MARKER GENES

- Selectable markers – for the selection of transgenic cells, tissues, plants with chemicals that are otherwise toxic to plant
- Reporter genes – will cause a visible color change in the transgenic plants (to see where transgenes are expressed in plant tissues)

- *Marker-free plants (a requirement to remove marker genes from transgenic plants!!)*

An example of a construct with and without reporter gene (GFP)

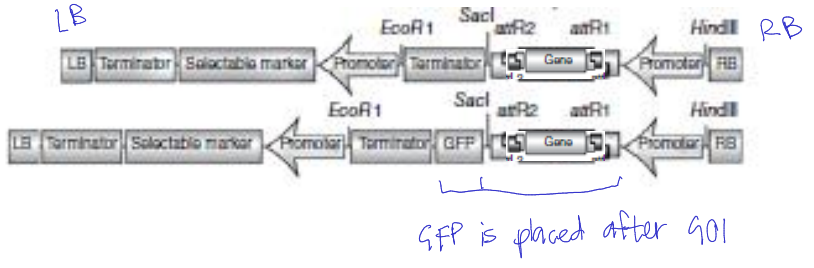

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TABLE 10.4. Categories of Marker Genes and Selective Agents used in Plants

Category	Marker genes	Source of genes	Selective agent
Selectable marker genes:			
Antibiotic-resistant	*nptII, neo, aphII*	*Escherichia coli* Tn*5* (bacterial)	Kanamycin
	hpt, hph, aphIV	*E. coli* (bacterial)	Hygromycin
Herbicide-resistant	*bar*	*Streptomyces hygroscopicus* (bacterial)	Phosphinothricin
	pat	*Streptomyces viridochromogenes* (bacterial)	Phosphinothricin
	CP4 EPSPS	*Agrobacterium* sp. strain CP4 (bacterial)	Glyphosate
Nutritional inhibitor-related	*manA*	*E. coli* (bacterial)	Mannose
	xylA	*S. rubiginosus*; *Thermoanaerobacterium thermosulfurogenes* (bacterial)	D-xylose
Hormone-related	*ipt*	*Agrobacterium tumefaciens* (bacterial)	N/A[a]
Ablation	*codA*	*E. coli* (bacterial)	5-Fluorocytosine
Reporter genes:			
Enzymatic	*uidA, gusA*	*E. coli* (bacterial)	MUG, X-gluc
	Luc	various	luciferin
Fluorescent proteins	*gfp*	*Aequorea victoria* (jellyfish)	N/A
	pporRFP	*Porites porites* (hard coral)	N/A
	mOrange	*Discosoma* sp. (soft coral)	N/A

[a] Not applicable.

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Reporter gene *β-glucoronidase* (*GUS*)

Vir: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3212524/

3

Bioluminiscence

The enzyme luciferase uses **luciferin as substrate and produces bioluminescent light.**

protein

The half-life of the luciferase protein in plant cells is lower than that for GUS and may reflect transcriptional activity more accurately

Figure 10.9. Luminescence detected in transgenic tobacco transformed with the firefly luciferase gene driven by the 35S promoter and watered with a solution of luciferin, the luciferase substrate. (*Source*: From Ow et al. (1986). Reproduced with permission of AAAS.) (*See insert for color representation of the figure.*)

