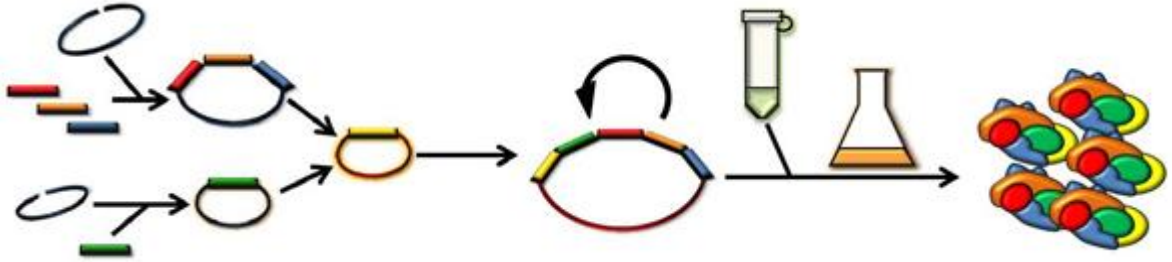
LOSCHMIDT LABORATORIES

Bi9540 Biotechnology and practical use of algae and fungi

Lecture 12 – Fungal and algal platforms for recombinant expression



Advantages of fungal platforms

- As eukaryotes, yeasts and filamentous fungi have many of the advantages of higher-eukaryotic cells, such as post-translational modifications.
- *Yeast cell* growth is faster, easier and less expensive than other eukaryotic cells, and generally gives higher expression levels.
- Three main species of yeast are used for the production of recombinant proteins – *Saccharomyces cerevisiae, Pichia pastoris and Schizosaccharomyces pombe.*

Expression system	*Classification*	*Development of system*	*Disulfide bonds*	*Glycosylation*	*Secretion*	*Costs of fermentation*	*Use of antibiotics*	*Safety costs*	*Processes developed*	*Products on market*
Mammalian cells	higher eukaryote	completely developed	yes	yes; typically human-like	possible	high	not required	high costs	industrial scale	yes
Plant cells	higher eukaryote	completely developed	yes	yes; terminal fucose	possible; size-restrictions	moderate	not required	low costs	pilot scale	no
Sordaria macrospora	filamentous fungus	earlys stages	yes	yes; exact features yet unknown	possible	low	not required	low costs expected	lab scale	no
Aspergillus sojae	filamentous fungus	completely developed	yes	yes; exact features yet unknown	possible	low	not required	low costs	pilot scale	no
Arxula adeninivorans	dimorphic yeast	early stages	yes	yes; exact features yet unknown	possible	low	not required	low costs expected	lab scale	no
Yarrowia lipolytica	dimorphic yeast	early stages	yes	yes; exact features yet unknown	possible	low	not required	low costs expected	lab scale	no
Pichia pastoris	methylo-trophic yeast	completely developed	yes	yes; no terminal α1, 3 mannose	possible	low	not required	low costs	industrial scale	yes
Hansenula polymorpha	methylo-trophic yeast	completely developed	yes	yes; no terminal α1, 3 mannose	possible	low	not required	low costs	industrial scale	yes
Staphylococcus carnosus	gram-positive bacterium	completely developed	limited	no	possible	low	typically required	low costs	pilot scale	no
Pseudomonas fluorescens	gram-negative bacterium	completely developed	(yes); in the periplasm	no	periplasmic secretion	promoter-de-pendent low to moderate	not required	low costs	pilot scale	no
Escherichia coli	gram-negative bacterium	completely developed	(yes); in the periplasm	no	periplasmic secretion	promoter-de-pendent low to moderate	typically required	low costs	industrial scale	yes

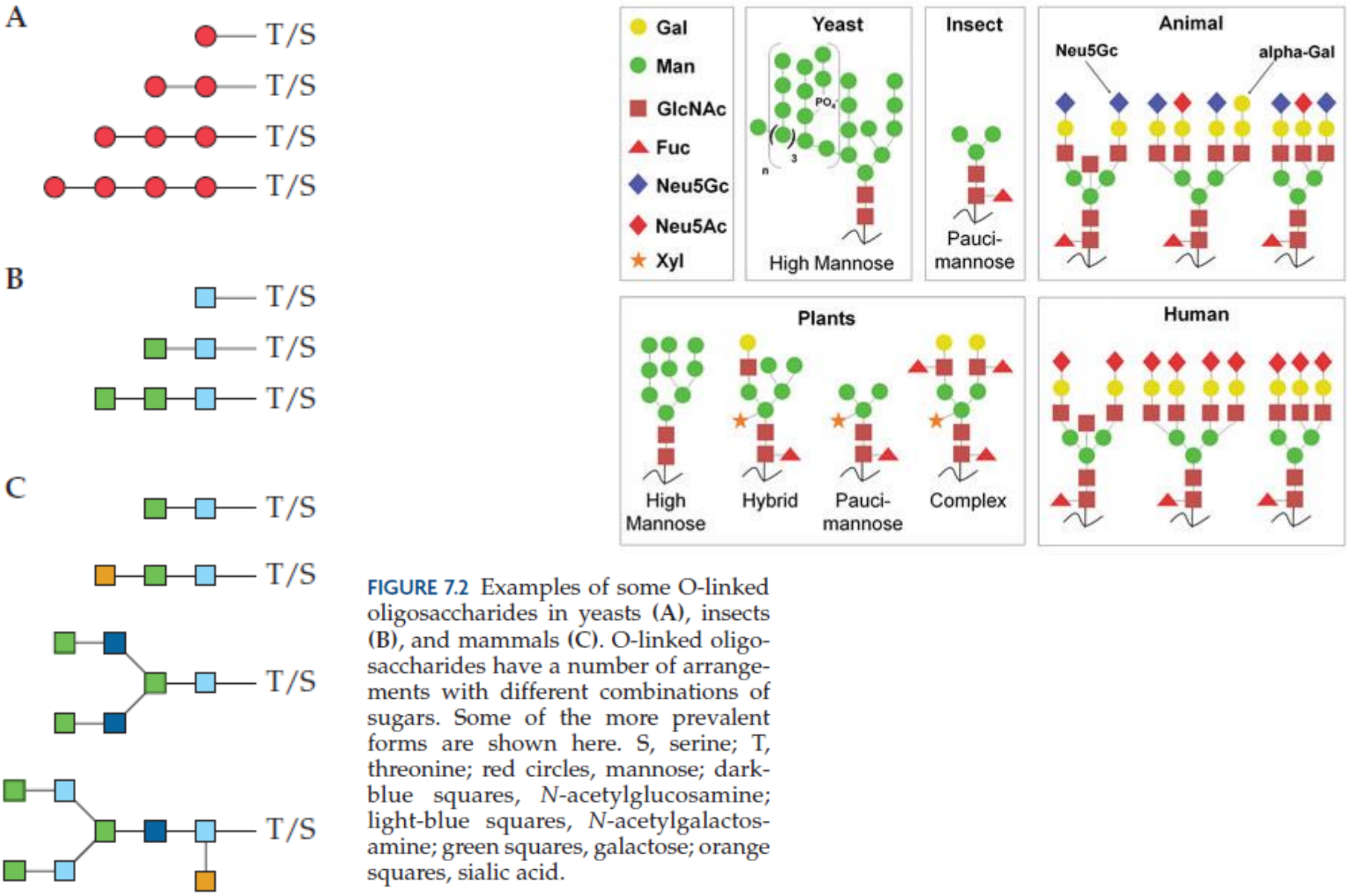


FIGURE 7.2 Examples of some O-linked oligosaccharides in yeasts (A), insects (B), and mammals (C). O-linked oligosaccharides have a number of arrangements with different combinations of sugars. Some of the more prevalent forms are shown here. S, serine; T, threonine; red circles, mannose; dark-blue squares, *N*-acetylglucosamine; light-blue squares, *N*-acetylgalactosamine; green squares, galactose; orange squares, sialic acid.

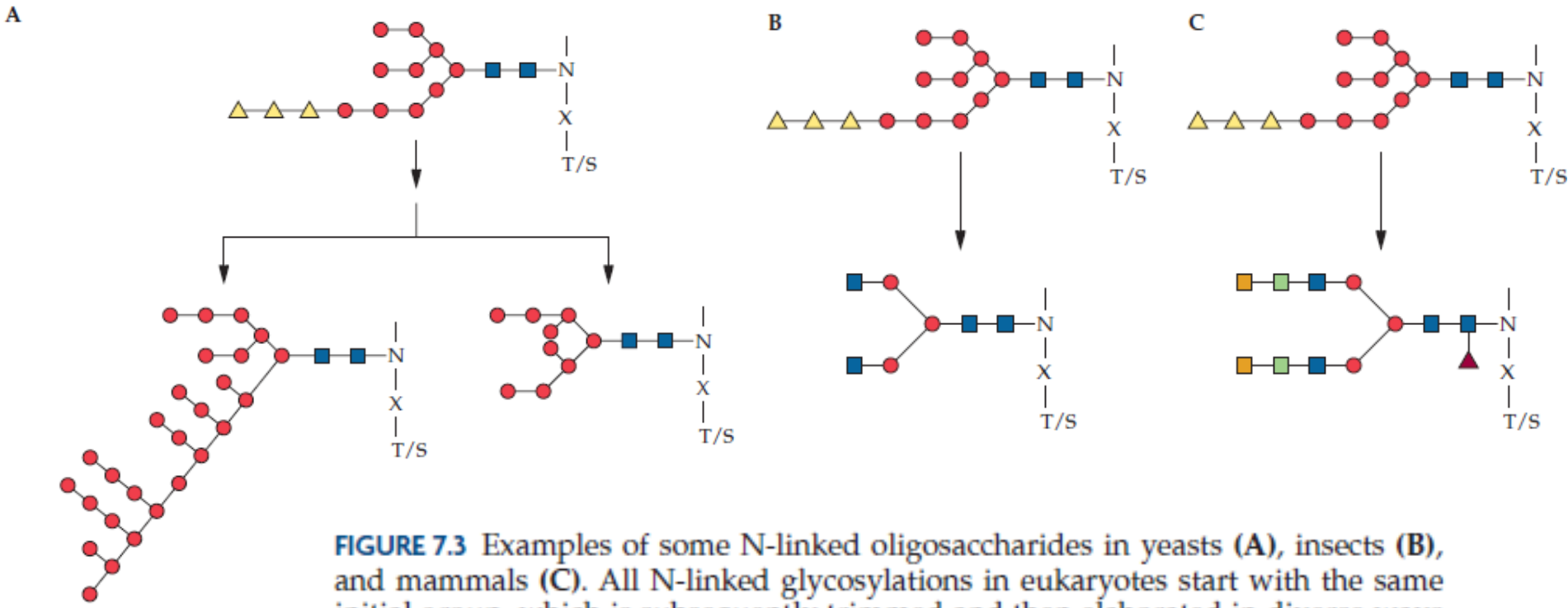
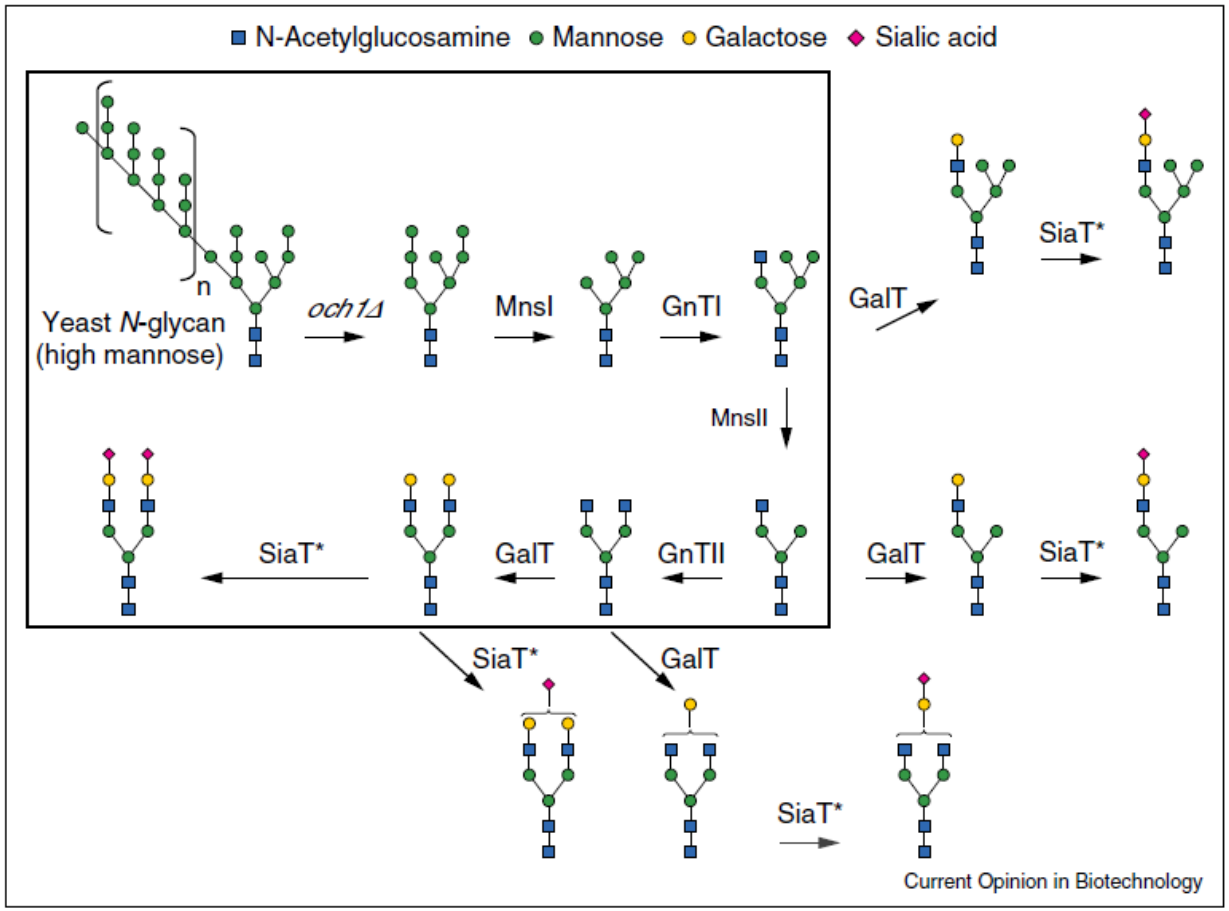


FIGURE 7.3 Examples of some N-linked oligosaccharides in yeasts **(A)**, insects **(B)**, and mammals **(C)**. All N-linked glycosylations in eukaryotes start with the same initial group, which is subsequently trimmed and then elaborated in diverse ways within and among species. Some yeast sites have 15 or fewer mannose units (core series), and others have more (outer-chain family). In *S. cerevisiae*, the chains frequently have 50 or more mannose units. An asparagine (N) residue next to any amino acid (X) followed by either threonine (T) or serine (S) can be targeted for glycosylation. Red circles, mannose; dark blue squares, *N*-acetylglucosamine; yellow triangles, glucose; green squares, galactose; orange squares, sialic acid; maroon triangle, fucose.



Yeast *N*-glycan engineering. The *N*-glycosylation pathway of glycoengineered *Pichia pastoris* was previously reviewed [34]. Glycoproteins harboring predetermined glycoforms [78] are obtained depending on the glycoengineered yeast host used, each of which contains a unique set of gene deletions and glycosylation enzymes, as indicated by arrows. The main glycosylation pathway to obtain mammalian biantennary glycans is shown in the upper left rectangle. As indicated by (*), sialic acid linkages may be exclusively α-2,6 or α-2,3 depending on the chosen sialyltransferase. Other yeast modifications (*e.g.* beta-linked mannose, mannosylphosphate) are not depicted in the figure.

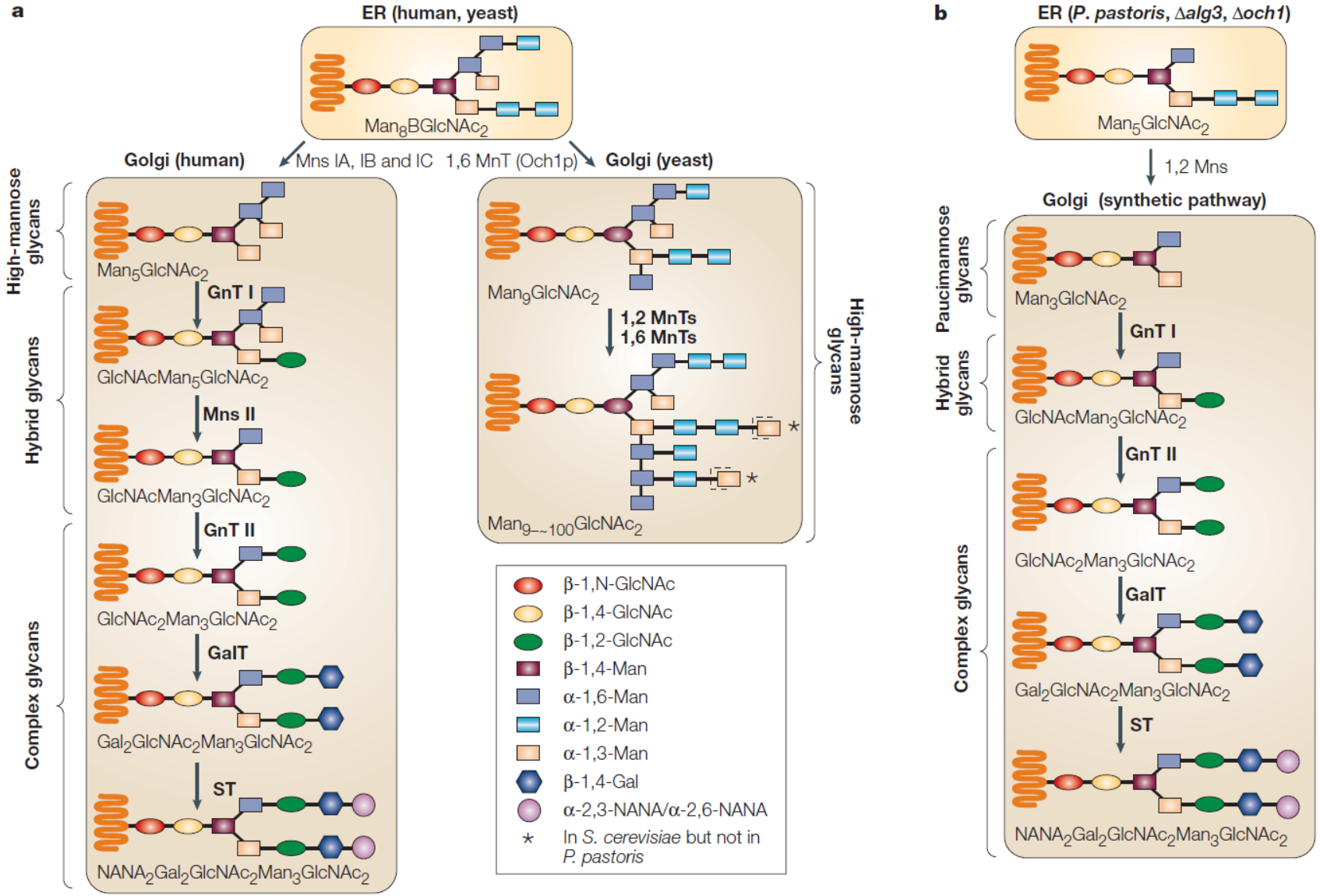


Figure 1 | **Major *N*-glycosylation pathways in humans and yeast. a** | Representative pathway of *N*-glycosylation pathways in humans (left) provides a template for humanizing *N*-glycosylation pathways in yeast (right). **b** | Early oligosaccharide assembly mutants can be used to recreate synthetic glycosylation pathways that lead to complex *N*-glycosylation in yeast (see main text). ER, endoplasmic reticulum; GalT, galactosyltransferase; GlcNAc, *N*-acetylglucosamine; GnT I, *N*-acetylglucosaminyl transferase I; GnT II, *N*-acetylglucosaminyl transferase II, Man, mannose; Mns II, mannosidase II; MnTs, mannosyltransferase; NANA, *N*-acetylneuraminic acid; ST, sialyltransferase.

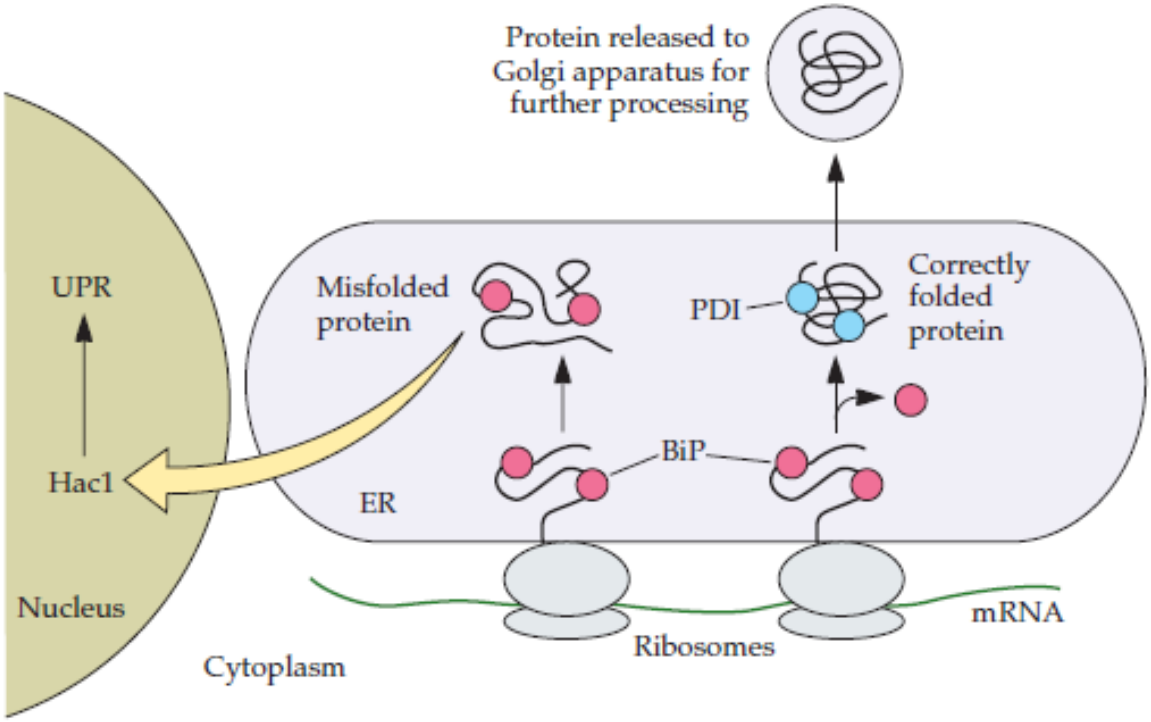


FIGURE 7.8 Summary of protein folding in the endoplasmic reticulum of yeast cells. During synthesis on ribosomes associated with the endoplasmic reticulum (ER), nascent proteins are bound by the chaperones BiP and calnexin, which aid in the correct folding of the protein. Protein disulfide isomerases (PDI) catalyze the formation of disulfide bonds between cysteine amino acids that are nearby in the folded protein. Quality control systems ensure that only correctly folded proteins are released from the ER. Proteins released from the ER are transported to the Golgi apparatus for further processing. Prolonged binding of BiP to misfolded proteins leads to activation of the *S. cerevisiae* transcription factor Hac1, which controls the expression of several proteins that mediate the unfolded-protein response (UPR). Adapted from Gasser et al., *Microb. Cell Fact.* **7**:11–29, 2008.

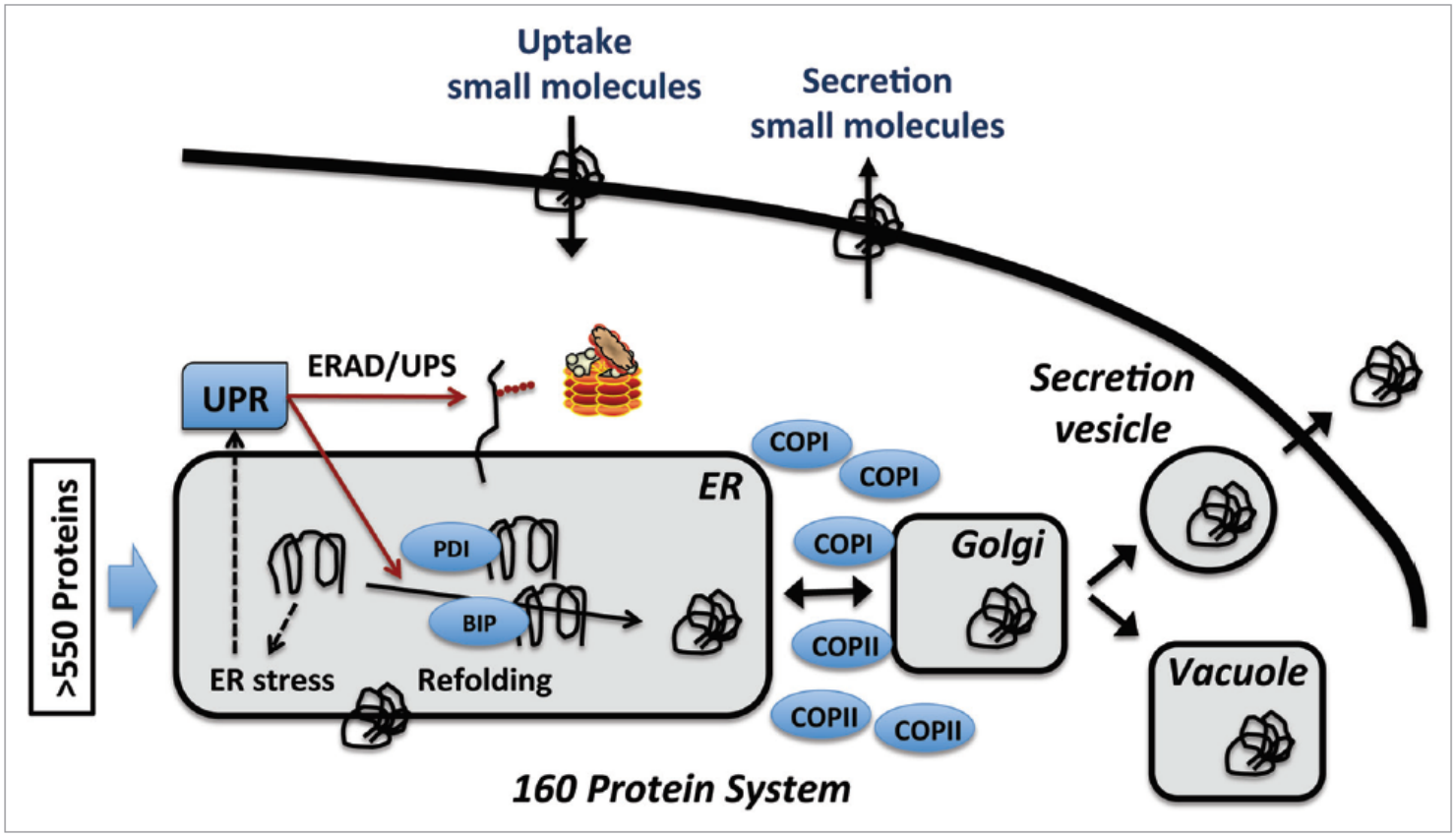


Figure 1. Schematic overview of the secretory pathway in yeast. Proteins targeted for secretion enter the endoplasmic reticulum (ER). If they fold correctly they can enter the secretory pathway, whereas misfolded protein cause ER stress leading to the activation of the unfolded protein response (UPR) that results in activation of a very large number of cellular processes, including activation of chaperones and foldases (like BIP and PDI) that assist with refolding. UPR is also upregulating ER-associated degradation (ERAD) where the unfolded proteins are exported from the ER, ubiquitinated and hereby targeted for degradation by the proteasome (ubiquitin-proteasome system, UPS). Correctly folded proteins can be exported to the Golgi for further processing (including additional glycosylation). The COPI- and COPII-complexes facilitate the ER-Golgi transfer, and from the Golgi the protein may be secreted via the endosome or be targeted to the vacuole for storage and/or degradation. Different colors represent different types of vesicular compartments of the secretory pathway.

