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

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)

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
Components of Gene Cloning

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

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...

Types of Vectors

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

Cosmids

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

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
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)

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) ↓

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)
Phage Biology

The most common organism in the world

• 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

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)
Sticky Ends Key to Recombinant DNA

Cloning DNA

Flow of Genetic Information

DNA → RNA → Protein

Reverse Transcriptase
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

Reading Assignment
Next:
- Gene Transformation Methods

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
**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

**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)

**Chimeric Gene Construct (Transgene)**

- 5' Enhancer → Promoter → Coding Sequence → poly (A) signal → 3'

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

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

Figure 6.14: Gene transfer system of a plant genome by T-DNA from the Ti plasmid.
(a) A nick forms at one end of the T-DNA; after the boundary has dissociated to a wound site on the plant, the T-DNA replicates divergently and integrates the additonal regions of single-stranded T-DNA. A second nick forms after recombination. (b) The single-stranded DNA then interacts with the cell and integrates into the plant genome.
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

Problems with Multiple Copies

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

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)
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%