Cloning in yeast and other fungi,
Gene manipulation of animals and plants,
Analysis of transcriptome,
Expression analysis of protein

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Cloning in yeast and other fungi

• Like Escherichia coli, fungi are not naturally transformable and artificial means have to be used for introducing foreign DNA.
  – One method involves the use of spheroplasts (i.e. wall-less cells).
  – Electroporation provides a simpler and more convenient alternative to the use of spheroplasts.
  – DNA can also be introduced into yeasts and filamentous fungi by conjugation.

• Enterobacterial plasmids, such as R751 (IncPβ) and F (IncF), could facilitate plasmid transfer from E. coli to S. cerevisiae and Schizosaccharomyces pombe.
Restriction Enzyme Mediated Integration (REMI)

• Schiestl and Petes (1991) developed a method for forcing illegitimate recombination by transforming yeast with BamH1-generated fragments in the presence of the BamH1 enzyme.

• Not only did this increase the frequency of transformants but the transformants which were obtained had the exogenous DNA integrated into genomic BamH1 sites.

• This technique, which is sometimes referred to as restriction enzyme mediated integration (REMI), has been extended to other fungi, such as Cochliobolus, Ustilago and Aspergillus.
TRANSFORMATION OF COMPETENT YEAST PREPARED WITH LITHIUM ACETATE

- Grow yeast cells to early log phase, i.e. to 5 to 7 $\times 10^6$ cells/ml in YPD or to 1 to 2 $\times 10^6$ cells/ml in Synthetic Medium.
- Centrifuge cells in a tabletop centrifuge at 2,000 X g for 5 min.
- Decant supernatant and resuspend cells in ddH2O.
- Centrifuge cells in a tabletop centrifuge at 2,000 X g for 5 min.
- Decant Supernatant and resuspend cells in 1X Lithium Acetate at 5 $\times 10^8$ cells/ml.
- Incubate at 30°C on a roller drum for 1 to 24 hr (usually 1 to 2 hr is sufficient)
- For each transformation, in a sterile microcentrifuge tube, add:
  - 100 µl of cells (5 $\times 10^7$ cells)
  - 40 µg of Carrier DNA
  - 10 µl or less of transforming DNA (0.1 to 5 µg) in TE Buffer
  - Also set up a negative control tube lacking transforming DNA
- Incubate at 30°C for 30 min.
- Add 0.7 ml of LiPEG and invert several times to mix.
- Incubate at 30°C for 60 min (longer is acceptable).
- Incubate in a 42°C water bath for 5 min.
- Plate about 200 to 250 µl per plate of the PEG/cells mixture directly onto selective medium (SC Plates).
Major Organisms

- *Saccharomyces cerevisiae* and
- the two filamentous fungi
  - *Aspergillus nidulans*, and
  - *Neurospora crassa*
Four techniques suitable for the genetic transformation of filamentous fungi

• Treatment of protoplasts with polyethylene glycol,

• Electroporation,

• Biolistic methods

• *Agrobacterium tumefaciens*. 
### Table 3
Examples of vectors of filamentous fungi

<table>
<thead>
<tr>
<th>Fungal sub-division</th>
<th>Species</th>
<th>Vector designation</th>
<th>Transformation frequency/µg DNA</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygomycotina</td>
<td>Mucor circinilloides</td>
<td>pMCL1302</td>
<td>600</td>
<td>17</td>
</tr>
<tr>
<td>Ascomycotina</td>
<td>Aspergillus nidulans</td>
<td>pHY201</td>
<td>20–300</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>(Emericella nidulans)</td>
<td>pFB6</td>
<td>1–10</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pDJB2</td>
<td>5000</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Neurospora crassa</td>
<td>pVK88</td>
<td>5–30</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pRAL1</td>
<td>50,000</td>
<td>26</td>
</tr>
<tr>
<td>Basidiomycotina</td>
<td>Ustilago maydis</td>
<td>pMP81</td>
<td>1–10</td>
<td>22</td>
</tr>
<tr>
<td>Deuteromycotina</td>
<td>Acremonium chrysogenum</td>
<td>—</td>
<td>1–2</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>Penicillium chrysogenum</td>
<td>pGB82</td>
<td>1–10</td>
<td>48</td>
</tr>
</tbody>
</table>

