staggered cuts (leaving “sticky ends”) (a) ↓ or blunt cuts (b) ↓



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## Role of Restriction Enzymes

- Only found in prokaryotes
  - (endonucleases are ubiquitous)
- Over 100 different enzymes have been isolated from bacteria
- Why to bacteria produce these enzymes?
  - Protection from foreign DNA → phages (bacterial viruses)

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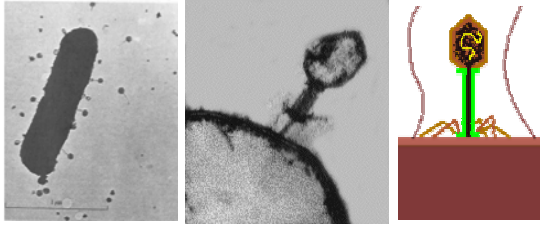
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## Phage Biology

The most common organism in the world



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- How do bacteria prevent restriction enzymes from damaging its own DNA?
  - Methylation, which can also occur in eukaryotic (plant) DNA
  - Methylation may prevent gene expression, and may play a role in tissue specific gene expression (zein and glutelin gene in maize: demethylated in endosperm)
  - Transgene silencing often associated with methylation

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## Ligation

- When sticky ends are created on the vector and the rDNA, the ends are compatible and complementary
- Can be added as linkers or adapters
- DNA Ligase
  - Seals single stranded nicks between adjacent nucleotides in a duplex DNA chain (catalyze the formation of a phosphodiester bond between adjacent 3' hydroxyl and 5' phosphate termini in DNA)

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## Sticky Ends Key to Recombinant DNA

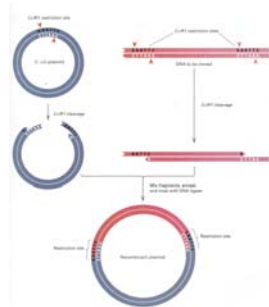


Figure 6.13 Identification of recombinant DNA molecules. A specific restriction enzyme (EcoRI) is used to create sticky ends on both strands of a plasmid vector. The sticky ends of one fragment are then ligated to the sticky ends of another fragment. The recombinant DNA is then identified by the presence of a specific DNA sequence. The sticky ends are then ligated together by the action of DNA ligase. The recombinant DNA is then identified by the presence of a specific DNA sequence. (Copyright 2004 Pearson Education, Inc. All rights reserved.)

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## Cloning DNA

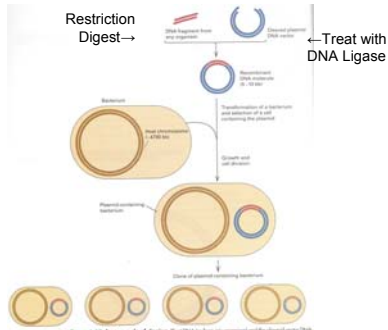


Figure 6.14 An example of cloning. Plasmid DNA is first digested and the pieces of vector DNA are joined by the same type of restriction enzyme and sealed together by DNA ligase. Clones (living cells) are then grown and selected for recombinant plasmids. The plasmid is replicated and the genes are expressed. (Copyright 2004 Pearson Education, Inc. All rights reserved.)

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## Flow of Genetic Information

**DNA** ↔ **RNA** → **Protein**  
Reverse Transcriptase

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## Transcriptional Control Sequences

- Promoter Element
  - Site for initial RNA polymerase binding
  - Plants and animals: TATA-Box required at about 25 bp upstream of start codon
- Enhancer Sequences
  - Usually located a few hundred bp upstream
  - Stimulate the expression of genes
- Transcription Factors
  - Mediate the binding of RNA polymerase

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## Reading Assignment

- Michael Schroda, Christoph F. Beck and Olivier Vallon. 2002. Sequence elements within an HSP70 promoter counteract transcriptional transgene silencing in *Chlamydomonas*. *The Plant Journal* 31(4): 445-455.