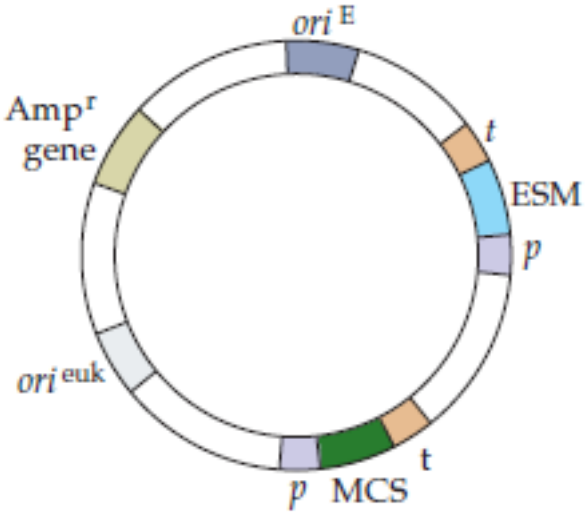


FIGURE 7.4 Generalized eukaryotic expression vector. The major features of a eukaryotic expression vector are a eukaryotic transcription unit with a promoter *(p)*, a multiple cloning site (MCS) for a gene of interest, and a DNA segment with termination and polyadenylation signals *(t)*; a eukaryotic selectable marker (ESM) gene system; an origin of replication that functions in the eukaryotic cell (ori^{euk}); an origin of replication that functions in *E. coli* (ori^{E}); and an *E. coli* selectable marker (Amp^r) gene.

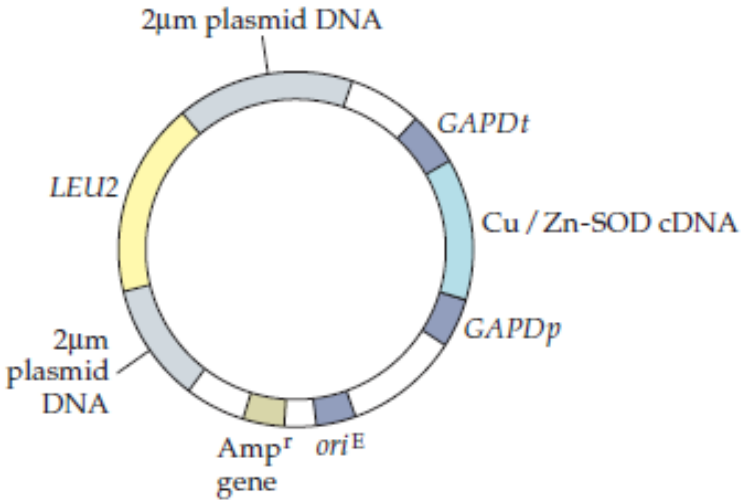


FIGURE 7.7 *S. cerevisiae* expression vector. The cDNA for human Cu/Zn-SOD was cloned between the promoter *(GAPDp)* and termination–polyadenylation sequence *(GAPDt)* of the *S. cerevisiae* glyceraldehyde phosphate dehydrogenase gene. The *LEU2* gene that was cloned between segments of the yeast 2µm plasmid DNA encodes a functional enzyme of the leucine biosynthesis pathway. The yeast origin of replication is included in the 2µm plasmid DNA. The ampicillin resistance (Amp^r) gene and the *E. coli* origin of replication (ori^{E}) are derived from plasmid pBR322.

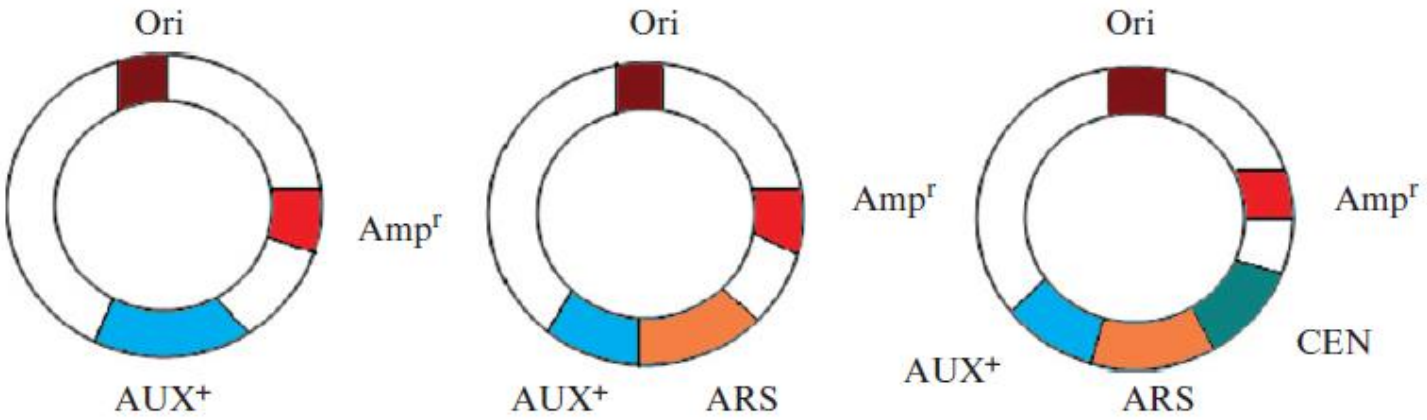


Fig. 16.1 Yeast transforming vectors. Ori: origin of replication; Amp^r: ampicillin resistant gene; AUX^+: wild type allele of yeast auxotrophic marker; ARS: autonomous replication sequence; CEN: centromere sequences from yeast

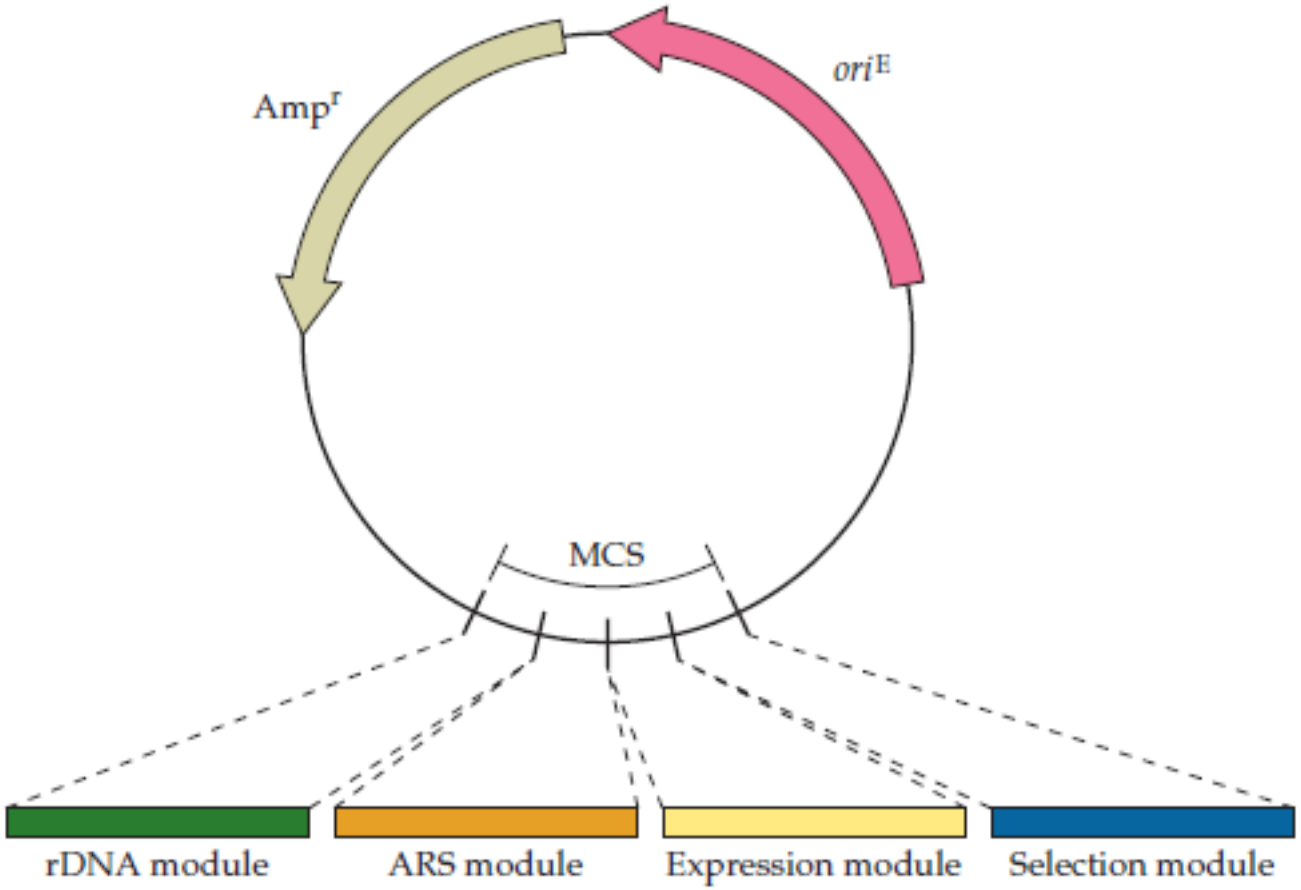
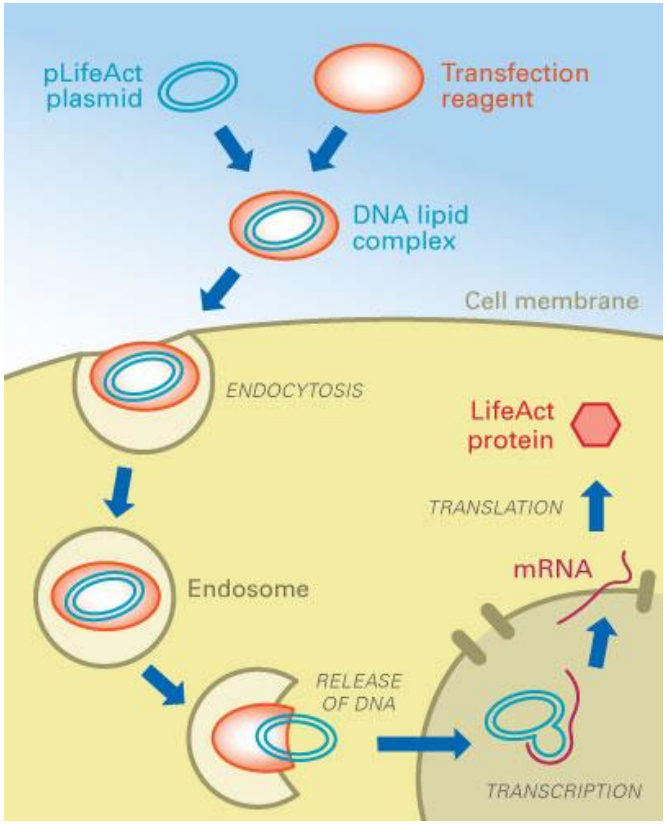
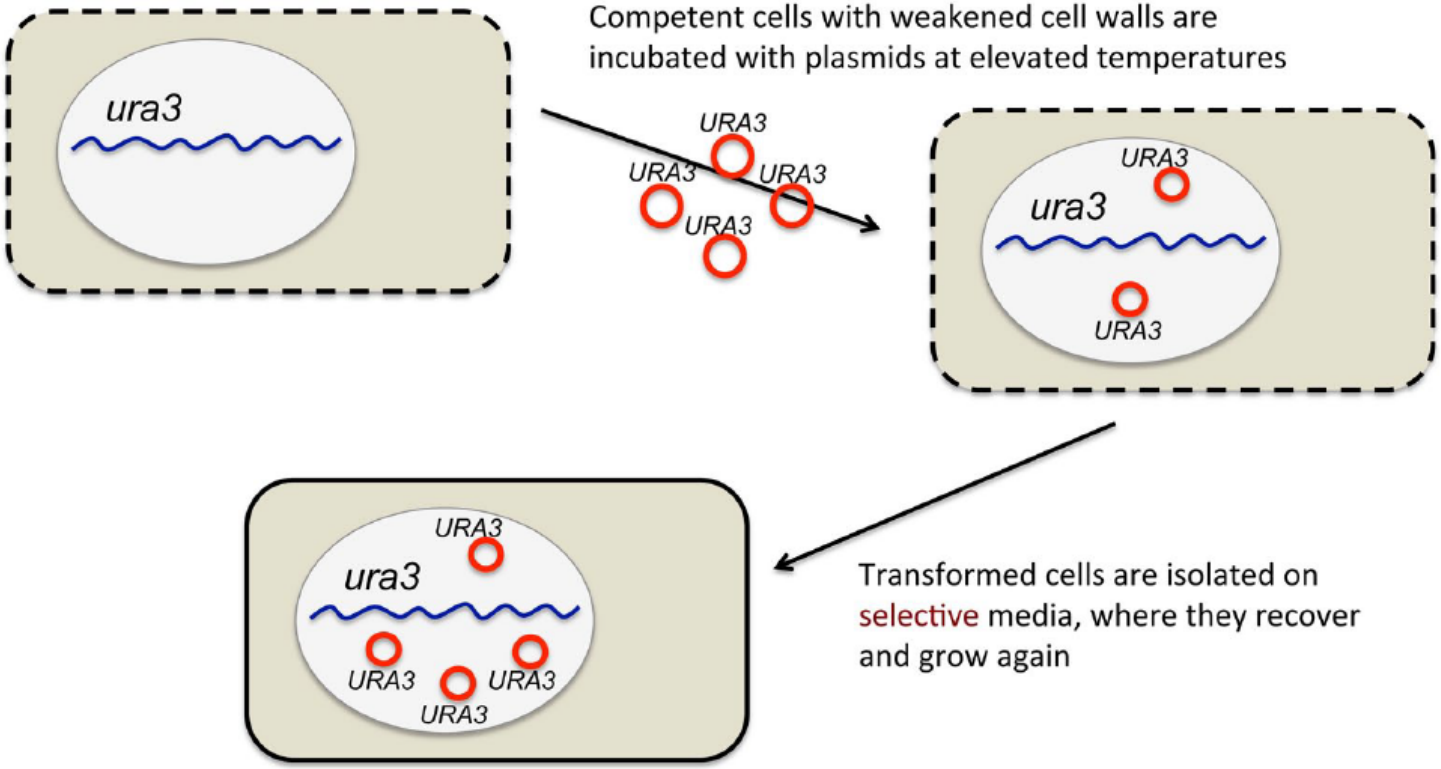


FIGURE 7.13 A wide-range yeast vector system for expression of heterologous genes in several different yeast hosts. The basic vector contains a multiple cloning site (MCS) for insertion of selected modules containing appropriate sequences for chromosomal integration (rDNA module), replication (ARS module), selection (Selection module), and expression (Expression module) of a target gene in a variety of yeast host cells (Table 7.3 shows examples of interchangeable modules). Sequences for maintenance (ori^E) and selection (Amp^r) of the vector in *E. coli* are also included.

Methods of DNA transfer

- Chemical transformation of intact cells and spheroplasts
- Electroporation
- Micromanipulation
- Biolistic method
- Glass beads method
- Magnetic beads method
- Liposome transformation
- Agrobacterium-based method





Transformation and plasmid complementation

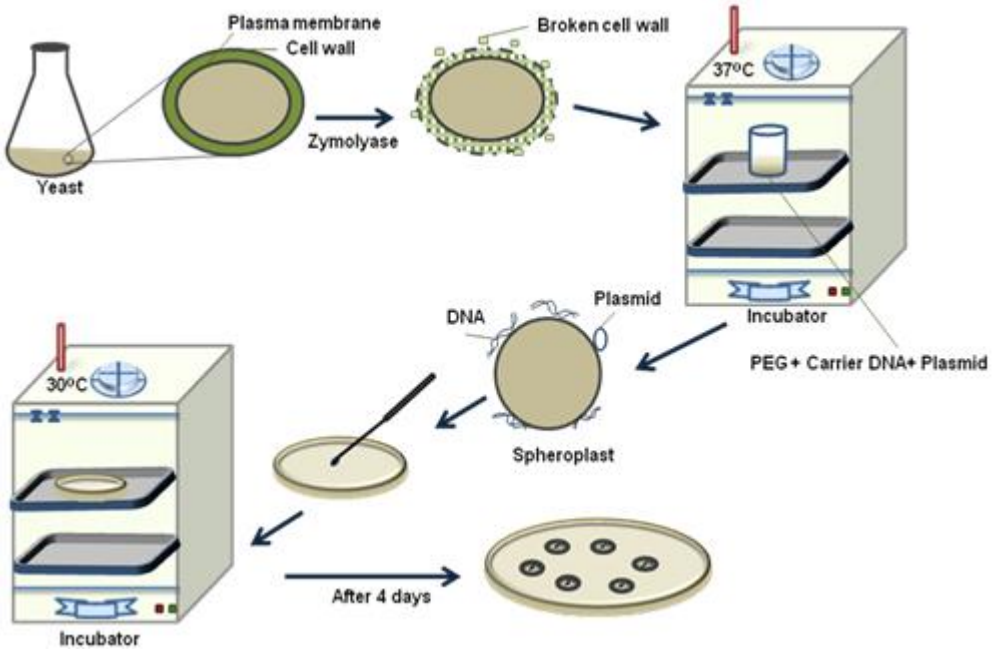
Competent *ura3* yeast cells are transformed incubating cells with a plasmid containing the yeast *URA3* gene at an elevated promoter (top). Transformed cells are selected on media that does not contail uracil (bottom).

Table 1. Protocol for the spheroplast method developed by Burgers and Percival[7]

Centrifuge cells and spheroplasts at 400–600 g and 200–300 g, respectively.

1. Grow the cells overnight with vigorous aeration in 50 ml of YPD (1% yeast extract, 2% bactopeptone, and 2% dextrose) to a concentration of about 3 x 10^7 cells/ml and harvest.
2. Wash the cells successively with 20 ml of sterile water and 20 ml of 1 M sorbitol by resuspension, followed by 5-min spins. Resuspend them in 20 ml of SCEM [1 M sorbitol, 0.1 M sodium citrate (pH 5.8), 10 mM EDTA and 30 mM 2-mercaptoethanol], add 1,000 U of lyticase, and incubate at 30°C with occasional inversion.
3. After spheroplasting, measure the decrease in the OD_{800} of a 10-fold dilution of spheroplasts in water. Harvest the spheroplasts for 3–4 min when the spheroplasting proceeds to 90% (~15–20 min).
4. Gently resuspend the spheroplasts in 20 ml of 1 M sorbitol by using a 1-ml pipette and pellet for 3–4 min. Then, gently resuspend them in 20 ml of STC [1 M sorbitol, 10 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$] and pellet again for 3–4 min. Resuspend this pellet in 2 ml of STC.
5. Mix aliquots (100 µl) with plasmid DNA and carrier DNA (calf thymus or *E. coli*) added to a total of 5 µg of DNA in <10 µl.
6. After 10 min at room temperature, add 1 ml of PEG [10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and 20% PEG 8000; filter-sterilized], gently resuspend the spheroplasts, and harvest them for 4 min after another 10 min.
7. Resuspend the pellet in 150 µl of SOS (1 M sorbitol, 6.5 mM $CaC1_2$, 0.25% yeast extract and 0.5% bactopeptone; filter-sterilized) and leave at 30°C for 20–40 min. Dilutions of the spheroplasts are made in the same medium.
8. Add 8 ml of TOP [1 M sorbitol and 2.5% agar in selective SD medium (0.67% yeast nitrogen base and 2% glucose)] kept at 45–46°C. Invert the tube quickly several times to mix and plate the suspension immediately on selective SORB plates (SD plates containing 0.9 M sorbitol and 3% glucose).

Spheroplast transformation



Lithium acetate transformation

Table 2. Original protocol for the lithium method developed by Ito et al.[2]

1. Grow the yeast cells aerobically on 100 ml of YPD medium at 30°C with reciprocation. At the mid-log phase, harvest the cells by centrifugation, wash once with TE [10 mM Tris-HCl (pH 8.0) and 1.0 mM EDTA] and suspend in TE to a final concentration of 2 x 10^8 cells/ml.
2. To a 0.5-ml portion of this cell suspension, add an equal volume of 0.2 M metal ions (LiAc). After 1 h at 30°C with shaking (140 rpm; stroke, 7.0 cm), incubate 0.1 ml of the cell suspension statically with 15 μl of a plasmid DNA solution (670 μg/ml) at 30°C for 30 min.
3. Add an equal volume of 70% PEG 4000 dissolved in water and sterilized at 120°C for 15 min and mix thoroughly on a vortex mixer. After standing for 1 h at 30°C, incubate the suspension at 42°C for 5 min.
4. Immediately cool the cells to room temperature, wash twice with water, and suspend in 1.0 ml of water.
5. For selecting the yeast transformants, directly spread 0.1 ml of the cell suspension on selective solid medium.

Table 3. Protocol for the LiAc/single-stranded carrier DNA/PEG method developed by Gietz and Woods[11]

1. Inoculate the yeast strain into 5 ml of liquid medium (2x YPAD or synthetic complete [SC] selection medium) and incubate overnight at 30°C. Place a bottle of double-strength YPAD broth (2x YPAD) and 250 ml culture flask in the incubator as well.
2. Determine the titer of the yeast culture by measuring the OD_{600} of a solution of 10 μl of the cells added to 1.0 ml of water in a spectrophotometer cuvette. For many yeast strains, a suspension containing 1 x 10^6 cells/ml will give an OD_{600} of 0.1.
3. Transfer 50 ml of the pre-warmed 2x YPAD to the pre-warmed culture flask and add 2.5 x 10^8 cells to give a density of 5 x 10^6 cells/ml. Incubate the flask on a rotary or reciprocating shaker at 30°C and 200 rpm. (Note: It is important to allow the cells to complete at least 2 divisions. Transformation efficiency remains constant for 3 to 4 cell divisions).
4. When the cell titer is at least 2 x 10^7 cells/ml, which should take about 4 h, harvest the cells by centrifugation, wash the cells in 25 ml of sterile water, and wash again in 1 ml of sterile water.
5. Add water to a final volume of 1.0 ml and vigorous vortex-mixing to resuspend the cells. Pipette 100 μl samples (~10^8 cells) into 1.5-ml microcentrifuge tubes, one for each transformation, centrifuge at top speed for 30 s, and discard the supernatant.
6. Add 360 μl of transformation mix, consisting of 240 μl PEG 3350 [50% (w/v)], 36 μl LiAc (1.0 M), 50 μl boiled single-stranded DNA (2.0 mg/ml), and 34 μl plasmid DNA plus water, to each transformation tube and resuspend the cells by vigorous vortex-mixing.
7. Incubate the tubes in a 42°C water bath for 40 min. [Note: The optimum time can vary for different yeast strains].
8. Microcentrifuge at top speed for 30 s and remove the transformation mix with a micropipette. Pipette 1.0 ml of sterile water into each tube, stir the pellet with a micropipette tip, and vortex.
9. Plate appropriate dilutions of the cell suspension onto SC selection medium.

