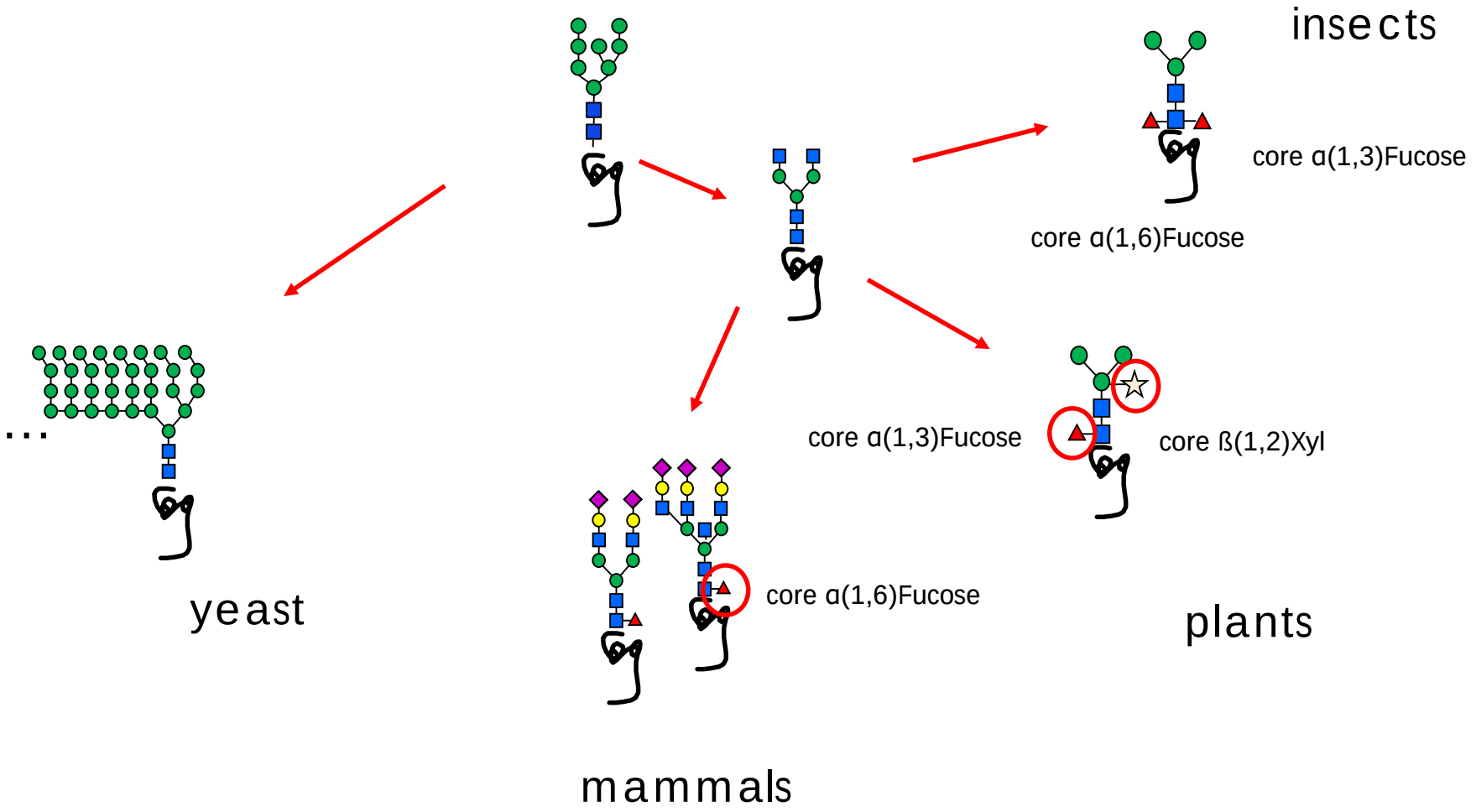
Recombinant protein expression in eukaryotes
Plants, insect cells, mammalian cells

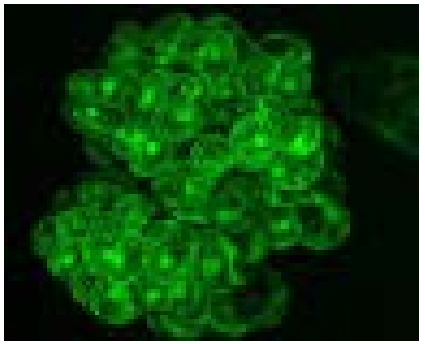
Lecture 4

Diversity of N-glycans in eukaryotic expression systems



Plants as expression factories

- Easily scaled up with standard agricultural practice
- Extensive infrastructure
- Economical approach for production and storage
- Safe with respect to human viruses or pathogens

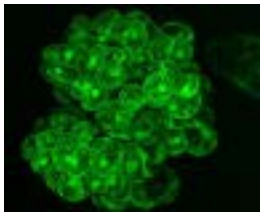


Expression in plants

- Post-translational modifications such as protein processing and glycosylation may differ form those of animal cells
- Insertion of DNA into genome is random, requires extensive screening
- Long lead times required for seed productions
- There are two general approaches to production:
  - Transient infection
  - Establishment of transgenic plants/plant cells

Plant expression systems

System	Advantages	Products
Single cell cultures	Scale, control of growth, ease of purification	Proteins, secondary metabolites
Leaves	Scalability, Harvest	Antibodies, Vaccines
Root extrusion cultures	Continuous system, ease of purification	Proteins, secondary metabolites
Seeds	Scalability, natural protein storage system	Recombinant proteins



Methods for generating transgenic plants

TABLE 18.1 Plant cell DNA-delivery methods

Method	Comment
Ti plasmid-mediated gene transfer	An excellent and highly effective system that is limited to a few kinds of plants
Microprojectile bombardment	Used with a wide range of plants and tissues; easy and inexpensive
Viral vectors	Not an effective way to deliver DNA to plant cells
Direct gene transfer into plant protoplasts	Can be used only with plant cell protoplasts that can be regenerated into viable plants
Microinjection	Has limited usefulness because only one cell can be injected at a time; requires the services of a highly skilled individual
Electroporation	Generally limited to plant cell protoplasts that can be regenerated into viable plants
Liposome fusion	Can be used only with plant cell protoplasts that can be regenerated into viable plants

All methods follow a similar pathway:

- Cloning of the gene into a suitable expression vector
- Transformation into plants or plant cells in culture
- Regeneration of plant
- Establishment of transgenic lines for production (or breed into cultivars for agriculture)

