Marker and reporter genes used for plant transformation

Mitesh Shrestha
Plant Biotechnology

• In order to achieve **high food productivity** and to ensure **nutritional quality**, genetic engineering methods in generating transgenics with useful agronomic traits are assuming significance.

• Plant biotechnology is based on the **delivery, integration and expression of defined genes into plant cells**, which can be grown to generate transformed plants.

• Efficiency of stable gene transfer is not high even in the most successful transfer systems and only a fraction of the cells exposed integrate the DNA construct into their genomes. Therefore, **systems to select the transformed cells**, tissues or organisms from the non-transformed ones are **indispensable** and selectable marker genes are vital to the plant transformation process.
Marker and Reporter genes

- A reporter or marker gene is a gene, which produces a specific phenotype, in turn enables the differentiation of the cells possessing this particular gene from those without this gene. Hence, the transformed cells can be selected easily among the thousands of non-transformed cells.

- Reporter genes form specific protein products, which are easily detectable and quantifiable, sometimes even without destroying the tissue.

- Reporter genes are an invaluable tool to track and study another associated gene in bacterial and mammalian cell culture, animals and plants. One can easily find out the expression patterns of a gene within the cell by fusing its promoter with one of the several reporter genes and transfecting inside the living cells.

- So, it is very useful in for the monitoring and detection of plant transformation, for studying the activities of regulatory elements such as promoter and enhancer.
Features of an ideal reporter gene

• Easily quantifiable
• Relatively rapid degradation of the enzyme
• High signal to noise ratio (Low endogenous background)
• Should not be toxic to cells
• Products of the reporter gene should be resistant to the chemicals used in the processing
• Assay should be sensitive and reliable.
Types of reporter genes

• Selectable marker
  – The **cells that contain this type of marker gene** show the ability to **survive under selective conditions**. These selective conditions would otherwise **result in the death of the cells lacking that specific gene**. Most commonly used selective agents are antibiotics. Out of the millions and billions of cells, only few of them get transformed by the foreign DNA. It is practically impossible to check every individual cell, so a **selective agent is required to eliminate the non transformed cells**, leaving only the desired ones.

• Scorable marker
  – Expression of this kind of marker gene results in a **quantifiable phenotype** i.e., it will make the cells containing it to look different. The main principle behind the use of these reporter genes for the study of molecular processes in living cells means that in natural genes, synthetic modification have introduced in order to either simplify the **detection of the product** or to distinguish it from similar genes in the genome. These reporter genes were assayed at the level of protein.
Selectable markers

• Typically used to recover transgenic plant cells from a sea of non-transgenic cells
• Antibiotic resistance markers and herbicide resistance markers are most common

Scorable markers (reporter genes)

• Can help visualize transient expression
• Can help visualize if tissue is stably transgenic
• Useful for cellular and ecological studies
### Selectable marker

<table>
<thead>
<tr>
<th>Selectable marker gene (encoded enzyme)</th>
<th>Abbreviation</th>
<th>Source of gene</th>
<th>Substrate(s) used for selection</th>
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<tr>
<td><strong>Antibiotic resistance</strong></td>
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<tr>
<td>Neomycin phosphotransferase II</td>
<td>nptII</td>
<td><em>E. coli</em></td>
<td>Kanamycin, geneticin (G418)</td>
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<td>Neomycin phosphotransferase III</td>
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<td>Hygromycin phosphotransferase</td>
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<td><em>E. coli</em></td>
<td>Hygromycin</td>
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<td>Bleomycin resistance</td>
<td>ble</td>
<td><em>E. coli</em></td>
<td>Bleomycin</td>
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<td>Aminoglycoside adenyltransferase</td>
<td>aadA</td>
<td><em>Shigella flexneri</em></td>
<td>Streptomycin, spectinomycin</td>
</tr>
<tr>
<td><strong>Antimetabolite markers</strong></td>
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<tr>
<td>Dihydrofolate reductase</td>
<td>dhfr</td>
<td>Mouse</td>
<td>Methotrexate</td>
</tr>
<tr>
<td>Dihydropyrrrole synthase</td>
<td>dhps/sul</td>
<td><em>E. coli</em></td>
<td>Sulfonamides</td>
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<td><strong>Herbicide resistance</strong></td>
<td></td>
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</tr>
<tr>
<td>Phosphinothricin acetyltransferase</td>
<td>bar/pat</td>
<td><em>Streptomyces hygroscopicus</em></td>
<td>Glufosinate, L-phosphinothricin, Bialophos</td>
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<td>Enolpyruvyl shikimate phosphate synthase</td>
<td>epsps/arOa</td>
<td><em>Agrobacterium sp</em></td>
<td>Glyphosate</td>
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<tr>
<td>Acetolactase synthase</td>
<td>als</td>
<td><em>Arabidopsis sp</em></td>
<td>Sulfonylureas</td>
</tr>
<tr>
<td>Glyphosate oxidoreductase</td>
<td>gox</td>
<td><em>Achromobacter LBAA</em></td>
<td>Glyphosate</td>
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<tr>
<td>Bromoxynil nitrilase</td>
<td>bxn</td>
<td><em>Klebsiella pneumoniae</em></td>
<td>Bromoxynil</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Glucuronidase</td>
<td>gus/uidA</td>
<td><em>E. coli</em></td>
<td>Cytokinin glucuronide</td>
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<td>Xylose isomerase</td>
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<td><em>Thermoanaerobacterium thermosulfurogenes</em></td>
<td>Xylose</td>
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<td><em>E. coli</em></td>
<td>Mannose</td>
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<tr>
<td>Betaine aldehyde dehydrogenase</td>
<td>badh</td>
<td>Spinach</td>
<td>Betaine aldehyde</td>
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</table>
Positive selection systems

• Those that promote the growth of transformed cells.

