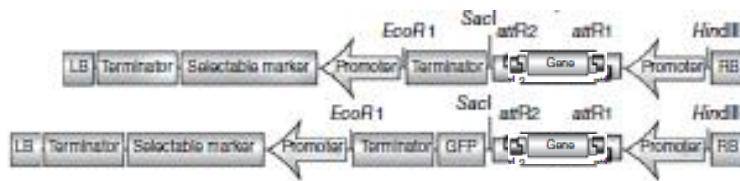


## MARKER GENES

- 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)



1

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

| Category                        | Marker genes                              | Source of genes   | Selective agent  |
|---------------------------------|---|---|--|
| <i>Selectable marker genes:</i> |   |   |  |
| Antibiotic-resistant            | <i>nptII, neo, aphII, hpt, hph, aphIV</i> | <i>Escherichia coli</i> Tn5 (bacterial)   | Kanamycin  |
| Herbicide-resistant             | <i>bar, pat, CP4 EPSPS</i>                | <i>Streptomyces hygroscopicus</i> (bacterial)<br><i>Streptomyces viridochromogenes</i> (bacterial)<br><i>Agrobacterium</i> sp. strain CP4 (bacterial) | Phosphinotrichicin<br>Phosphinotrichicin<br>Glyphosate |
| Nutritional inhibitor-related   | <i>manA, xylA</i>                         | <i>E. coli</i> (bacterial)<br><i>S. rubiginosus; Thermoanaerobacterium thermosulfurogenes</i> (bacterial)   | Mannose<br>D-xylose                                    |
| Hormone-related Ablation        | <i>ipt, codA</i>                          | <i>Agrobacterium tumefaciens</i> (bacterial)<br><i>E. coli</i> (bacterial)  | N/A <sup>a</sup><br>5-Fluorocytosine                   |
| <i>Reporter genes:</i>          |   |   |  |
| Enzymatic                       | <i>uidA, gusA, Luc</i>                    | <i>E. coli</i> (bacterial)<br>various   | MUG, X-gluc<br>luciferin                               |
| Fluorescent proteins            | <i>gfp, pporRFP, mOrange</i>              | <i>Aequorea victoria</i> (jellyfish)<br><i>Porites porites</i> (hard coral)<br><i>Discosoma</i> sp. (soft coral)                                      | N/A<br>N/A   |

<sup>a</sup>Not applicable.

Reporter gene  $\beta$ -glucuronidase (GUS)



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

3

#### Bioluminescence

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

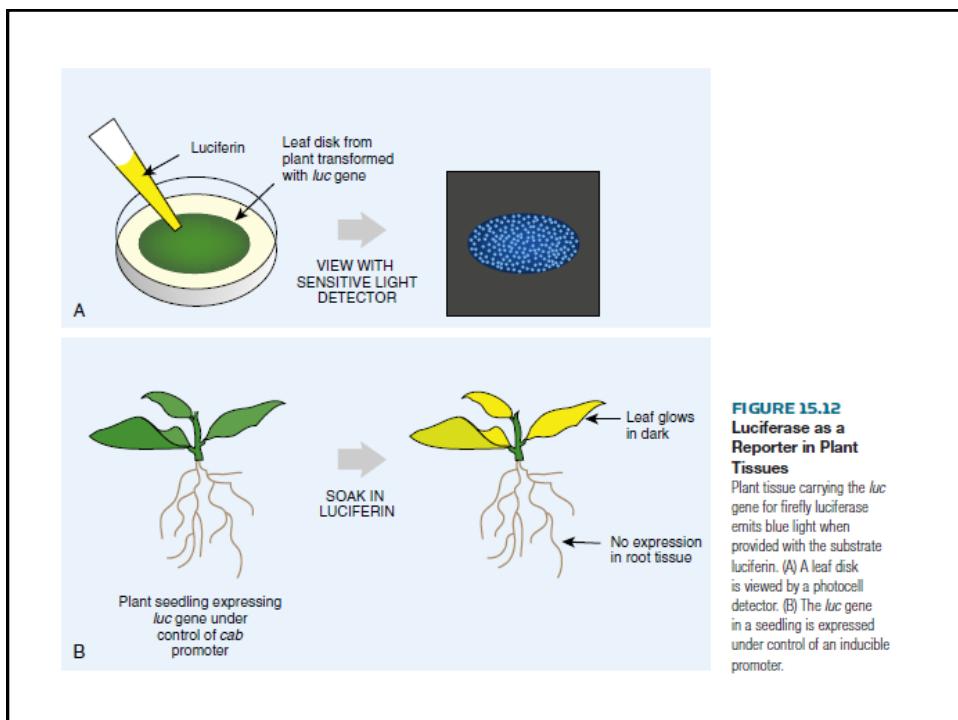
The half-life of the luciferase protein in plant cells is lower than that for GUS and may reflect transcriptional activity more accurately