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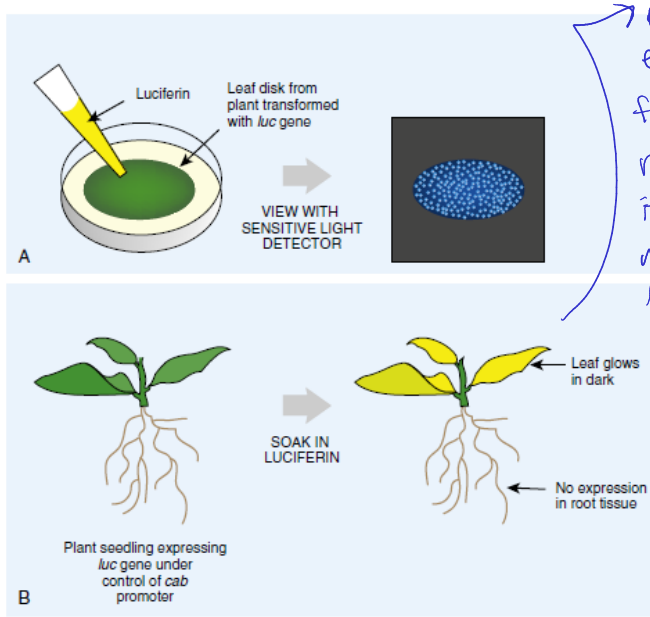

→ expose plant to enzyme; luciferin protein will react with luciferase and emmit flourescent light

FIGURE 15.12 Luciferase as a Reporter in Plant Tissues
Plant tissue carrying the *luc* gene for firefly luciferase emits blue light when provided with the substrate luciferin. (A) A leaf disk is viewed by a photocell detector. (B) The *luc* gene in a seedling is expressed under control of an inducible promoter.

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Fluorescent proteins

- FPs have become the most important reporter gene system for plants.
- They require[1] no external substrate for detection, and there have been no[2] reports of detrimental effects on the fitness of plants that express them
- [3] Simultaneously monitoring of several FPs
- [4] great sensitivity at the subcellular level (confocal laser scanning microscopy with real-time detection in living cells)

2 types of flourescent rep-genes/proteins

1. luciferin + luciferase
2. flourescent proteins

↑ better!

4 reasons above

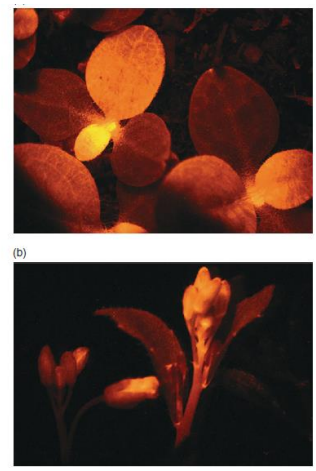

Figure 10.11. Orange fluorescent proteins whose genes were cloned from corals and expressed in tobacco (a) and *Arabidopsis* (b) plants. (*See insert for color representation of the figure.*)

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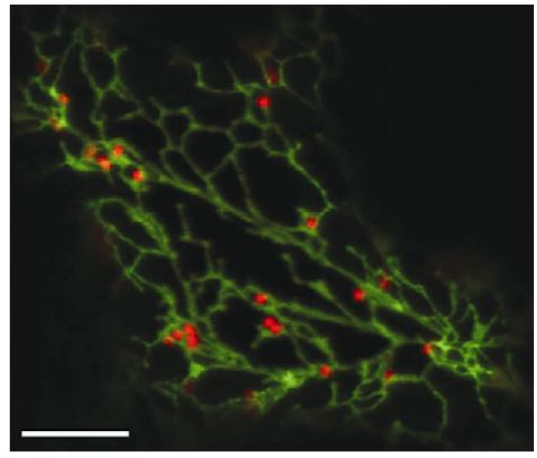

Figure 10.10 Confocal laser scanning microscopy of leaf mesophyll cells transiently expressing peptides fused to green fluorescent protein or GFP (green image) and yellow fluorescent protein (red image). GFP is fused to the HDEL tetrapeptide (spGFP-HDEL) to achieve ER retention and thus reveals the cortical ER network in leaf cells. The proximity of the Golgi to the ER network is revealed by the yellow FP fused to a Golgi glycosylation enzyme (ST-YFP). (Bar = 10 µm.) (*Source*: From Brandizzi et al. 2004.) (*See insert for color representation of the figure.*)

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Method for removing the selectable marker (marker-free plants)