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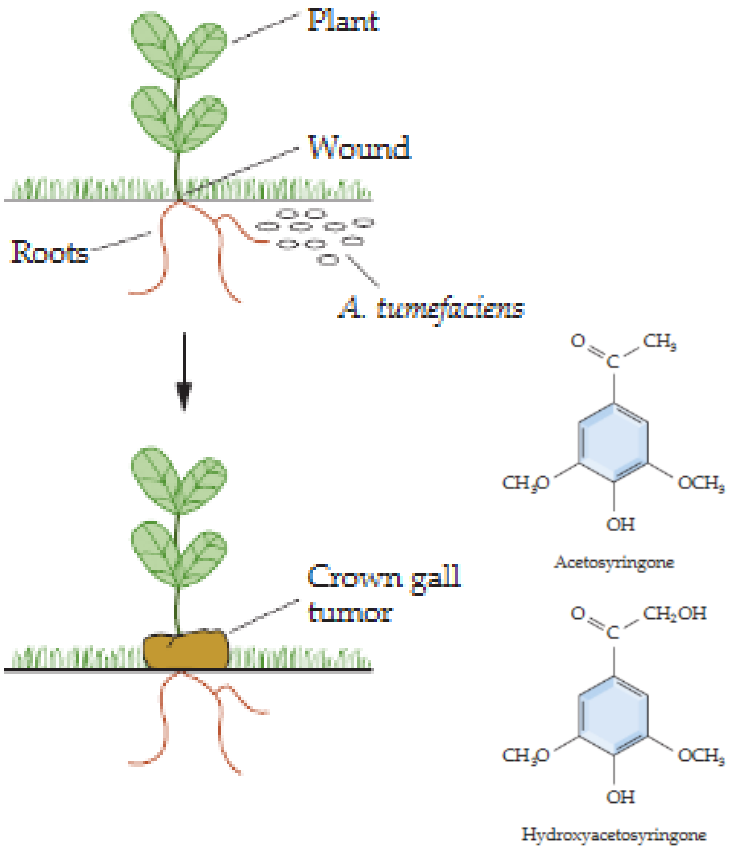
Agrobacterium tumefaciens infection



- *A. tumefaciens* infects wounded plant cells
- In response to infection plants produces Acetosyringone
- Acetosyringone induces virulence genes (*vir*) on Ti-plasmid of *Agrobacterium*
- Expression of *vir* genes enables the transfer of T-DNA into plant
- T-DNA encodes plant hormones inducing tumor formation and opine genes

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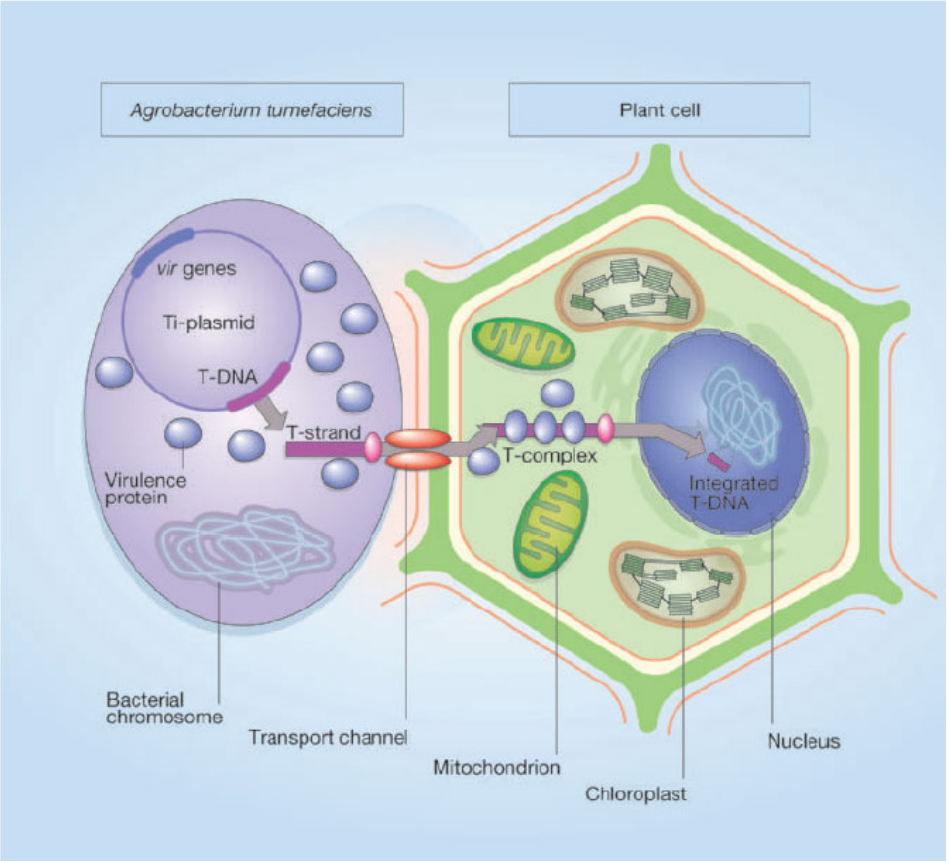
A. tumefaciens infection

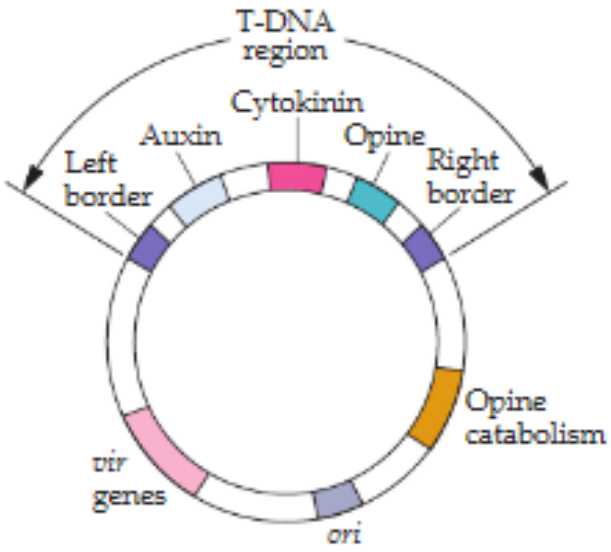


- **Progression of infection**

1. Chemical perception of host
2. Induction of virulence genes
3. Formation of infection apparatus
4. Preparation of T-DNA
5. Export T-DNA to plant cell
6. Import T-DNA to plant nucleus
7. Integration of T-DNA into host genome
8. Expression of T-DNA

Agrobacteria can infect a number of other host organisms including plants, yeast and fungi

The Ti-plasmid



- Vir proteins mediate T-DNA processing and transfer from the bacterium to the host cell
- T-DNA region is flanked by short sequences defining the stretch of DNA being transformed into the plant cell
- T-DNA region encodes genes for synthesis of plant hormones and opine
- Plant hormones induce tumor formation and keep transformed cells in a dedifferentiated state
- Opines can be catabolized by *Agrobacterium* and serve as C- and N-source

