Selectable Markers & Markers for Screening

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Chapter 15

Gene Clone and DNA Analysis in Agriculture

Chapter 15.3 p341-344
**Selectable markers & Markers for screening**

- A **selectable marker** will protect the organism from a **selective agent** that would normally kill it or prevent its growth. In most applications, only one in a several million or billion cells will take up DNA. Rather than checking every single cell, scientists use a selective agent to kill all cells that do not contain the foreign DNA, leaving only the desired ones.

- **Antibiotics** are the most common selective agents. In bacteria, antibiotics are used almost exclusively. In plants, antibiotics that kill the chloroplast are often used as well, although tolerance to salts and growth-inhibiting hormones is becoming more popular.

- A **marker for screening** will make cells containing the gene look different. There are three types of screening commonly used:
  - **Green fluorescent protein (GFP)** makes cells glow green under UV light. A specialized microscope is required to see individual cells. Yellow and red versions are also available, so scientists can look at multiple genes at once. It is commonly used to measure gene expression.
  - **GUS assay** (using β-glucuronidase) is an excellent method for detecting a single cell by staining it blue without using any complicated equipment. The drawback is that the cells are killed in the process. It is particularly common in plant science.
  - **Blue/white screening** is used in bacteria. The **lacZ** gene makes cells turn blue in special media (e.g. X-gal). A colony of cells with the gene can be seen with the naked eye.


LacZ gene

- f1 (+) origin 135–441
- β-galactosidase α-fragment 460–816
- multiple cloning site 653–760
- lac promoter 817–938
- pUC origin 1158–1825
- ampicillin resistance (bla) ORF 1976–2833

**pBluescript II SK (+) Multiple Cloning Site Region**
(sequence shown 598–826)

```
TTGTAAACGACGGCCAGTGAACGGCGCTGAATACGACTCACTATAGGGGAATTGTAGTGACCG
```

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bsp106</td>
<td>GGATCC</td>
</tr>
<tr>
<td>CiaI</td>
<td>TCTAGA</td>
</tr>
<tr>
<td>HindIII</td>
<td>GTCTAGA</td>
</tr>
<tr>
<td>EcoRI</td>
<td>TCTAGA</td>
</tr>
<tr>
<td>PstI</td>
<td>CTAGA</td>
</tr>
<tr>
<td>SmaI</td>
<td>TGAG</td>
</tr>
<tr>
<td>BamHI</td>
<td>GGATCC</td>
</tr>
<tr>
<td>SpeI</td>
<td>TCTAGA</td>
</tr>
<tr>
<td>XbaI</td>
<td>TCTAGA</td>
</tr>
</tbody>
</table>

**T3 Promoter**

```
...CAGCTTTTTGTCCCCATTTAGATGGAGTTATGGAATTCGCGCTTQQCCTGAATCACTATGTCATAGCT
```

**T3 primer binding site**

**β-gal α-fragment**

```
...CAGCTTTTTGTCCCCATTTAGATGGAGTTATGGAATTCGCGCTTQQCCTGAATCACTATGTCATAGCT
```

**T3 primer binding site**

**M13 Reverse primer binding site**

```
M13 –20 primer binding site
```

**M13 –20 primer binding site**

```
TTGTAAACGACGGCCAGTGAACGGCGCTGAATACGACTCACTATAGGGGAATTGTAGTGACCG
```

**T7 Promoter**

```
GGATCC
```

**T7 primer binding site**

```
CTAGA
```

**SK primer binding site**

```
TCTAGA
```

**β-galactosidase α-fragment**

```
GGATCC
```

**Kpn I**

**Sac I**

**pUC ori**

**ampicillin resistance (bla)**

**lacZ**

**f1 (+) ori**

**3.0 kb**
Selectable Markers
Selectable Markers

- About **50** selectable marker genes
- **Six negative SMG**: codA, aux2, tms2, dhIA, CYP105A, and cue
- *nptII*, *hpt*, and *bar* contribute to production of over 95% transgenic plants
- *pmi* (**the E. coli manA**): mannose-dependent SMG

Marker genes listed in US field test notifications and release permits for the years 2001 and 2002 (data extracted from ISB, 2003)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Number of records in 2001 and 2002</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neomycin phosphotransferase II (NPT II)</td>
<td>949</td>
</tr>
<tr>
<td>Hygromycin B phosphotransferase (hpt)</td>
<td>65</td>
</tr>
<tr>
<td>Phosphinothricin N-acetyltransferase (PAT)</td>
<td>327</td>
</tr>
<tr>
<td>5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase</td>
<td>507</td>
</tr>
<tr>
<td>Acetolactate synthase or acetohydroxyacid synthase</td>
<td>5</td>
</tr>
<tr>
<td>Nitrilase</td>
<td>0</td>
</tr>
<tr>
<td>Cyanamide hydratase</td>
<td>2</td>
</tr>
<tr>
<td>Glucuronidase (GUS)</td>
<td>91</td>
</tr>
<tr>
<td>Luciferase</td>
<td>4</td>
</tr>
<tr>
<td>Green fluorescent protein (GFP)</td>
<td>20</td>
</tr>
</tbody>
</table>

NPT II—Kanamycin (Km) resistance

- NPT II = neomycin phosphotransferase II
- Normally, plant cells are sensitive to Km.
- Km inhibits protein synthesis and protein translocation across membranes.

