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Production of Courseware e-Content for Postgraduate Subjects

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1. Details of Module and its Structure

Module Detail	
Subject Name	<Botany >
Paper Name	<Plant Genetic Engineering >
Module Name/Title	< Selectable and Screenable Markers>
Module Id	<>
Pre-requisites	A basic idea about plant genetic engineering
Objectives	To make the students aware of Selectable and Screenable markers to know whether the transgene has been transferred, where it is located, and when it is expressed.

Structure of Module / Syllabus of a module (Define Topic / Sub-topic of module)	
< Selectable and Screenable Markers >	<Sub-topic Name1>, <Sub-topic Name2>
Keywords	selectable, screenable markers,

2. Development Team

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TABLE OF CONTENTS (for textual content)

1. INTRODUCTION

Marker systems are tools for studying the transfer of genes into an experimental organism. In gene transfer studies, a foreign gene, called a transgene, is introduced into an organism, in a process called transformation. A common problem for researchers is to determine quickly and easily if the target cells of the organism have actually taken up the transgene. A marker allows the researcher to determine whether the transgene has been transferred, where it is located, and when it is expressed (used to make protein). Marker systems exist in two broad categories: selectable markers and screenable markers.

- Selectable markers are typically genes for antibiotic resistance, which give the transformed organism (usually a single cell) the ability to live in the presence of an antibiotic.
- Screenable markers, also called reporter genes or scorable genes, typically cause a color change or other visible change in the tissue of the transformed organism. This allows the

investigator to quickly screen a large group of cells for the ones that have been transformed.

Selectable and screenable markers are essential for genetic engineering in both prokaryotes and eukaryotes, and are often built into engineered DNA plasmids used for genetic transformation.

2. SELECTABLE MARKERS

A selectable marker gene encodes a product that allows the transformed cell to survive and grow under conditions that kill or restrict the growth of non transformed cells. Most such genes used in plants are dominant selectable markers that confer resistance to antibiotics or herbicides (Table 1).

Important aspects of the marker gene that has to be considered are:

1. Its structure (nucleic acid sequence), which will determine factors such as regulation of transcription (constitutive, environmental or developmental expression), rate of transcription, transcript stability and efficiency of translation.
2. The gene product itself, which is obviously responsible for the dominant expression of a suitable selective phenotype.

The selectable functions on most general transformation vectors are prokaryotic antibiotic-resistance enzymes which have been engineered to be expressed constitutively in plant cells. In some experiments, enzymes affording protection against specific herbicides have also been used successfully as dominant marker genes. The enzyme coding sequence is normally fused to promoters isolated from the T-DNA region of *Agrobacterium tumefaciens* or Cauliflower Mosaic Virus (CaMV) genome at the 5' end, and a polyadenylation signal, often again from a T-DNA gene, at the 3' end.

Table 1 : Selectable markers and selective agents used in plant transformations

Selectable marker	Source	Principle of selection
npt-II (neomycin phosphotransferase)	*Escherichia coli*	Confers resistance to the aminoglycoside antibiotics neomycin, kanamycin and G148 (geneticin)
hpt (hygromycin phosphotransferase)	*Klebsiella* spp.	Confers resistance to hygromycin B
bar and pat (*(phosphinothricin acetyltransferase)*	*Streptomyces hygroscopicus*	Confers resistance to phosphinothricin (glufosinate) and the herbicides bialaphos and Basta
epsps (5-enolpyruvylshikimate-3-phosphate synthase)	*Agrobacterium tumefaciens strain CP4*	Confers resistance to the herbicide glyphosate *N*-(phosphonomethyl)glycine - Round-up
Ble (glycopeptide- binding protein)	*Streptalloteichus hindustantus*	Confers resistance to the glycopeptide antibiotics bleomycin and pheomycin (and the derivative Zeocin)
dhfr (dihydrofolate)	Mouse	Confers resistance to methotrexate
dhfr (dihydrofolate reductase)	Mouse	Confers resistance to methotrexate
csr1–1 (acetolactate synthase)	*Arabidopsis thaliana*	Confers resistance to chlorsulphuron
tms2 (indoleacetic acid hydrolase)	*Agrobacterium tumefaciens*	Confers sensitivity to naphthalene acetamide (NAM)

Some of the common selectable markers are discussed in detail.