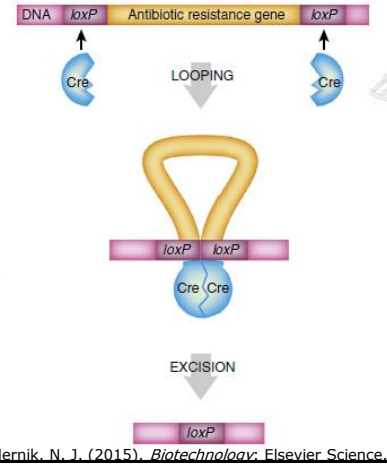
- It is based on the Bacteriophage's P1 system for genetic recombination - the *Cre/loxP* system

Cre stands for "**c**auses **re**combination"
recombinase recognizes a specific 34 base-pair DNA sequence, the *loxP site*

Cre recombinase catalyzes recombination between two loxP sites

How Cre recombinase can be added to the plant cells?
a) cross-pollination of two different plants – one plant carrying the transgene plus a selectable marker that is flanked by two loxP sites and another plant carrying the cre gene.
b) Cre recombinase is included in the construct under the inducbile promoter

FIGURE 15.13
Cre/*loxP* System of Bacteriophage P1
The Cre protein binds to *loxP* recognition sites in the DNA. Two nearby *loxP* sites are brought together, and recombination between them eliminates the intervening DNA. A single *loxP* "scar" site remains in the target DNA molecule.



Clark, D. P., & Pazdernik, N. J. (2015). *Biotechnology*: Elsevier Science.

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a) cross-pollination…

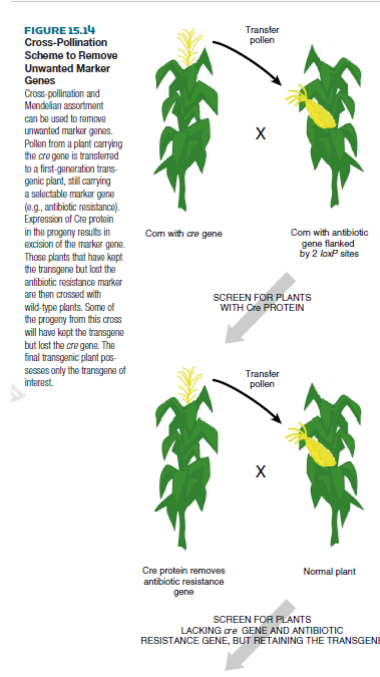

FIGURE 15.14 Cross-Pollination Scheme to Remove Unwanted Marker Genes
Cross-pollination and Mendelian assortment can be used to remove unwanted marker genes. Pollen from a plant carrying the *cre* gene is transferred to a first-generation transgenic plant, still carrying a selectable marker gene (e.g., antibiotic resistance). Expression of Cre protein in the progeny results in excision of the marker gene. Those plants that have kept the transgene but lost the antibiotic resistance marker are then crossed with wild-type plants. Some of the progeny from this cross will have kept the transgene but lost the *cre* gene. The final transgenic plant possesses only the transgene of interest.

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b) Cre recombinase under the inducible promoter…
- Addition of *β*-estradiol causes a series of events, resulting in the expression of Cre recombinase