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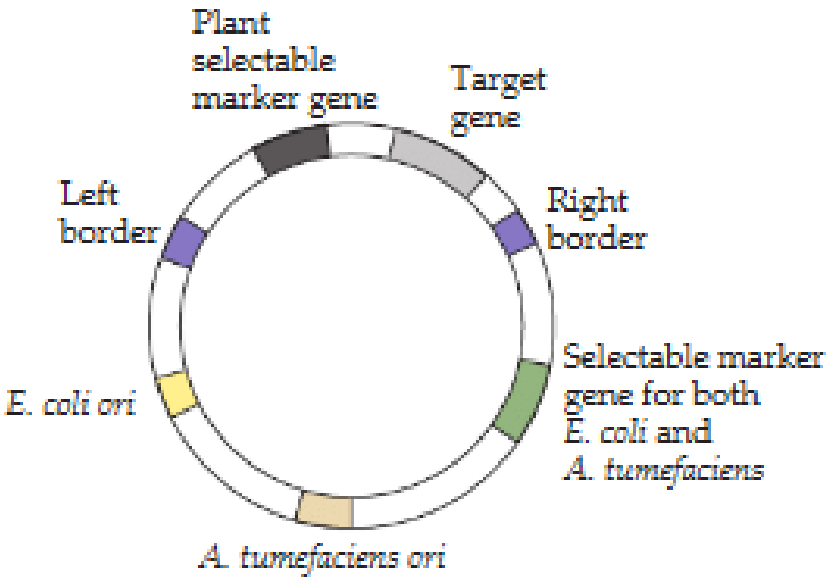
A disarmed Ti-plasmid for plant transformation



- Gene required for infection (*vir*) need to be provided in trans
- The region between RB and LB defines the region where target genes and selection markers can be introduced

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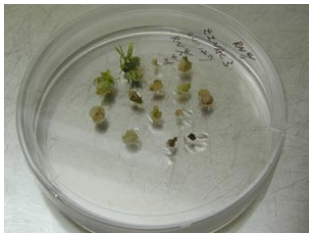
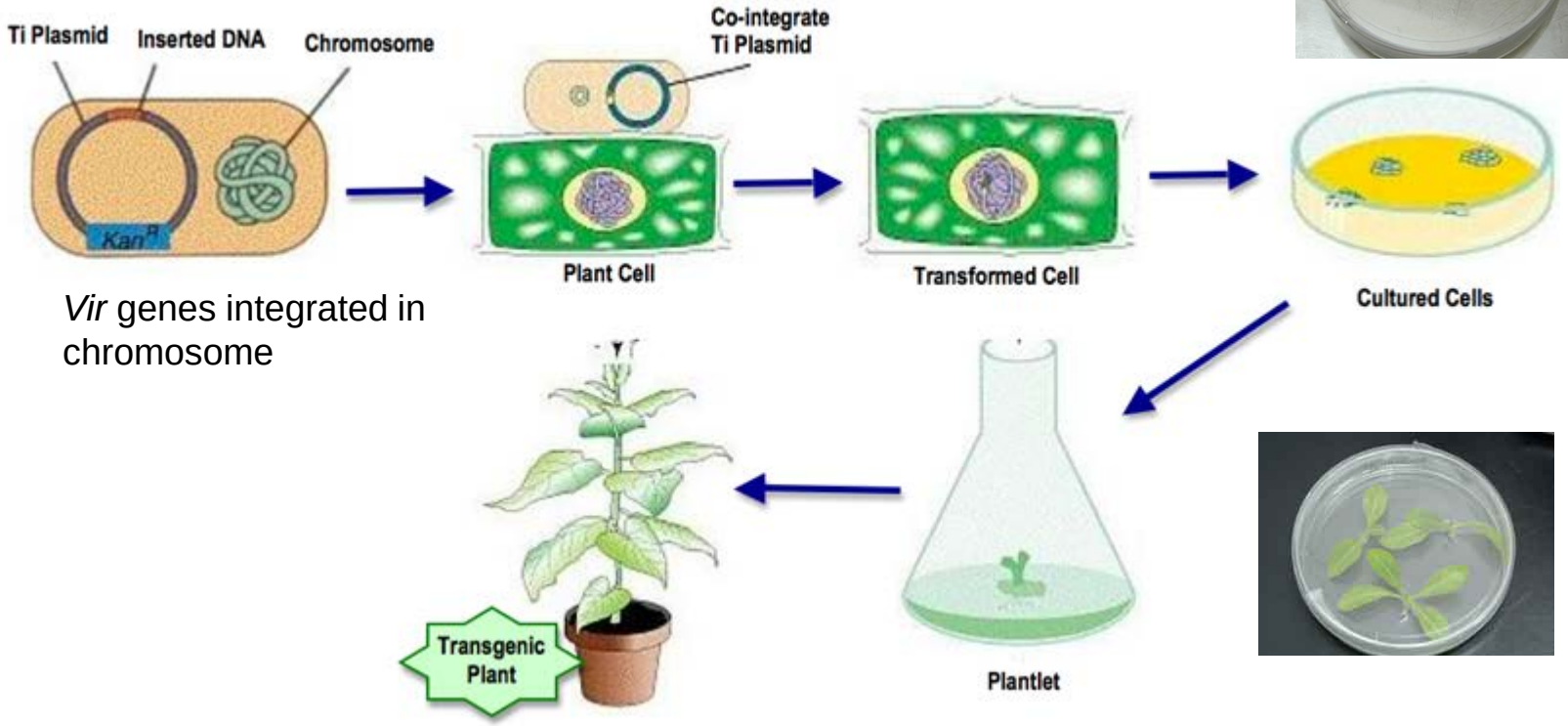
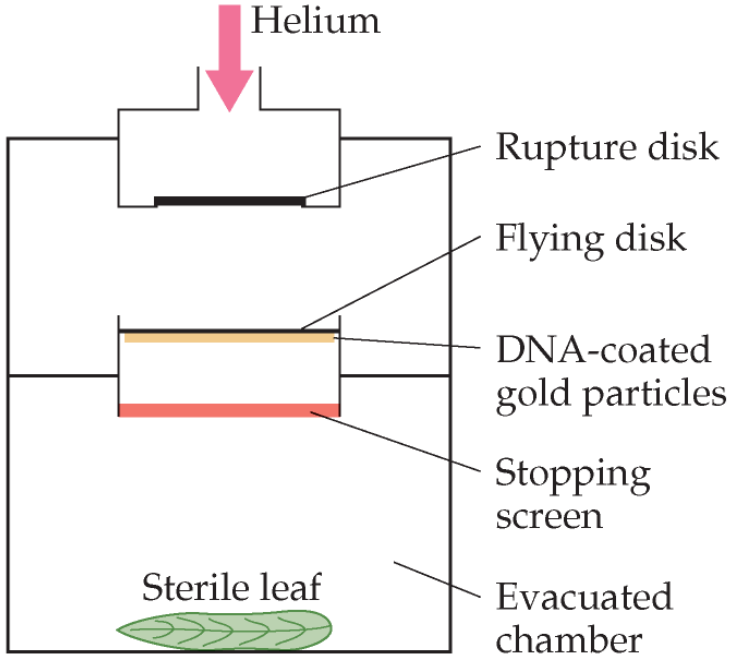
Infection, transformation and plant regeneration process





- *Agrobacteria* infection allows stable transformation of plants and plant cells
- Dipping of whole plants allows transient transformation with *A*. *tumefaciens*. e.g. highly efficient for *N*. *benthamiana*

Biolistic plant transformation (Gene gun)



- DNA is precipitated on the surface of gold particles
- Loaded gold particles are located on lower side of flying disk
- Helium flow builds up pressure which breaks rupture disk
- Flying disk is propelled forward until reaching stopping screen
- DNA-coated particles penetrate stopping screen and bombard plant material