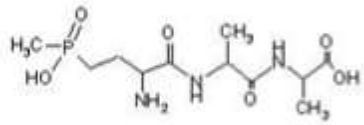
2.1. *npt*-II

- Neomycin phosphotransferase is a bacterial enzyme that confers resistance to the antibiotic **Kanamycin.**
- Kanamycin works by inhibiting protein synthesis in prokaryotes and eukaryotes.
- *npt*-II provides resistance by phosphorylating kanamycin, making it inactive.
- Very popular selection system for many plant species because most plant species are extremely sensitive to kanamycin.

2.2. *bar* and *pat*

- It consists of a glutamic acid analogue moiety, called phosphinothricin [PTC or glufosinate =2-amino-4-(hydroxymethylphosphinyl) butanoate] and two alanine residues.
- Bialaphos is an inhibitor of the key enzyme in the nitrogen assimilation pathway, glutamine synthetase (GS).
- A *bar* (bialaphos resistance) gene encodes a phosphinothricin acetyl transferase (PAT) enzyme
- It becomes active after removal of the alanine residues by intracellullar peptidases.
- The remaining glufosinate compound inhibits GS and as a result, leads to accumulation of toxic levels of ammonia in both bacteria and plant cells.
- The biochemical and toxicological characteristics of glufosinate have made it a popular, nonselective herbicide, which has been commercialized under the names Basta®, Buster® and Liberty® by Bayer Crop Science (formerly Aventis).
- Treatment of genetically modified plants carrying a *bar* gene with glufosinate or bialaphos provides a very efficient means of selection in genetic transformation protocols.

Bialaphos



phosphinothricin-alanyl-alanine

2.3. *hpt*

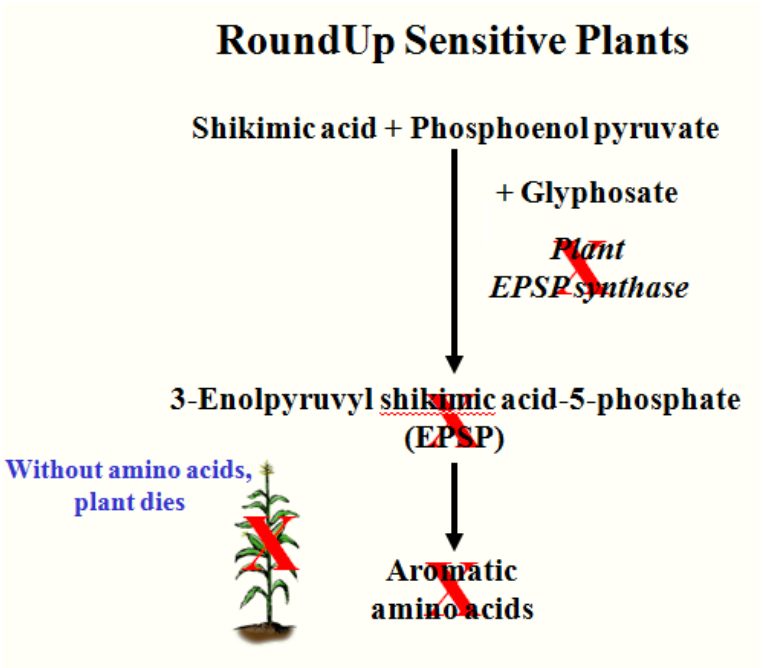
- The gene codes for hygromycin phosphotransferase (HPT), which detoxifies the aminocyclitol antibiotic hygromycin B.
- A large number of plants have been transformed with the *hpt* gene and hygromycin B has proved very effective in the selection of a wide range of plants, especially monocotyledons.
- Most plants exhibit higher sensitivity to hygromycin B than to kanamycin, for instance cereals.