- Expression of the NPTII in plant cells results in synthesis of NPTII enzyme
- The enzyme detoxifies Km by phosphorylation

\[
\text{ATP} \quad \text{ADP} \quad \text{Km} \quad \text{Km-PO}_4
\]

(Disrupts plant growth) \quad \text{NPTII enzyme} \quad (Unable to disrupts plant growth)
Example 1: Km-selection

Dose experiment on Km-resistance

Km=0 (mg/L)  Km=10  Km=20

Km=30  Km=50  Km=100
Example 1: Km-selection

<table>
<thead>
<tr>
<th>Km</th>
<th>Non-transformed Explants</th>
<th>Transformed Explants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Km = 0 mg/L</td>
<td><img src="image1.jpg" alt="Image" /></td>
<td><img src="image2.jpg" alt="Image" /></td>
</tr>
<tr>
<td>Km = 25 mg/L</td>
<td><img src="image3.jpg" alt="Image" /></td>
<td><img src="image4.jpg" alt="Image" /></td>
</tr>
</tbody>
</table>

Inoculation  
Selection & Regeneration

Bar-Phosphinothricin (PPT) resistance

- PPT normally acts to inhibit glutamine synthetase, causing a fatal accumulation of ammonia.
- PAT belongs to the family of acetyltransferases. It detoxifies PPT by catalysing the addition of an acetyl group to the free amino group.

Non-trangenic

- Glutamate
- ATP, NH₃ → ADP, PI
- Glutamine Synthase
- Inhibiting GS
- Rapid accumulation of ammonia which lead to death of the plant cell

Trangenic

- PPT
- PAT
- Acetyl-PPT

Rapid accumulation of ammonia which lead to death of the plant cell
Example 2: PPT-selection

PPT = 0.2 mg/L
Chlorophenol red assay

2 wk, 5 mg/L ppt

WT

Transgenic

Example 2: PPT-resistance

PPT=7500 ppm, 1 week

Song et al. JASHS. (2008)
Example 2: PPT-resistance
The hygromycin phosphotransferase (denoted *hpt*, *hph* or *aphIV*) gene was originally derived from *Escherichia coli*. The gene codes for hygromycin phosphotransferase (HPT), which detoxifies the aminocyclitol antibiotic hygromycin B. A large number of plants have been transformed with the *hpt* gene and hygromycin B has proved very effective in the selection of a wide range of plants, including monocotyledonous.

Most plants exhibit higher sensitivity to hygromycin B than to kanamycin, for instance cereals. Likewise, the *hpt* gene is used widely in selection of transformed mammalian cells.

Like kanamycin and other aminoglycoside antibiotics, hygromycin B inhibits protein synthesis by interfering with mRNA translation and causing mistranslocation of mRNA.
Example 3: Hyg-selection

<table>
<thead>
<tr>
<th>Hyg-Selection (50 mg/L)</th>
<th>Hyg-Selection &amp; Regeneration (50 mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image of hygromycin selection" /></td>
<td><img src="image2.png" alt="Image of hygromycin selection and regeneration" /></td>
</tr>
</tbody>
</table>
### NPTII, HPT, and Bar

<table>
<thead>
<tr>
<th>Condition</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>50 mg/L Kanamycin, 250 mg/L Timentin</td>
</tr>
<tr>
<td>Glufosinate</td>
<td>5 mg/L Glufosinate, 250 mg/L Timentin</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>50 mg/L Hygromycin, 250 mg/L Timentin</td>
</tr>
</tbody>
</table>

The images show the effects of the different conditions on plant growth.
Friendly SMG

The PMI Technology

Advantages:

Non toxic
Rapid and efficient
Trait stacking
Address public concern

maize, rice, wheat, barley, cassava, sugar beet, watermelon, tomato, squash, cabbage, sunflower, oilseed rape, sweet orange, almond, papaya, and apple
1 - **PMI**, phosphomannose isomerase activity is low in some plants, and apparently absent in others, grasses like wheat, rice, corn, and sorghum. Gene for this enzyme is also known as manA.

2 - **M6PR**, mannose-6-phosphate reductase, is present in some higher plant families.

3 - non-specific phosphatases, **Pases**, are present in nearly all higher plants. This releases phosphate otherwise sequestered in mannose and mannitol phosphate.