Often used for monocotyledons such as rice, maize which are not amenable for *A. tumefaciens* infection

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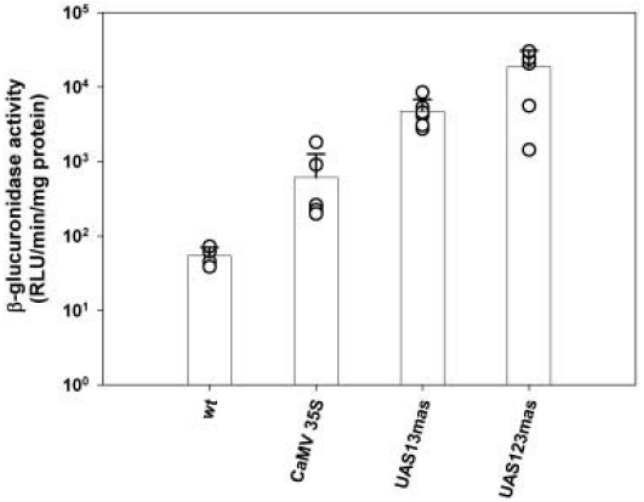
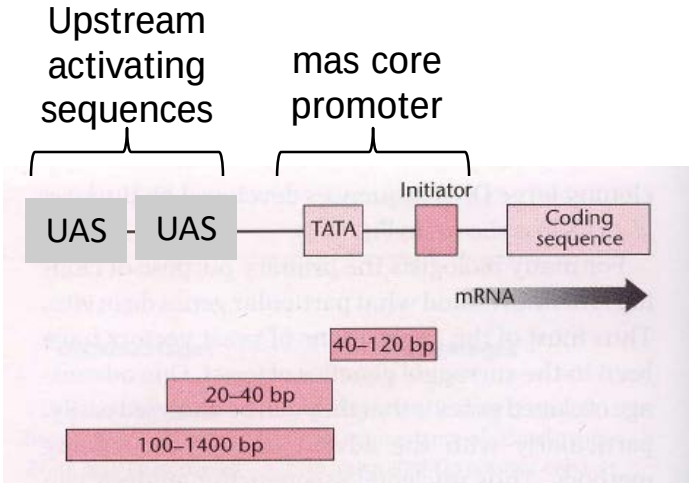
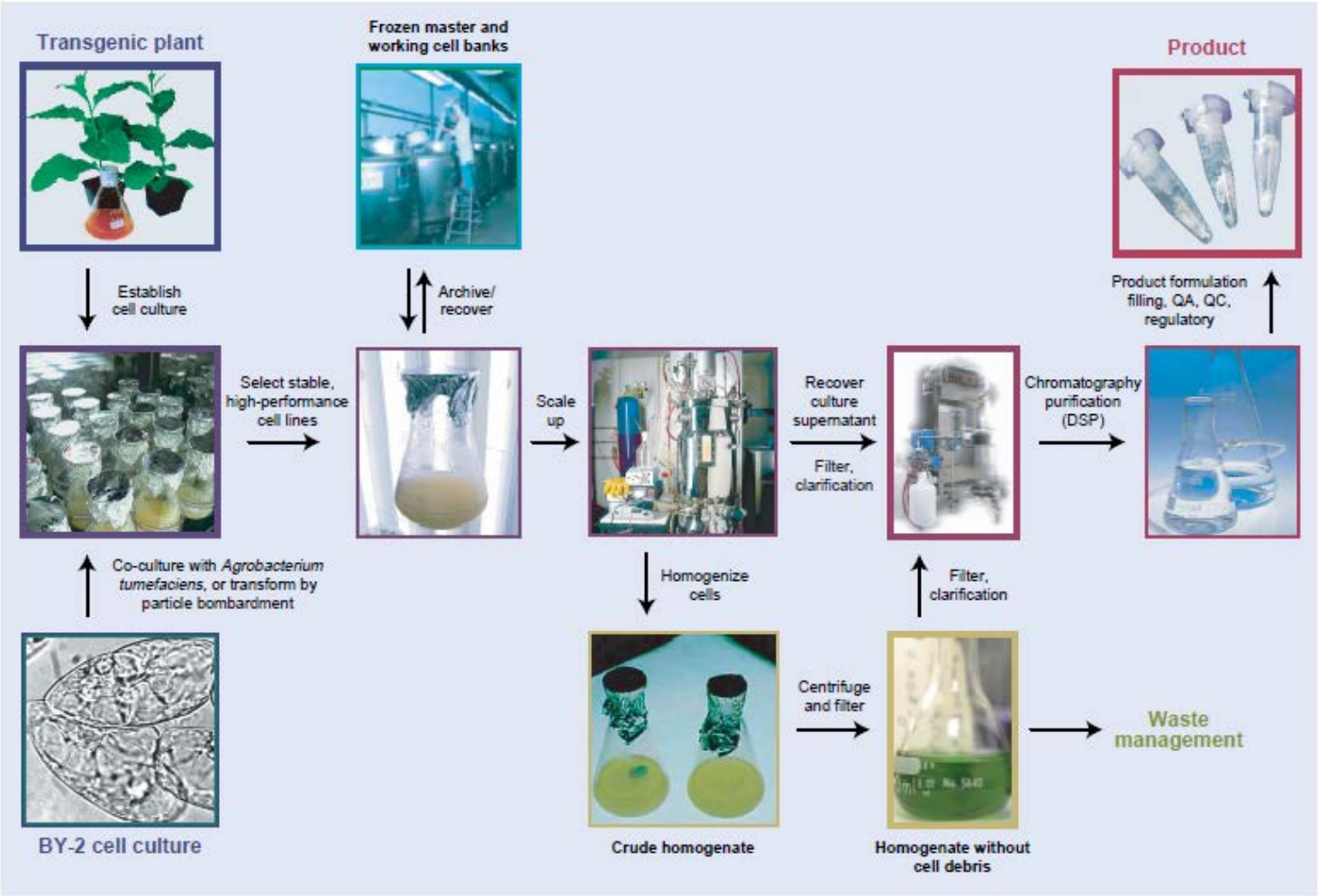
Plant promoters and engineering

- Promoters often derived from
  - Viruses
    - CaMV 35S native and engineered
  - *A. tumefaciens*
    - Mannopine or octopine synthase promoter (Mas, Ocs)
  - Promoters from seed storage proteins