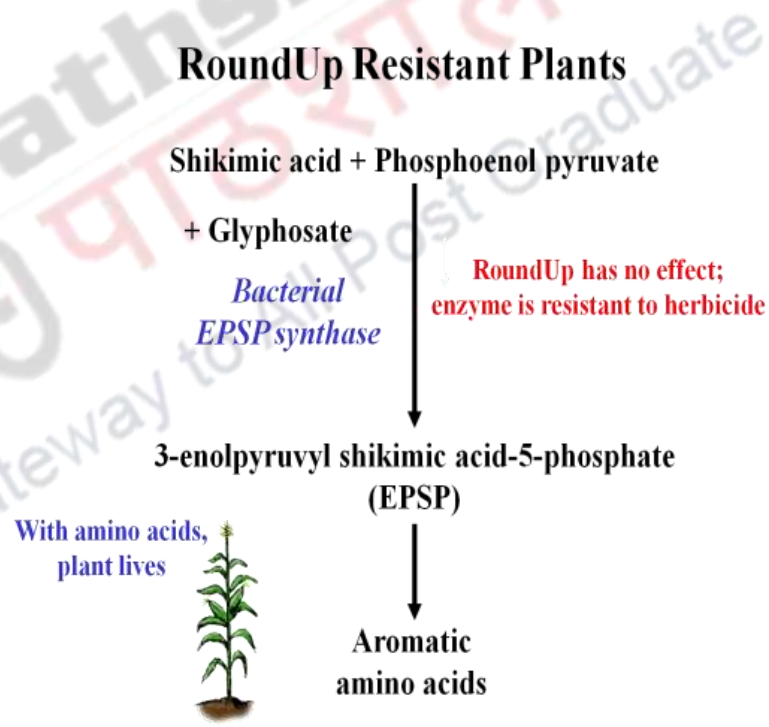
- The sequence of the *hpt* gene has been modified for its use in plant transformation. Deletions and substitutions of amino acid residues close to the carboxy (C)-terminus of the enzyme have increased the level of resistance in certain plants, such as tobacco.
- At the same time, the hydrophilic C-terminus of the enzyme has been maintained and may be essential for the strong activity of HPT.
- HPT activity can be checked using an enzymatic assay. A non-destructive callus induction test can be used to verify hygromycin resistance.

Hygromicin resistant rice callus

2.4. *epsps*

- Glyphosate (*N*-[phosphonomethyl]glycine) is a broad-spectrum herbicide that is the active ingredient of the commercial Roundup® formulations.
- It acts as an inhibitor of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) which is essential in the shikimate pathway for the biosynthesis of the aromatic amino acids.
- The chemical binds to the active site of the EPSPS enzyme. This enzyme is a key to the development of a group of amino acids called the aromatic amino acids.
- When this enzyme is bound by glyphosate, it can not synthesize those amino acids, and the plants die because protein synthesis is severely disrupted.
- Glyphosate will not bind to a particular genetically-engineered version of EPSPS. Therefore RoundUp Ready crops with this altered enzyme will survive when sprayed with the herbicide.





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EPSPS synthesizes 3-enolpyruvly shikimic acid-5-phosphate is the essential precursor to aromatic amino acids. When plants are sprayed with a glyphosate-containing herbicide, such as RoundUp, this important precursor is not synthesized, and consequently the plant is starved of aromatic amino acids. The result is plant death.

An engineered version of EPSPS one that was discovered in a bacterium, is introduced into the plant. This enzyme cannot be bound by glyphosate. Therefore, if a field is sprayed with the herbicide, the introduced version of the gene produces a functional enzyme. The EPSP precursor is synthesized normally, and the plant produces enough aromatic amino acids to survive.

2.5 Other Dominant Selectable Markers

A wide range of dominant selectable markers are available for plants, including genes providing resistance to other antibiotics e.g. bleomycin, pheleomycin etc.,

Phleomycin and Bleomycin are novel antibiotics that belong to the bleomycin family of glycopeptides that act by site-specific, single- and double-stranded DNA cleavage.

3. SCREENABLE MARKERS

Screenable marker systems employ a gene whose protein product is easily detectable in the cell, either because it produces a visible pigment or because it fluoresces under appropriate conditions. Visible markers rarely affect the studied trait of interest, but they provide a powerful tool for identifying transformed cells before the gene of interest can be identified in the culture. They can also identify the tissues that have (and have not) been transformed in a multicellular organism such as a plant.