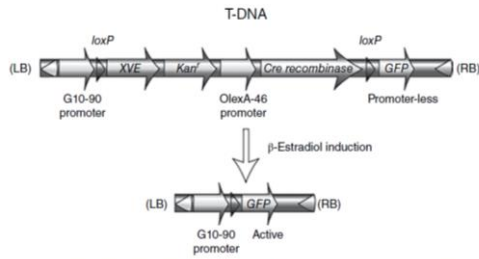

Figure 8.17. Excision of selectable marker gene following T-DNA insertion into the plant genome. XVE is a chimeric transcription factor. It contains three functional domains: a LexA DNA-binding domain (X), the VP16 activation domain (V), and the estrogen receptor-binding domain (E). The G10-90 promoter drives the constitutive and ubiquitous expression of XVE in transformed plant cells. The XVE protein is then bound as a monomer in the cytosol of the cell by a chaperone protein HSP90, and the target gene is transcriptionally inactive. Application of *β*-estradiol causes a conformational change in E, which leads to the release of HSP90 and dimerization of the receptor. On dimerization, the receptor is activated, allowing the protein to translocate to the nucleus of the cell where it binds O*LexA* binding sites of the promoter that is placed upstream of the Cre recombinase. The VP16 activation domain activates RNA polymerase II, leading to the transcription of the Cre recombinase gene. Cre recombinase allows recombination to occur between the *LoxP* sites removing all intervening genes, including the selectable marker gene.

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PMID: 28871269

A Chemical-Induced, Seed-Soaking Activation Procedure for Regulated Gene Expression in Rice

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Abstract

Inducible gene expression has emerged as a powerful tool for plant functional genomics. The estrogen receptor-based, chemical-inducible system XVE has been used in many plant species, but the limited systemic movement of inducer β-estradiol in transgenic rice plants has prohibited a wide use of the XVE system in this important food crop. Here, we constructed an improved chemical-regulated, site-specific recombination system by employing the XVE transactivator in combination with a Cre/*loxP*-FRT system, and optimized a seed-soaking procedure for XVE induction in rice. By using a *gus* gene and an hpRNAi cassette targeted for *OsPDS* as reporters, we demonstrated that soaking transgenic seeds with estradiol solution could induce highly efficient site-specific recombination in germinating embryos, resulting in constitutive and high-level expression of target gene or RNAi cassette in intact rice plants from induced seeds. The strategy reported here thereby provides a useful gene activation approach for effectively regulating gene expression in rice.

Keywords: DNA recombination; Oryza sativa; XVE system; regulated gene expression; seed-soaking.

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Components for Efficient Gene Expression in Plants

- Failure to obtain gene expression using cistrons (gene and promoter sequences) from other species led to the first chimeric genes that used the 5' and 3' nopaline synthase (nos) regulatory sequences: the nos promoter (contains sequences that resemble CAAT and TATA boxes) and nos terminator (contains AATAA polyadenylation signal).
- UTR regions (5' in 3') – often the omega sequence from the 5' UTR of the tobacco mosaic virus (TMV) is used to enchance translation in dicot plants. Omega contains a poly(CAA) region, which serves as a binding site for the heat-shock protein, HSP101, which is required for translational enhancement.
- *Codon usage* → species can differ in the usage of codons to express certain amino acids, e.g. AAA can be more frequently used than AAG, although both encode the same AA.
- Positional effect…

Thus codons must be appropriately altered in transgenes between bacteria/plant to make expression more efficient.

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Promoters

A constitutive promoter allows constant expression of a gene in all tissues and at all developmental stages.

- The most frequently used constitutive promoters are those of the 35S transcript in the cauliflower mosaic virus (CaMV) or of genes (i.e., the nopaline synthase (nos) gene) found in the T-DNA of Ti (tumor-inducing) plasmids
- ZmUbi1 (from Zea mays) and OsAct2 (from Oryza sativa) promoters are the most widely used in monocot crops.

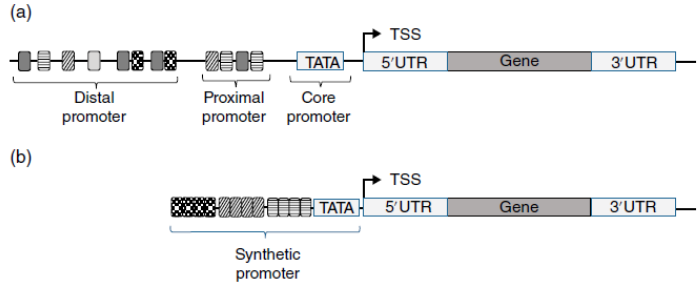

Figure 10.1. Examples of promoters that could be used to regulate the expression of a transgene. In the first example (a) a native plant promoter is shown, which contains a TATA-box in the core region, a few key motifs in the proximal region and many more distant motifs in the distal region. These motifs are the binding sites for various transcription factors, activators, or repressors. In the second example (b) a synthetic promoter has essentially the same features but is in a compressed state. The untranslated regions (UTRs) are shown as is the transcription start site (TSS).