Next:

- Gene Transformation Methods

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## Genetic Transformation

- The directed desirable transfer of foreign DNA into an organism and the stable integration and expression of the foreign DNA in the genome
- Transgene: the transferred DNA sequence
- Transgenic: the organism that develops after successful DNA transfer

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## Transient & Stable Gene Expression

- **Transient Gene Expression**
  - Transferred DNA is only expressed for a short period of time
  - Only a small amount of transferred DNA introduced into cells is stably integrated into the chromosome of the cell
  - Most is lost with time and cell division
- **Stable Gene Expression**
  - DNA is integrated into plant nuclear or plastid genome, expression occurs in regenerated plants, and inherited in subsequent generations

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## Marker Genes

- Easy assay; no DNA extraction
- **Reporter Genes**
  - Usually based on protein quantification
  - Detection with high sensitivity
  - Low endogenous background
  - Should have quantifiable assay
  - Assay should be non-destructive
  - Assay should be cheap and easy
  - e.g.: GUS, GFP, others
- **Selectable Marker Genes**
  - Antibiotic Resistance Genes (*NPT-II*)
  - Herbicide Resistance/Tolerance Genes
  - Antimetabolite Markers (enzyme blockers with altered enzyme gene; e.g.: *dhfr*)

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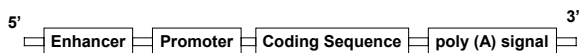
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## Chimeric Gene Construct (Transgene)



Plant Viral promoters  
rely on transcription  
and translation factors  
already present in plants

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## Gene Transfer Methods

- Vector Mediated Gene Transfer
  - Agrobacterium mediated transformation
  - Viral mediated transformation
- Vectorless or Direct DNA Transfer
  - Electroporation
  - Particle Bombardment
  - Microinjection
  - DNA transfer via pollen

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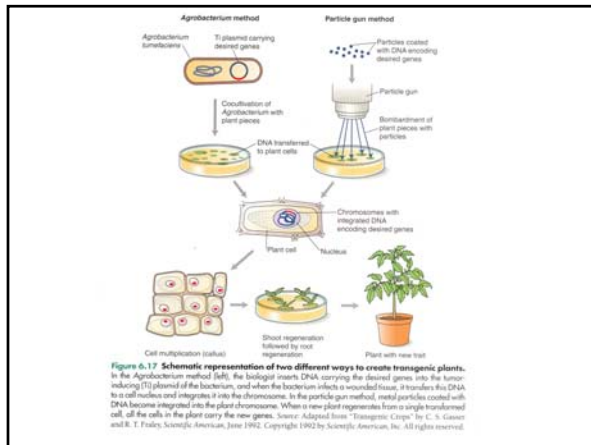
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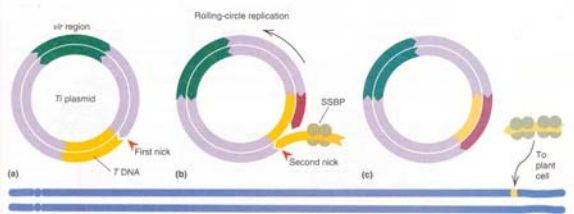
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## T-DNA on the Ti Plasmid in Agrobacteria is Transferred to Plants



**Figure 6.16 Genetic transformation of a plant genome by T-DNA from the Ti plasmid.** (a) A nick forms at one end of the T-DNA after the bacteria have attached to a wound site on the plant. (b) Replication elongates one end and displaces the other end where the first nick was formed. The single-stranded binding protein (SSBP) that is coded by one of the vir genes then stabilizes this displaced piece of single-stranded DNA. A second nick terminates replication. (c) The single-stranded DNA that is coated with the SSBP is transferred into a plant cell and integrates into the plant genome. Source: D. Hartl and E. W. Jones (1998), *Genetics: Principles and Analysis*, 4th ed. (Boston: Jones and Bartlett), p. 382.

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## Direct Gene Transfer

- Usually creates multiple copies of the insert
- These copies are usually tightly linked
  - Cannot be separated by crossing
- Tightly linked multiple copies will segregate 3:1, just like a single insert
- May have two or more sets of tightly linked inserts that will segregate like two or more genes

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## Problems with Multiple Copies

- Difficult to breed with
- Difficult to track phenotype with markers
- Regulatory Concerns/Explanations
- Gene Silencing

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## Causes of Gene Silencing

- Documented causes of gene silencing
  - Homology dependant gene silencing
    - Hypermethylation due to multiple copies of homologous genes
  - Suppression by antisense genes
    - Antisense RNA may block expression by inhibition of transcription or translation
  - Position effect
    - Integration into suppressive environment such as hypermethylation region, heterochromatin region, etc...
- May occur after one to four or five generations
- Appears to be more frequent in polyploid species (Wheat)

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## Sometime have Deleterious Effects

- Are these caused by transgene expression or insertional effects?
  - Frequency varies by method and species
    - Agrobacterium mediated transformation at Seminis:
      - » Lettuce = ~48%
      - » Tomato = ~25%

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