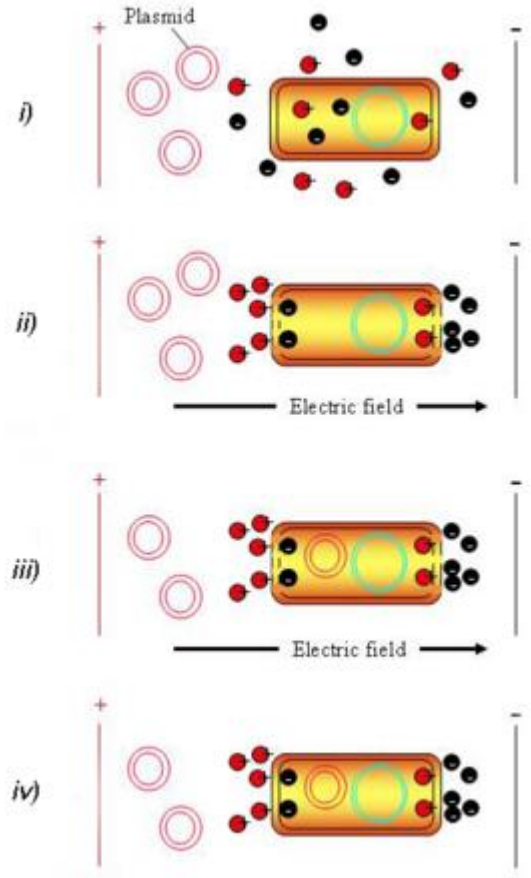
Table 5. Effects of LiAc, heat shock and PEG on the transformation of intact cells and spheroplasts

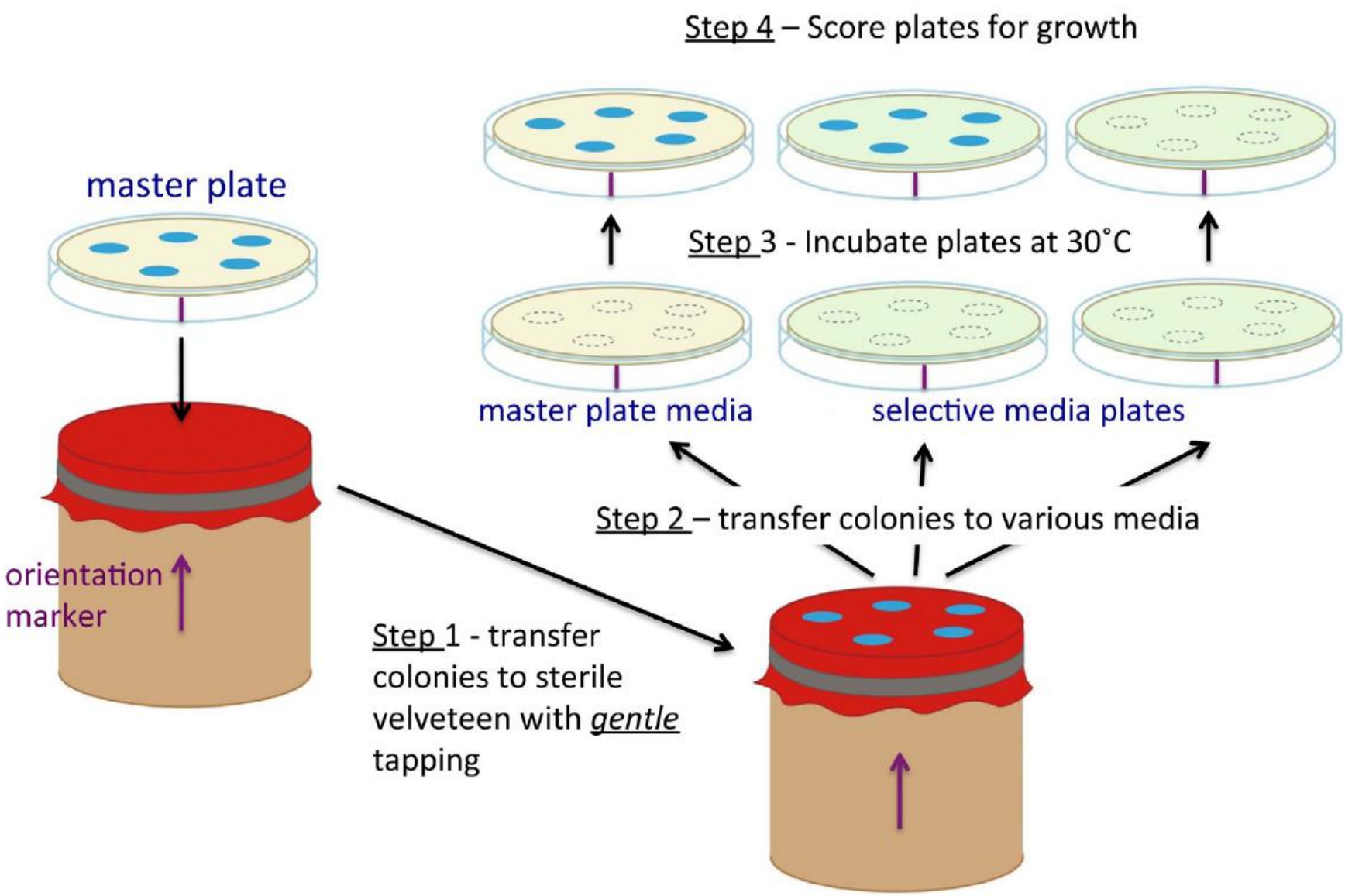
	Intact cells	Spheroplasts
LiAc	Enhances the transformation efficiency and frequency (although not indispensable).[2,38]	No effect on the transformation frequency.[38]
	Increases the permeability of intact cells.[37]	
Heat shock	Enhances the transformation efficiency (although not indispensable).[2,15]	No effect on the transformation efficiency.[74]
	Increases the permeability of intact cells.[37]	
PEG	Indispensable for transformation efficiency.[2,15]	Not indispensable for transformation frequency but enhances the frequency.[38]
	Pre-incubation enhances the transformation efficiency and frequency.[36]	
	Increases the permeability of intact cells.[37]	
	Indispensable for DNA attachment.[38]	Indispensable for DNA attachment.[38]

Electroporation

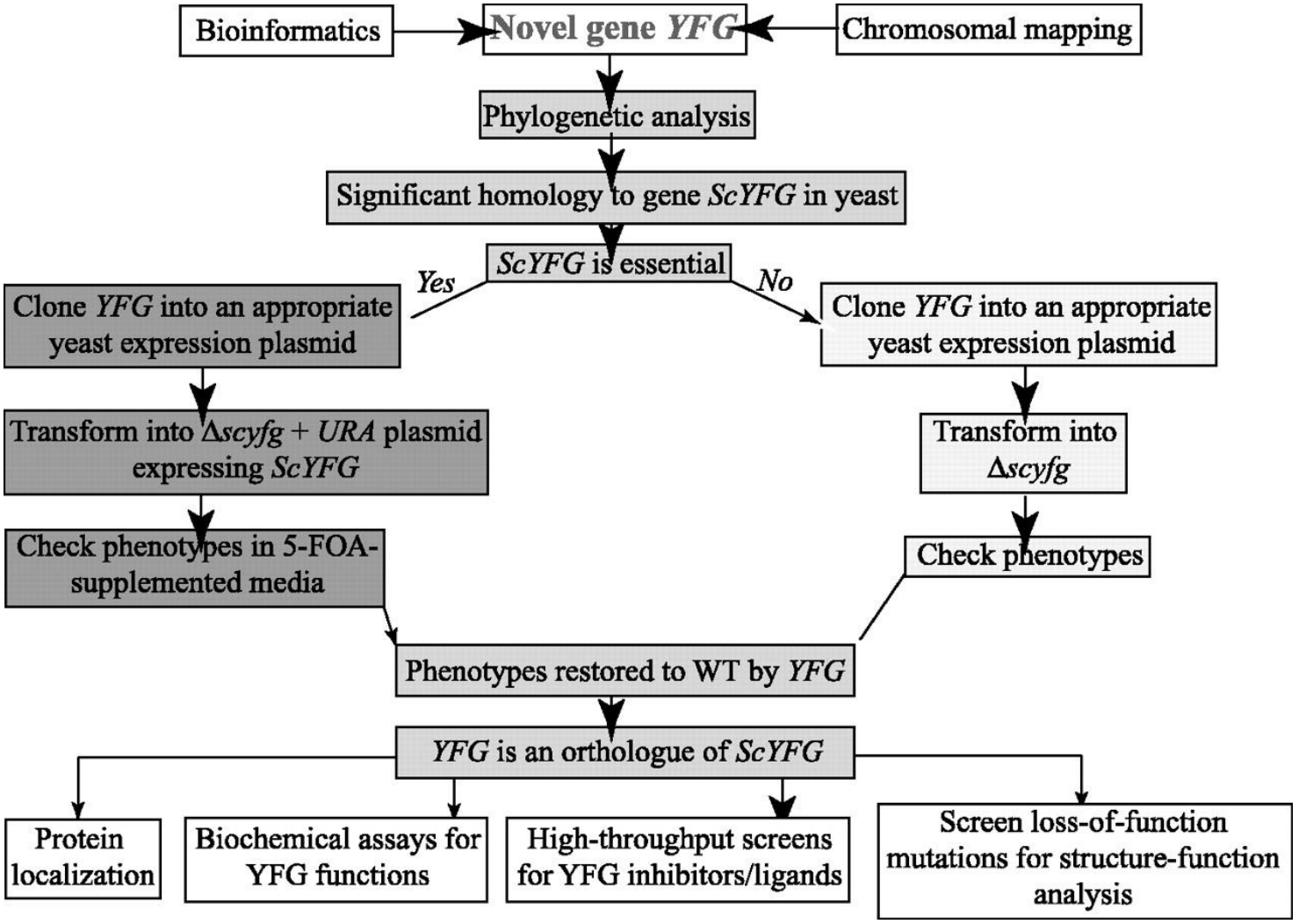
Table 4. Protocol for electroporation of frozen competent cells developed by Suga and Hatakeyama[18]

1. Grow *S. pombe* cells in SD medium supplemented with appropriate nutrients to a density of approximately 1 x 10^7 cells/ml at 30°C. Grow *S. cerevisiae* cells in YPD medium to a density of approximately 1 x 10^7 cells/ml at 30°C.
2. Place the cultures on ice for 15 min just before harvesting. Collect the cells by centrifugation and wash the resulting pellet thrice with ice-cold sterilized water. Suspend this pellet in ice-cold freezing buffer containing 0.6–2.5 M sorbitol, 5–10 mM $CaCl_2$ and 10 mM 2-(4-[2-hydroxyethyl]-1-piperazinyl)ethanesulphonic acid (HEPES; pH 7.5) to give a density of approximately 5 x 10^8 cells/ml.
3. Dispense aliquots (0.1 ml) of the cell suspension in 1.5-ml microcentrifuge tubes, slowly freeze them, and store by placing them directly in a -80°C freezer (cooling rate = ~10°C/min).
4. For each electroporation, quickly thaw the frozen competent cells in a water bath at 30°C (warming rate = ~200°C/min) and wash once with 1 ml of ice-cold 1.0 M sorbitol by centrifugation. Resuspend the final pellet in 1.0 M sorbitol to give a density of 1–2 x 10^9 cells/ml.
5. Mix the cell suspension with 0.5–10.0 ng of purified plasmid DNA and then transfer to a chilled cuvette with a 0.2-cm electrode gap. Apply a high electric pulse to the cell suspension, by using the Bio-Rad Gene Pulser II with Pulse Controller Plus.
6. Immediately dilute the electroporated cells in 1 ml of ice-cold 1.0 M sorbitol and spread an aliquot (0.1–0.2 ml) on minimal selection plates. For *S. cerevisiae*, the minimal selection plates contain 1.0 M sorbitol as an osmotic stabilizer.
7. The transformant colonies appear within 4–6 days at 30°C.




Step 4 – Score plates for growth
master plate
Step 3 - Incubate plates at 30˚C
master plate media
selective media plates
Step 2 – transfer colonies to various media
orientation marker
Step 1 - transfer colonies to sterile velveteen with gentle tapping

Strategies for heterologous expression in yeast



Therapeutic proteins

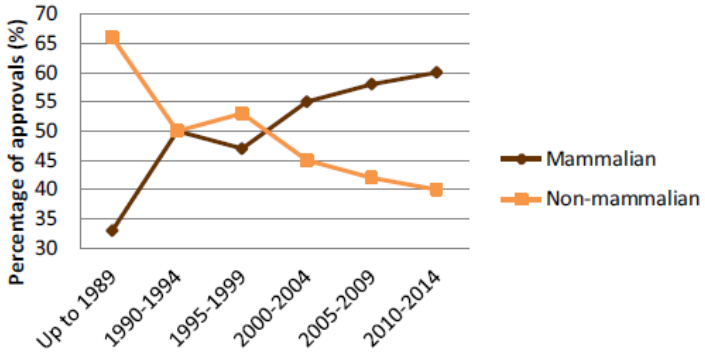


Fig. 1 Number of recombinant protein products approved for use as drugs in humans, depending on the type of production platform

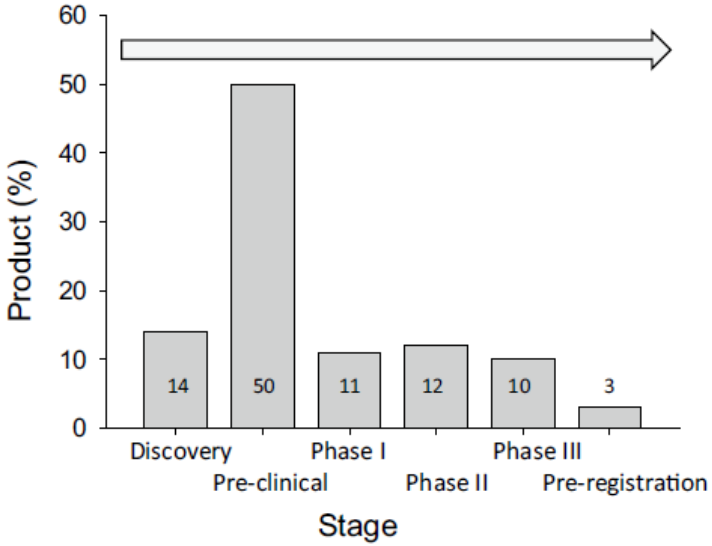


Fig. 2 Workflow involved in the development of a new drugs and approximate percentage (*bars* and *numbers*) of recombinant proteins currently in each step [9]

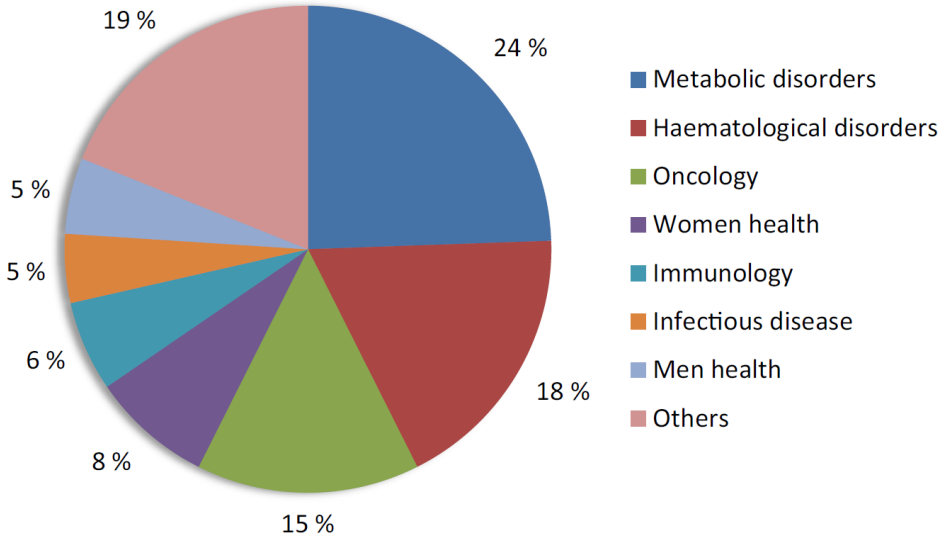
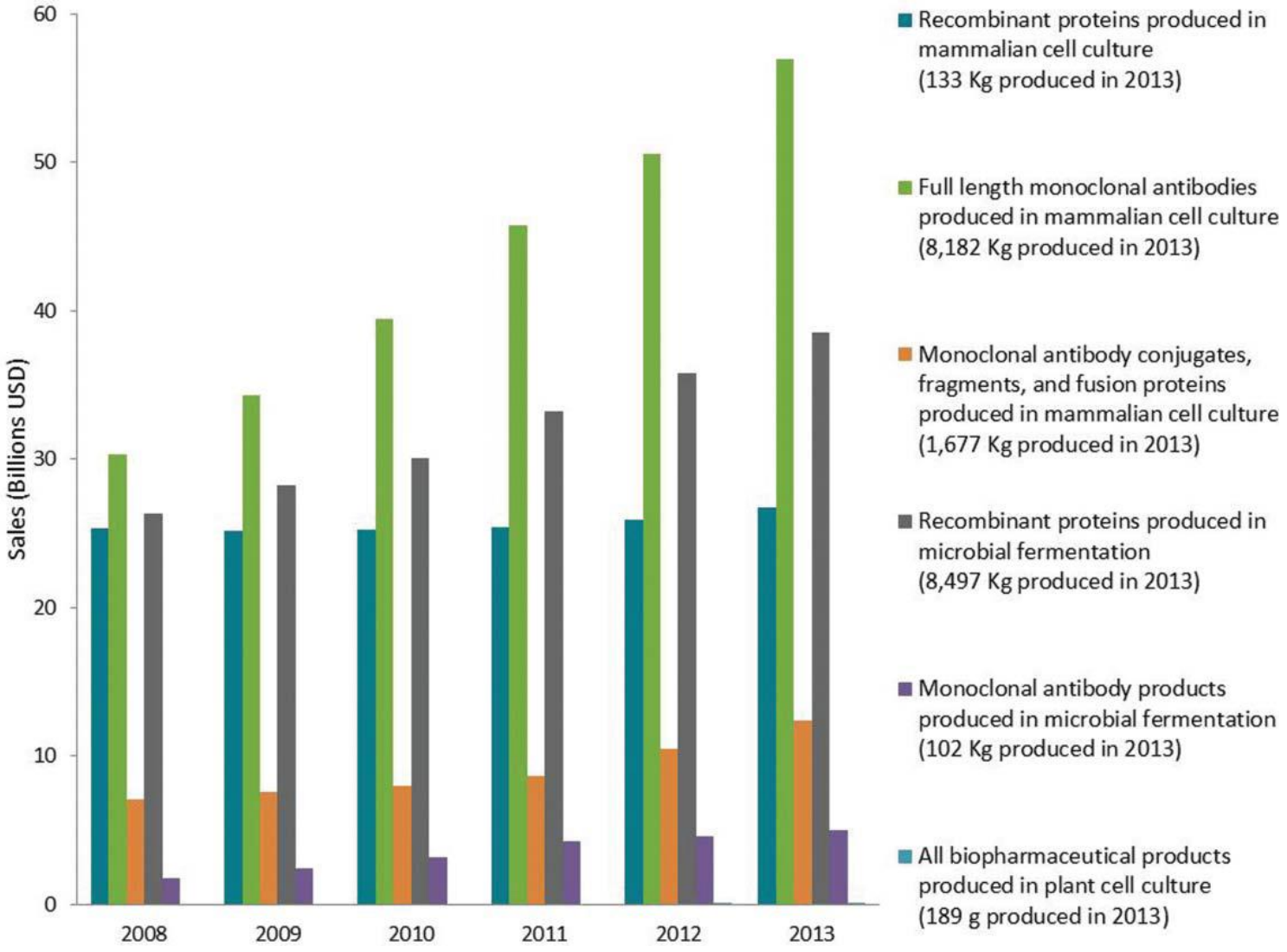


Fig. 3 Amount of marketed recombinant proteins (expressed in percentages) applied to each therapeutic area. Coloured in pink, other therapeutic areas (<5 % each) include diseases related to cardiology, central nervous system, ophthalmology and dermatology among others

Biopharmaceuticals production



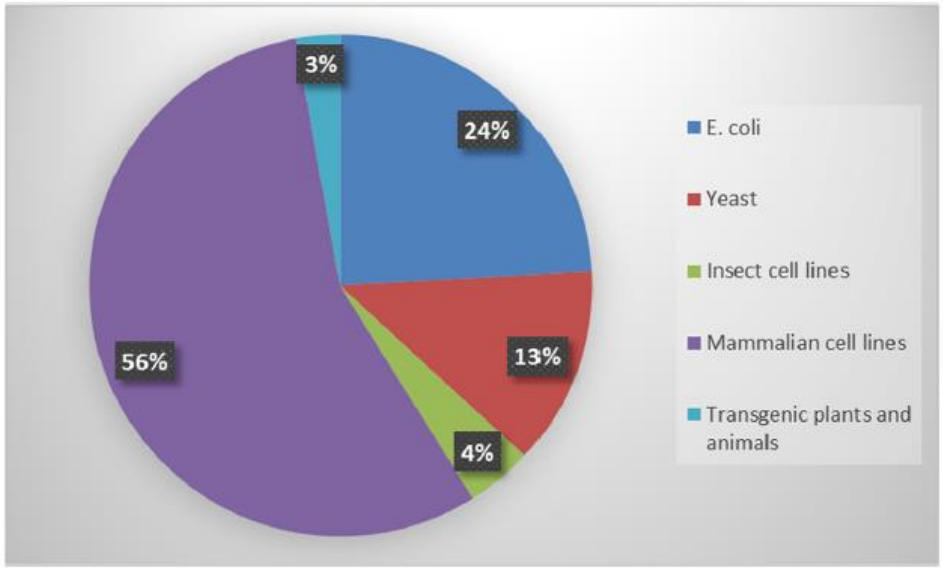


Figure 1 Percentage of biopharmaceuticals produced in different expression systems [5-13].

Table 1 Comparison of human insulin production systems [70]

Source	*E. coli*	*E. coli*	*S. cerevisiae*	*P. pastoris*
Destination of product	Cytoplasm	Secreted	Secreted	Secreted
Biomass cell dry weight (g/l)	80, in bioreactor with fed-batch culture	1.2, in shake flask with batch culture	5, in shake flask with batch culture	59, in bioreactor with fed-batch culture
Typical spec. growth rate (1/h)	0.08– 0.12	not specified	< 0.33	<0.03
Typical spec. production rate (mg/gh)	14.2	3.4	0.21	0.375
Product concentration (g/L)	4.34	0.009	0.075	3.075
Productivity (mg/l h)	1,085	4.01	1.04	17
Reference	[71]	[72]	[19]	[73]

HPV vaccine

- Virus-like particles
- Cervarix , Silgard, Gardasil, …
- Available in CZE since 2006

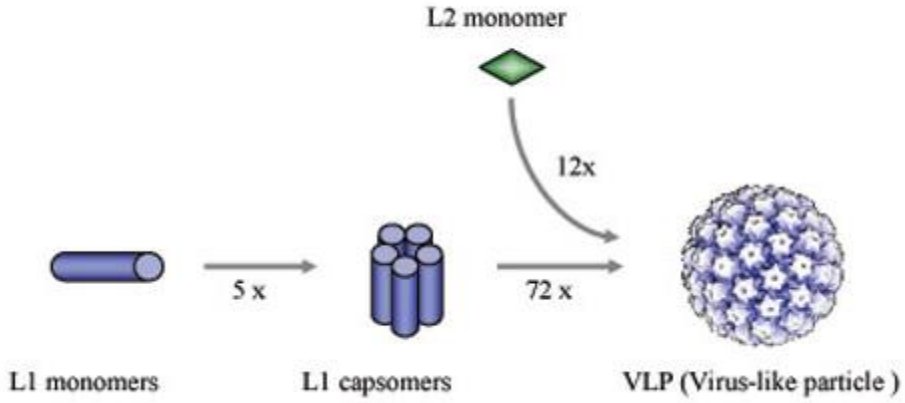
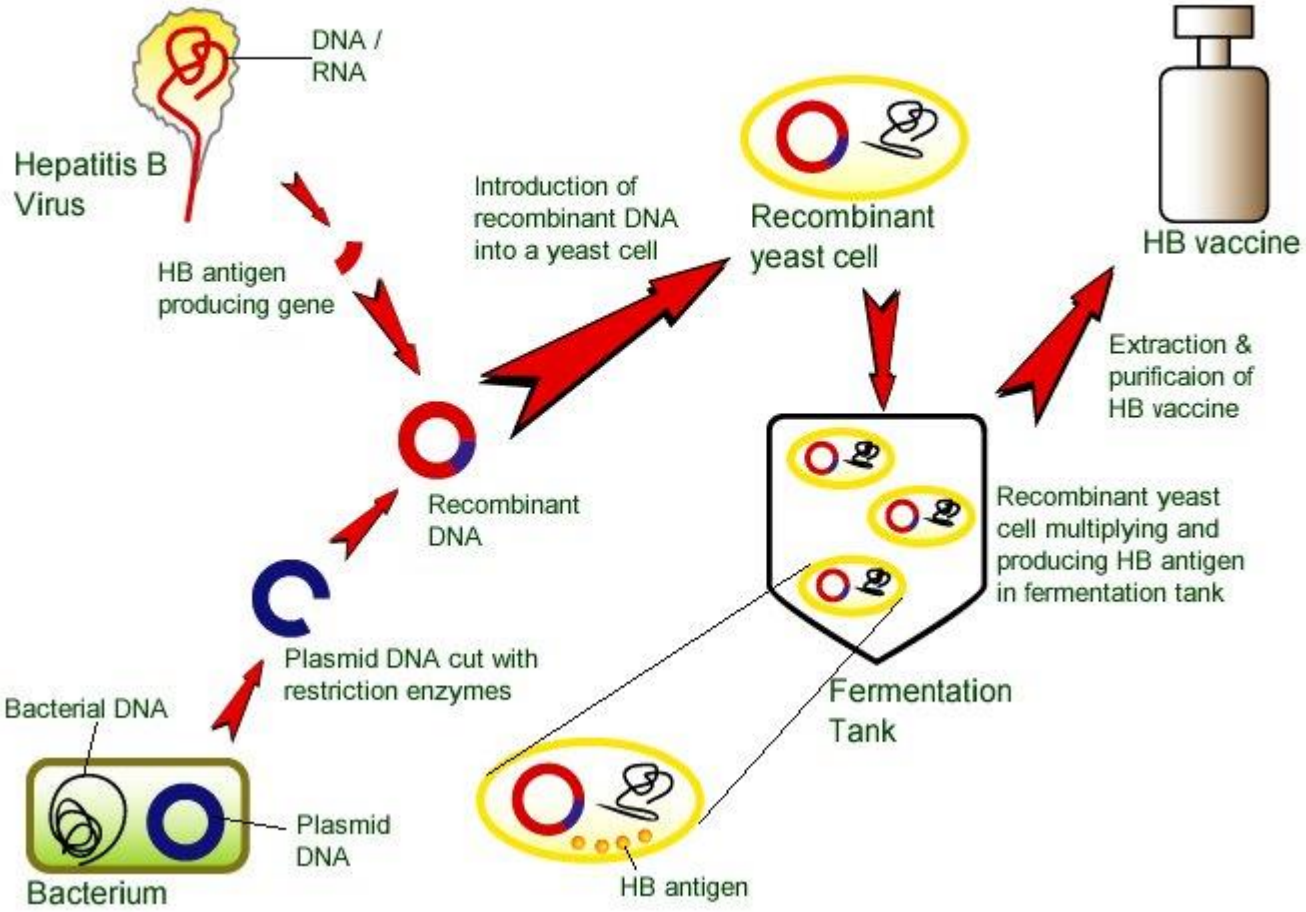


Table 1 Comparisons between Cervarix and Gardasil

Category	Cervarix	Gardasil
HPV types Included	HPV 16 and 18	HPV 16, 18, 6, 11
Production system	Insect cells infected with recombinant baculovirus	Yeast
Adjuvant	ASO4 (aluminium salt + MPL (3-O-desacyl-4'-monophosphoryl lipid A))	Alum
Diseases covered	Anogenital cancers, including cervical, vulval, vaginal, and anal cancers and their associated precursor lesions (and a subset of head and neck cancers)	Anogenital cancers, including cervical, vulval, vaginal, and anal cancers and their associated precursor lesions (and a subset of head and neck cancers) Genital warts and laryngeal papillomas
Available data regarding Length of protection	5.5 years	At least 5 years
Dose	0.5 mL dose containing 20 µg HPV 16 L1 and 20 µg HPV18 L1	0.5 mL dose containing 20 µg HPV6 L1, 40 µg HPV 11 L1, 40 µg HPV16 L1 and 20 µg HPV18 L1
Recommended administration Route and regimen	Three intramuscular injections at 0, 1, and 6 months	Three intramuscular injections at 0, 2, and 6 months
Recommended age group for vaccination	10–25	9–26
Price (US$)	Approx. $100 per dose	Approx. $120 per dose

Production of Recombinant HB Vaccine



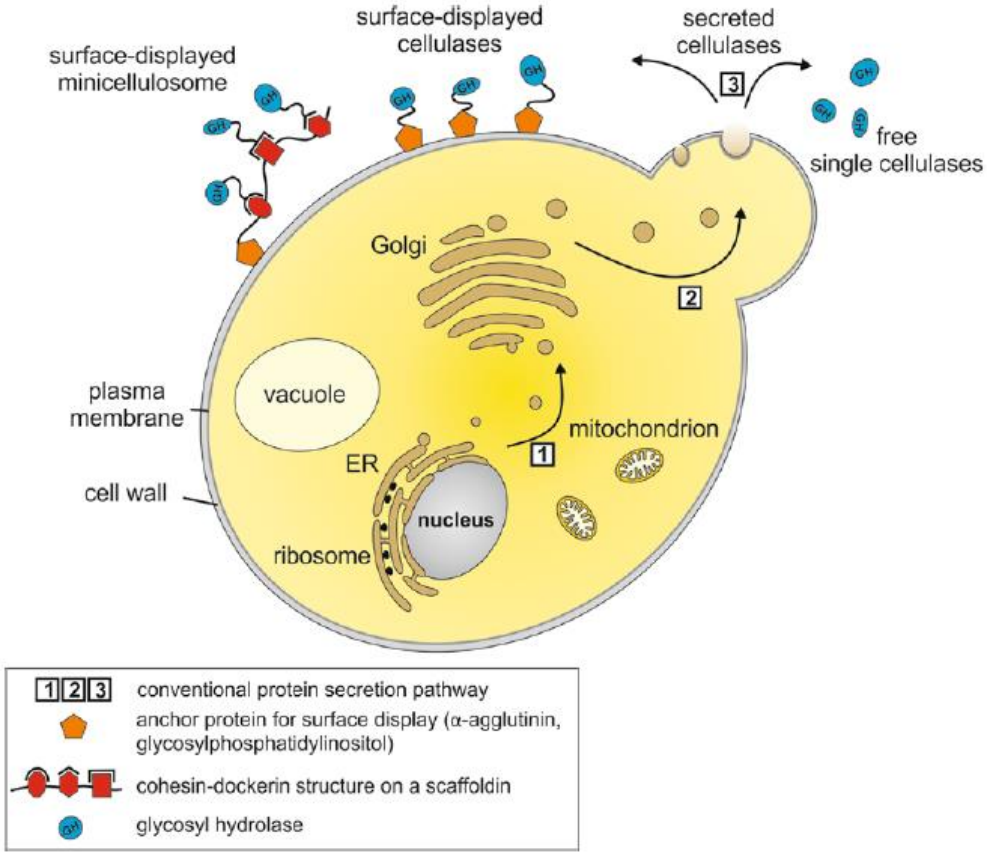


Figure 2 Illustration of a single yeast cell showing the main cell compartments involved in recombinant protein expression. Arrows indicate the secretory pathway, whereby cellulases are expressed on the ER (1) and transferred via the Golgi apparatus (2) to the medium (3) in secretory vesicles. Cellulases are either free in solution or surface-displayed via an anchor protein (such as α-agglutinin) as single cellulases or as mini-cellulosomes. ER: endoplasmic reticulum; GH: glycosyl hydrolase.