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Tissue specific promoters

TABLE 10.1. The Most Widely Used Tissue-Specific Promoters in Plants

Promoter type	Promoter name	Gene function	Species	References
Green tissue	Cab3	Chlorophyll a-/b-binding protein	*Arabidopsis*	Mitra et al. (1989)
	rbcS	Ribulose bisphosphate carboxylase small subunit	*Arabidopsis*	De Almeida et al. (1989)
	PEPC	Phosphoenolpyruvate carboxylase	Maize	Ku et al. (1999)
Vascular tissue	PP2	Phloem protein 2	Pumpkin	Guo et al. (2004)
	Pfn2	Profilin 2	*Arabidopsis*	Christensen et al. (1996)
Root	EIR1	Ethylene-insensitive root1	*Arabidopsis*	Luschnig et al. (1998)
	NAC10	*N*AM, *A*TAF1-2, *C*UC2	Rice	Jeong et al. (2000)
Pollen	Lat52;59	Late anthogenesis	Tomato	Twell et al. (1991)
	TA29	Tobacco anther-specific protein TA29	Tobacco	Koltunow et al. (1990)
	Zm13	Pollen specific	Maize	Hamilton et al. (1998)
Seed	napA	Napin storage protein	*Brassica napus*	Rask et al. (1998)
	GluB-1	Glutelin storage protein	Rice	Wu et al. (2000)

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Inducible promoters

TABLE 10.2. The Most Widely-used Inducible Promoters in Plants

Promoter inducibility	Promoter name	Gene function	Species	References
Pathogen	PR1	Pathogenesis-related 1	*Arabidopsis*	Lebel et al. (1998)
	NPR1	Nonexpressor of PR1	*Arabidopsis*	Yu et al. (2001)
	VSP1	Vegetative storage protein 1	*Arabidopsis*	Guerineau et al. (2003)
	PcPR1-1	Pathogenesis-related 1	Parsley	Rushton et al. (2002)
	PcPAL1	Phenylalanine ammonia-lyase 1	Parsley	Lois et al. (1989)
	PR2-d	Pathogenesis-related 2-d	Tobacco	Shah et al. (1996)
	NtGlnP	Glucanase 2	Tobacco	
Light	CHS	Chalcone synthase	Parsley	Weisshaar et al. (1991)
	LHCP	Light-harvesting chlorophyll a/b protein	Pea	Simpson et al. (1985)
	Rca	Rubisco activase	Spinach	Orozco and Ogren (1993)
Wound	MPI	Maize proteinase inhibitor	Maize	Cordero et al. (1994)
	Pin2	Proteinase inhibitor II	Potato	Thornburg et al. (1987)
Drought	ERD1	Early responsive to dehydration stress 1	*Arabidopsis*	Tran et al. (2004)
Salt	RD29A, B	Responsive to desiccation 29A, B	*Arabidopsis*	Yamaguchi-Shinozaki and Shinozaki (1994)
Cold	Cor15A	Cold-regulated 15A	*Arabidopsis*	Stockinger et al. (1997)
	CBF2/DREB1C	C-repeat/DRE binding factor 1	*Arabidopsis*	Zarka et al. (2003)
ABA	HVA22	ABA-inducible	Wheat	Shen et al. (1993)
	Osem	Rice homolog of Em	Rice	Hattori et al. (1995)
ABA, Drought	RD22	Responsive to desiccation	*Arabidopsis*	Abe et al. (1997)
	Em	Late embryogenesis	Wheat	Guiltinan et al. (1990)
	RD29A,B	Responsive to desiccation 29A, B	*Arabidopsis*	Yamaguchi-Shinozaki and Shinozaki (1994)
Ethanol	AlcA	Alcohol-regulated	*Aspergillus nidulans*	Caddick et al. (1998)