**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.)

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

4



**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.

5

### Fluorescent proteins

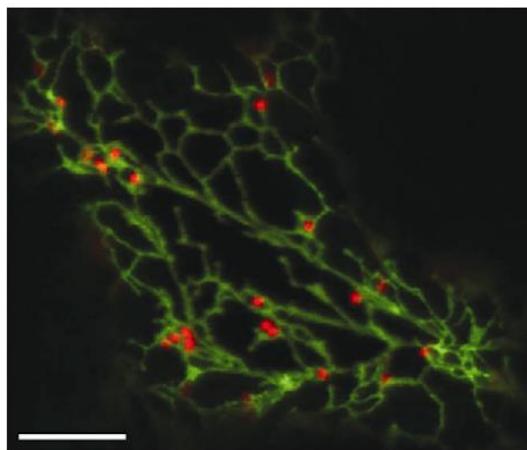
- FPs have become the most important reporter gene system for plants.
- They require no external substrate for detection, and there have been no reports of detrimental effects on the fitness of plants that express them
- Simultaneously monitoring of several FPs
- great sensitivity at the subcellular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



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.)

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

6



**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 $\mu$ m.) (Source: From Brandizzi et al. 2004.) (See insert for color representation of the figure.)

7

### 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 “causes recombination” recombinase recognizes a specific 34 base-pair DNA sequence, the *loxP* site

Cre recombinase catalyzes recombination between two *loxP* sites

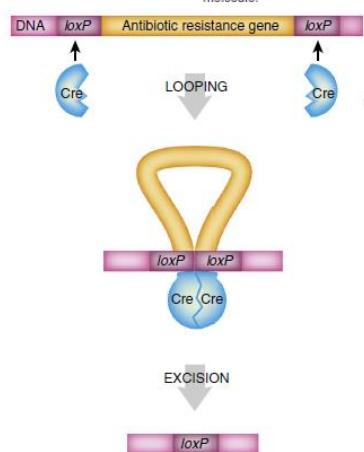
How Cre recombinase can be added to the plant cells?

- 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.
- Cre recombinase is included in the construct under the inducible promoter

**FIGURE 15.13**

### *Cre/loxP* System of Bacteriophage P1

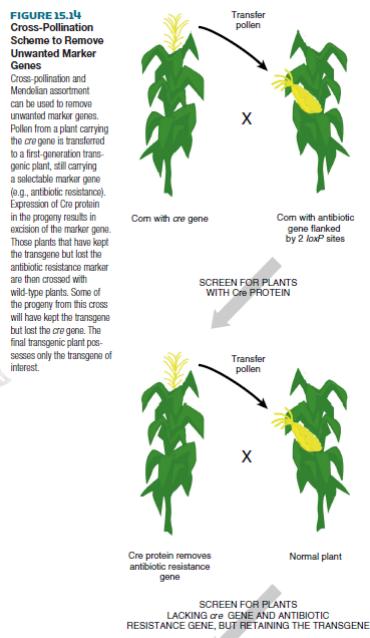
The Cre protein binds to *loxP* recognition sites in the DNA. Two nearby *loxP* sites are brought together, and recombination between them eliminates the intervening DNA. A single *loxP* “scar” site remains in the target DNA molecule.