Saccharomyces cerevisiae

Favorable public acceptance
GRAS status
The most well studied of simple eukaryotes
Amenable to both classical genetics and modern recombinant DNA techniques
Versatile vector systems (episomal, integrative, copy-number regulated) are available (Invitrogen)
A wide range of mutant strains
Well-established fermentation and downstream processing
Hypermannosylation with immunogenic terminal α-1,3-linked mannose residues
Genome sequencing: Reference strain S288C; 12 157 Kb (6273 ORFs); Accession number PRJNA128

Table 16.2 Some commonly used *S. cerevisiae* vectors and their important features

Vector	Yeast sequences	Copy number/ cell	Transformation frequency/µg DNA	Stability	Advantages	Disadvantages	Reference
Yip	Homologous DNA	≥ 1	10^1–10^2	Less than 1%	• Provide most stable maintenance of cloned genes • Integrated YIp behave as genetic marker • Used to introduce inversions, deletions & transpositions	• Transformation frequency low	Hinnen et al., 1978
Yep (2µ based)	ORUI, STB, REP1, REP2, FLP	50–200	10^4	1%	• High copy no. plasmid • High transformation frequency • Readily recovered from yeast cells • Useful for complementation studies	• Novel recombinants can generate in vivo by recombination with endogeneous 2µm plasmids	Futcher and Cox, 1984
YCp	ARS/CEN	1–2	10^2–10^4	Less than 1%	• High transformation frequency • Useful for complementation studies • Show Mendelian segregation at meiosis • Low copy no is useful if product of gene is deleterious to cells	• Low copy no. • Recovery of vector is more difficult than YEp & YRp plasmids	Clarke and Carbon, 1980
YRp	ARS	1–20	10^3–10^4	20%	• High copy no. • Readily recovered from yeast	• Transformants are unstable	Murray, 1987
Ty/YIp	Ty & DNA	Depends on the vectors used to introduce Ty into the	≤ 20	Stable	• Amplification following chromosomal integration	• Needs to be introduced into cell in another vector	Sakai et al., 1991; Shuster et al., 1990
YAC	TEL, ARS, CEN			Stability depends upon length longer the YAC is more stable it is	• Very long DNA molecules > 40 Kb can be cloned	• Difficult to map by standard techniques	Sambrook and Russell, 2001

TABLE 7.2 Promoters for *S. cerevisiae* expression vectors

Promoter	Expression conditions	Status
Acid phosphatase *(PH05)*	Phosphate-deficient medium	Inducible medium
Alcohol dehydrogenase I *(ADHI)*	2–5% Glucose	Constitutive
Alcohol dehydrogenase II *(ADHII)*	0.1–0.2% Glucose	Inducible
Cytochrome c_1 *(CYC1)*	Glucose	Repressible
Gal-1-P Glc-1-P uridyltransferase	Galactose	Inducible
Galactokinase *(GAL1)*	Galactose	Inducible
Glyceraldehyde-3-phosphate dehydrogenase *(GAPD, GAPDH)*	2–5% Glucose	Constitutive
Metallothionein *(CUP1)*	0.03–0.1 mM Copper	Inducible
Phosphoglycerate kinase *(PGK)*	2–5% Glucose	Constitutive
Triosephosphate isomerase *(TPI)*	2–5% Glucose	Constitutive
UDP-galactose epimerase *(GAL10)*	Galactose	Inducible

Table 16.3 Promoter systems used for expression of heterologous proteins in *S. cerevisiae*

Promoter	Gene	Protein encoded	Regulation	Strength	References
Constitutive	*ADH1*	Alcohol dehydrogenase1		+++	Hitzeman et al., 1981; Bennetzen and Hall, 1982
	PYK1	Pyruvate kinase	20-fold induced by glucose	+++	Burke et al., 1983
	PGK1	Phosphoglycerate kinase		++++	Tuite et al., 1982; Dobson et al., 1982
	ENO	Enolase	10-fold induced by glucose		Holland et al., 1981
Regulated	*ADH2*	Alcohol dehydrogenase 2	1000 fold-induced by galactose	++	Johnston and Davis, 1984
	GAL1.10.7	Galactose metabolic enzymes	100-fold repressed by glucose	+++	
	GALS	Galactokinase variant	-	+++	Mumberg et al., 1995
	MET25	O-acetyl homoserine sulphydrylase	200-fold repressed by phosphate	+	
	CUP1	Copper metallothionein	20- fold induced by Cu^{2+}	+	Karin et al., 1984
	PHO5	Acid phosphatase	100 to 200-fold induction with inorganic phosphate	++	Meyhack et al., 1982; Kramer et al., 1984
	tetO-CYC1	Tetracycline promoter	1000-fold induction with tetracycline	+++	Gari et al., 1997
Heterologous	*CaMV*	Cauliflower mosaic virus 35S promoter	RAS/cAMP pathway		Ruth et al., 1992
	ARE	Androgen response element	Dihydrotestosterone/testosterone		Eldridge et al., 2007

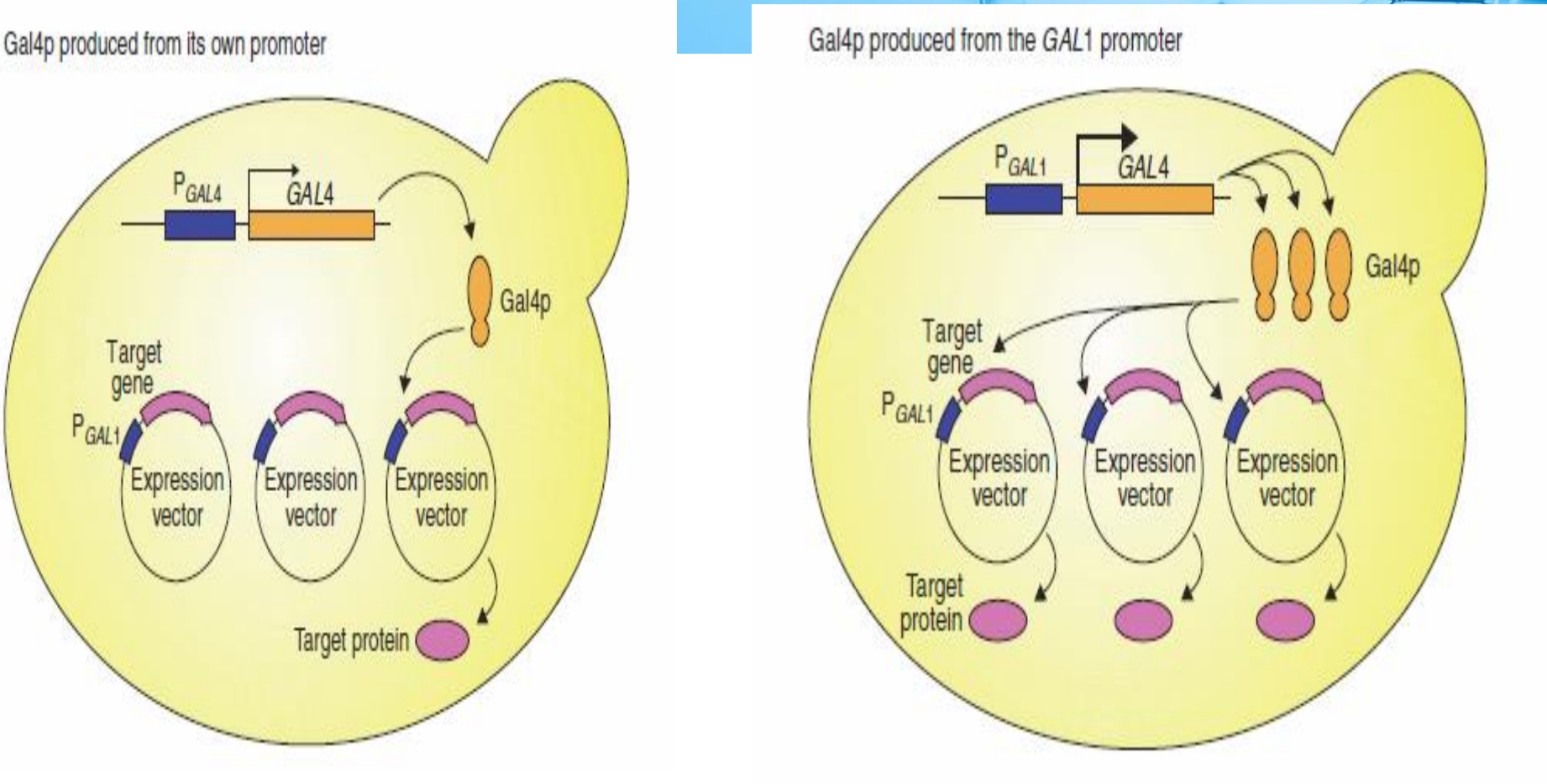


Figure: Galactose inducible gene expression in yeast. The expression of genes from multicopy vectors under the control of the GAL1 promoter (PGAL1*) can be increased* substantially if the gene encoding the transcriptional activator of GAL1, GAL4, is also placed under the control of PGAL1. In this case, induction by galactose will produce more Gal4p and consequently more of the target protein.

Table 16.1 Selectable markers used in *S. cerevisiae* transformation

Markers	Marker type: dominant (D)/ Auxotrophic(A)	Comments	References
URA3	A	(a) Selection possible in casamino acid (CAA)	
		(b) Counter selection with 5- fluoro-ortic acid (5-FOA)	Boeke et al., 1989
		(c) URA3-d for high copy no. selection	Loison et al., 1989
LYS2	A	(a) Counter selection using α-amino adipate	Barnes and Thorner, 1986; Chattoo et al., 1979; Fleig et al., 1986
TRP1	A	Selection in CAA	
HIS3	A		
LEU2	A	LEU2-d for high copy no. selection	
Cm^r(Chloramphenicol-resistance) gene	D	(a) Selection using chloramphenicol in glycerol medium	Hadfield et al., 1986
		(b) Effective only using yeast promoter	
Herpes simplex virus thymidine Kinase gene [HSV TK]	D	(a) Thymidine/ Sulphanilamide/ amethopterin selection	Zealey et al., 1988
		(b) The level of resistance dependent on gene dosage	
S. pombe triose phosphate isomerase gene	D	(a) Marker used in *S. cerevisiae* tpi^- host	Kawasaki, 1986
		(b) autoselection in glucose	
Tn903 Kam^r	D	(a) Selection using G418	Hadfield et al., 1990

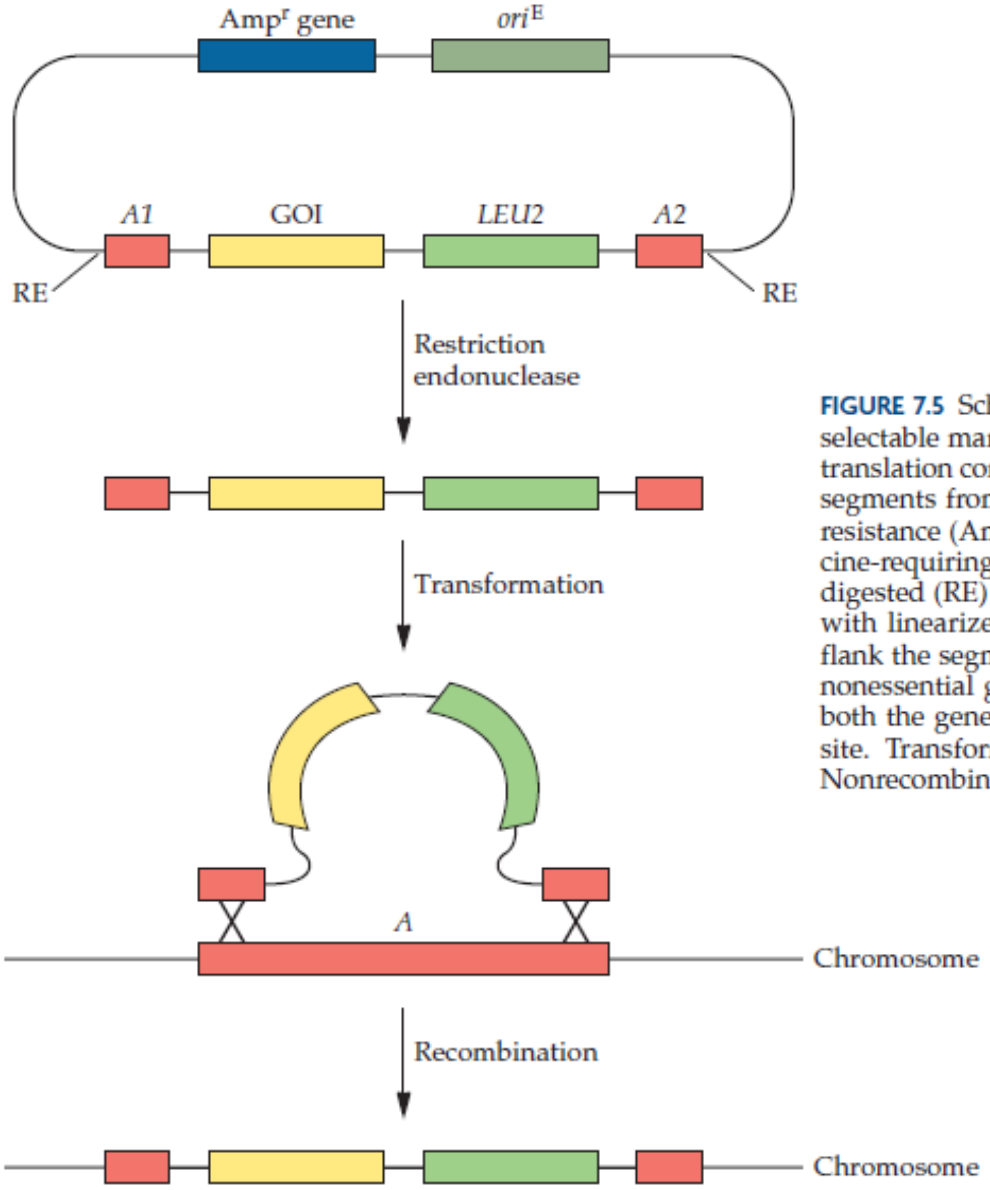


FIGURE 7.5 Schematic representation of integration of DNA with a YIp vector. A selectable marker gene *(LEU2)* and a gene of interest (GOI) with transcription and translation control elements (not shown) are inserted into a YIp vector between two segments from the ends of a nonessential yeast gene *(A1* and *A2)*. The ampicillin resistance (Amp^r) gene and the origin of replication (*ori*^{E}) function in *E. coli*. A leucine-requiring *(leu2)* yeast strain is transformed with restriction endonuclease-digested (RE) vector DNA because chromosomal DNA is more likely to recombine with linearized DNA than with circular DNA. The restriction endonuclease sites flank the segments from the nonessential gene. The DNA sequences at the ends of nonessential gene *A* undergo recombination (×) that leads to the incorporation of both the gene of interest and the *LEU2* gene into the corresponding chromosome site. Transformants grow on medium that is not supplemented with leucine. Nonrecombined DNA is degraded.

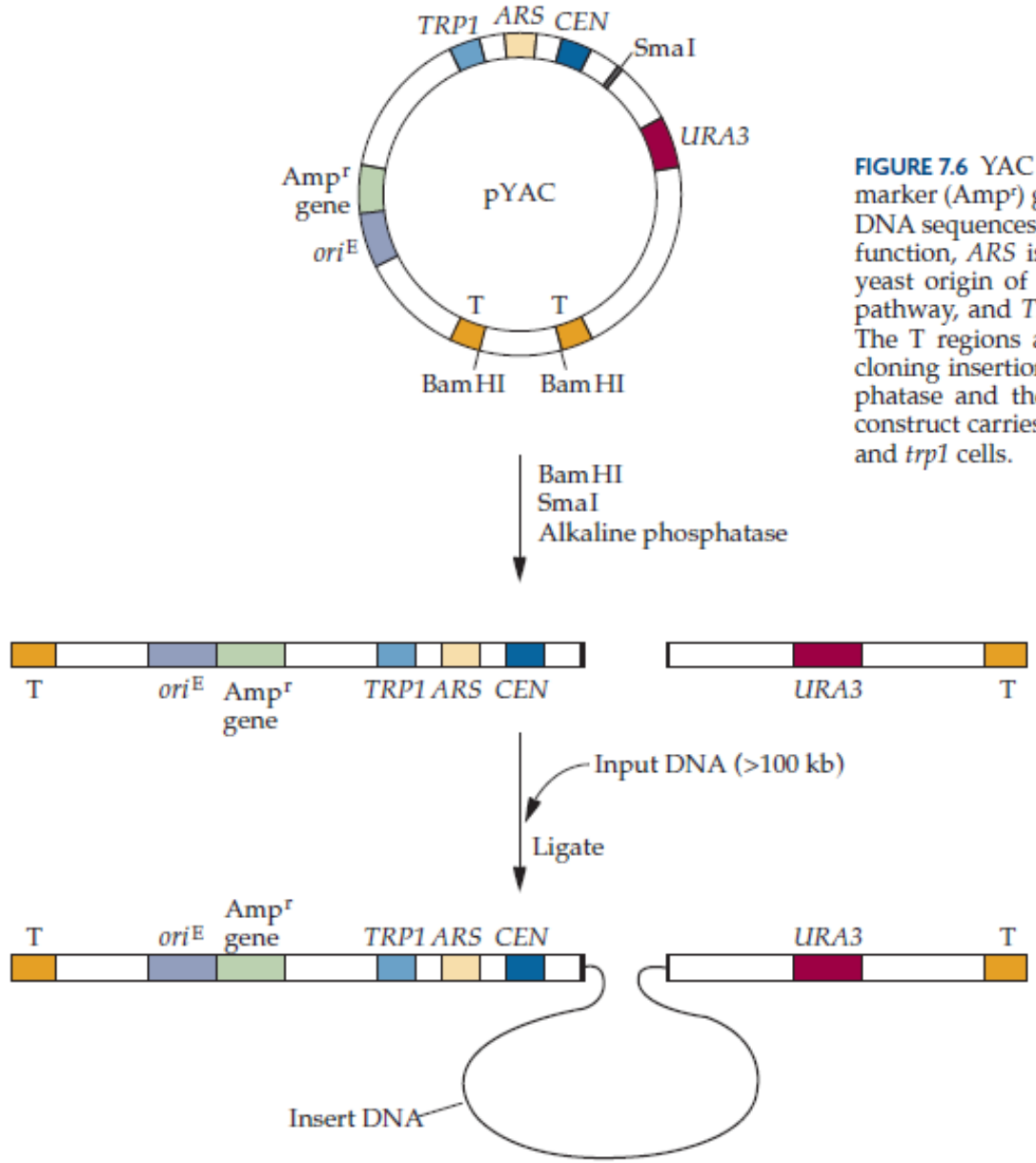


FIGURE 7.6 YAC cloning system. The YAC plasmid (pYAC) has an *E. coli* selectable marker (Amp^r) gene; an origin of replication that functions in *E. coli* (*ori*^{E}); and yeast DNA sequences, including *URA3*, *CEN*, *TRP1*, and *ARS*. *CEN* provides centromere function, *ARS* is a yeast autonomous replicating sequence that is equivalent to a yeast origin of replication, *URA3* is a functional gene of the uracil biosynthesis pathway, and *TRP1* is a functional gene of the tryptophan biosynthesis pathway. The T regions are yeast chromosome telomeric sequences. The SmaI site is the cloning insertion site. pYAC is first treated with SmaI, BamHI, and alkaline phosphatase and then ligated with size-fractionated (100-kb) input DNA. The final construct carries cloned DNA and can be stably maintained in double-mutant *ura3* and *trp1* cells.

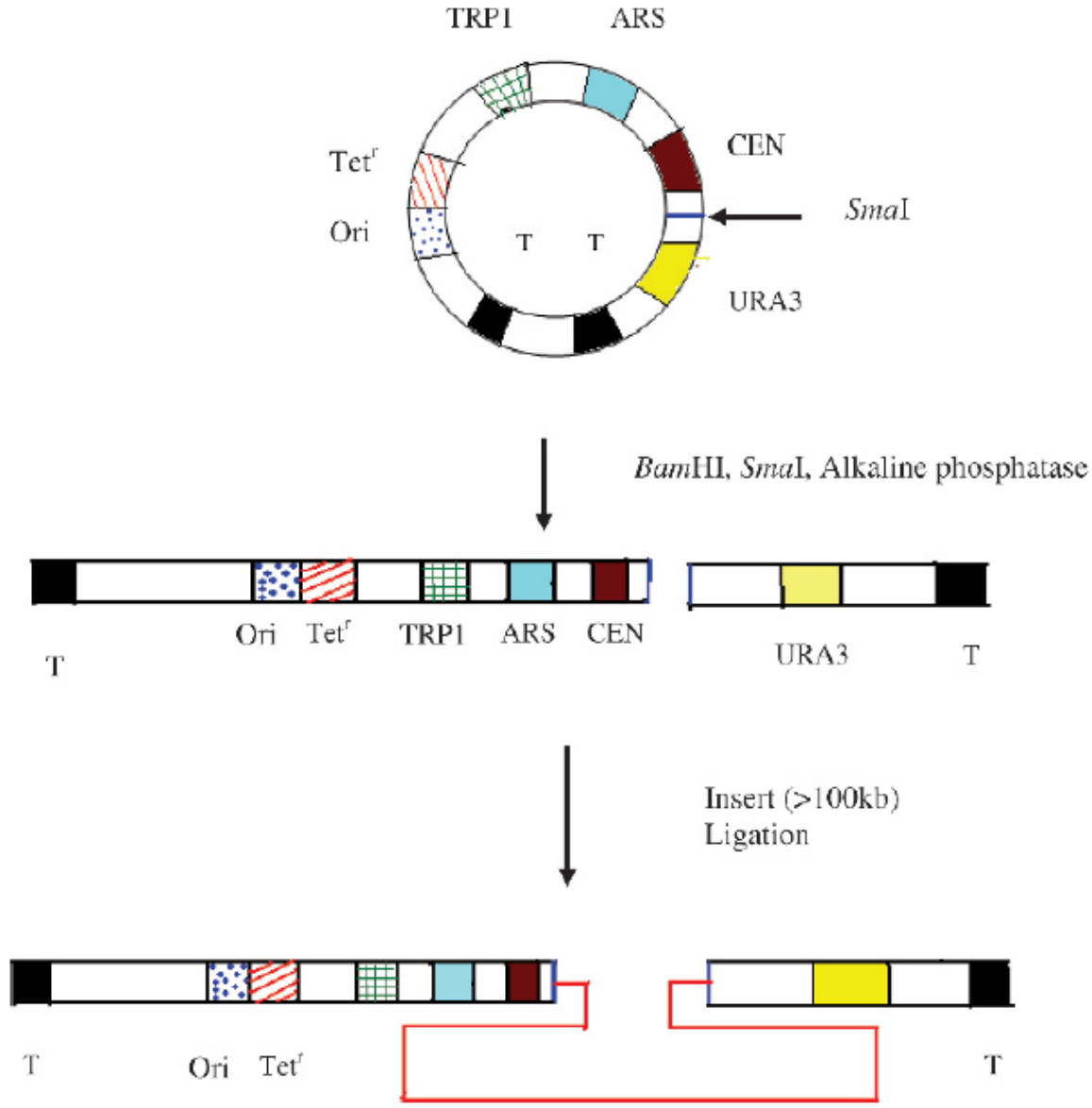
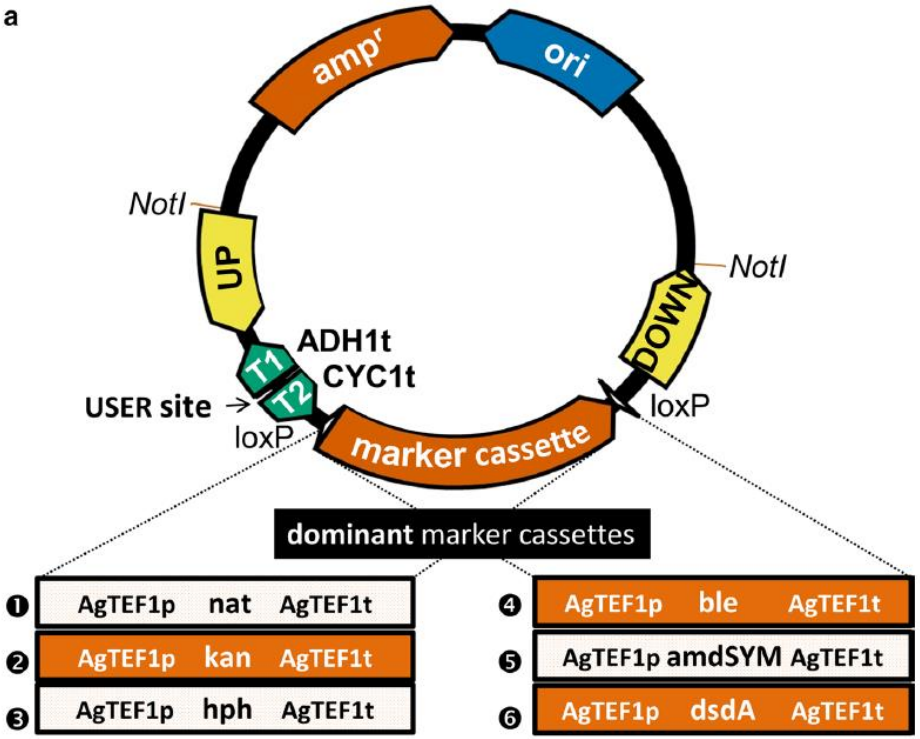


Fig. 16.2 YAC cloning system- pYAC has *E.coli* ori and selectable marker gene (Tet^r); and yeast DNA sequences TRP1, URA3, ARS and CEN. T represents telomeric sequences. URA3 is a gene in uracil biosynthesis pathway and TRP1is a gene of Tryptophan biosynthesis pathway

Fig. 1 New set of Easy-Clone2.0 vectors with dominant markers. **a** Schematic illustration of the vector structure and the dominant marker cassettes. **b** Table of the vectors presenting combinations of particular vector (integration site) with the dominant markers

a



b

marker / integration site		① natMX	② kanMX	③ hphMX	④ bleMX	⑤ amdSYM	⑥ dsdAMX
Chr X	X-2	pCfB2193		pCfB2513			
	X-3		pCfB2223				
	X-4			pCfB2194			
Chr XI	XI-1	pCfB2375					
	XI-2		pCfB2224				
	XI-3			pCfB2195			
	XI-5				pCfB2196	pCfB2399	
Chr XII	XII-1	pCfB2197					pCfB2400
	XII-2		pCfB2225				
	XII-4			pCfB2198			
	XII-5			pCfB2337			

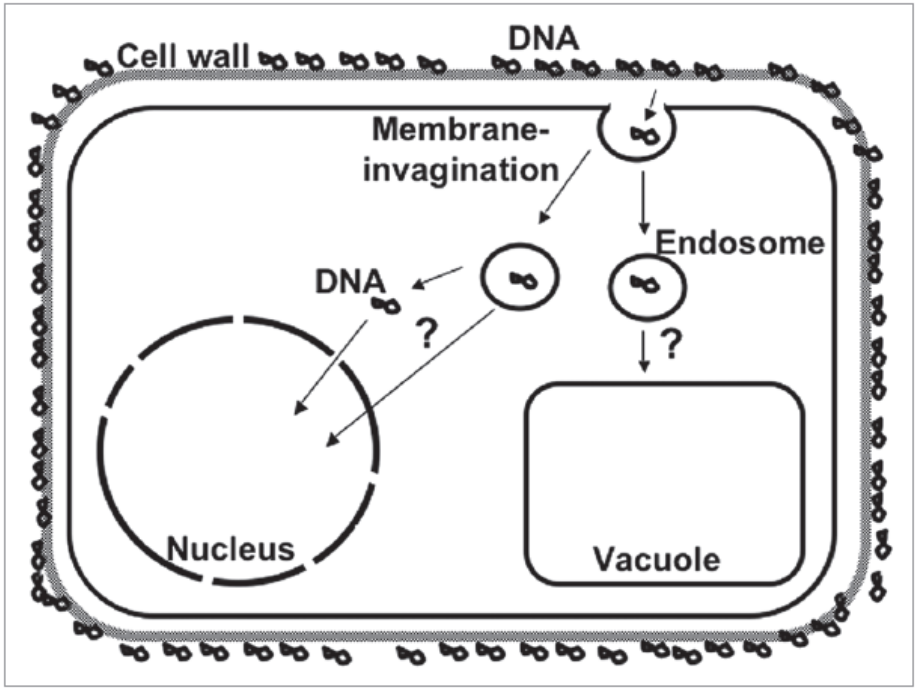


Figure 2. Putative mechanism of *S. cerevisiae* transformation. DNA initially attaches to the cell wall. PEG is indispensable for this attachment and for successful transformation of intact cells. PEG also possibly acts on the membrane to increase the transformation frequency and efficiency as well as the permeability to YOYO-1. The attached DNA passes through the cell wall. LiAc and heat shock help DNA to pass through the cell wall. DNA then enters into the cell via endocytotic membrane invagination. Some DNA in the endosomes is delivered to the vacuoles and digested. However, the manner in which DNA escapes digestion, reaches the nucleus and enters it through the nuclear pore is still unclear.