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Codon usage effect

Different organisms favor different codons for the same amino acid, a phenomenon known **as codon bias**, and have different levels of the corresponding tRNAs. If a bacterial transgene uses codons that require tRNA molecules that are rare in the plant, the rate of protein synthesis will be limited. This is particularly a problem for transgenes, which often need to be expressed at high levels. Therefore, the Bt toxin gene was altered by changing many of the bases in the third position of redundant codons. Almost 20% of its bases were altered to make the gene more plant-like in codon usage

In practice, different organisms favor different codons for the same amino acid. For example, both AAA and AAG encode the amino acid lysine. In E. coli, AAA is used 75% of the time and AAG only 25%. In contrast, Rhodobacter does the exact opposite and uses AAG 75% of the time, even though E. coli and Rhodobacter are both gram-negative bacteria.

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ASSAYS FOR TRANSGENICITY, INSERT COPY NUMBER, AND SEGREGATION

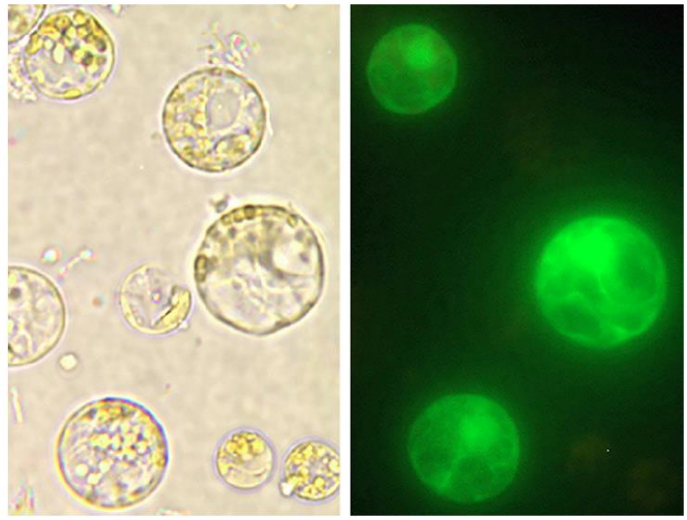

Figure 11.9. Maize protoplasts, electroporated with a *gfp* gene, showing bright field (left) and with GFP filters (right). Courtesy of JC Jang. (*See insert for color representation of the figure.*)

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Segregation analysis

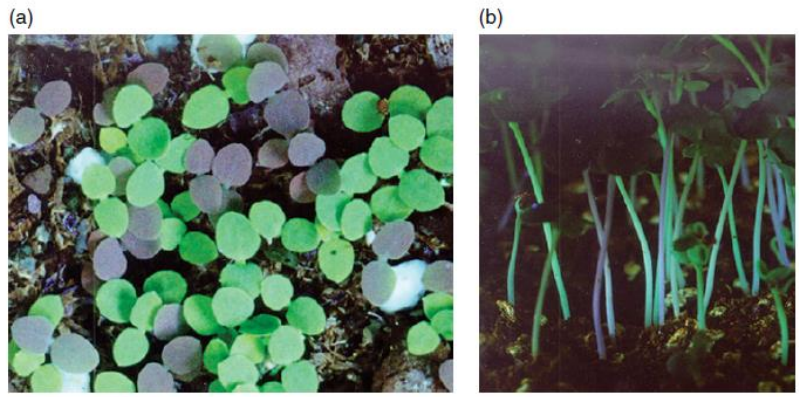

Figure 12.8. Segregation analysis of T1 transgenic (a) tobacco and (b) canola seedlings that have a single insert of a green fluorescent protein (GFP) gene. Under a UV light, the transgenic plants fluoresce green and the non-transgenic plants fluoresce red. The transgene presence and the single insert into the genome are confirmed by the Mendelian 3 : 1 segregation pattern in both of these cases. (*Source*: Reproduced with permission from Harper et al. (1999). (*See insert for color representation of the figure.*)