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

8

### a) cross-pollination...

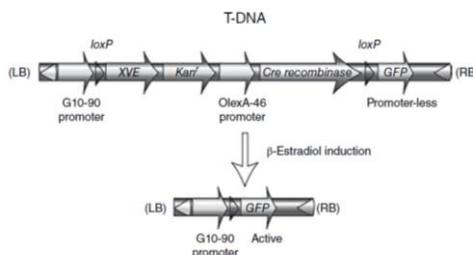


Clark, D. P., & Pazdernik, N. J. (2015). *Biotechnology*. Elsevier Science.

9

### b) Cre recombinase under the inducible promoter...

- Addition of  $\beta$ -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  $\beta$ -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 OLexA 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.

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*. Wiley.

10

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

Zaijie Chen,<sup>1,2,†</sup> Qianqian Cheng,<sup>2,†</sup> Changquan Hu,<sup>2</sup> Xinrui Guo,<sup>2</sup> Ziqiang Chen,<sup>2</sup> Yan Lin,<sup>2</sup> Taijiao Hu,<sup>2</sup> Maria Bellizzi,<sup>3</sup> Guodong Lu,<sup>1</sup> Guo-Liang Wang,<sup>3</sup> Zonghua Wang,<sup>1,\*</sup> Songbiao Chen,<sup>2,\*</sup> and Feng Wang<sup>2,\*</sup>

### 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.

11

## Components for Efficient Gene Expression in Plants

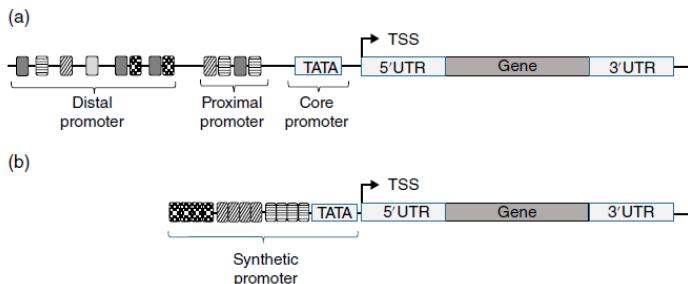
- Failure to obtain gene expression using cistrons (gene and promoter sequences) from other species led to the first chimeric genes that used the 5' and 3' nopaline synthase (*nos*) regulatory sequences: the *nos* promoter (contains sequences that resemble CAAT and TATA boxes) and *nos* terminator (contains 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 enhance 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
- Positional effect...

12

### 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).

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

13

### 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                 | <i>Arabidopsis</i>    | Mitra et al. (1989)       |
|                 | rbcS          | Ribulose bisphosphate carboxylase small subunit | <i>Arabidopsis</i>    | De Almeida et al. (1989)  |
| Vascular tissue | PEPC          | Phosphoenolpyruvate carboxylase                 | Maize                 | Ku et al. (1999)          |
|                 | PP2           | Phloem protein 2                                | Pumpkin               | Guo et al. (2004)         |
| Root            | Pfn2          | Profilin 2                                      | <i>Arabidopsis</i>    | Christensen et al. (1996) |
|                 | EIR1          | Ethylene-insensitive root1                      | <i>Arabidopsis</i>    | Luschnig et al. (1998)    |
| Pollen          | NAC10         | NAM, ATAF1-2, CUC2                              | Rice                  | Jeong et al. (2000)       |
|                 | Lat52;59      | Late anthergenesis                              | Tomato                | Twell et al. (1991)       |
| Seed            | TA29          | Tobacco anther-specific protein TA29            | Tobacco               | Koltunow et al. (1990)    |
|                 | Zm13          | Pollen specific                                 | Maize                 | Hamilton et al. (1998)    |
|                 | napA          | Napin storage protein                           | <i>Brassica napus</i> | Rask et al. (1998)        |
|                 | GluB-1        | Glutelin storage protein                        | Rice                  | Wu et al. (2000)          |