S. cerevisiae medium	Components (L^{-1})
YPD (rich medium)	10 g bacto yeast extract 20 g bacto peptone 20 g glucose
YPG (rich medium with non-fermentable carbon source)	10 g bacto yeast extract 20 g bacto peptone 30 mL glycerol
CSM (complete synthetic medium)	1.7 g bacto yeast nitrogen base (without amino acids) 5 g ammonium sulphate 20 g glucose 100 mL 10× amino acid solution (see Table 2)
2× CBS (Centralbureau voor Schimmelcultures medium)	10 g ammonium sulphate 6 g potassium dihydrogen phosphate 1 g magnesium sulphate heptahydrate 20 g glucose 100 mL 1 M MES, pH 6 200 mL 10× amino acid solution (see Table 2) 2 mL vitamin solution (see Table 2) 2 mL trace element solution (see Table 2)

Table 16.4 Eukaryotic therapeutic proteins expressed in *S. cerevisiae*

Recombinant protein	Commercial name	Company	Therapeutic indication
Recombinant blood factor			
Hirudin/lepirudin	Refludan	Hoechst Marion Roussel (US)	Anticoagulant for heparin-associated thrombocytopenia
Hirudin/desirudin	Revasac	Canyon pharmaceuticals	Prevention of venous thrombosis
Recombinant hormone			
Insulin	Novolog	Novo Nordisk	Diabetes mellitus
Insulin	Exubera	Pfizer(New York) Aventis(Kent, UK)	Diabetes mellitus
Insulin	Apidra	Aventis (Germany)	Diabetes mellitus
Insulin	Liprolog	Eli Lilly	Diabetes mellitus
Somatotropin	Valtropin	Biopartners	Growth disturbances in children and adults
Glucagon	Glucagen	Novo Nordisk	Hyperglycemia
Recombinant enzyme			
r urate oxidase	Fasturtec	Sanofi-Synthetalco	Hyperuricemia
Recombinant Vaccine			
Hepatitis B	Ambirix	Glaxo Smith Kline	Immunization against hepatitis A and B
Hepatitis B	Pediarix	Glaxo Smith Kline	Immunization against hepatitis B
Hepatitis B	HBVAXPRO	Aventis Pharma	Immunization against hepatitis A and B
Hepatitis B	Infanrix-Penta	Glaxo Smith Kline	Immunization against diphtheria, tetanus, pertussis, polio and hepatitis B
Hepatitis B	Procomvax	Aventis Pasteur	Immunization against *H. influenzae* type b and hepatitis B
Hepatitis B	Primavax	Aventis Pasteur	Immunization against diphtheria, tetanus and hepatitis B
Hepatitis B	Twinrix	Glaxo SmithKline	Immunization against hepatitis A & B

Table 2. Commercial biopharmaceuticals produced by yeast

System	Protein*	Brand name	Therapeutic area	Company
S. cerevisiae	Hepatitis (or plus other infectious disease) vaccines (I)	Comvax	*H. influenzae* type B and hepatitis B infection in infants	Merck
		Recombivax	Hepatitis B	
		Euvax B		Sanofi Pasteur (France)
		Engerix-B		GlaxoSmithKline (GSK)
		Fendrix		
		Ambirix	Hepatitis A and B	
		Twinrix		
		Pediarix8	Various conditions inducing hepatitis B in children	
		Tritanrix-HB	Diphtheria, tetanus, pertussis, and hepatitis B	
		Infanrix Hep B		
		Infanrix-Penta	Diphtheria, tetanus, pertussis, polio, and hepatitis B	
		Infanrix-Hexa	Diphtheria, tetanus, pertussis, hepatitis B, polio, and *H. influenzae* type B	
		Hexavac		Aventis Pasteur
		Procomvax	*H. influenzae* type B and hepatitis B	
		Primavax	Diphtheria, tetanus, and hepatitis B	
		HBVaxPro	Hepatitis B in children and adolescents	Aventis Pharma

Lepirudin (S)	Refludan	Heparin-induced thrombocytopenia type II	Hoechst Marion Rousse (USA), Behringwerke AG (Germany)
Desirudin (S)	Revasc	Venous thrombosis	Canyon Pharmaceuticals (UK)
Insulin (S)	Actrapid, Velosulin, Monotard, Insulatard, Protaphane, Mixtard, Actraphane, Ultratard	Diabetes mellitus	Novo Nordisk
Insulin aspart (S)	Novolog, Novolog FlexPen, Novolog Penfill, NovoRapid, NovoRapid Penfill, Novomix 30, Novolog mix 70/30		
Insulin detemir (S)	Levemir, Levemir FlexPen		
GLP-1 (S)	Victoza	Type 2 diabetes	
Glucagon (S)	GlucaGen	Hypoglycemia	
	Glucagon		Eli Lilly
GM-CSF (S)	Leukine	Cancer, bone marrow transplant	Berlex Laboratories
	Leucomax		Novartis
HGH (S)	Valtropin	Dwarfism, pituitary turner syndrome	Biopartners
PDGF (I)	Regranex	Lower extremity diabetic neuropathic ulcers	Ortho-McNeil Pharmaceutical (USA), Janssen-Cilag
	GEM 125	Periodontal defects	Luitpold Pharmaceuticals (USA) BioMimetic Pharmaceuticals (USA)
HPV vaccine (I)	Gardasil	Cervical cancer caused by human papillomavirus (HPV)	Merck, Sanofi Pasteur, Merck Sharp & Dohme
Rasburicase (I)	Fasturtec, Elitex	Hyperuricemia	Sanofi-Synthelabo (France), Sanofi-Aventis (France)

Pichia pastoris

GRAS status

Tightly regulated, methanol-inducible *AOX* promoters

A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes

Can grow rapidly on inexpensive media at high cell densities (up to 150 g DCW L^{-1})

Integrated vectors developed that help genetic stability of the recombinant elements, even in continuous and large-scale fermentation processes

Well-established commercial vector systems and host strains (Invitrogen)

A lesser extent of hypermannosylation compared to *S. cerevisiae;* No terminal α-1,3-linked mannose residues

Genome sequencing: Reference strain GS115; 9216 Kb (5040 ORFs); Accession number PRJNA39439, PRJEA37871

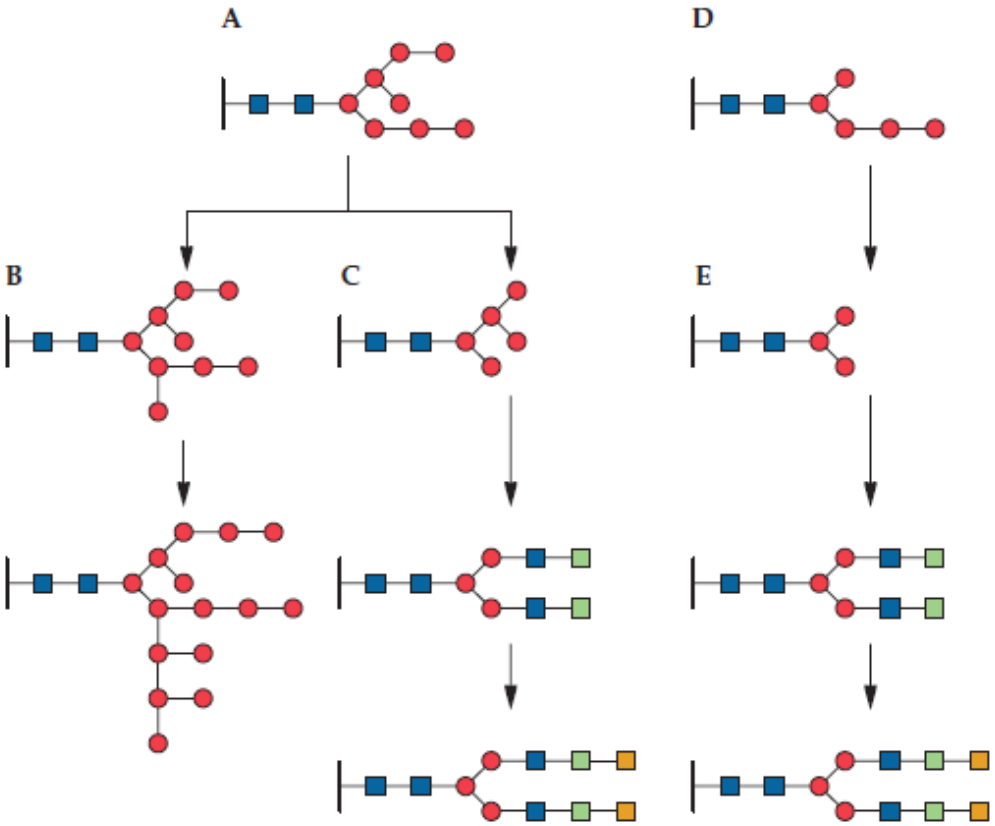


FIGURE 7.9 Differential processing of glycoproteins in *P. pastoris*, humans, and "humanized" *P. pastoris*. Initial additions of sugar residues to glycoproteins in the endoplasmic reticulum are similar in human and *P. pastoris* cells **(A).** However, further N glycosylation in the Golgi apparatus differs significantly between the two cell types. N-glycans are hypermannosylated in *P. pastoris* **(B),** while in humans, mannose residues are trimmed and specific sugars are added, leading to termination of the oligosaccharide in sialic acid **(C).** *P. pastoris* cells have been engineered to produce enzymes that process glycoproteins in a manner similar to that of human cells. In "humanized" *P. pastoris*, a recombinant glycoprotein produced in the endoplasmic reticulum **(D)** is transported to the Golgi apparatus, where it is further processed to yield a properly sialylated glycoprotein **(E).** Blue squares, *N*-acetylglucosamine; red circles, mannose; green squares, galactose; orange squares, sialic acid. Adapted from Hamilton and Gerngross, *Curr. Opin. Biotechnol.* **18**:387–392, 2007.

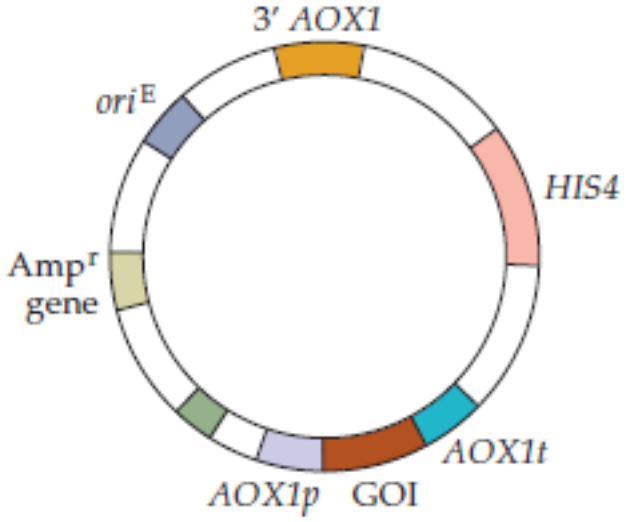


FIGURE 7.10 *P. pastoris* integrating expression vector. The gene of interest (GOI) is cloned between the promoter *(AOX1p)* and termination-polyadenylation sequence *(AOX1t)* of the *P. pastoris* alcohol oxidase 1 gene. The *HIS4* gene encodes a functional histidinol dehydrogenase of the histidine biosynthesis pathway. The ampicillin resistance (Amp^r) gene and an origin of replication (ori^E) function in *E. coli*. The segment marked 3′ *AOX1* is a piece of DNA from the 3′ end of the alcohol oxidase 1 gene of *P. pastoris*. A double recombination event between the *AOX1p* and 3′ *AOX1* regions of the vector and the homologous segments of chromosome DNA results in the insertion of the DNA carrying the gene of interest and the *HIS4* gene.

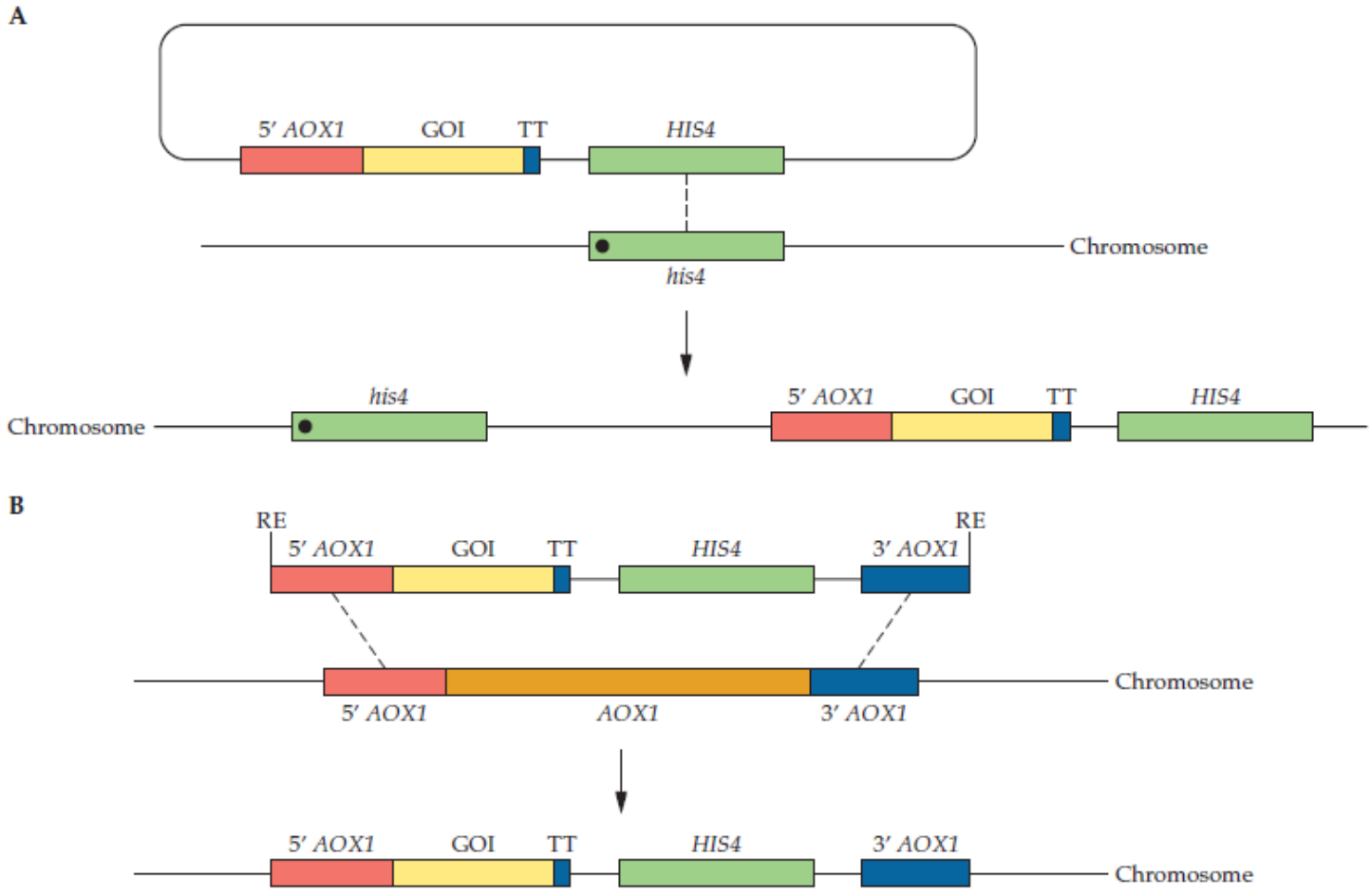


FIGURE 7.11 Integration of DNA into a specific *P. pastoris* chromosome site by single **(A)** or double **(B)** recombination. **(A)** A single recombination (dashed line) between the *HIS4* gene of an intact circular plasmid and a chromosome *his4* mutant gene results in the integration of the entire vector, including the gene of interest (GOI) with the *AOX1* promoter in the 5′ *AOX1* DNA segment and the transcription–polyadenylation sequence from the *AOX1* gene (TT), into the chromosome. The inserted DNA is flanked by recombined mutant *his4* and functional *HIS4* genes. The dot in the *his4* gene represents the mutation. **(B)** A double recombination (dashed lines) between the cloned 5′ *AOX1* and 3′ *AOX1* DNA segments of a restriction endonuclease (RE) linearized DNA fragment from the vector and the corresponding chromosome regions results in the integration of the gene of interest (GOI) with the *AOX1* promoter in the 5′ *AOX1* segment, the termination–polyadenylation sequence from the *AOX1* gene (TT), and a functional *HIS4* gene. The chromosome *AOX1* coding region is lost as a result of the recombination event.

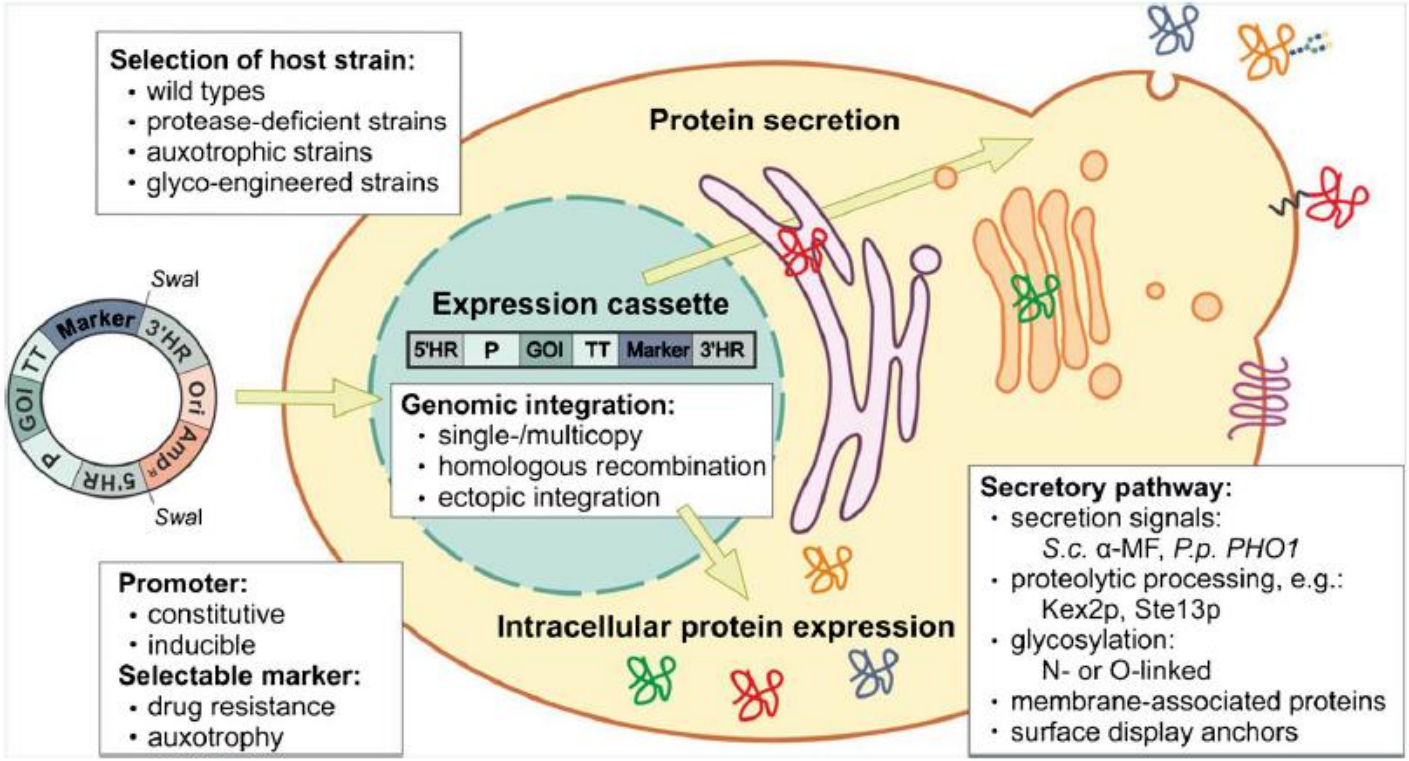


Fig. 1 General considerations for heterologous gene expression in *P. pastoris*. Expression plasmids harbouring the gene(s) of interest (*GOI*) are linearized prior to transformation. Selectable markers (e.g., Amp^R) and origin of replication (*Ori*) are required for plasmid propagation in *E. coli*. The expression level of the protein of interest may depend on (i) the chromosomal integration locus, which is targeted by the 5′ and 3′ homologous regions (5′HR and 3′HR), and (ii) on the gene copy number. A representative promoter (*P*) and transcription terminator (*TT*) pair are shown. Proper signal sequences will guide recombinant protein for intracellular or secretory expression, and will govern membrane integration or membrane anchoring

Table 1 The most prominently used and very recently established promoters for heterologous expression in *P. pastoris*

Inducible	Corresponding gene	Regulation	Reference
AOX1	Alcohol oxidase 1	Inducible with MeOH	(Tschopp et al. 1987a)
DAS	Dihydroxyacetone synthase	Inducible with MeOH	(Ellis et al. 1985; Tschopp et al. 1987a)
FLD1	Formaldehyde dehydrogenase 1	Inducible with MeOH or methylamine	(Shen et al. 1998)
ICL1	Isocitrate lyase	Repressed by glucose, induction in absence of glucose/by addition of ethanol	(Menendez et al. 2003)
PHO89	Putative Na^+/phosphate symporter	Induction upon phosphate starvation	(Ahn et al. 2009)
THI11	Thiamine biosynthesis gene	Repressed by thiamin	(Stadlmayr et al. 2010)
ADH1	Alcohol dehydrogenase	Repressed on glucose and methanol, induced on glycerol and ethanol	(Cregg and Tolstorukov 2012)
ENO1	Enolase	Repressed on glucose, methanol and ethanol, induced on glycerol	(Cregg and Tolstorukov 2012)
GUT1	Glycerol kinase	Repressed on methanol, induced on glucose, glycerol and ethanol	(Cregg and Tolstorukov 2012)
Constitutive	Corresponding gene	Regulation	Reference
GAP	Glyceraldehyde-3-P dehydrogenase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol	(Waterham et al. 1997)
TEF1	Translation elongation factor 1	Constitutive expression on glycerol and glucose	(Ahn et al. 2007)
PGK1	3-Phosphoglycerate kinase	Constitutive expression on glucose, to a lesser extent on glycerol and methanol	(de Almeida et al. 2005)
GCW14	Potential glycosyl phosphatidyl inositol (GPI)-anchored protein	Constitutive expression on glycerol, glucose and methanol	(Liang et al. 2013b)
G1	High affinity glucose transporter	Repressed on glycerol, induced upon glucose limitation	(Prielhofer et al. 2013)
G6	Putative aldehyde dehydrogenase	Repressed on glycerol, induced upon glucose limitation	(Prielhofer et al. 2013)

Table 2 Commercial vector systems

Supplier	Promoter	Signal sequences	Selection in yeast	Selection in bacteria	Comments
Life Technologies™	*AOX1, FLD1, GAP*	*S. cerevisiae* α-MF; *P. pastoris PHO1*	Blasticidin, G418, Zeocin™, *HIS4*	Zeocin™, Ampicillin, Blasticidin	c-myc epitope, V5 epitope, C-terminal 6× His-tag available for detection/purification
Life Technologies –PichiaPink™	*AOX1*	α-MF; set of eight different signal sequences – not ready to use[a]	*ADE2*	Ampicillin	Low- and high-copy vectors available, *TRP2* sequence for targeting
BioGrammatics	*AOX1*	α-MF	Zeocin™, G418, Nourseothricin	Ampicillin	Intracellular or secreted expression
BioGrammatics – GlycoSwitch®	*GAP*	–	Zeocin™, G418, Hygromycin, *HIS4*, Nourseothricin	Zeocin™, Ampicillin, Kanamycin, Nurseothricin	Human GlcNAc transferase I, rat Mannosidase II, human Gal transferase I
DNA2.0	*AOX1*	Ten different signal sequences – ready to use[b]	Zeocin™, G418	Zeocin™, Ampicillin	Intracellular or secreted

[a] The different secretion signals have to be cloned into the vector by a three-way ligation step

[b] The α-MF secretion signal is provided once with Kex2p (KR) and Ste13p cleavage sites (EAEA), once lacking EA repeats, and once as truncated version (pre-region only)

Table 16.5 Commonly used *P. pastoris* expression vectors and their important features

Vector	Comments	Marker	Reference
pA0815	Expression cassette is between *Bam*HI & *Bgl*II for generation of multi copy expression vector	HIS4	Thill et al., 1990
pPIC3K	MCS for foreign gene expression; G418 selection for multicopy strains	Kan^r	Scorer et al., 1993b
pHIL-D2	*Not*I sites are present for *AOX1* gene replacement	*HIS4*	Sreekrishna, 1993
PHW 010	Constitutive promoter *GAP* controls the expression	*HIS4*	Waterham et al., 1997
pP1C9K	*AOX1* is fused to α-MF prepro signal sequence; *Not*I, *Sna*I1, *Eco*RI, *Xho*I, *Avr* II restriction sites for foreign gene insertion	*HIS4* & Kan^r	Scorer et al., 1993b
pGAP	*GAP* promoter fused to α-MF pre-pro signal sequence	ble^r	Invitrogen (Carlsbad CA)
pPICZα	MCS for cloning, *AOX1* promoter fused to α-MF pre-pro signal sequence	ble^r	Higgins and Cregg, 1998