*Selection of transformants for each vector are as follows: pMCL1302, leucine independence; pHY201, tryptophan independence; pFB6 and pDJB2, uridine independence; pVK88 and pRAL1, aromatic amino acids independence; pMP81, neomycin resistance; vector for A. chrysogenum, G418 resistance; pGB83, unspecified amino acid independence.*
Yeast episomal plasmids

- YEps were first constructed by Beggs (1978) by recombining an E. coli cloning vector with the naturally occurring yeast 2 µm plasmid.
- This plasmid is 6.3 kb in size, has a copy number of 50–100 per haploid cell and has no known function.
Fig. 9.2 Schematic representation of a typical yeast episomal plasmid (YEp 24). The plasmid can replicate both in *E. coli* (due to the presence of the pBR 322 origin of replication) and in *S. cerevisiae* (due to the presence of the yeast 2 \( \mu \)m origin of replication). The ampicillin and tetracycline determinants are derived from pBR 322 and the URA3 gene from yeast.
Yeast replicating plasmids

- YRps were initially constructed by Struhl et al. (1979).
- They isolated chromosomal fragments of DNA which carry sequences that enable E. coli vectors to replicate in yeast cells.
- Such sequences are known as **ARS (autonomously replicating sequence)**.
- **An ARS is quite different from a centromere**: the former acts as an origin of replication whereas the latter is involved in chromosome segregation.
- Although plasmids containing an ARS transform yeast very efficiently, the resulting transformants are exceedingly unstable.
- For unknown reasons, Yrps tend to remain associated with the mother cell and are not efficiently distributed to the daughter cell. (Note: *S. cerevisiae* does not undergo binary fission but buds off daughter cells instead.)
- Occasional stable transformants are found and these appear to be cases in which the entire YRp has integrated into a homologous region on a chromosome in a manner identical to that of Yips.
Yeast centromere plasmids

• Using a YRp vector, Clarke and Carbon (1980) isolated a number of hybrid plasmids containing DNA segments from around the centromere-linked leu2, cdc10 and pgk loci on chromosome III of yeast.

• As expected for plasmids carrying an ars, most of the recombinants were unstable in yeast. However, one of them was maintained stably through mitosis and meiosis.

• The stability segment was confined to a 1.6 kb region lying between the leu2 and cdc10 loci and its presence on plasmids carrying either of two ars tested resulted in those plasmids behaving like minichromosomes.

• Genetic markers on the minichromosomes acted as linked markers segregating in the first meiotic division as centromere-linked genes and were unlinked to genes on other chromosomes.

• Structurally, plasmid-borne centromere sequences have the same distinctive chromatin structure that occurs in the centromere region of yeast chromosomes (Bloom & Carbon 1982).

• Functionally Ycps exhibit three characteristics of chromosomes in yeast cells.
  – First, they are **mitotically stable** in the absence of selective pressure.
  – Secondly, they **segregate during meiosis** in a Mendelian manner.
  – Finally, they are found **at low copy number** in the host cell.
Yeast integrative plasmids (Ylps)
Disadvantages of using Ylp as a cloning vector:

- **Transformation frequency** - It is the number of transformants that can be obtained per microgram of plasmid DNA. e.g. YEps have the highest transformation frequency, providing between 10,000 and 100,000 transformed cells per µg. YRps are also quite productive, giving between 1000 and 10,000 transformants per µg, but a **Ylp yields less than 1000 transformants per µg.**

- **Copy number** - YEps and YRps have the highest copy numbers: 20–50 and 5–100, respectively. In contrast, a **Ylp is usually present at just one copy per cell.**

- In spite of the above demerits Ylp is preferred as it produces a very stable recombinants, as loss of a Ylp that has become integrated into a chromosome occurs at only a very low frequency. **Ylp is the vector of choice if the needs of the experiment dictate that the recombinant yeast cells must retain the cloned gene for long periods in culture.**
Yeast artificial chromosomes

• All three autonomous plasmid vectors described above are maintained in yeast as circular DNA molecules – even the YCp vectors, which possess yeast centromeres.

• Thus, none of these vectors resembles the normal yeast chromosomes which have a linear structure.