Table 2 : Screenable markers and selective agents used

Screenable marker	Source	Principle of selection
gusA (β-glucuronidase GUS)	*Escherichia coli*	Catalyses hydrolysis of β-glucuronides; variety of colorimetric, fluorometric and chemiluminescent assay formats; can be used for *in vitro* and *in vivo* assays
lux (Firefly luciferase)	The firefly *Photinus pyralis*	Light produced in the presence of luciferase, its substrate luciferin, Mg^{2+}, oxygen and ATP
gfp (green fluorescent protein)	The jellyfish *Aqueorea victoria*	Spontaneous fluorescence under UV or blue light.

Screenable marker genes are included on many transformation vectors for two reasons.

1. To allow independent verification of the transformed status of tissues growing on media containing selective antibiotics or herbicides.
2. As a principal means of identifying transformants in conditions where transformation frequencies are high.

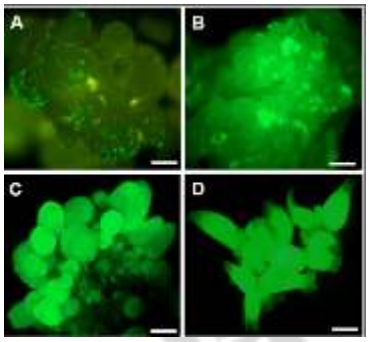
A specific use of a screenable marker is as a reporter gene; both in the development of transformation systems using transient expression assays to monitor success or to test out DNA sequences which may be able to regulate gene expression in stably transformed tissues.

The most commonly used screenable markers are:

3.1 Green fluorescent protein (GFP)

- GFP is a fluorescent protein from the **Jellyfish** - ***Aequorea victoria***.
- In the Jellyfish, it works with ***Aqueorin***, a blue fluorescent protein that converts ATP into light in the presence of calcium.
- When aqueorin gives off the blue light, it is absorbed by GFP which then fluoresces green light.
- Best use of GFP is for examinations of protein levels and protein distribution in intact cells.

- Because of its smaller size as compared with luciferase and GUS, the GFP protein is better for making protein fusions.
- Assay can be done on ***LIVE*** tissue and is ***not destructive*** to the sample.

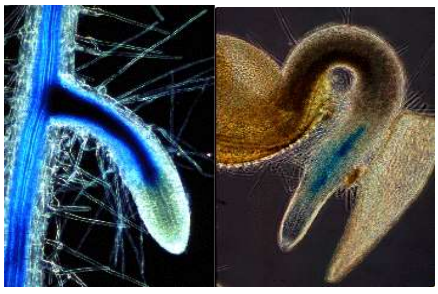


GFP fluorescence in embryogenic callus cultures transformed with *A. tumefaciens*

3.2 GUS assay

3.3 GUS encodes the β-glucuronidase enzyme from *E. coli* and it has been extensively used as a reporter gene in transgenic plants.

- The main advantage of the GUS reporter gene system is the stable expression of GUS enzyme in plant cells. GUS activity can readily be detected by chromogenic or fluorogenic enzyme substrates.
- Various β-glucuronic acid substrates are available for detection of GUS expression.
- The most widely used fluorogenic substrate for detection of β-glucuronidase activity *in vitro* is 4-methylumbelliferyl β-D-glucuronide (MUG, 4-methylumbelliferyl-beta D-glucuronic acid dihydrate, M-5700).



Transgenic plants that harbor the AGL12::GUS fusions show root-specific expression