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

14

## 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                   | <i>Arabidopsis</i>          | Lebel et al. (1998)                      |
|                       | NPR1          | Nonexpressor of PR1                      | <i>Arabidopsis</i>          | Yu et al. (2001)                         |
|                       | VSP1          | Vegetative storage protein 1             | <i>Arabidopsis</i>          | Guerineau et al. (2003)                  |
|                       | PePR1-1       | Pathogenesis-related 1                   | Parsley                     | Rushton et al. (2002)                    |
|                       | PePAL1        | Phenylalanine ammonia-lyase 1            | Parsley                     | Lois et al. (1989)                       |
| Light                 | PR2-d         | Pathogenesis-related 2-d                 | Tobacco                     | Shah et al. (1996)                       |
|                       | NtGnP         | Glucanase 2                              | Tobacco                     |  |
|                       | CHS           | Chalcone synthase                        | Parsley                     | Weissshaar et al. (1991)                 |
| Wound                 | LHCP          | Light-harvesting chlorophyll a/b protein | Pea                         | Simpson et al. (1985)                    |
|                       | Rca           | Rubisco activase                         | Spinach                     | Orozco and Ogren (1993)                  |
|                       | MPI           | Maize proteinase inhibitor               | Maize                       | Cordero et al. (1994)                    |
| Drought               | Pin2          | Proteinase inhibitor II                  | Potato                      | Thornburg et al. (1987)                  |
|                       | ERD1          | Early responsive to dehydration stress 1 | <i>Arabidopsis</i>          | Tran et al. (2004)                       |
|                       | RD29A, B      | Responsive to desiccation 29A, B         | <i>Arabidopsis</i>          | Yamaguchi-Shinozaki and Shinozaki (1994) |
| Cold                  | Cor15A        | Cold-regulated 15A                       | <i>Arabidopsis</i>          | Stockinger et al. (1997)                 |
|                       | CBF2/DREB1C   | C-repeat/DRE binding factor 1            | <i>Arabidopsis</i>          | 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                | <i>Arabidopsis</i>          | Abe et al. (1997)                        |
|                       | Em            | Late embryogenesis                       | Wheat                       | Guiltnan et al. (1990)                   |
| Ethanol               | RD29A,B       | Responsive to desiccation 29A, B         | <i>Arabidopsis</i>          | Yamaguchi-Shinozaki and Shinozaki (1994) |
|                       | AlcA          | Alcohol-regulated                        | <i>Aspergillus nidulans</i> | Caddick et al. (1998)                    |

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

15

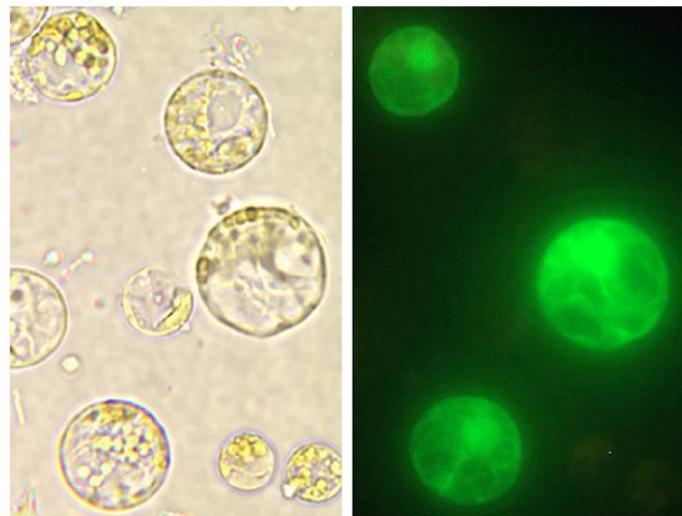
## Codon usage effect

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.