• The ends of all yeast chromosomes, like those of all other linear eukaryotic chromosomes, have unique structures that are called *telomeres*.
Yeast artificial chromosomes

• The key components of a chromosome are:
  – **The centromere**, which is required for the chromosome to be distributed correctly to daughter cells during cell division.
  – **Two telomeres**, the structures at the ends of a chromosome, which are needed in order for the ends to be replicated correctly and which also prevent the chromosome from being nibbled away by exonucleases.
  – **The origins of replication**, which are the positions along the chromosome at which DNA replication initiates, similar to the origin of replication of a plasmid.
Figure: Construction of a yeast artificial chromosome (YAC)
Fig. 9.3 Construction of a yeast artificial chromosome containing large pieces of cloned DNA. Key regions of the pYAC vector are as follows: TEL, yeast telomeres; ARS 1, autonomously replicating sequence; CEN 4, centromere from chromosome 4; URA 3 and TRP 1, yeast marker genes; Amp, ampicillin-resistance determinant of pBR322; ori, origin of replication of pBR322.
Retrovirus-like vectors

• The genome of S. cerevisiae contains **30–40 copies of a 5.9 kb** mobile genetic element called Ty.
• This transposable element shares many structural and functional features with retroviruses and the copia element of Drosophila.
• Ty consists of a central region containing **two long open reading frames (ORFs)** flanked by two identical terminal 334 bp repeats called delta.
• Each delta element contains a promoter as well as sequences recognized by the transposing enzyme.
• New copies of the transposon arise by a replicative process, in which the Ty transcript is converted to a progeny DNA molecule by a Ty-encoded reverse transcriptase. The complementary DNA can transpose to many sites in the host DNA.
Retrovirus-like vectors

• The Ty element has been modified in vitro by replacing its delta promoter sequence with promoters derived from the phosphoglycerate kinase or galactose-utilization genes.

• When such constraints are introduced into yeast on high-copy-number vectors, the Ty element is overexpressed. This results in the formation of large numbers of virus-like particles (VLPs), which accumulate in the cytoplasm.

• The particles, which have a diameter of 60–80 nm, have reverse-transcriptase activity. The major structural components of VLPs are proteins produced by proteolysis of the primary translation product of ORF 1.

• Adams et al. (1987) have shown that fusion proteins can be produced in cells by inserting part of a gene from human immunodeficiency virus (HIV) into ORF 1. Such fusion proteins formed hybrid HIV:Ty-VLPs.
**Fig. 9.4** Structure of a typical Ty element. ORF 1 and ORF 2 represent the two open reading frames. The delta sequences are indicated by LTR (long terminal repeats).
Fig. 9.6 Structure of the multicopy plasmid used for inserting a modified Ty element, carrying a cloned gene, into the yeast chromosome. pGAL and P are yeast promoters, δ represents the long terminal repeats (delta sequences) and the red region represents the cloned gene. (See text for details.)
Fig. 9.5 Ty virus-like particles (magnification 80,000) carrying the entire HIV1 TAT coding region. (Photograph courtesy of Dr S. Kingsman.)
# Table 9.1 Properties of the different yeast vectors.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Transformation frequency</th>
<th>Copy no./cell</th>
<th>Loss in non-selective medium</th>
<th>Disadvantages</th>
<th>Advantages</th>
</tr>
</thead>
</table>
| YIp    | $10^2$ transformants per µg DNA | 1             | Much less than 1% per generation | 1 Low transformation frequency  
2 Can only be recovered from yeast by cutting chromosomal DNA with restriction endonuclease which does not cleave original vector containing cloned gene | 1 Of all vectors, this kind give most stable maintenance of cloned genes  
2 An integrated YIp plasmid behaves as an ordinary genetic marker, e.g. a diploid heterozygous for an integrated plasmid segregates the plasmid in a Mendelian fashion  
3 Most useful for surrogate genetics of yeast, e.g. can be used to introduce deletions, inversions and transpositions (see Botstein & Davis 1982) |
| YEp    | $10^3$–$10^5$ transformants per µg DNA | 25–200        | 1% per generation | Novel recombinants generated *in vivo* by recombination with endogenous 2 µm plasmid | 1 Readily recovered from yeast  
2 High copy number  
3 High transformation frequency  
4 Very useful for complementation studies |
| YRp    | $10^4$ transformants per µg DNA | 1–20          | Much greater than 1% per generation but can get chromosomal integration | Instability of transformants | 1 Readily recovered from yeast  
2 High copy number. Note that the copy number is usually less than that of YE p vectors but this may be useful if cloning gene whose product is deleterious to the cell if produced in excess  
3 High transformation frequency  
4 Very useful for complementation studies  
5 Can integrate into the chromosome |
| YCp    | $10^4$ transformants per µg DNA | 1–2           | Less than 1% per generation | Low copy number makes recovery from yeast more difficult than that with YE p or YRp vectors | 1 Low copy number is useful if product of cloned gene is deleterious to cell  
2 High transformation frequency  
3 Very useful for complementation studies  
4 At meiosis generally shows Mendelian segregation |
| YAC    |                          | 1–2           | Depends on length: the longer the YAC the more stable it is | Difficult to map by standard techniques | 1 High-capacity cloning system permitting DNA molecules greater than 40 kb to be cloned  
2 Can amplify large DNA molecules in a simple genetic background |
| Ty     |                          | ~20           | Stable, since integrated into chromosome | Needs to be introduced into cell in another vector | Can get amplification following chromosomal integration |
Table 9.2 Common fungal promoters used for manipulation of gene expression.