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QPCR analysis

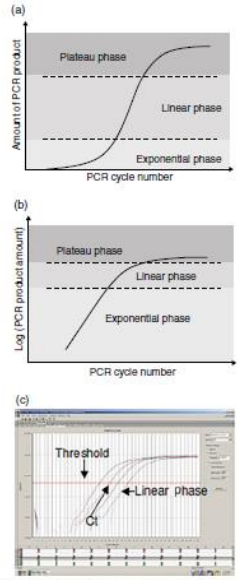

Figure 12.3. The dynamics of qPCR and analysis. (a) Theoretical plot of PCR cycle number versus PCR product showing the phases of DNA amplification. (b) Another view of the phases, but where PRC product is expressed in logarithmic terms. (c) The same scheme as panel (b), but with actual data of four samples are shown. The amount of target template decreases in the samples going from left to right as shown by respectively increasing cycle threshold (Ct) numbers. Ct is defined as the cycle at the boundary between exponential and linear phases. By knowing exactly how much DNA is in certain samples in the beginning, we can infer the amounts of DNA (and hence copy numbers) in the unknowns. This figure is reprinted with permission from Yuan et al. 2006.

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Southern (DNA) Blot Analysis

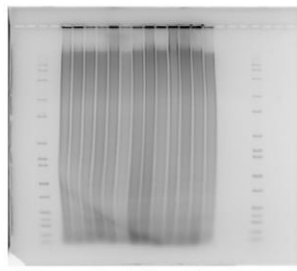

Figure 12.4. Thirteen samples of plant genomic DNA are completely digested by a restriction endonuclease and subjected to agarose gel electrophoresis to separate the DNA fragments according to size. The DNA is stained. Flanking these samples are "apparently" empty lanes and flanking these lanes are DNA size markers. One or more of the apparently empty lanes contains cut plasmid DNA that can be used as a positive control in the Southern blot analysis. The DNA will be transferred to a nylon membrane that can be probed by a labeled DNA molecule of interest.

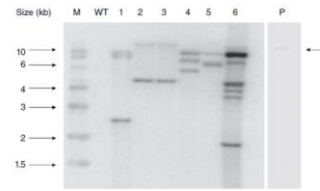

Figure 12.6. The raw data of part of a Southern blot experiment (superfluous lanes were removed for simplification). BamHI-digested genomic DNA was loaded in each of the plant lanes: WT (non-transgenic wild-type) and 1–6 (each putative independent transgenic T0 plants). M represents a DNA marker and P represents the plasmid control sample containing the gene of interest, which will bind to the DNA used as a probe; the arrow points to the faint band. It appears as if lanes 1–6 represent 5 independent transgenic plant events.

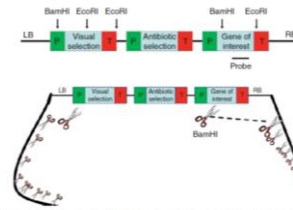

Figure 12.7. A schematic showing the T-DNA construct (top) and rationale behind the choices and setup of the experiment whose results are shown in Figure 12.7. In this vector, the BamHI and EcoRI restriction sites are shown, as well as the location of the probe DNA (top). When the T-DNA gets integrated into a plant genomic locus on a chromosome (bottom), the scissors represent actual cutting sites and some of the DNA fragments generated. Only the fragment represented by the dashed line will be hybridized by the probe in the Southern hybridization. Thanks to Mai Huber for assistance with this figure.

https://www.youtube.com/watch?v=3I9wzwj0b_A

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