16

## ASSAYS FOR TRANSGENICITY, INSERT COPY NUMBER, AND SEGREGATION

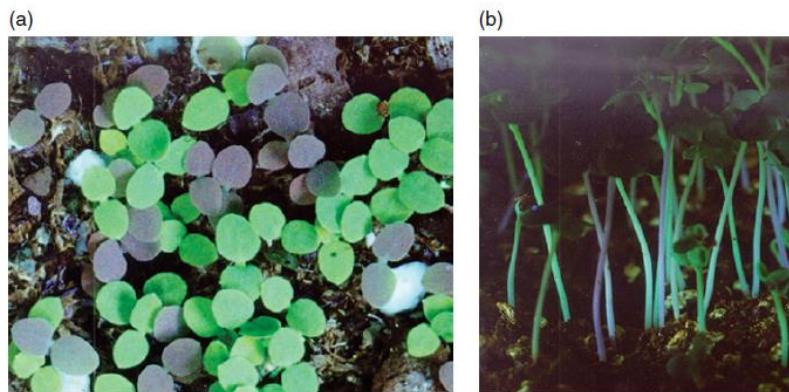


**Figure 11.9.** Maize protoplasts, electroporated with a *gfp* gene, showing bright field (left) and with GFP filters (right). Courtesy of JC Jang. (See insert for color representation of the figure.)

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

17

## Segregation analysis

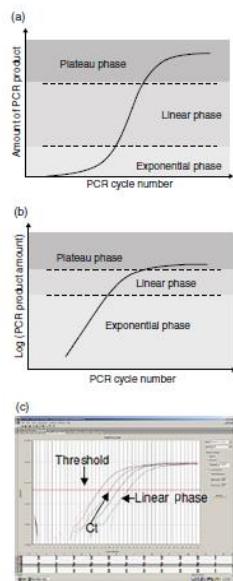


**Figure 12.8.** Segregation analysis of T1 transgenic (a) tobacco and (b) canola seedlings that have a single insert of a green fluorescent protein (GFP) gene. Under a UV light, the transgenic plants fluoresce green and the non-transgenic plants fluoresce red. The transgene presence and the single insert 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.)

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

18

## QPCR analysis

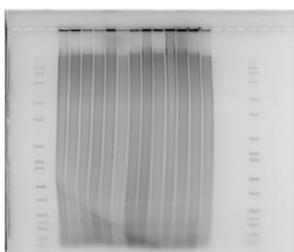


**Figure 12.3.** The dynamics of qPCR and analysis. (a) Theoretical plot of PCR product showing the phases of DNA amplification. (b) Another view of the phases, but where PCR product is expressed in logarithmic terms. (c) The same scheme as panel (b), but with actual data of four samples are shown. The amount of target template decreases in the samples going from left to right as shown by respectively increasing cycle threshold ( $C_t$ ) numbers.  $C_t$  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.

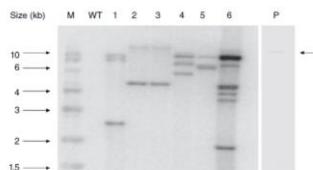
Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

19

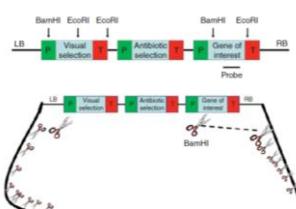
## Southern (DNA) Blot Analysis



**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 Hafer for assistance with this figure.

[https://www.youtube.com/watch?v=3I9wzwj0b\\_A](https://www.youtube.com/watch?v=3I9wzwj0b_A)

Stewart, C. N. (2016). *Plant Biotechnology and Genetics: Principles, Techniques, and Applications*: Wiley.

20