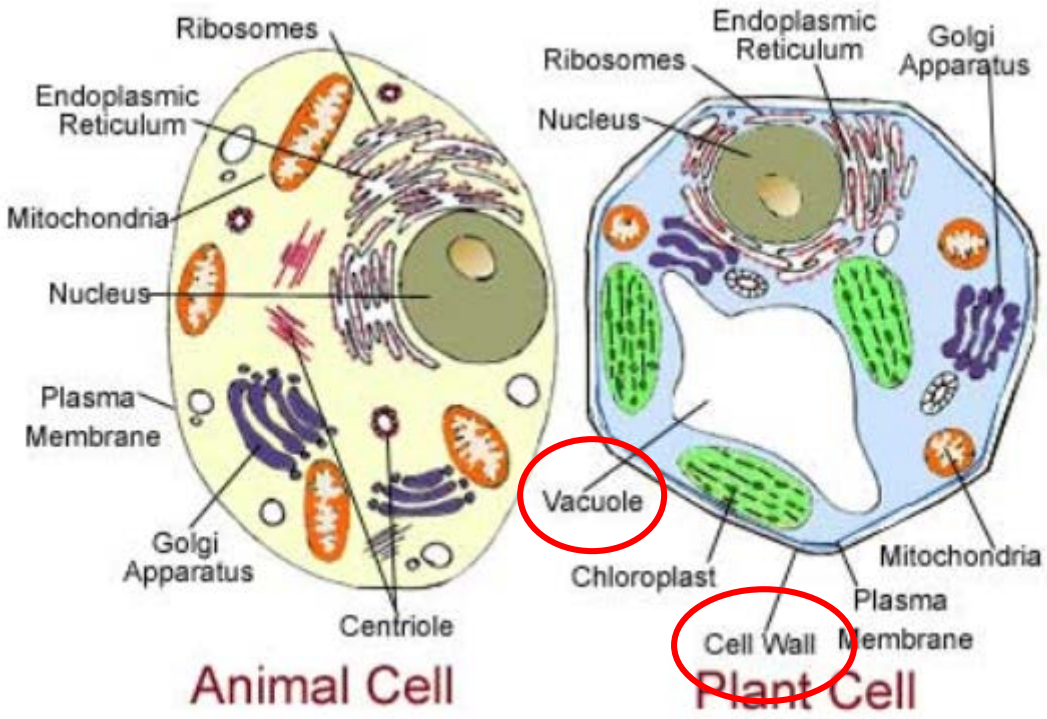


Overview of biopharmaceutical production in plant cells



Hellwig et al. 2004, Nat. Biotech.

Structural differences between plant and mammalian cells impacting protein expression

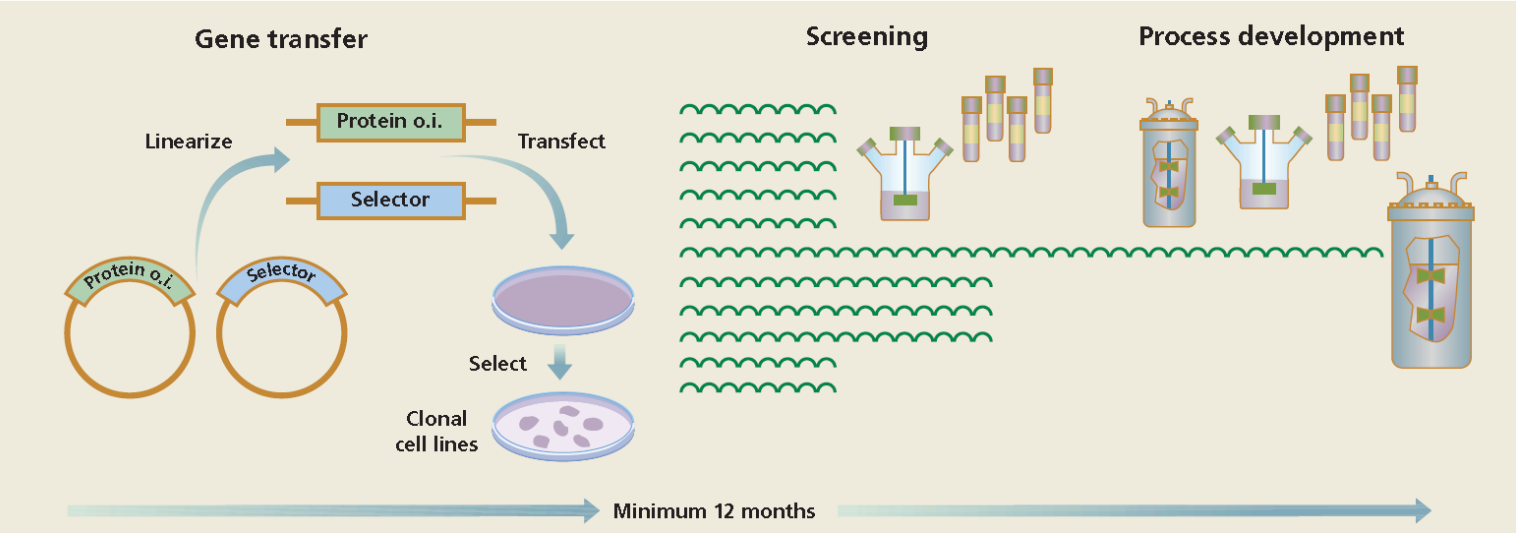


- Vacuole contains many proteases -> released when cells lyse
- Cell wall is a physical barrier restricting release of secreted proteins

Protein expression in mammalian cells

A typical development process for a cell line

- Transfection and selection of transfected cells
- Cell line screening
- Clonal selection to identify high producers
- Process development
  - Media optimization
  - Scale-up
- Cell banking



Wurm, 2004, *Nature Biotechnol. 22:1393-1398*

Industrially relevant mammalian cell lines

Origin	Cell line	Source	Serum Free Media	Mode	Reference	Products
Hamster	CHODG44, DUXB11	Gibco #12613-014; Lawrence Chasin (Columbia University)	CD CHO DG44 (Gibco)	Stable	1	Rituxan, Herceptin, Avastin, Xolair, Enbrel
	CHO-K1	ATCC #CCL-61	CD-CHO (Gibco), proCHO-5(lonza)	Stable	2	
	CHO-S	Gibco #11619-012	CD-CHO (Gibco)	Stable	3	
Human Aminocyte	CAP	Cevec	PEM (Gibco)	Stable	4	None
Human Retina	PER.C6	Crucell	Excell VPRO (Sigma), CDM4PERMab (HyClone)	Stable	5	None
Mouse Myeloma	NS0	ECACC #85110503	CDM4NSO (HyClone)	Stable	6	Synagis, Zenapax,
	Sp2/0	ATCC-CRL-1581	SFM4MAB (HyClone)	Stable	7	Remicade Erbitux
Duck	EB66	Vivalis	Excell Ebx (Sigma)	Stable	8	None
Hamster	BHK	ATCC #CRL-8544	OptiPro SFM	Stable	9	Factor VIII
Hamster	Freestyle CHO-S	Invitrogen	Freestyle CHO (Gibco)	Transient	3	None
	CHO-T	Acyte	CHO-S SFM II (Gibco)	Transient	10	None
	CHO3E7	CNRC #L-11992	Freestyle CHO (Gibco)	Transient	11	None
Human	Freestyle HEK293-F	Invitrogen	293 Freestyle (Gibco)	Transient	3	None
	HEK 293 6E	CNRC #L-11266	293 Freestyle (Gibco)	Transient	11	None
	HEK 293 T	ATCC #CRL-11268	293 Freestyle (Gibco)	Transient	12	None
Human Aminocyte	CAP-T	Cevec	293 Freestyle (Invitrogen)	Transient	13	None

Critical points of mammalian cells

- Slow growth, doubling time 24 hours
- More demanding growth requirements (CO_2 and normally requiring serum)
- Can become contaminated with human pathogens
- Grow from microtiter-plate scale to bioreactors