<table>
<thead>
<tr>
<th>Species</th>
<th>Promoter</th>
<th>Gene</th>
<th>Regulation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
<td>Glucose-induced</td>
</tr>
<tr>
<td></td>
<td>GAL1</td>
<td>Galactokinase</td>
<td>Galactose-induced</td>
</tr>
<tr>
<td></td>
<td>PHOS</td>
<td>Acid phosphatase</td>
<td>Phosphate-repressed</td>
</tr>
<tr>
<td></td>
<td>ADH2</td>
<td>Alcohol dehydrogenase II</td>
<td>Glucose-repressed</td>
</tr>
<tr>
<td></td>
<td>CUP1</td>
<td>Copper metallothionein</td>
<td>Copper-induced</td>
</tr>
<tr>
<td></td>
<td>MFα1</td>
<td>Mating factor α1</td>
<td>Constitutive but temperature-induced variant available</td>
</tr>
<tr>
<td><strong>Candida albicans</strong></td>
<td>MET3</td>
<td>ATP sulphur lyase</td>
<td>Repressed by methionine and cysteine</td>
</tr>
<tr>
<td><strong>Methanol utilizers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Candida boidinii</em></td>
<td>AOD1</td>
<td>Alcohol oxidase</td>
<td>Methanol-induced</td>
</tr>
<tr>
<td><em>Hansenula polymorpha</em></td>
<td>MOX</td>
<td>Alcohol oxidase</td>
<td>Methanol-induced</td>
</tr>
<tr>
<td><em>Pichia methanolica</em></td>
<td>AUG1</td>
<td>Alcohol oxidase</td>
<td>Methanol-induced</td>
</tr>
<tr>
<td><em>Pichia pastoris</em></td>
<td>AOX1</td>
<td>Alcohol oxidase</td>
<td>Methanol-induced</td>
</tr>
<tr>
<td></td>
<td>GAP</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Strong constitutive</td>
</tr>
<tr>
<td></td>
<td>FLD1</td>
<td>Formaldehyde dehydrogenase</td>
<td>Methanol- or methylamine-induced</td>
</tr>
<tr>
<td></td>
<td>PEX8</td>
<td>Peroxin</td>
<td>Methanol-induced</td>
</tr>
<tr>
<td></td>
<td>YPT1</td>
<td>Secretory GTPase</td>
<td>Medium constitutive</td>
</tr>
<tr>
<td><strong>Lactose utilizer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Kluveromyces lactis</em></td>
<td>LAC4</td>
<td>β-Galactosidase</td>
<td>Lactose-induced</td>
</tr>
<tr>
<td></td>
<td>PGK</td>
<td>Phosphoglycerate kinase</td>
<td>Strong constitutive</td>
</tr>
<tr>
<td></td>
<td>ADH4</td>
<td>Alcohol dehydrogenase</td>
<td>Ethanol-induced</td>
</tr>
<tr>
<td><strong>Starch utilizer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Schwanniomyces occidentalis</em></td>
<td>AMY1</td>
<td>α-Amylase</td>
<td>Maltose- or starch-induced</td>
</tr>
<tr>
<td></td>
<td>GAM1</td>
<td>Glucoamylase</td>
<td>Maltose- or starch-induced</td>
</tr>
<tr>
<td><strong>Xylose utilizer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pichia stipitis</em></td>
<td>XYL1</td>
<td>Xylose-induced</td>
<td>Xylose-induced</td>
</tr>
<tr>
<td><strong>Alkane utilizer</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Yarrowia lipolytica</em></td>
<td>XPR2</td>
<td>Extracellular protease</td>
<td>Peptone-induced</td>
</tr>
<tr>
<td></td>
<td>TEF</td>
<td>Translation elongation factor</td>
<td>Strong constitutive</td>
</tr>
<tr>
<td></td>
<td>RPS7</td>
<td>Ribosomal protein S7</td>
<td>Strong constitutive</td>
</tr>
<tr>
<td>Method</td>
<td>Procedure</td>
<td>Advantages</td>
<td>Disadvantages</td>
</tr>
<tr>
<td>-----------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Electroporation</td>
<td>Electric pulses induce membrane permeabilization providing a local driving force for ionic and molecular transport through the pores.</td>
<td>Can be applied to any fungi in vivo or frozen. Efficient protocols: simple, fast, and easily optimized.</td>
<td>Depends on the electrophysiological characteristics of the fungus. Low transformation efficiency. Medium cost.</td>
</tr>
<tr>
<td>Biolistics</td>
<td>Small particles covered with genes are accelerated to penetrate the cell wall.</td>
<td>Simple. No pretreatment of the cell wall required. Independent of the physiological properties of the fungi. Transformation with multiple transgenes is possible.</td>
<td>DNA can be damaged. Produces multiple copies of introduced genes. Complex protocols due to projectile preparation. Low transformation efficiency. Expensive.</td>
</tr>
<tr>
<td>Agitation with glass beads</td>
<td>Agitation of the fungal cells with glass beads in the presence of carrier and plasmid DNA allows DNA absorption.</td>
<td>Simple, fast and safe protocol. Inexpensive.</td>
<td>May cause cell disruption. Cells require osmotic support. Low transformation efficiency.</td>
</tr>
<tr>
<td>Vacuum infiltration</td>
<td>Vacuum generates a negative atmospheric pressure that causes the air spaces between the cells to decrease allowing the infiltration of bacteria such as Agrobacterium.</td>
<td>Simple and fast. In vitro fungi regeneration. Medium efficiency.</td>
<td>Requires the use of bacteria which may have unwanted consequences.</td>
</tr>
<tr>
<td>Shock waves</td>
<td>Acoustic cavitation changes the permeability of the membrane facilitating the absorption of DNA.</td>
<td>Simple, good reproducibility, safe. High efficiency. Low operating costs.</td>
<td>Relatively high cost of the shock wave source. Expertise in shock wave physics required.</td>
</tr>
</tbody>
</table>
Gene manipulation of animals and plants