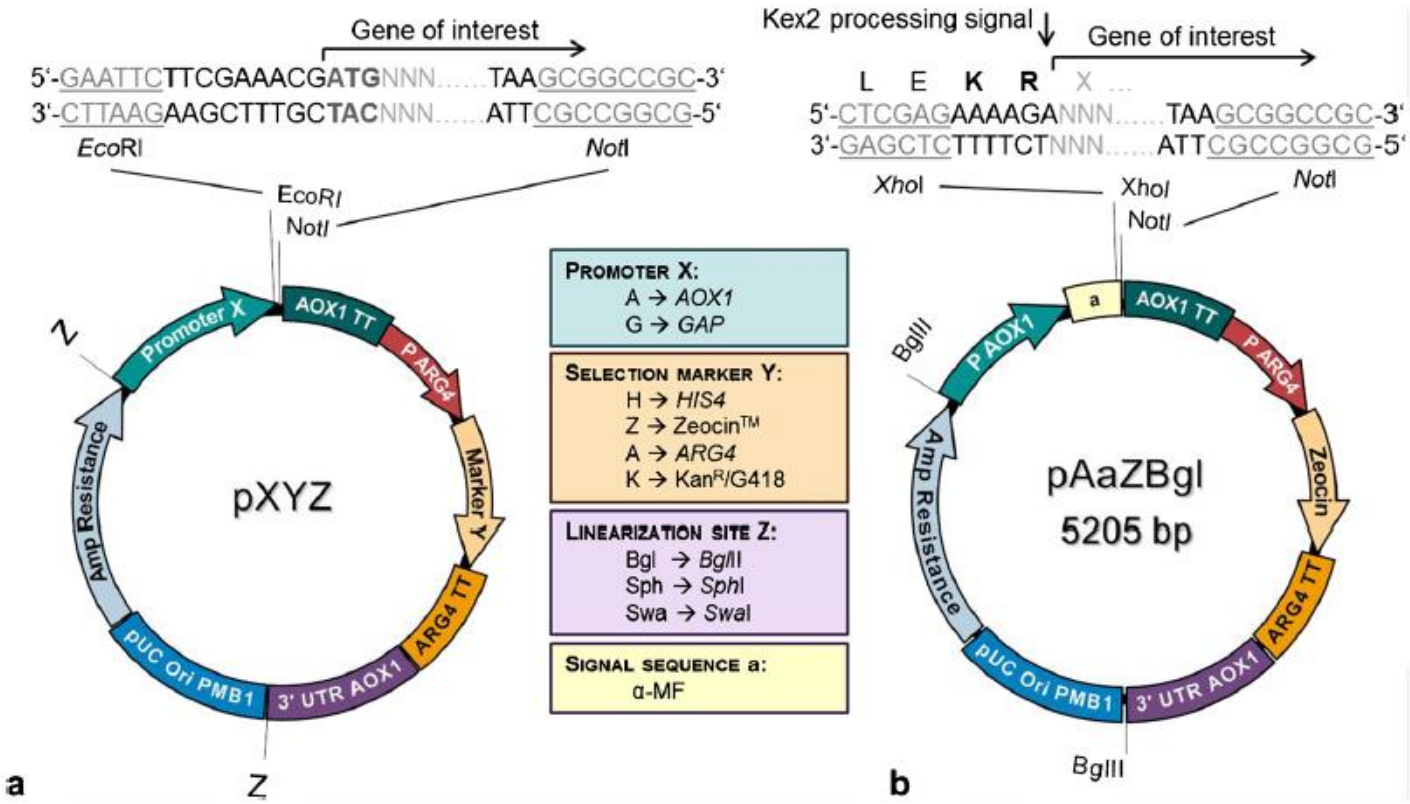


Fig. 2 Novel '*Pichia* Pool' plasmid sets for intracellular and secretory expression. **a** General features of pXYZ vector for intracellular expression. Letters refer to the choice of promoters (*X*), selection markers (*Y*), and restriction enzymes (*Z*) for linearization. Available elements are shown in boxes. The vector backbone harbours an ampicillin resistance marker and origin of replication for maintenance of the plasmid in *E. coli*. The GOI is *Eco*RI–*Not*I cloned directly after the promoter of choice. The Kozak consensus sequence for yeast (i.e., CGAAACG), should be restored between the *Eco*RI cloning site and the start codon of the GOI in order to achieve optimal translation. In addition, sequence variation within this region will allow fine-tuning translation initiation efficiency. Expression in *P. pastoris* is driven either by the methanol inducible *AOX1* or the constitutive *GAP* promoter. Positive clones can be selected for by antibiotic resistance (i.e., to Zeocin™ or geneticin sulphate) or by selection for His or Arg prototrophy. Selection marker expression is uniformly driven by the *ARG4* promoter–terminator pair. **b** Plasmid pAaZBgl from '*Pichia* Pool' is shown as an example of a vector made for secretory expression encoding *S. cerevisiae* α-MF signal sequence in front of the GOI cloning site. The Kex2 processing site AAAAGA should be restored between the *Xho*I cloning site and the fusion point of the GOI

Table 3 Signal sequences used to secrete the protein into the extracellular space

Secretion signal	Source	Target protein(s)	Length	Reference
α-MF	*S.c.* α-mating factor	Most commonly used secretion signal in *P. pastoris*	85 aa, with or without EA repeats	(Brake et al. 1984)
PHO1	*P.p.* acid phosphatase	Mouse 5-HT5A, porcine pepsinogen,	15 aa	(Payne et al. 1995; Weiss et al. 1995; Yoshimasu et al. 2002)
SUC2	*S.c.* Invertase	Human interferon, α-amylase, α-1-antitrypsin	19 aa	(Moir and Dumais 1987; Paifer et al. 1994; Tschopp et al. 1987b)
PHA-E	Phytohemagglutinin	GNA, GFP and native protein	21 aa	(Raemaekers et al. 1999)
KILM1	Kl toxin	CM cellulase	44 aa	(Skipper et al. 1985)
pGKL	pGKL killer protein	Mouse α-amylase	20 aa	(Kato et al. 2001)
CLY and CLY-L8	C-lysozyme and syn. leucin-rich peptide	Human lysozyme	18 and 16 aa	(Oka et al. 1999)
K28 pre-pro-toxin	K28 virus toxin	Green fluorescent protein	36 aa	(Eiden-Plach et al. 2004)
Scw, Dse and Exg	*P.p.* Endogenous signal peptides	CALB and EGFP	19, 20 and 23 aa	(Liang et al. 2013a)
Pp Pir1	*P.p.* Pir1p	EGFP and Human α1-antitrypsin	61 aa	(Khasa et al. 2011)
HBFI and HBFII	Hydrophobins of *Trichoderma reesei*	EGFP	16 and 15 aa	(Kottmeier et al. 2011)

Table 4 *P. pastoris* host strains

Strain	Genotype	Phenotype	Source
Wild-type strains			
CBS7435 (NRRL Y-11430)	WT	WT	Centraalbureau voor Schimmelcultures, the Netherlands
CBS704 (DSMZ 70382)	WT	WT	Centraalbureau voor Schimmelcultures, the Netherlands
X-33	WT	WT	Life Technologies™
Auxotrophic strains			
GS115	*his4*	His^-	Life Technologies™
PichiaPink™ 1	*ade2*	Ade^-	Life Technologies™
KM71	*his4, aox1::ARG4, arg4*	His^-, Mut^S	Life Technologies™
KM71H	*aox1::ARG4, arg4*	Mut^S	Life Technologies™
BG09	*arg4::nourseo*^{R} *Δlys2::hyg*^{R}	Lys^-, Arg^-, $Nourseothricin^R$, $Hygromycin^R$	BioGrammatics
GS190	*arg4*	Arg^-	(Cregg et al. 1998)
GS200	*arg4 his4*	His^-, Arg^-	(Waterham et al. 1996)
JC220	*ade1*	Ade^-	(Cregg et al. 1998)
JC254	*ura3*	Ura^-	(Cregg et al. 1998)
JC227	*ade1 arg4*	Ade^- Arg^-	(Lin-Cereghino et al. 2001)
JC300-JC308	Combinations of *ade1 arg4 his4 ura3*	Combinations of Ade^-, Arg^-, His^-, Ura^-	(Lin-Cereghino et al. 2001)
YJN165	*ura5*	Ura^-	(Nett and Gerngross 2003)
CBS7435 *his4*^{a}	*his4*	His^-	(Näätsaari et al. 2012)
CBS7435 Mut^S *his4*^{a}	*aox1, his4*	Mut^S, His^-	(Näätsaari et al. 2012)
CBS7435 Mut^S *arg4*^{a}	*aox1, arg4*	Mut^S, Arg^-	(Näätsaari et al. 2012)
CBS7435 *met2*^{a}	*met2*	Met^-	(*Pp*7030)^b
CBS7435 *met2 arg4*^{a}	*met2 arg4*	Met^- Arg^-	(*Pp*7031)^b
CBS7435 *met2 his4*^{a}	*met2 his4*	Met^- His^-	(*Pp*7032)^b
CBS7435 *lys2*^{a}	*lys2*	Lys^-	(*Pp*7033)^b
CBS7435 *lys2 arg4*^{a}	*lys2 arg4*	Lys^- Arg^-	(*Pp*7034)^b
CBS7435 *lys2 his4*^{a}	*lys2 his4*	Lys^- His^-	(*Pp*7035)^b
CBS7435 *pro3*^{a}	*pro3*	Pro^-	(*Pp*7036)^b
CBS7435 *tyr1*^{a}	*tyr1*	Tyr^-	(*Pp*7037)^b

Protease-deficient strains			
SMD1163	*his4 pep4 prb1*	His$^-$	(Gleeson et al. 1998)
SMD1165	*his4 prb1*	His$^-$	(Gleeson et al. 1998)
SMD1168	*his4 pep4::URA3 ura3*	His$^-$	Life Technologies™
SMD1168H	*pep4*		Life Technologies™
SMD1168 kex1::SUC2	*pep4::URA3 kex1::SUC2 his4 ura3*	His$^-$	(Boehm et al. 1999)
PichiaPink 2-4	Combinations of *prb1/pep4*	Ade$^-$	Life Technologies™
BG21	*sub2*		BioGrammatics
CBS7435 *prc1*[a]	*prc1*		(*Pp*6676)[b]
CBS7435 *sub2*[a]	*sub2*		(*Pp*6668)[b]
CBS7435 *sub2*[a]	*his4 pep4*	His$^-$	(*Pp*6911)[b]
CBS7435 *prb1*[a]	*prb1*		(*Pp*6912)[b]
CBS7435 *his4 pep4 prb1*	*his4 pep4 prb1*	His$^-$	(*Pp*7013)[b]
Glyco-engineered strains			
$SuperMan_5$	*his4 och1::pGAPTrα1,2-mannosidase*	His$^-$, Blasticidin^R	BioGrammatics
	och1::pGAPTrα1,2-mannosidase	Blasticidin^R	BioGrammatics
	pep4 och1::pGAPTrα1,2-mannosidase	Blasticidin^R	BioGrammatics
Other strains			
GS241	*fld1*	Growth defect on methanol as sole C-source or methylamine as sole N-source	(Shen et al. 1998)
MS105	*his4 fld1*	See GS241; His$^-$	(Shen et al. 1998)
MC100-3	*his4 arg4 aox1::ScARG4 aox2::PpHIS4*	Mut$^-$	(Cregg et al. 1989)
CBS7435 *ku70*[a]	*ku70*	WT	(Näätsaari et al. 2012)
CBS7435 *ku70 his4*[a]	*ku70, his4*	His$^-$	(Näätsaari et al. 2012)
CBS7435 *ku70 gut1*	*ku70, gut1*	Growth defect on glycerol; Zeocin^R	(Näätsaari et al. 2012)
CBS7435 *ku70 ade1*	*ku70, ade1*	Ade$^-$, Zeocin^R	(Näätsaari et al. 2012)

P. pastoris medium	Components (L^{-1})
BMGY (buffered glycerol-complex medium)	10 g bacto yeast extract 20 g bacto peptone 100 mL 10× YNB (see Table 2) 100 mL 1 M potassium phosphate (pH 6) 2 mL 500× biotin (see Table 2) 100 mL 10× glycerol (see Table 2)
BMMY (buffered methanol-complex medium)	10 g bacto yeast extract 20 g bacto peptone 100 mL 10× YNB (see Table 2) 100 mL 1 M potassium phosphate (see Table 2) 2 mL 500× biotin (see Table 2) 100 mL 10× methanol (see Table 2)
BSM (basal salts medium)	26.7 mL phosphoric acid 0.93 g calcium sulphate 18.2 g potassium sulphate 14.9 g magnesium sulphate heptahydrate 4.13 g potassium hydroxide 40 g glycerol 4.35 mL PTM_1 salts (see Table 2)

System	Protein*	Brand name	Therapeutic area	Company
P. pastoris	Ecallantide (I)	Kalbitor	Hereditary angioedema	Dyax (USA)
	Insulin (S)	Insugen	Type 2 diabetes	Biocon (India)
	Human serum albumin (S)	Medway	Blood volume expansion	Mitsubishi Tanabe Pharma (Japan)
	Hepatitis vaccine (I)	Shanvac	Hepatitis B	Shantha/Sanofi (India)
	IFN-α 2b (S)	Shanferon	Hepatitis C, cancer	Shantha/Sanofi (India)
	Ocriplasmin (I)	Jetrea	Vitreomacular adhesion (VMA)	ThromboGenics (Belgium)
	Anti-IL-6R Ab (I)	Nanobody ALX-0061	Rheumatoid arthritis	Ablynx (Belgium)
	Anti-RSV Ab (S)	Nanobody ALX00171	Respiratory syncytial virus (RSV) infection	Ablynx (Belgium)
	HB-EGF (I)	–	Treatment of interstitial cystitis/bladder pain syndrome (IC/BPS)	Trillium (Canada)
	Collagen (I)	–	Medical research reagents/ dermal filler	Fibrogen (USA)

Hansenula polymorpha

GRAS status

Stringently regulated strong promoters (*MOX*, *FMDH*, etc.)

A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes

Stable, multicopy integration of foreign DNA into chromosomal locations

Thermotolerant (growth up to 45 °C), resistant to heavy metals and oxidative stress

Can assimilate nitrates

A lesser extent of hypermannosylation compared to *S. cerevisiae*; No terminal α-1,3-linked mannose residues

Genome sequencing: Reference strain DL1; 9056 Kb (5325 ORFs); Accession number PRJNA60503

H. polymorpha	HBV vaccine (I)	Hepavax-Gene	Hepatitis B	Rhein Biotech (Germany), Green Cross Vaccine Corp (Korea)

Yarrowia lipolytica

An oleaginous yeast, based on its ability to accumulate large amounts of lipids

GRAS status

Can grow in hydrophobic environments, that is able to metabolize triglycerides, fatty acids, *n*-alkanes, and *n*-paraffins as carbon sources for the bioremediation of environments contaminated with oil spills

Can secrete a variety of proteins via cotranslational translocation and efficient secretion signal recognition similar to higher eukaryotes

Availability of a commercial expression kit (YEASTERN BIOTECH CO., LTD.)

Salt tolerance

A lesser extent of hypermannosylation compared to *S. cerevisiae*; a lack of the immunogenic terminal α-1,3-mannose linkages

Genome sequencing: Reference strain CLIB122; 20 503 Kb (7042 ORFs); Accession number PRJNA12414

Y. lipolytica	Pancrelipase (S)	Creon, Ultresa, Viokase	Exocrine pancreatic insufficiency	Aptalis Pharma

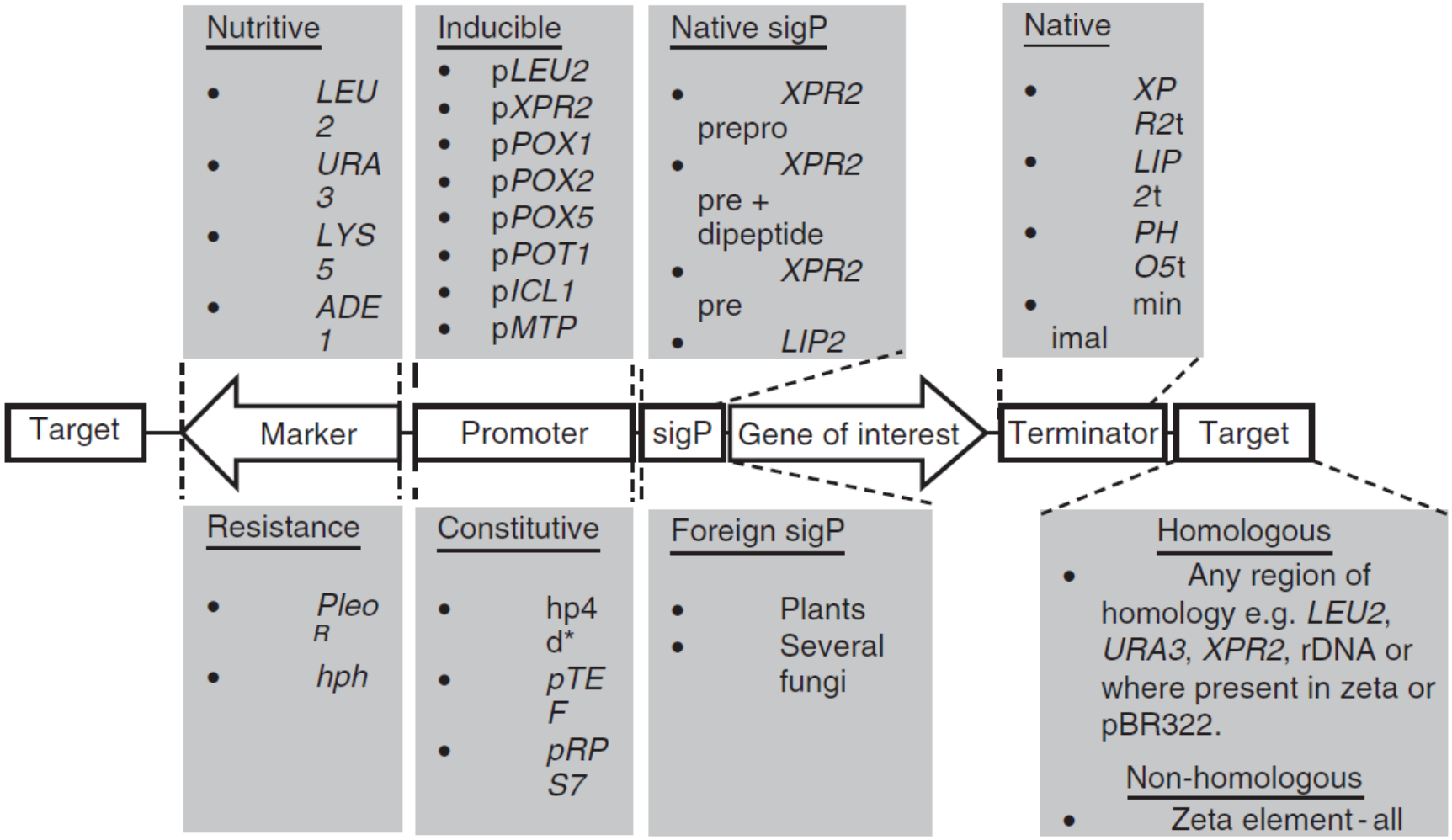


Fig. 18.2 Graphical representation of a typical auto-cloning integrative expression construct used for transformation of *Y. lipolytica*. All elements listed have been reviewed and described in detail by Madzak and co-workers (2004)

Kluyveromyces lactis

GRAS status

A Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes

Lactose-fermenting present in milk and whey, and the strong, lactose-inducible *LAC4* promoter

Very high cell density (> 100 g DCW L^{-1})

Able to use both integrative and episomal expression vectors

An available easy-to-use reagent kit for *K. lactis* protein expression (New England Biolabs)

Terminal *N*-acetylglucosamine and no mannose phosphate

Genome sequencing: Reference strain NRRL Y-1140; 10 689 Kb (5502 ORFs); Accession number PRJNA12377

Schizosascharomyces pombe

A fission yeast, reflecting proliferation of higher eukaryotic cells

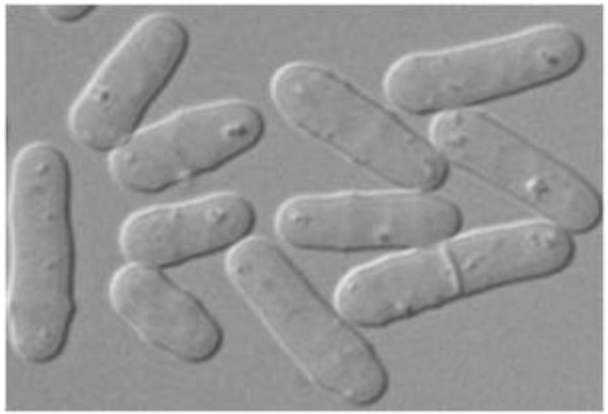
Many cellular processes similar to those of higher eukaryotes, such as mRNA splicing, post-translational modification (including protein galactosylation), cell cycle control, etc.

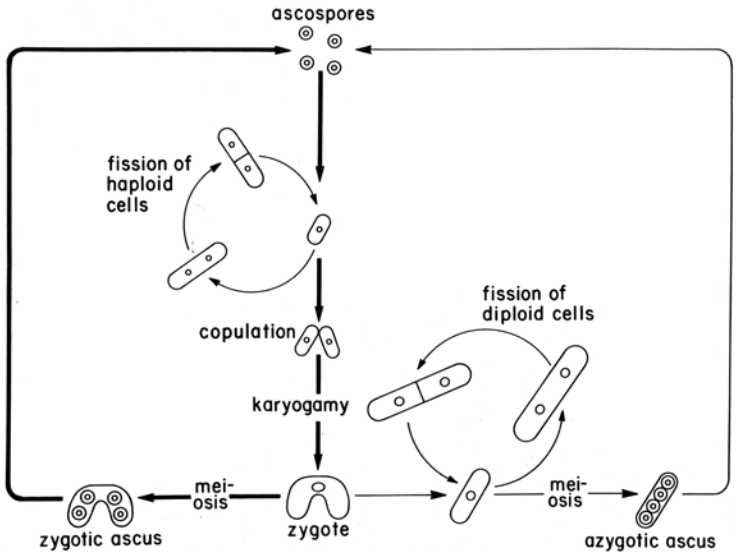
Transcription start site similar to that in higher eukaryotes

Expression vectors for high-level expression developed

Presence of galactose in both *O*- and *N*-linked glycans

Genome sequencing: Reference strain 972h-; 12 554 Kb (5364 ORFs); Accession number PRJNA127





Arxula adeninivorans

- Relatively new expression system
- Genome sequence published in 2014
- Dimorphic fungus
  - Budding yeast form up to 42 °C
  - Mycelial form at higher temperatures
- Can grow on variety of substrates including n-alkanes, purines and starch

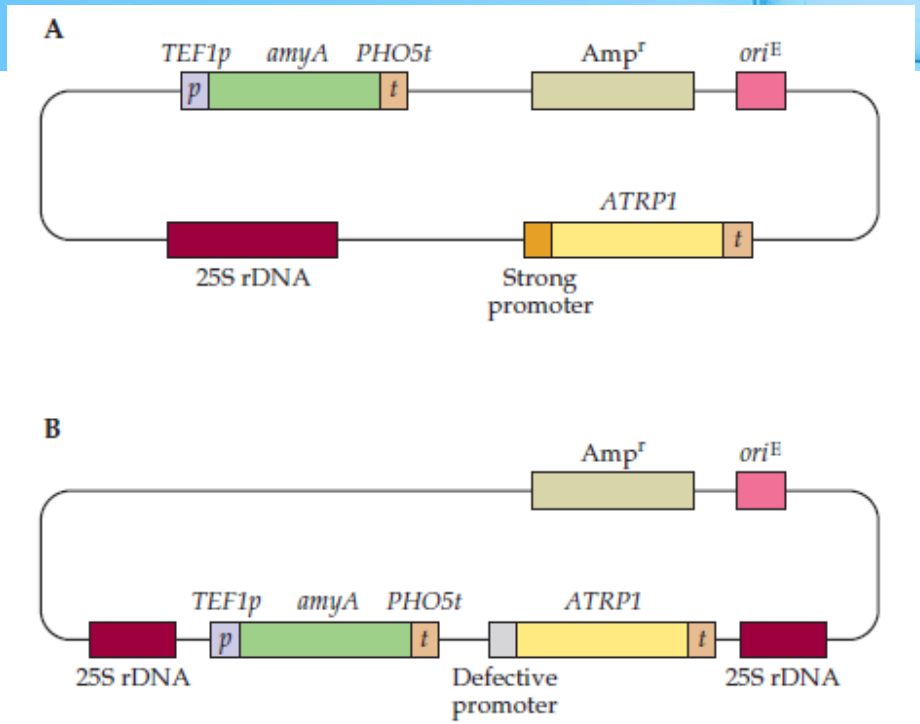


FIGURE 7.12 Constructs for stable integration of target genes into a chromosome of the yeast *A. adeninivorans*. **(A)** The target gene (e.g., the *amyA* gene) is inserted into a vector between the *TEF1* promoter *(p)* and the *PHO5* terminator *(t)*, and the vector is introduced into a strain of *A. adeninivorans* that is unable to synthesize tryptophan. The vector is integrated into a chromosome by homologous recombination between chromosomal and vector 25S rDNA sequences, and expression of the *ATRP1* gene driven by a strong promoter restores tryptophan biosynthesis, enabling survival of the yeast on media lacking tryptophan. **(B)** Expression of low levels of ATRP1 from a defective promoter results in chromosomal integration of multiple copies of the expression cassette. In this construct, the expression cassette, which consists of the target gene and the *ATRP1* gene, is inserted in the middle of the 25S rDNA sequence so that, following a double recombination event, only the expression cassette is integrated into the *A. adeninivorans* genome. Sequences for maintainance (ori^E) and selection (Amp^r) in *E. coli* are included on the vector.