Some desirable properties of mammalian cells

- Ability to grow in serum-free medium is advantage
  - Reduces costs
  - Facilitates purification of target protein
  - Reduces the risk of contamination with animal-derived medium
- Growth in suspension
- Receptive to transfection

Transient transfection

- Gene to protein in days
- Testing expression
- Functional studies
- Low yield depending on transfection efficiency

Stable transfection

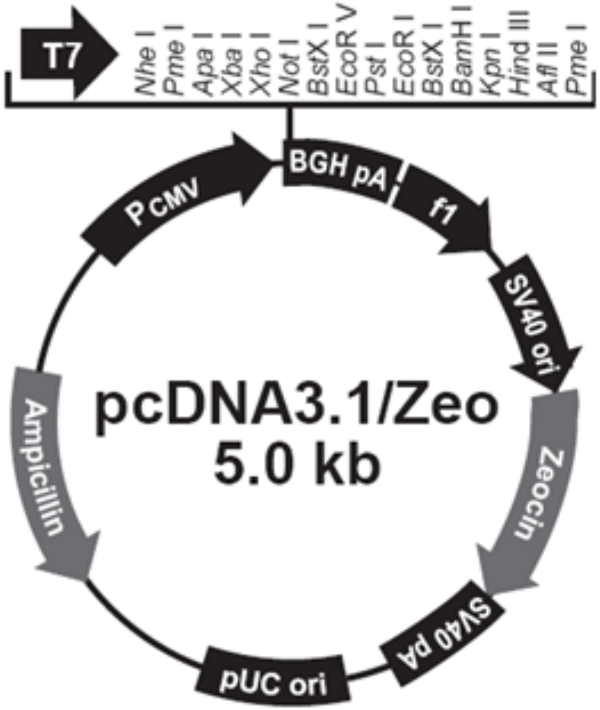
- Gene to protein in ≥ 2 months
- Complex process
- Gene of interest integrates into genome of host cell
  - Selection of best expressing clones required
- High yields possible (from few mg/L to g/L possible)
- Stock of cells expressing desired recombinant protein

Introducing a gene into mammalian cells

- Complexing of DNA with Ca^{2+} and uptake of complex by cells
- Liposomes – packaging of DNA into lipid-based vesicles
- Electroporation
- Viral vectors

Mammalian cell expression vectors

- eukaryotic origin of replication from an animal virus, e.g. Simian virus 40 (SV40)
- promoter sequences that drive both the cloned gene(s) and the selectable marker gene(s)
- multiple cloning sites to permit cloning
- resistance genes for selection of stable cell lines (Neomycin, Blasticidin, Hygromycin or Zeocin)
- Prokaryotic sequences to permit growth and maintenance in *E. coli*



Reminder: Cap-Independent Initiation

- Some mRNAs can initiate translation by a cap-independent process
- ribosome is guided to the correct initiation codon by structural motifs in the 5'-untranslated region of the mRNA
  - motifs usually referred to as internal ribosome entry sites (IRES)

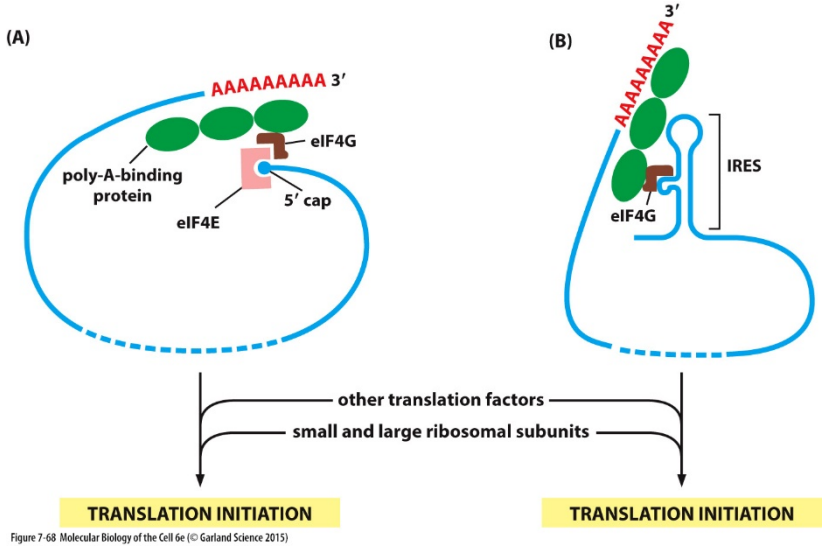
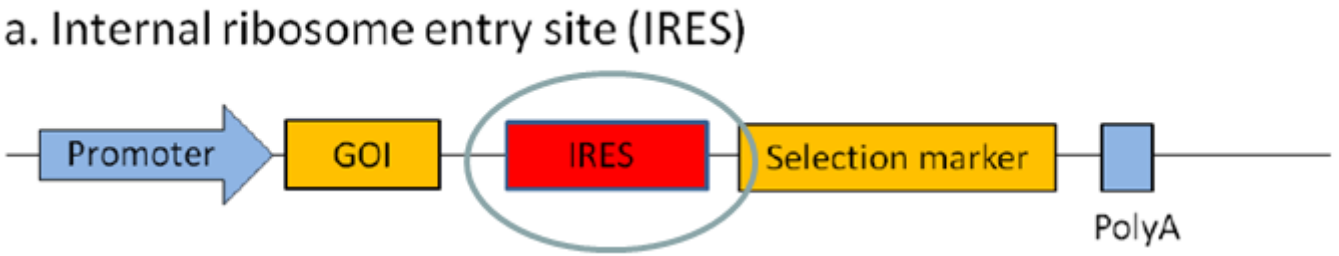


Figure 7-68 Molecular Biology of the Cell 6e (© Garland Science 2015)

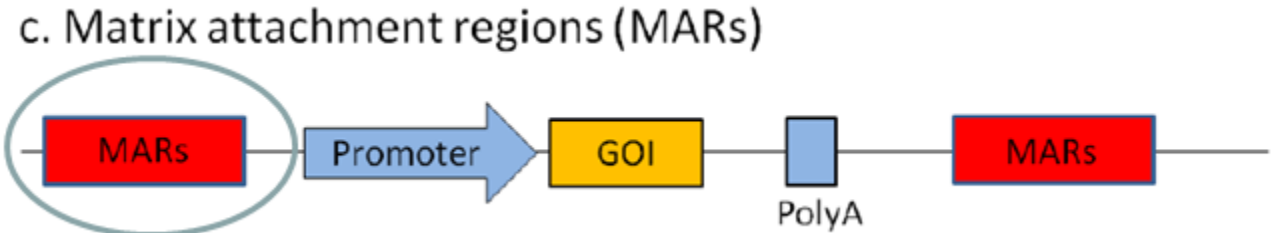
Internal ribosome entry site (IRES)

- Multiple promoters in one vector may result in transcriptional interference
- These problems can be solved with the applications of IRES elements.
- Expression of multiple genes such as selection marker and gene of interest can be linked by insertion of an IRES element between the two genes
- Both genes are dependent on the same promoter for transcription into a single mRNA.