- Modification to produce desired traits in plants, animals, and microbes used for food began about 10,000 years ago.
- These changes, along with natural evolutionary changes, have resulted in common food species that are now genetically different from their ancestors.
- Advantageous outcomes of these genetic modifications include increased food production, reliability, and yields; enhanced taste and nutritional value; and decreased losses due to various biotic and abiotic stresses, such as fungal and bacterial pathogens.
PLANT GENETIC MODIFICATION

- Techniques Other than Genetic Engineering
  - Simple Selection
  - Crossing
  - Interspecies Crossing
  - Embryo Rescue
  - Somatic Hybridization
  - Somatic Variational Variation
  - Mutation Breeding: Induced Chemical and X-ray Mutagenesis
  - Cell Selection
PLANT GENETIC MODIFICATION

• Genetic Engineering
  – Microbial Vectors
  – Microprojectile Bombardment
  – Electroporation
  – Microinjection
  – Transposons/Transposable Elements
ANIMAL GENETIC MODIFICATION

• Techniques Other than Genetic Engineering
  – Domestication and Artificial Selection
  – Assisted Reproductive Procedures
ANIMAL GENETIC MODIFICATION

• Techniques Fundamental to Genetic Engineering in Livestock
  – Although the following are not methods to generate modifications per se, they are considered modern methods that support the overall breeding and selection system for propagating desired genotypes for animals expressing desired traits.
    • Embryo Recovery and Transfer and Superovulation
    • In Vitro Maturation and Fertilization of Oocytes
    • Embryo Splitting
ANIMAL GENETIC MODIFICATION

• Genetic Engineering
  – Transfection
  – Retroviral Vectors
  – Transposons
  – Knock-In and Knock-Out Technology
Analysis of transcriptome

• Whole transcriptome analysis is of growing importance in understanding how altered expression of genetic variants contributes to complex diseases such as cancer, diabetes, and heart disease. Analysis of genome wide differential RNA expression provides researchers with greater insights into biological pathways and molecular mechanisms that regulate cell fate, development, and disease progression.

• Microarray

• Real Time PCR

• RNA – seq
Expression analysis of protein

• Real-Time PCR

• Immunostaining

• Western Blotting