Other yeast expression platforms

- *Endomyces (Dipodascus) magnusii*
- *Candida boidinii*
- *Pichia methanolica*
- *Pichia stipitis*
- *Schwanniomyces occidentalis*
- *Debaryomyces hansenii*

Filamentous fungi

Table 3 Advantages of fungal expression systems

High yield
Stable production strains
Cost effectiveness
High density growth
Easy scale-up
Safety
High expression levels
Rapid growth in chemically defined media
Product processing similar to mammalian cells
Can handle S–S rich proteins
Can assist protein folding
Can glycosylate protein

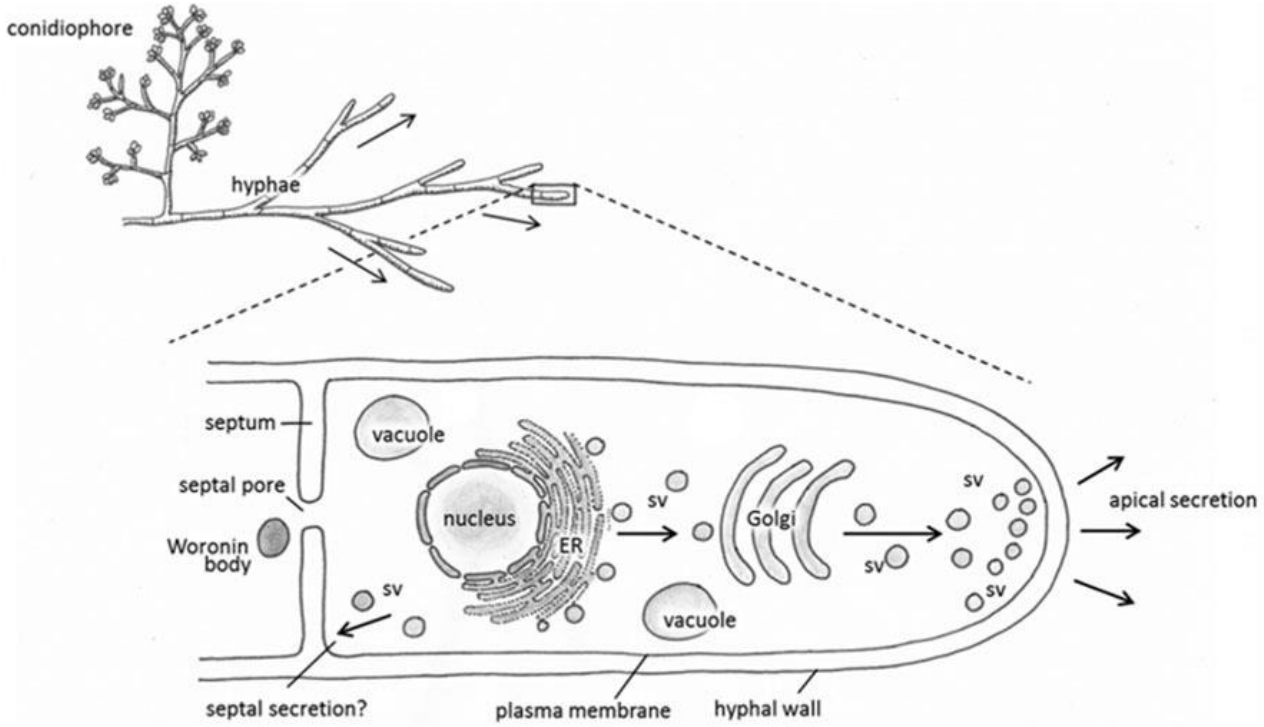


Table 1.1 Transformation methods used in filamentous fungi

Method	Advantage	Disadvantage
PEG-mediated protoplast fusion	No requirement for special equipment	Optimization of making and regenerating the protoplast takes time.
Electroporation	Time saving, easily performed	Need to prepare the competent cell
Agrobacterium tumefaciens-mediated transformation (ATMT)	Possibility of recovering T-DNA flanking sequences by PCR-based techniques. Alleviating protoplast preparation	The transformation process takes long time.
Biolistic transformation	No requirement for the protoplast. Transformation protocol is relatively simple	The equipment is expensive

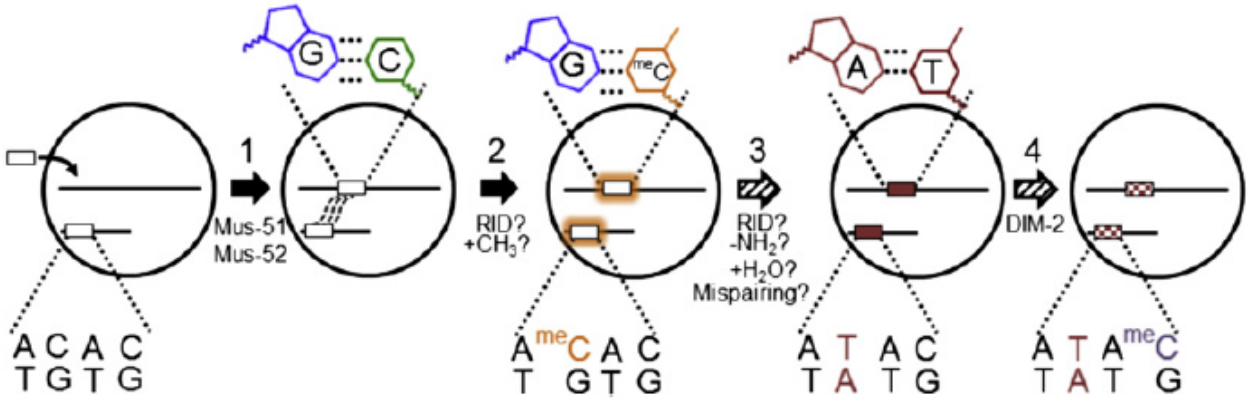


Figure 1.1 ***Popular model of the RIP mechanism in*** **Neurospora crassa.** The events of RIP in *N. crassa* are shown as numbered arrows. Proteins known or predicted to mediate the events are indicated. Circles represent a single nucleus. For simplicity, only two chromosomes (black lines) are shown. The open rectangle on the short chromosome indicates a native gene, and a short sequence of its dsDNA is indicated below. The open rectangle outside the nucleus indicates a transgene with high similarity to the native gene. In event 1, the transgene DNA is transformed into the cell's nucleus and ectopically integrated into the long chromosome. Integration could occur also in multiple copies or in the same chromosome as the transgene. A normal G:C base pair is symbolized above the nucleus. Letters G, C, A, and T represent normal nitrogen bases guanine, cytosine, adenine, and thymine, and ^{me}C represents 5-methylated cytosine. Wavy lines indicate an unknown mechanism that senses high similarity between the transgene and the native gene. In events 2 and 3, the putative DNA methyltransferase RID is predicted to facilitate methylation and/or deamination (orange glow) of the base cytosine to generate uracil (leading from the G:C-to-A:T transition: closed, brown rectangles). Dashed arrows for events 3 and 4 represent the possibility of multiple steps or several cell cycles. During vegetative growth, DIM-2 methylates many cytosines that survived RIP mutagenesis of the duplications (checkered, brown rectangles). (For interpretation of the references to color in this figure legend, the reader is referred to the online version of this book.)

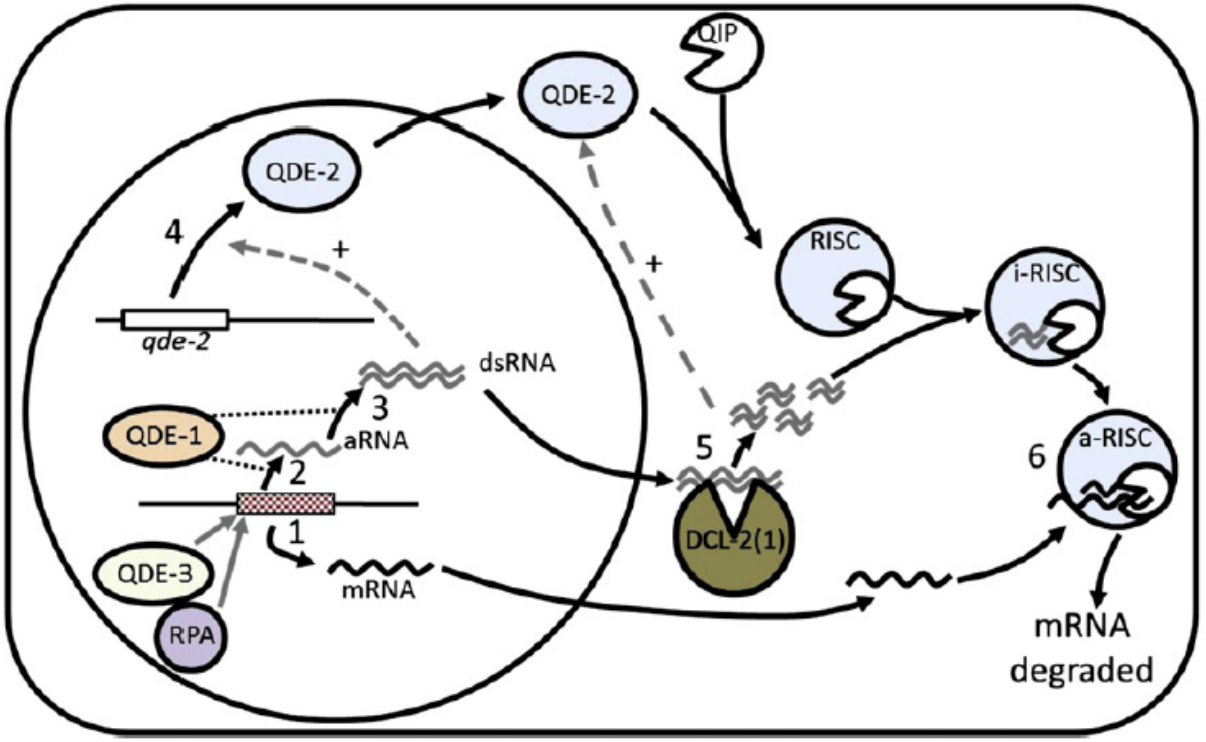


Figure 1.2 ***Model for the quelling mechanism in Neurospora crassa.*** Major events in the quelling mechanism are represented with black arrows and numerals. 1. Host RNA polymerase generates a normal transcript of a transgene (checkered box). 2. The DNA-dependent RNA polymerase activity of QDE-1 generates an aRNA from the transgene. DNA helicase QDE-3 and RPA are involved. 3. The RdRP activity of QDE-1 generates a double-stranded RNA (dsRNA) molecule from the aRNA. 4. The dsRNA induces transcription of the *qde-2* gene. 5. Dicer-like proteins DCL-2 and/or DCL-1 cleave the dsRNA into siRNA duplex molecules. QDE-2 accumulation is positively regulated by either this event or the molecules involved (Choudhary et al., 2007). The siRNA duplex molecules are loaded onto an inactive RISC (i-RISC) molecule, which contains QDE-2 and QIP. 6. The slicer activity of QDE-2 and the exonuclease activity of QIP cooperate to remove the passenger strand of the siRNA duplex, leaving only the guide strand. The guide strand directs the active RISC (a-RISC) molecule to the complementary mRNA transcript, which is then degraded. The precise locations of some events have not been verified. (For color version of this figure, the reader is referred to the online version of this book.)

Table 1.2 Examples of utilization of inducible and constitutive promoters for heterologous gene expression in filamentous fungi

Organism	Promoter	Heterologous gene	Yield	References
T. reesei	*cbh1*	Bovine chymosin	40 mg/L	Harkki et al. (1989)
T. reesei	*cbh1*	*Phlebia radiata* laccase	20 mg/L	M. Saloheimo and Niku-Paavola (1991)
T. reesei	*cbh1*	*Hormoconis resinae* glucoamylase	700 mg/L	Joutsjoki et al. (1993)
T. reesei	*cbh1*	Fab antibody fragment	150 mg/L	Keranen and Penttila (1995)
T. reesei	*cbh1*	Barley endopeptidase B	500 mg/L	Saarelainen et al. (1997)
T. reesei	*cbh1*	*A. niger* acid phosphatase	≈500 mg/L	Miettinen-Oinonen et al. (1997)
T. reesei	*cbh1*	*A. niger* lipase	310 mg/L	Qin et al. (2012)
T. reesei	*cbh1*	*T. harzianum* endochitinase	130 mg/L	Margolles-Clark, Hayes, Harman, & Penttila (1996b)
A. nidulans	*glaA*	Bovine chymosin	146 μg/g dry weight of mycelia	Cullen et al. (1987a)
A. oryzae	*glaA*	Bovine chymosin	150 mg/kg wheat bran	Tsuchiya, et al. (1994)
A. awamori	*glaA*	Bovine chymosin	47.5 mg/L	M. Ward, Wilson, Kodama, Rey, & Berka (1990)
A. nidulans	*glaA*	Cytotoxin restrictocin	0.3 mg/L	Brandhorst, Yang, & Kenealy (1994)
A. nidulans	*glaA*	Human interleukin-6	0.025 mg/L	Carrez et al. (1990)
A. awamori	*glaA*	Human lactoferrin	2000 mg/L	P. P. Ward et al. (1995)
A. niger	*glaA*	*Phanerochaete chrysosporium* manganese peroxidases	100 mg/L	Punt et al. (2002)
A. niger	*glaA*	Human tissue plasminogen activator	25 mg/L	Wiebe et al. (2001)
A. oryzae	*α-amylase*	Human lactoferrin	25 mg/L	P. P. Ward et al. (1992a)
A. nidulans	*alcA*	Human lactoferrin	5 mg/L	P. P. Ward , May, Headon, & Conneely (1992b)

A. nidulans	*alcA*	Human interferon α2	1 mg/L	Gwynne, Buxton, Williams, Garven, & Davies (1987)
A. nidulans	*alcA*	*Cellulomonas fimi* endoglucanase	20 mg/L	Gwynne et al. (1987)
A. niger	*alcA*	Human interleukin-6	100–500 mg/L	Hintz, Kalsner, Plawinski, Guo, & Lagosky (1995)
A. niger	*gpdA*	*P. chrysosporium* manganese peroxidases	15–25 mg/L	Punt et al. (2002)
A. niger	*gpdA*	Human tissue plasminogen activator	12 mg/L	Wiebe et al. (2001)
A. awamori	*gpdA*	Sweet protein thaumatin II	9.6 mg/L	Moralejo, Cardoza, Gutierrez, & Martin (1999)
A. awamori	*B2*	Sweet protein thaumatin II	7.7 mg/L	Moralejo et al. (1999)
A. awamori	*pcbC*	Sweet protein thaumatin II	1.6 mg/L	Moralejo et al. (1999)
A. awamori	*gdhA*	Sweet protein thaumatin II	10 mg/L	Moralejo et al. (1999)
A. awamori	*B2* & *gdhA*	Sweet protein thaumatin II	14 mg/L	Moralejo et al. (1999)
A. nidulans	*tpi*	Human tissue plasminogen activator	0.1 mg/L	Upshall et al. (1987)
A. nidulans	*alcA*	Human tissue plasminogen activator	1 mg/L	Upshall et al. (1987)
N. crassa	*ccg-1*	Bovine RNase A	0.11 mg/L	Allgaier et al. (2010)
N. crassa	*gla-1*	*Zea mays* zeamatin	0.01 mg/L	Rasmussen-Wilson, Palas, Wolf, Taft, & Selitrennikoff (1997)
N. crassa	*cfp*	Bovine RNase A	0.36 mg/L	Allgaier et al. (2010)
N. crassa	*enol*	Bovine RNase A	0.09 mg/L	Allgaier et al. (2010)

T. reesei: *Trichoderma reesei; T. harzianum*: *Trichoderma harzianum; A. nidulans*: *Aspergillus nidulans; A. niger*: *Aspergillus niger; A. oryzae*: *Aspergillus oryzae; A. awamori*: *Aspergillus awamori; N. crassa*: *Neurospora crassa; cbh1*: cellobiohydrolase 1; *glaA/gla-1*: glucoamylase A; *alcA*: alcohol dehydrogenase; *pcbC*: penicillin biosynthesis gene; *gdhA*: glutamate dehydrogenase gene; *B2*: the wide-spectrum esterase gene *(cesB) of Acremonium chrysogenum*; *tpi*: triose-phosphate isomerase; *ccg-1*: clock-controlled gene; *cfp*: cytoplasmic filament protein; *enol*: enolase.

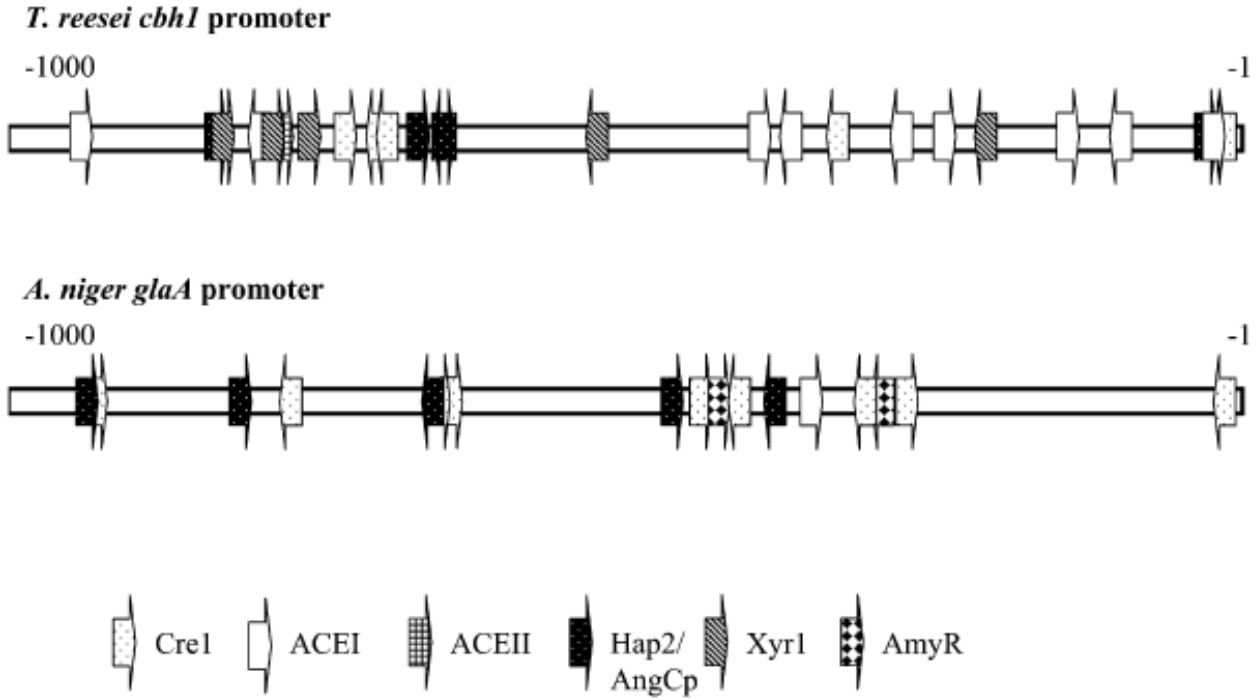


Figure 1.3 *Putative binding sites on the* Trichoderma reesei cbh1 *and* Aspergillus niger glaA *promoters*. A 1.0-kb DNA fragment upstream of the ATG codon was arbitrarily selected. The binding motifs of the transcription factors are as follows: Cre1, 5′-SYGGRG-3′, where S = C/G, Y = C/T, and R = A/G; ACEI, 5′-AGGCA-3′; ACEII, 5′-GGCTAATAA-3′; Xyr1, 5′-GGCTAA-3′; Hap2 and AngCP, 5′-CCAAT-3′; and AmyR: 5′-CGGNNNNNNNNCGG-3′ and 5′-CGGAAATTTAA-3′.

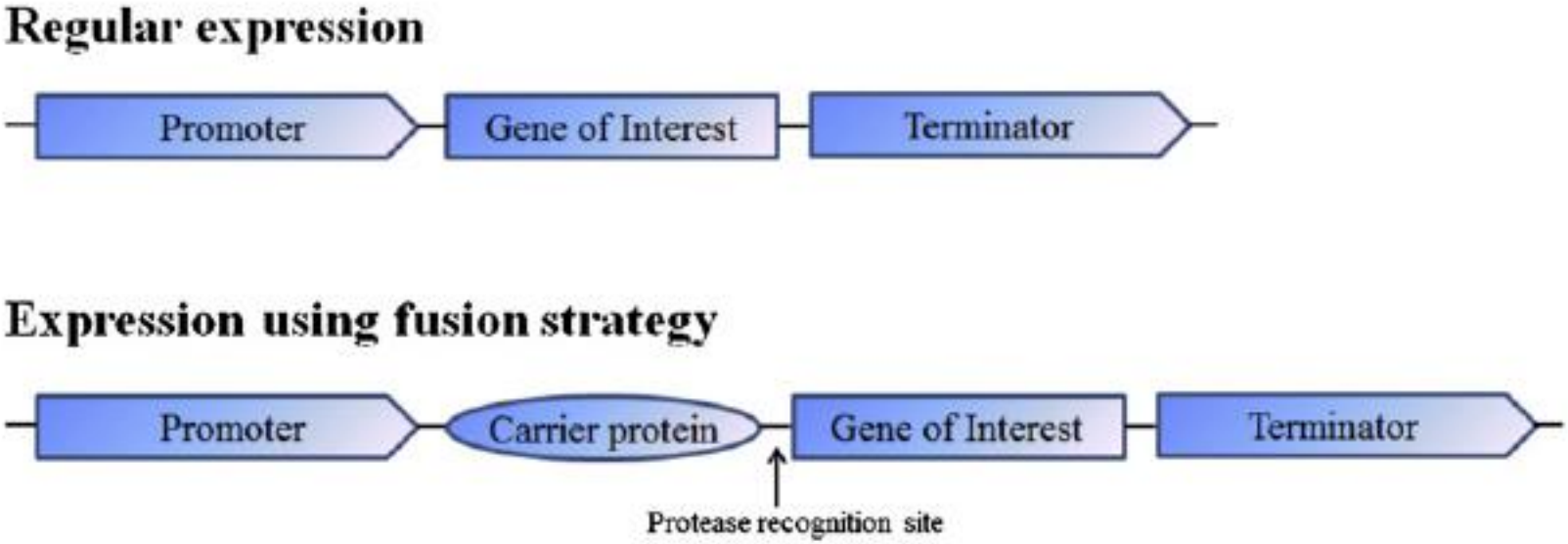


Figure 1.4 Schematic diagram showing the fusion strategy for expression of heterologous genes in filamentous fungi. (For color version of this figure, the reader is referred to the online version of this book.)

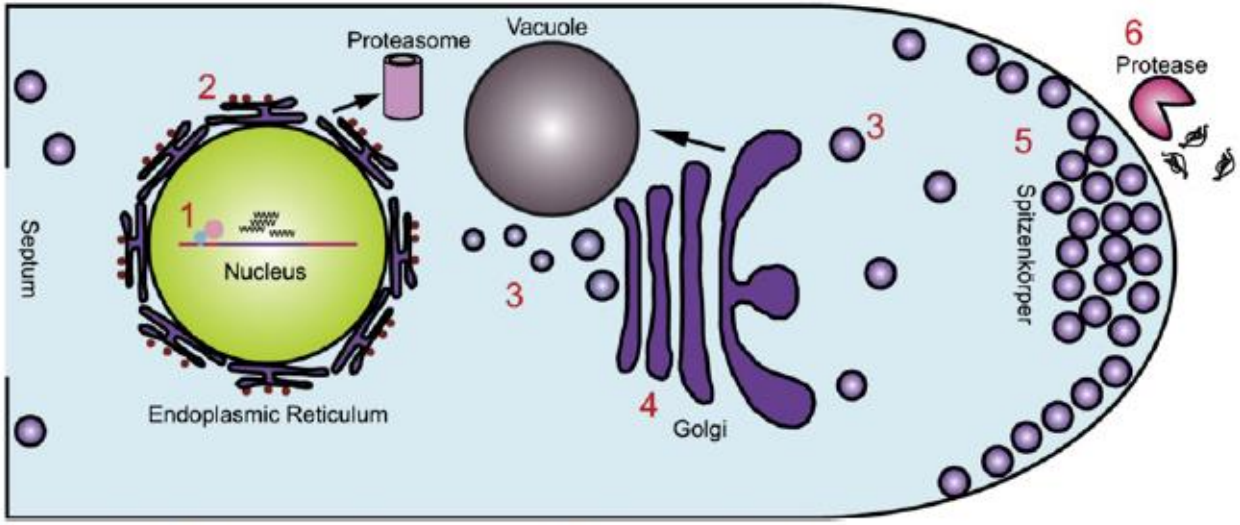


Figure 1.5 Pathways in filamentous fungi that can be engineered for enhanced heterologous gene expression. The numbers indicate the pathways that can be engineered. 1. Transcription/host defense/copy numbers/genome integration locus/intron structure; 2. signal peptide/codon usage/mRNA stability/protein quality control; 3. vesicle sorting; 4. glycosylation; 5. hyphal growth/membrane fusion; and 6. extracellular proteolysis. (For color version of this figure, the reader is referred to the online version of this book.)

FIGURE 7.14 Construct for expression and secretion of the human α_1-proteinase inhibitor in the filamentous fungus *A. niger*. The expression cassette includes the strong constitutive promoter *gpdAp*, the transcriptional terminator from the *TrpC* gene *(TrpCt)*, the cDNA encoding glucoamylase to facilitate secretion, and the coding sequence for the Kex2 recognition site for in vivo removal of the glucoamylase fusion protein by the host Kex2 endoprotease.