Matrix attachment regions (MARs)

- MARs are genomic DNA sequences which serve as attachment points within the DNA that facilitate the anchoring of chromatin to the nuclear matrix during interphase
- MARs maintain a transcriptionally active chromatin structure (euchromatin) through formation of chromatin loops
- When used as cis acting elements or by flanking the transgene MARs have been shown to promote gene expression and increase the occurrence of high producing clones



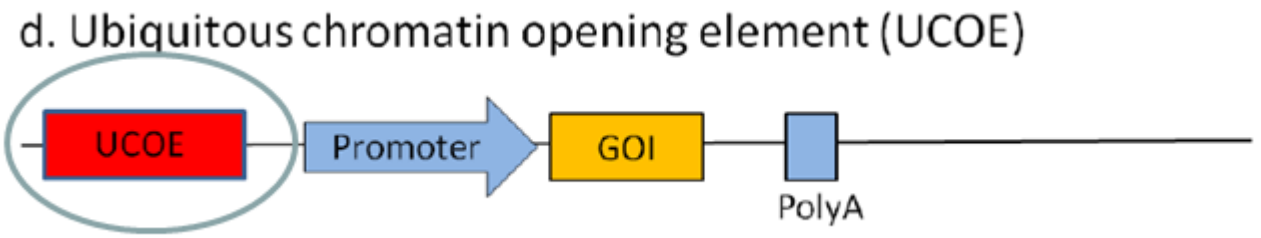
Biotechnol J. 2018 Mar;13(3):e1700232. doi: 10.1002/biot.201700232. Epub 2017 Dec 7.

Engineered and Natural Promoters and Chromatin-Modifying Elements for Recombinant Protein Expression in CHO Cells.

Romanova N[1], Noll T[1,2].

Ubiquitous chromatin opening element (UCOE)

- UCOE is an insulator element against heterochromatin expansion
- It abolishes integration position dependent effects and maintains the chromatin in an “open” configuration to increase accessibility of the DNA region to transcription machinery



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Engineered and Natural Promoters and Chromatin-Modifying Elements for Recombinant Protein Expression in CHO Cells.

Romanova N[1], Noll T[1,2].

Spodoptera frugiperda
(fall armyworm)

Expression using insect cells
The Baculovirus Expression Vector System (BEVS)

Bombyx mori (common silkworm)

Expression in insect cells

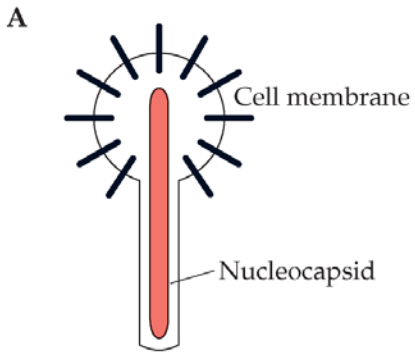
- BEVS has been widely used in research and scientific industrial communities for the production of high levels of proteins (up to 1000 mg/mL)
- Proteins can be targeted to organelles or exported outside for harvesting
- Correct folding, S-S bonds
- Post-translational modifications “similar” to those in mammalian cells

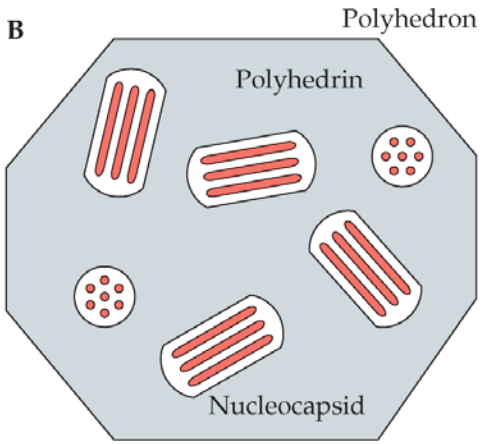
Expression in insect cells

- Disadvantages
  - More difficult to work with compared to lower eukaryotes
  - Slow generation time
  - Culturing is costly (compared to bacteria, yeast) but less than for mammalian cells

Expression in Insect cells

- 2 forms of baculovirus are formed during infection cycle
  - single virus particle
  - in protein matrix (polyhedron) trapped clusters of viruses
- during late stage of infection massive amount of polyhedron produced -> strong promoter
  - polyhedron not required for virus production
  - polyhedron promoter optimal for heterologous protein production in insect cells





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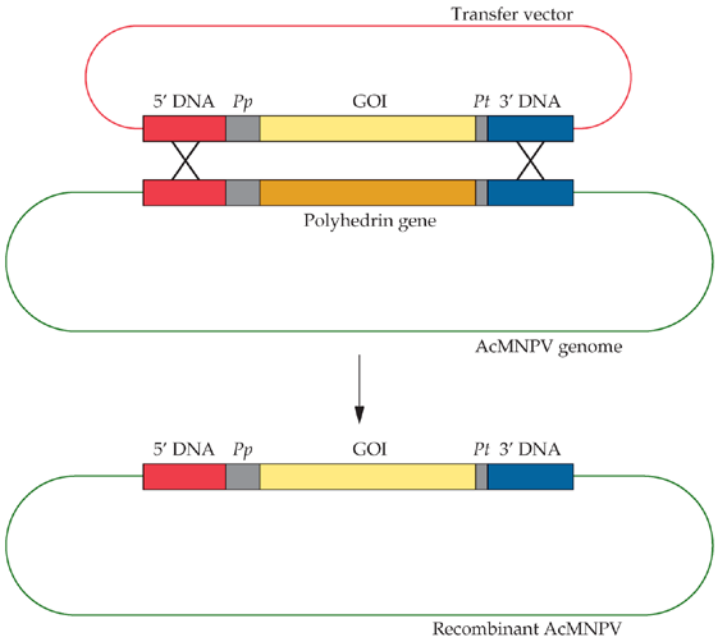
Expression using Baculovirus