4 - **Mtd**, mannitol dehydrogenase, is present in nearly all higher plants.
Other SMG

- Isopentyl transferases
- Histidine kinase homologue
- Hairy root-inducing genes
Markers for Screening
**GUS: beta-glucuronidase**

5-bromo-4-chloro-indolyl glucuronide (X-gluc): Colorless

4-methyl-umbelliferyl-beta-D-glucuronide (■-gluc)

**Beta-glucuronidase**

\[ \text{GUS} + \text{X-Gluc} \xrightarrow{37^\circ C} \text{X} + \text{Gluc} \xrightarrow{O_2} \text{The GUS histochemical staining assay} \]

- Hydrolysis of the X-Gluc substrate by the GUS enzyme
- Dimerization of the Gluc product by reaction with O2

**Beta-glucuronidase**

\[ \text{GUS} + \text{■-Gluc} \xrightarrow{37^\circ C} \text{■} + \text{Gluc} \]

- Hydrolysis of the X-Gluc substrate by the GUS enzyme
- Fluorescent of the released fluorescent product

Ruijter et al., 2003, Plant Biology, 5:103-115
The GUS Histochemical Staining Assay

Blueberry

Sweetpotato

Celery

The GUS Histochemical Staining Assay

Rice

Evaluation of different promoters
Luciferase (LUC)

A conditional non-selectable marker gene
Firefly Luciferase (ff-LUC)

In the absence of CoA the protein is inactivated by complex formation with oxyluciferin and the reaction is non-enzymatic (the "so-called" flash reaction):

\[ \text{Oxyluciferin} + \text{O}_2, \text{ATP} \rightarrow \text{Oxyluciferin-}^{\text{AMP + PPI + CO}_2} \]

In the presence of CoA the luciferase protein is rapidly released from the complex, resulting in an enzymatic reaction:

\[ \text{Oxyluciferin-}^{\text{AMP + PPI + CO}_2} + \text{CoA} \rightarrow \text{CoA} \rightarrow \text{Oxyluciferin-}^{\text{AMP + PPI + CO}_2} \]
Green Fluorescent Protein (GFP)

The great advantage of GFP as a non-conditional reporter is the direct visualization of GFP in living cells in real time without invasive procedures such as the application or penetration of cells with substrate and products that may diffuse within or among cells. Both considerations provide a significant improvement over GUS and LUC as reporter genes.
In 1994 GFP was cloned. Now GFP is found in laboratories all over the world where it is used in every conceivable plant and animal. The GFP gene can be introduced into organisms and maintained in their genome through breeding, or local injection with a viral vector which can be used to introduce the gene.
The GFP is composed of 238 amino acids, originally isolated from the jellyfish Aequorea victoria that fluoresces green when exposed to blue light.

Martin Chalfie, Osamu Shimomura and Roger Y. Tsien were awarded the 2008 Nobel Prize in Chemistry on 10 December 2008 for their discovery and development of the green fluorescent protein.
Formation of the chromophore requires molecular oxygen

Fluorescent of GFP

O$_2$
A Transgenic embryoid of Valencia sweet orange; \( \text{bar} = 200 \, \mu m \).
B Transgenic plant of Valencia sweet orange in the greenhouse.
C GFP expression in shoot tip of a transgenic Valencia plant; \( \text{bar} = 1 \, \text{mm} \)

Marker-free Strategies
Concerns about the SMG

Although no adverse biosafety effects have been reported for the marker genes that have been adopted for widespread use, biosafety concerns should help direct which markers will be chosen for future crop development. Common sense dictates that marker genes conferring resistance to significant therapeutic antibiotics could not be used.
Marker-free Strategies

1. Co-transformation and segregation of marker genes
2. Transposon-mediated repositioning of genes
3. Intrachromosomal homologous recombination to remove SMG
4. Site-specific recombinase-mediated excision of marker genes
Co-transformation

Positive selection

Segregation

SMG-free transformants

None T-DNA

PCR
1. Co-transformation and segregation of marker genes

   • Co-transformation with separate plasmids in one or two Agrobacterium strains

   • Co-transformation with single plasmids carrying multiple T-DNA regions

An advantage of Agrobacterium-mediated co-transformation technologies over biolistic transformation is that the co-transformation genes often integrate into different loci in plant genome.

4. Site-specific recombinase-mediated excision of marker genes

- The Cre-LoxP System (bacteriophage)

- The FLP-FRT System (yeast)
  The FLP-FRT system derived from the *Saccharomyces cerevisiae* 2 plasmid

- The R-RS System
  The R-RS system from *Zygosaccharomyces rouxii*

*Cre, FLP and R are the recombinases, and loxP, FRT and RS are the recombination sites.*
Figure 1. Recognition sites for recombinases shown to function in plants share a similar design. All comprise palindromes, which flank the six to eight innermost base pairs. Each recombinase binding element (RBE) is bound by a single recombinase subunit. Cleavage of the sites occurs at the borders between the RBEs and the core sequence. The core element is the site of strand exchange and confers directionality on the recombination site. Recombination requires two recombinase recognition sites bound by four identical recombinase subunits.
Marker-free Strategies

Integration
Selection with SMG

Excision of SMG


**Objective:**

To get familiar with using the *gusA* as a screening marker