3.3 Chloramphenicol Acetyltransferase or **CAT**

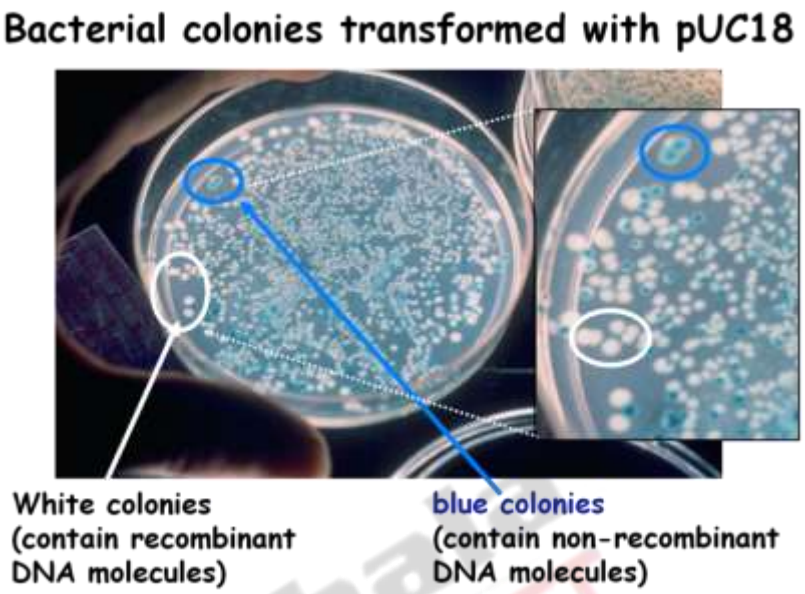
- Enzyme detoxifies Chloramphenicol by acetylation.
- Chloramphenicol is an antibiotic that inhibits protein synthesis on 70S ribosomes (prokaryotic).
- Main problem with CAT assays is that they work only in extracts and not in intact cells. Assay involves addition of 14C-chloramphenicol to extract and allowing acetylation by CAT.
- Chloramphenicol is acetylated in two positions, 1 or 3, producing three products, the 1 and 3 acetylation and the 1,3 double acetylation.
- Reaction products are separated from substrate by TLC and detected by autoradiography.

3.4 Luciferase

- *Luciferase* - class of enzymes that convert chemical energy into light.
- Most commonly used is from the **firefly** - ***Photinus pyralis.***
- Firefly luciferase encoded by single gene called the ***lux*** gene and requires the cofactor **Luciferin**.
- Luciferase/luciferin complex produces light by the hydrolysis of ATP.
- During Transformation, the expression of luciferase is detected by addition of luciferin to watering solutions of plants.
- *In vivo* detection of luciferase activity is accomplished by placing the plant or tissue on photographic film.
- For *in vitro* assays of plant extracts, luciferin and ATP are added to the extract the reaction is carried out in a scintillation counter which then measures light emission

3.5 Blue/white screening

Blue/white screening is used in bacteria. The **lacZ** gene makes cells turn blue in special media (containing X-gal). A colony of transformed cells can be seen with the naked eye.



4. APPLICATION OF REPORTER GENE/SCREENABLE MARKERS

4.1 Transformation and transfection assays

- Many methods of transfection and transformation - two ways of introducing a foreign or modified gene into an organism - are effective in only a small percentage of a population subjected to the techniques.
- Reporter genes used in this way are normally expressed under their own promoter independent from that of the introduced gene of interest.
- In the case of selectable-marker reporters such as CAT, the transfected population of bacteria can be grown on a substrate that contains chloramphenicol.
- Only those cells that have successfully taken up the construct containing the CAT gene will survive and multiply under these conditions.

4.2 Gene expression assays

- Reporter genes can also be used to assay for the expression of the gene of interest, which may produce a protein that has little obvious or immediate effect on the cell culture or organism.
- In these cases the reporter is directly attached to the gene of interest to create a gene fusion.
- The two genes are under the same promoter and are transcribed into a single polypeptide chain.
- In these cases it is important that both proteins be able to properly fold into their active conformations and interact with their substrates despite being fused.
- In building the DNA construct, a segment of DNA coding for a flexible polypeptide linker region is usually included so that the reporter and the gene product of will only minimally interfere with one another

4.3 Promoter assays

- Reporter genes can be used to assay for the activity of a particular promoter in a cell or organism.
- In this case there is no separate "gene of interest"; the reporter gene is simply placed under the control of the target promoter and the reporter gene product's activity is quantitatively measured.
- The results are normally reported relative to the activity under a "consensus" promoter known to induce strong gene expression.

Summary

Selectable and screenable markers are important tools in genetic engineering. Selectable markers enable selection of transformed bacterial cells or plant cells and tissues from the non-transformed ones. They are usually genes that confer antibiotic resistance to the transformed cells or tissues. Screenable markers are used to determine whether the gene of interest has been transferred, where it is located, and when it is expressed. These are

usually color markers that can be visualised in transformed cells. These include GUS markers, LUX markers or GFP markers. Screenable markers not only report on successful transformation, but also tell us whether the transformation is stable, whether the gene of interest is expressed and also about specificity of expression of a promoter.

These markers have greatly improved our understanding of the temporal and spatial expression of genes.

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