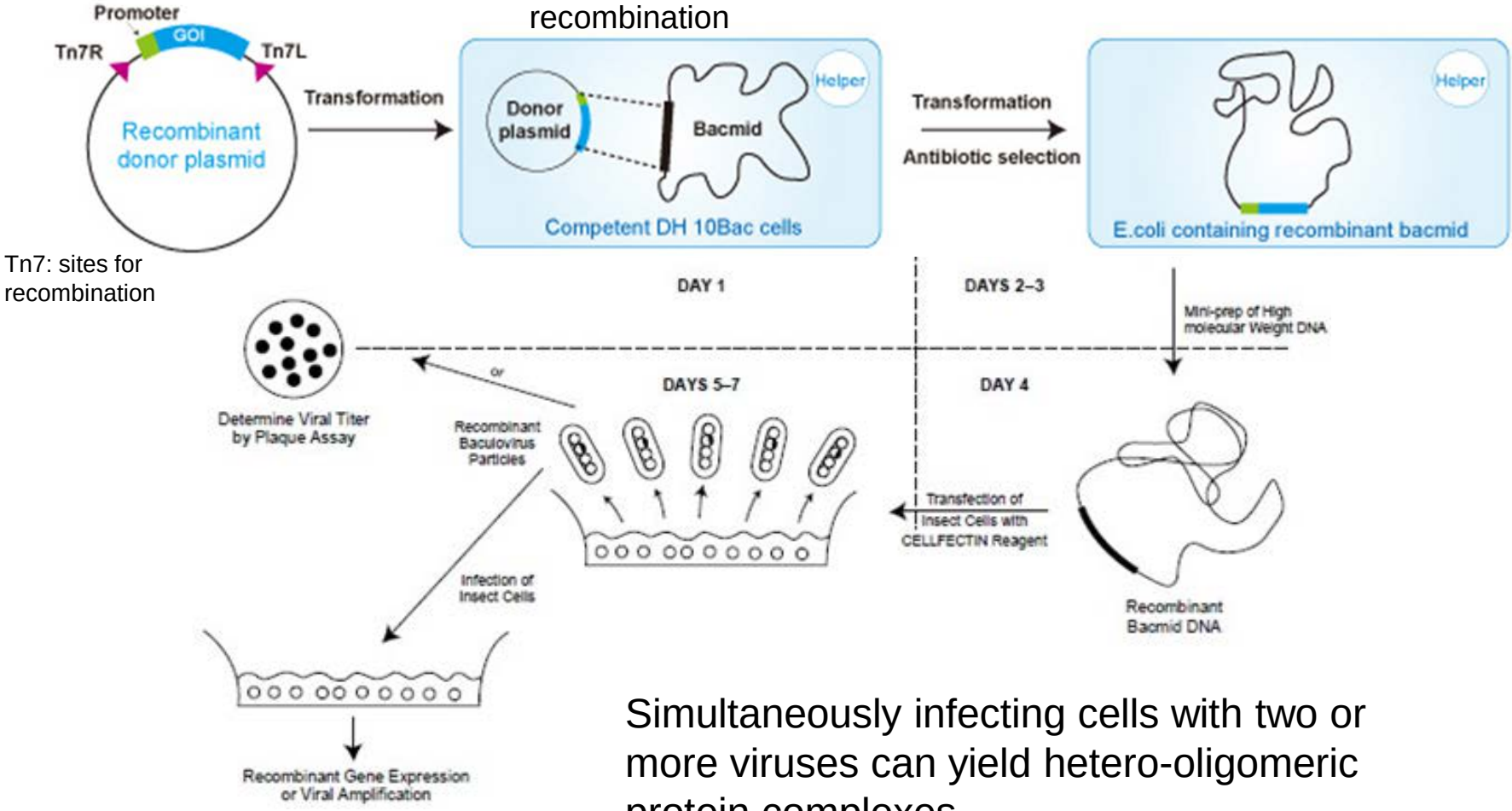
- Baculoviruses infect primarily insect cells
- Properties of baculovirus:
  - Rod shaped viruses that infect mostly insects, 40-50 nm in width and 200-400nm in length.
  - Genome is a large, double stranded, circular DNA of 80-200kb.
  - Replicate in nucleus
- Common baculovirus are:
  - Autographa californica (AcNPV) NC_001623 133,894 bp
  - Bombyx mori nucleopolyhedrovirus (BmNPV) L33180 128,413 bp

Cloning into Baculovirus expression vector

- Foreign gene cloned into a transfer vector based on *E*. *coli* plasmid that carries a segment of the DNA from AcNPV
- Homologous recombination of the transfer vector with insert DNA with viral genome leads to the cloned gene being transferred into the AcNPV DNA, here polyhedrin gene is replaced
- Gene of interest can replace any nonessential gene (polh, v-cath, chiA etc.) in recombinant vector

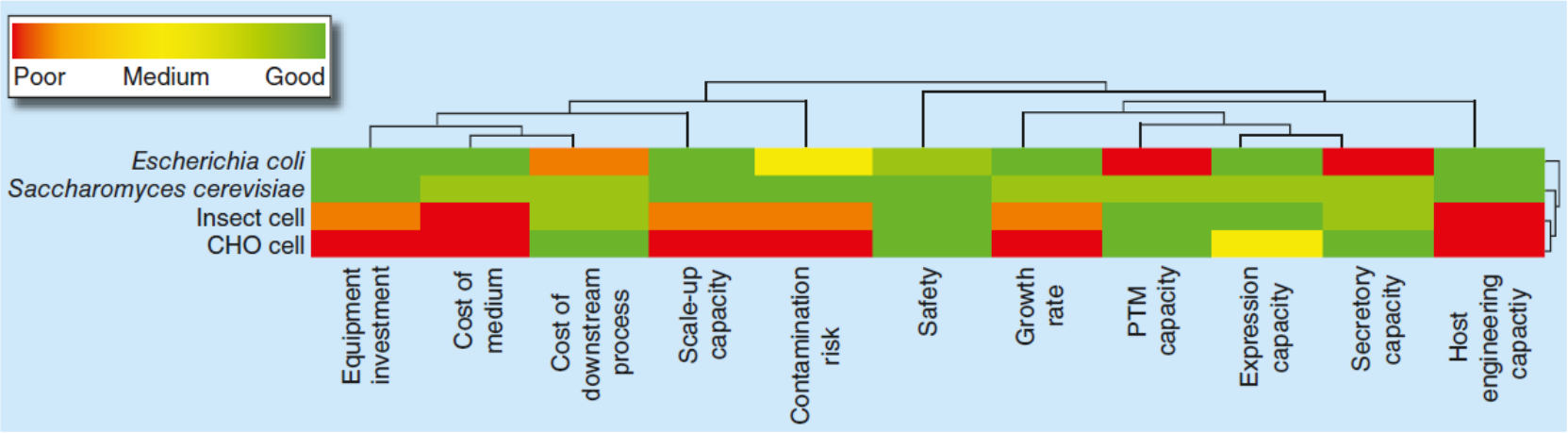


Preparation of vector and virus particles



Simultaneously infecting cells with two or more viruses can yield hetero-oligomeric protein complexes

Summarizing protein expression systems



Huang et al. (2014) Pharm Bioprocess. 2:167-182.

The cluster rules of assessment criteria are based on the industrial extent and host intrinsic property, such as upfront and production cost, scale and risk, strain producing capacities and safety.

Summarizing protein expression systems

- Due to the fast growth rate, *E*. *coli* is widely used for production of small and simple proteins. However, there are some limitations for using this expression system.
  - secretion of recombinant proteins is inefficient, which requires more complex downstream purification process
  - difficult for *E*. *coli* to perform complicated PTM
- CHO cell has a high similarity to human cells, and this impart CHO cell lines a good ability to perform PTM of proteins for human use.
- CHO cell shows other disadvantages
  - such as low growth rate
  - high contamination risk
  - manufacturers need to spend more time and money to maintain the production process

Summarizing protein expression systems

- *S. cerevisiae* and *P. pastoris* seems to be a good compromise because of the fast growth rate, low cost of medium and downstream processing, low contamination risk, and good secretory capacity.
  - Yeast can carry out N-linked glycosylation of a high mannose type
  - Glycoengineered yeasts are emerging that allow producing complex (human-like) glycoproteins

- Plant systems and insects cells are currently only minor players in recombinant protein expression