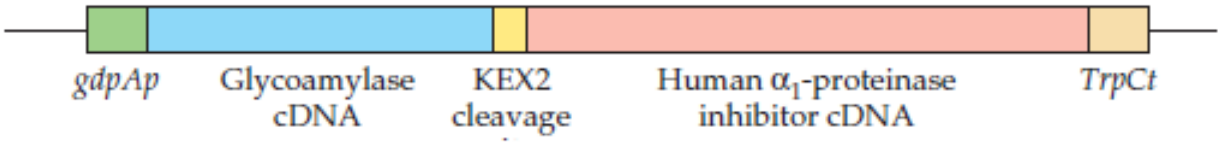


TABLE 7.4 Some recombinant proteins produced by filamentous fungal expression systems

Recombinant protein	Host cell	Main application
α-Amylase	*A. niger*, *Aspergillus oryzae*	Starch processing, food industry
Aspartyl protease	*A. nidulans*, *A. oryzae*	Food industry
Cellulase	*T. reesei*	Textile, pulp and paper industries
Chymosin	*A. niger*	Food industry
Immunoglobulin G	*A. niger*	Pharmaceutical industry
Insulin	*A. niger*	Pharmaceutical industry
Interleukin-6	*A. niger*	Pharmaceutical industry
Laccase	*A. niger*, *T. reesei*	Textile, pulp and paper industries
Manganese peroxidase	*A. niger*	Chemical industry
Lactoferrin	*A. oryzae*	Pharmaceutical industry
Lipase, thermophilic	*A. oryzae*	Detergent
Lysozyme	*A. niger*	Pharmaceutical industry
Phytase	*T. reesei*	Food industry
Xylanase	*A. niger*, *T. reesei*	Textile, pulp and paper, food industries

TABLE 7.5 Production of human interleukin-6 in filamentous fungi

Host cell	Relevant host trait	Promoter (donor)	Fusion partner	Yield (mg/liter)
A. nidulans		*glaA* (*A. niger*)		<0.1
		glaA (*A. niger*)	*glaA*	5
A. niger		*glaA*		<0.1
	Protease deficient	*gpdA*		<0.1
	Protease deficient	*gpdA*	*glaA*	2
	Protease deficient, nonacidifying	*gpdA*	*glaA*	10

Table 1 Distribution of industrial enzymes and production strains according to the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) list in 2004

Total enzymes	186
Homologous product	64.5%
Heterologous product	35.5%
Aspergillus strains	36.6%
Trichoderma strains	10.8%
Penicillium strains	8.1%
Kluyveromyces strains	1.6%
Saccharomyces strains	1.1%
Prokaryotic strains	30.6%

Table 4 Examples of some important heterologous proteins expressed in *Aspergillus*

Protein	Yield	Expression system	Reference
Glucoamylase from *A. niger*	4.6 g/l	*A. awamori*	Radzio and Kück (1997)
Human interleukin 6	150 mg/l	*A. niger*	Punt et al. (2002)
Alkaline protease from *Fusarium*	4 g/l	*A. chrysogenum*	Morita et al. (1994)
Chymosin (calf)	0.16 mg/l	*A. oryzae*	Dunn-Coleman et al. (1991)
Interferon-a 2 (human)	0.2 mg/l	*A. nidulans*	Macrae et al. (1993)
Lactoferrin (human)	2.0 g/l	*A. oryzae*	Ward et al. (1995)
Lysozyme (hen egg-white)	1.0 mg/l	*A. niger*	Archer et al. (1990); Gyamerah et al. (2002)

Enzyme	Host organism	Donor organism
Aminopeptidase	*Trichoderma reesei*	*Aspergillus* sp.
Arabinofuranosidase	*Aspergillus niger*	*Aspergillus* sp.
Catalase	*Aspergillus niger*	*Aspergillus* sp.
Cellulase	*Aspergillus oryzae*	*Humicola* sp.
Galactosidase (alpha)	*Saccharomyces cerevisiae*	Guar plant
Glucanase (beta)	*Trichoderma reesei*	*Trichoderma* sp.
Glucoamylase	*Aspergillus niger*	*Aspergillus* sp.
Glucose oxidase	*Aspergillus niger*	*Aspergillus* sp.
Laccase	*Aspergillus oryzae*	*Myceliopthora* sp.
Lactase or Galactosidase (beta)	*Aspergillus oryzae*	*Aspergillus* sp.
Lipase	*Aspergillus oryzae*	*Candida* sp.
Mannanase (endo-1, 4-beta)	*Trichoderma reesei*	*Trichoderma* sp.
Pectin lyase	*Aspergillus niger* var. *awamori*	*Aspergillus niger*
Phytase	*Aspergillus oryzae*	*Peniophora* sp.
Protease	*Aspergillus oryzae*	*Rhizomucor* sp.
Pullulanase	*Trichoderma longibrachiatum*	*Hormoconis* sp.
Xylanase	*Aspergillus niger* var. *awamori*	*Aspergillus* sp.
Chymosin	*A. niger* var. *awamori*	Calf

Microalgae

- Promising platforms for heterologous protein expression
- Several systems are currently in use
- Biggest obstacles are in transformation and cell biomass productivity
- Nuclear and chloroplast expression is available
- Limited number of species is being used, most of them belong to *Chlamydomonas*

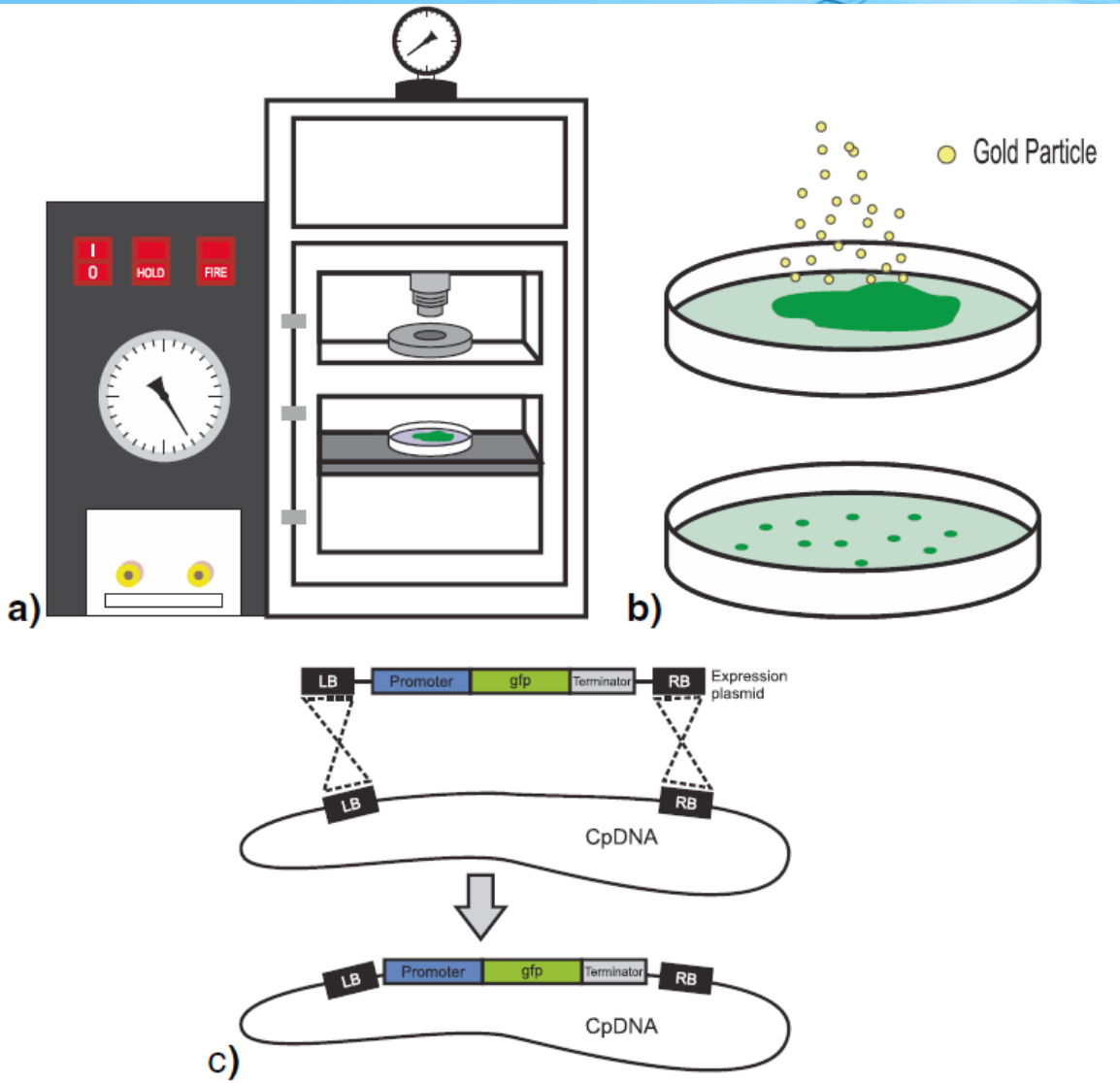


Figure 1 Chloroplast transformation in *Chlamydomonas reinhardtii*. **a)** Introduction of foreign DNA material into the chloroplast of *C. reinhardtii* is carried out using a particle bombardment device. The device uses helium gas to accelerate particles towards algae placed at the interior of a vacuum chamber. **b)** Gold or tungsten particles are coated with a plasmid carrying the genes of interest (in this case the green fluorescence protein GFP) and when accelerated penetrate the cells placed on top of selection medium. After a few weeks, transformed cells proliferate in the presence of a selection antibiotic. **c)** When the plasmid carrying the genes reaches de chloroplast, genes integrate into the plastid genome by homologous recombination between regions present in the plasmid (LB and RB) and in the chloroplast genome (CpDNA).

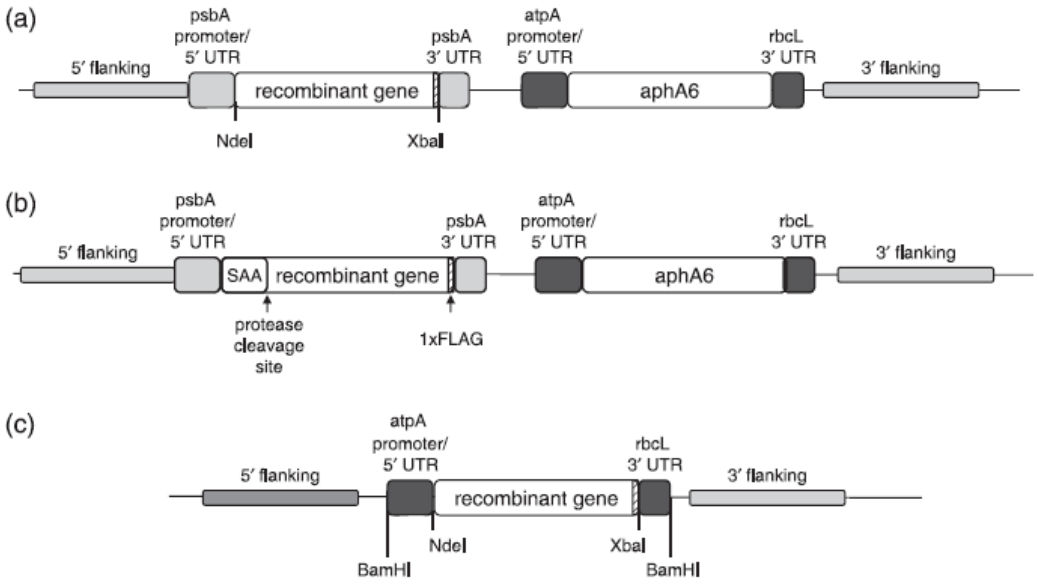
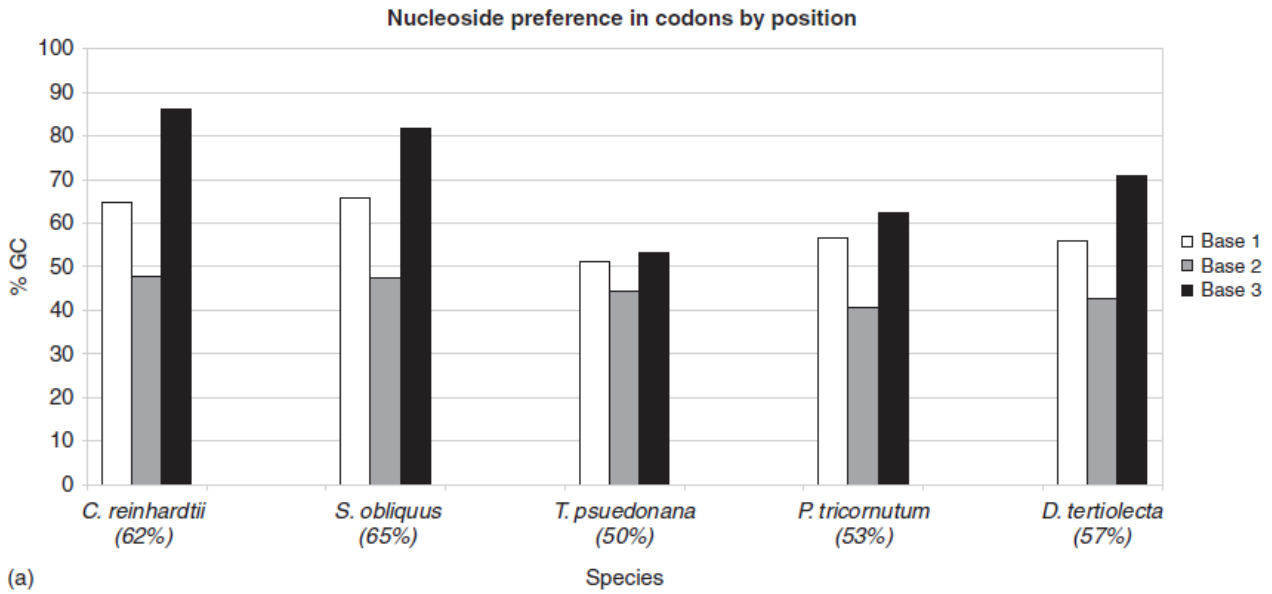


Figure 1 Introduction of the recombinant genes into the *Chlamydomonas reinhardtii* chloroplast genome. Schematic diagram of transformation vectors used, including relevant restriction sites. (a) and (b) pD1-Kan: Replacement of the endogenous *psbA* gene with the gene of interest (a), or with the gene of interest fused to the C-terminus of M-SAA (Manuell *et al.*, 2007) (b). The kanamycin-resistance gene *aphA6* under the control of the *atpA* promoter and 5′ untranslated region (UTR) is genetically linked to the gene of interest. Grey regions flanking the gene of interest and resistance gene correspond to regions of the chloroplast genome used for homologous recombination between the insertion plasmid and the *C. reinhardtii* chloroplast genome. (c) Schematic diagram of p322 (Franklin *et al.*, 2002) used to transform the genes of interest under the control of the *atpA* promoter and 5′ UTR and the *rbcL* 3′ UTR into the BamHI silent site near the *psbA* gene (Barnes *et al.*, 2005). (a–c) All recombinant proteins were C-terminally fused to the 1× FLAG-tag sequence (DYKDDDDKS) for Western blotting and purification.



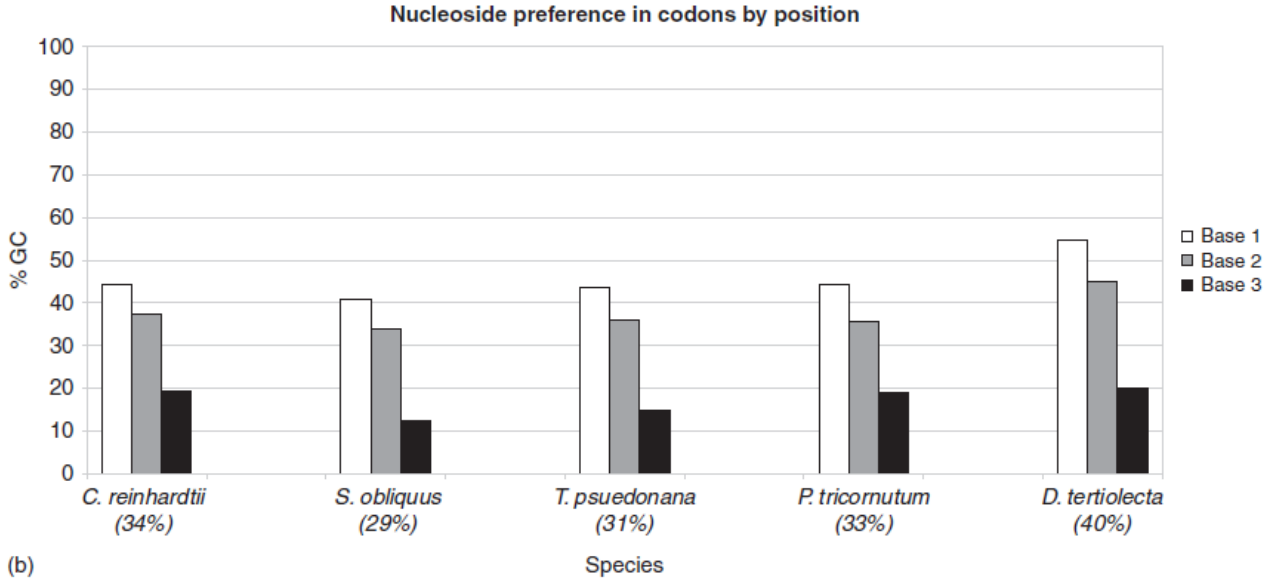


Figure 27.1. Nucleoside preference of codons by position. GC content of each position is presented based on all available coding sequences (CDSs) at the CUTG. The following photosynthetic microalgae (green algae, diatoms) are analyzed and include the average GC content in parenthesis for the (a) nucleus and (b) chloroplast: *Chlamydomonas reinhardtii*, *Scenedesmus obliquus*, *Thalassiosira pseudonana*, *Phaeodactylum tricornutum*, and *Dunaliella tertiolecta*.

Table 27.2. Nuclear and chloroplast promoters used to express transgenes in microalgae

Promoter	Source	Host	Description	Genome
vcp1	*Nannochloropsis oculata*	*Nannochloropsis oculata*	Violaxanthin/chlorophyll *a*-binding protein 1	Nuclear
vcp2	*Nannochloropsis oculata*	*Nannochloropsis oculata*	Violaxanthin/chlorophyll *a*-binding protein 2; bidirectional	Nuclear
psbD	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Photosystem II protein D2	Chloroplast
cyc6	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Cytochrome c6	Nuclear
psaD	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Photosystem I complex protein	Nuclear
hsp70A	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Heat shock protein 70A	Nuclear
psbA	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Photosystem II protein D1	Chloroplast
atpA	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	ATPase alpha subunit	Chloroplast
rbcL	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Ribulose bisphosphate carboxylase large subunit	Chloroplast
nia1	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Nitrate reductase	Nuclear
cop	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Chlamyopsin	Nuclear
p1′2′	*Agrobacterium tumefaciens*	*Amphidinium* sp., *Symbiodinium microdriaticum*	Bidirectional promoter	Nuclear
rbcs2	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Small subunit of ribulose bisphosphat carboxylase	Nuclear
fcp	*Phaeodactylum tricornutum*	*Phaeodactylum tricornutum*	Fucoxanthin chlorophyll-*a* or -*c* binding protein	Nuclear
acc1	*Cyclotella cryptica*	*Cyclotella cryptica*, *Navicula saprophila*	Acetyl-CoA carboxylase	Nuclear
CaMV 35S	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Cauliflower mosaic virus 35S	Nuclear
Nos	*Agrobacterium tumefaciens*	*Chlamydomonas*	Nopaline synthase	Nuclear
Nos	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Nopaline synthase from *Agrobacterium tumefaciens*	Nuclear
cabII-1	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	Chlorophyll-*ab* binding protein	Chloroplast
β-2-tub	*Chlamydomonas reinhardtii*	*Chlamydomonas reinhardtii*	*β*-2-tubulin	Nuclear

Table 27.3. Nuclear and chloroplast selectable markers used in microalgae

Marker	Selection	Microalgae transformed
Bsr	Blasticydin S resistance (deaminase)	*Nannochloropsis* sp. (strain W2J3B)
ARG9	Arginine prototrophy (chloroplast encoded)	*Chlamydomonas reinhardtii*
PDS	Norflurazon resistance (mutated endogenous phytoene desaturase)	*Chlorella zofingiensis*, *Haematococcus pluvialis*
aph7″	Hygromycin B resistance (aminoglycoside phosphotransferase)	*Chlamydomonas reinhardtii*
ALS	Sulfometuron methyl resistance (mutated endogenous acetolactase gene)	*Chlamydomonas reinhardtii*
aphVIII	paromomycin/kanamycin resistance (aminoglycoside phosphotransferase)	*Chlamydomonas reinhardtii*
act-2	Cycloheximide resistance (mutated endogenous ribosomal L41)	*Chlamydomonas reinhardtii*
aphA-6	Kanamycin/amikacin resistance (aminoglycoside phosphotransferase)	*Chlamydomonas reinhardtii*
nat	Nourseothricin resistance (acetyltransferase)	*Phaeodactylum tricornutum*
sat-1	Nourseothricin resistance (acetyltransferase)	*Phaeodactylum tricornutum*
hup1	Nutritional marker or trophic conversion (hexose transporter)	*Phaeodactylum tricornutum*, *Cylindrotheca fusiformis*
PPX1	Porphyric herbicide resistance (mutated endogenous protoporphyrinogen oxidase)	*Chlamydomonas reinhardtii*
hpt	Hygromycin B resistance (phosphotransferase)	*Amphidinium*, *Symbiodinium*, *Nannochloropsis* sp. (strain W2J3B)
ble	Zeocin resistance (stoichiometrically neutralizes phleomycins)	*Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*, *Nannochloropsis* sp. (strain W2J3B)
NIC7	Nicotinamide prototrophy	*Chlamydomonas reinhardtii*
THI-10	Thiamine prototrophy	*Chlamydomonas reinhardtii*
cat	Chloramphenicol resistance (acetyltransferase)	*Chlamydomonas reinhardtii*, *Phaeodactylum tricornutum*
CRY1-1	Cryptopleurine/emetine resistance (mutated endogenous ribosomal S14)	*Chlamydomonas reinhardtii*
nptII	Neomycin resistance (phosphotransferase)	*Chlamydomonas reinhardtii*, *Symbiodinium* sp., *Phaeodactylum tricornutum*, *Amphidinium* sp., *Cyclotella cryptica*, *Navicula saprophila*
aadA	Spectinomycin/Streptomycin resistance (adenylyltransferase)	*Chlamydomonas reinhardtii*
oee-1	Oxygen-evolving enhancer protein (restores photosynthesis)	*Chlamydomonas reinhardtii*
ARG7	Arginine prototrophy	*Chlamydomonas reinhardtii*
NIT1 (NIA1)	Nitrate prototrophy	*Chlamydomonas reinhardtii*

Protein	Biotechnological application	Bioassay	Genetic source	Platform organism	Compartment
α-HBsAg full-length IgG1 mAb (CL4mAb)	Binds hepatitis B surface antigen	HBsAg binding ELISA	*Homo sapiens*	*Phaeodactylum tricornutum*	Cytosol
Hepatitis B virus surface antigen (HBsAg)	Immunogen	α-HBsAg binding inhibition ELISA	Hepatitis B virus	*Phaeodactylum tricornutum*	Cytosol
C-terminal domain from the apical major antigen AMA1 fused to a truncated granule-bound starch synthase (GBSS)	Immunogen	Red blood cell entry inhibition assay and lethal dose mouse survivability	*Plasmodium berghei*	*Chlamydomonas reinhardtii*	Nuclear encoded, chloroplast directed
C-terminal domain from the Major Surface Protein (MSP1) fused to a truncated granule-bound starch synthase (GBSS)	Immunogen	Red blood cell entry inhibition assay and lethal dose mouse survivability	*Plasmodium falcipirum*	*Chlamydomonas reinhardtii*	Nuclear encoded, chloroplast directed
D2 fibronectin-binding domain of *Staphylococcus aureus* fused with the cholera toxin B subunit (CTB-D2)	Immunogen	IgA and IgG ELISA, pathogen load qtPCR, lethal dose survivability in mice	*Staphylococcus aureus*, *Vibrio cholerae*	*Chlamydomonas reinhardtii*	Chloroplast
High mobility group protein B1 (HMGB1)	Inflammatory cytokine	Fibroblast chemotaxis	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast
Vascular endothelial growth factor (VEGF)	Therapeutic angiogenesis	VEGF receptor binding ELISA	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast
α-PA83 full-length IgG1 mAb (83K7C)	Binds anthrax protective antigen 83 (PA83); anthrax neutralization	PA83 binding ELISA	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast

Bovine lactoferricin (LFB)	Digestive tract bacteriocidal	Fish-feeding survival assay	*Bos taurus*	*Nannochloropsis oculata*	Cytosol
Glutamic acid decarboxylase 65 (hGAD65)	Autoantigen	Sera immunoreactivity and spleen cell proliferation in NOD mice	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast
Bovine mammary-associated serum amyloid (M-SAA)	Intestinal infection protectant for livestock	Mucin induction assay	*Bos taurus*	*Chlamydomonas reinhardtii*	Chloroplast
Swine fever virus E2 viral protein (CSVF-E2)	Immunogen	Subcutaneous immunization in mice	Classical swine fever virus	*Chlamydomonas reinhardtii*	Chloroplast
Metalothionein-2 (hMT-2)	UV protectant	Cell survival after UV exposure	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast
A-glycoprotein D large single chain mAb (HSV8-lsc)	Binds herpes simplex virus glycoprotein D	HSV8 binding ELISA	*Homo sapiens*	*Chlamydomonas reinhardtii*	Chloroplast
Cholera toxin B subunit fused to FMD virus VP1 (CTBVP1)	Mucosal adjuvant fused to a livestock viral immunogen	GM1-ganglioside receptor binding ELISA	*Vibrio cholerae*, Foot-and-mouth disease virus	*Chlamydomonas reinhardtii*	Chloroplast
Flounder growth hormone (fGH)	Agricultural growth hormone	Dietary supplementation and growth promotion	*Panaeolus olivaceus*	*Chlorella ellipsoidea*, *Nannochloropsis oculata*	Cytosol

Table 1 Overview of recombinant proteins produces in the chloroplast of *Chlamydomonas reinhardtii*

Recombinant therapeutic protein	Yield	Relevant information
VP1-CTB; Protein VP1 from foot and mouth disease virus (FMDV) fused to cholera toxin B (CTB)	3-4% Total Soluble Protein (TSP)	Demonstrated that the *C. reinhardtii* chloroplast derived VP1-CTB could bind to GM1-ganglioside receptor *in vitro*
HSV-Isc; Large single chain (Isc) antibody directed against glycoprotein D protein from Herpes simplex virus (HSV)	Not reported	First report to show that the *C. reinhardtii* chloroplast can efficiently fold antibodies and form disulfide bonds
TRAIL; Tumor necrosis factor-related apoptosis-inducing ligand	0.43%-0.67% TSP	
M-SAA; Mammary-associated serum amyloid	3%-5% TSP	M-SAA was shown to generate mucin induction in a human intestinal epithelial cell line. Demonstrated that the *psbA* promoter yields high level or recombinant protein accumulation when the endogenous *psbA* gene is absent
CSFV-E2; Classical swine fever virus (CSFV) structural protein E2	1.5-2% TSP	Subcutaneous immunization of mice with E2 was shown to induced IgG antibodies
Human glutamic acid decarboxylase (hGAD65)	0.25-0.3% TSP	The protein was shown to immunoreact with sera from diabetic mice
IBDV-VP2; Infectious burial disease virus VP2 protein	4-0.8% Total cell protein (TCP)	This report looked at the expression of 11 proteins. Nine proteins showed some level of accumulation, while the rest could not be detected. It showed that there are variations in the level of expression even amongst lines obtained with the transformation construct. Authors postulated the existence of the transformosome, a state in which particular genomic characteristics, induced incidentally with transformation, affect, negatively or positively, the expression of the transgene
IHNV-G; Infectious haematopoietic necrosis virus	< 0.5% TCP	
IPNV-VP2; Infectious pancreatic necrosis virus	< 0.3% TCP	
VP2 protein	1-0.1% TCP	
IPNV-VP2 SBC; Infectious pancreatic necrosis virus	1-0.2% TCP	
Quorum sensing-regulated gene (LecA) p57	< 0.5 TCP	
PCV2; Porcine circovirus type 2	0.9-0.2% TCP	
VP-2C	< 0.5% TCP	
VP28	21-0.2% TCP	
HC-83K7C; Heavy chain human monoclonal antibody against anthrax protective antigen 83 (PA83)	0.01% dwt	It was shown that the heavy and light chains expressed in trans could assembled into a fully-functional monoclonal antibody against PA83
LC-83K7C; Light chain human monoclonal antibody against anthrax PA83		

CTB-D2; D2 fibronectin-binding domain of *Staphylococcus aureus* fused to the cholera toxin B subunit	0.7% TSP	First report to show that an orally-administered alga expressing an antigen in the chloroplast triggers a mucosal and systemic immune response in mice
14FN3; Domain 14 of human fibronectin	3%-0.15% TSP	This report looked at the expression of seven therapeutic proteins. For three of the proteins, a level of accumulation above 1% was observed, whereas for the rest of the proteins, erythropoietin, interferon β, and proinsulin no protein was detected. Biological activity was evaluated for VEGF and HMGB1
VEGF; Human vascular endothelial growth factor	2%-0.1% TSP	
HMGB1; High mobility group protein B1	2.5%-1% TSP	
acrV2 and vapA2; antigens from the fish pathogen *Aeromonas salmonicida*	0.8% and 0.3% TP respectively	Showed that the *psaA* promoter-exon1 element can be used to drive the expression of foreign genes in non-photosynthetic strains
***Escherichia coli* phytase gene (*appA*)**	N.D.	This study showed that algae expressing a bacterial phytase gene in the chloroplast could be lyophilized and administered orally to broiler chicks. The enzyme was active in the gut and reduce the fecal excretion of phytate.
Pfs25 and Pfs28; surface proteins from *Plasmodium falciparum*	0.5% and 0.2% TSP respectively	First report to show that Pfs25 and Pfs28 can be produced without glycosilation and in a correct conformation recognized by monoclonal antibodies specific to conformational epitopes
αCD22PE40; monomeric immunotoxin consisting on the single chain antibody that recognizes the CD22 surface protein from B-cells, fused to domains II and III of exotoxin A (PE40) from *Pseudomonas aeruginosa*	0.3%-0.4% TSP	First report to show that immunotoxins can be produced in an eukaryotic system without being toxic to the cell.
αCD22HCH23PE40; dimeric version of αCD22PE40	0.2%-0.3% TSP	
CtxB-Pfs25; *Plasmodium falciparum* surface protein 25 fused to the β subunit of the cholera toxin from *Vibrio cholera*	0.09% TSP	Demonstrated that the fusion protein can induced IgA antibodies when administered orally as part of a lyophilized powered. However, IgG antibodies could not be elicited with this route of administration
αCD22Gel; single chain antibody targeting the CD22 receptor from B-cells, fused to the eukaryotic ribosome inactivating protein, gelonin, from *Gelonium multiflorm*	0.2%-0.3% TSP	Demonstrated that immunotoxin can efficiently bind to cancerous B-cells in vitro and kill them without affecting non B-cells
αCD22CH23Gel; dimeric version of αCD22Gel	0.1% - 0.2% TSP	