• They may be divided into conditional-positive or non-conditional-positive selection systems.

• A conditional-positive selection system consists of a gene coding for a protein, usually an enzyme, that confers resistance to a specific substrate that is toxic to untransformed plant cells or that encourages growth and/or differentiation of the transformed cells.

• The nptII gene which confers resistance to kanamycin, inhibits protein synthesis, is the classic example of a system that is toxic to untransformed cells.

• The manA gene, which codes for phosphomannose isomerase, is an example of a conditional-positive selection system where the selection substrate is not toxic. In this system, the substrate mannose is unable to act as a carbon source for untransformed cells BUT it will promote the growth of cells transformed with manA.
Positive Selectable Marker Genes (SMGs)

• The following seven types of positive SMGs can be identified based on the selection strategy:

1. genes acting through chemical detoxification of a phytotoxin;
2. genes acting through removal or exclusion of a phytotoxin from the affected cell compartment;
3. genes acting through overexpression of the phytotoxinsensitive, or expression of an insensitive target molecule;
4. genes that confer pathogen resistance;
5. genes that confer heat tolerance;
6. genes that confer to the plant cells the ability to utilize as carbon sources carbohydrates that otherwise cannot be metabolized; in some cases these carbohydrates also exert phytotoxic effects;
7. genes that confer to the plant cell the ability to autonomously produce the growth regulators necessary for regeneration.
Non-conditional-positive selection systems

- Non-conditional-positive selection systems do not require external substrates yet promote the selective growth and differentiation of transformed material.
- An example is the ipt gene that enhances shoot development by modifying the plant hormone levels endogenously.
<table>
<thead>
<tr>
<th>Selective agent</th>
<th>Genes</th>
<th>Enzymes</th>
<th>Sources</th>
<th>Genomes</th>
<th>References</th>
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<td>Nuclear</td>
<td>Endo et al., 2001; Zuo et al., 2002a</td>
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<td><em>rol</em></td>
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<td><em>CKII</em></td>
<td>Histidine kinase</td>
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<tr>
<td></td>
<td></td>
<td>(cytokinin-independent 1)</td>
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</table>
Negative selection systems

- Negative selection systems have been described in plants for genes that result in the death of transformed cells.

- These are dominant selectable marker systems that may be described as conditional and non-conditional selection systems.

- When the selection system is not substrate dependent, it is a non-conditional-negative selection system. An example is the expression of a toxic protein, such as a ribonuclease to ablate specific cell types.

- When the action of the toxic gene requires a substrate to express toxicity, the system is a conditional negative selection system.
<table>
<thead>
<tr>
<th>Substrates</th>
<th>Genes</th>
<th>Enzymes</th>
<th>Sources</th>
<th>Genome</th>
<th>References</th>
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<tr>
<td>5-Fluorocytosine</td>
<td>codA</td>
<td>Cytosine deaminase</td>
<td><em>Escherichia coli</em></td>
<td>Nuclear, plastid</td>
<td>Stougaard, 1993; Serino and Maliga, 1997</td>
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<td>Naphthalene acetamide</td>
<td>aux2</td>
<td>Amido hydrolase</td>
<td><em>Agrobacterium rhizogenes</em></td>
<td>Nuclear</td>
<td>Becln et al., 1993</td>
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<td>Indole-3-acetamide</td>
<td>tms 2</td>
<td>Indoleacetic acid</td>
<td><em>Agrobacterium tumefaciens</em></td>
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<td>Depicker et al., 1988</td>
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<td>Dihaloalkanes</td>
<td>dhlA</td>
<td>Dehalogenase</td>
<td><em>Xanthobacter autotrophicus</em></td>
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<td>CYP105A</td>
<td>Cytochrome P450</td>
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<td>Allyl alcohol</td>
<td>cue</td>
<td>Alcohol dehydrogenase</td>
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<td>Crop</td>
<td>Identifier</td>
<td>Phenotypic trait</td>
<td>Selectable markers&lt;sup&gt;a&lt;/sup&gt; (gene–enzyme)</td>
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<td><em>Beta vulgaris</em> (sugar beet)</td>
<td>GTSB77 *(InVigor&lt;sup&gt;TM&lt;/sup&gt;)</td>
<td>Glyphosate herbicide resistance</td>
<td><em>uidA</em>–GUS</td>
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<td>T120-7</td>
<td>Phosphinothricin herbicide resistance, specifically glufosinate ammonium</td>
<td><em>neo</em>–NPTII</td>
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<td><em>Brassica napus</em> (canola, oilseed rape)</td>
<td>23-18-17, 23-198</td>
<td>High laurate and myristate canola</td>
<td><em>neo</em>–NPTII</td>
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<td>GT200 *(Roundup Ready&lt;sup&gt;®&lt;/sup&gt;)</td>
<td>Glyphosate herbicide resistance</td>
<td><em>CP4 epsps–EPSPS, goxv247–GOX</em></td>
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<tr>
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<td>GT73, RT73 *(Roundup Ready&lt;sup&gt;®&lt;/sup&gt;)</td>
<td>Glyphosate herbicide resistance</td>
<td><em>CP4 epsps–EPSPS, goxv247–GOX</em></td>
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<td>HCN10 <em>(Liberty-Link&lt;sup&gt;TM&lt;/sup&gt; Independence)</em></td>
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<td><em>pat</em>–PAT</td>
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<td></td>
<td>HCN92 <em>(Liberty Link&lt;sup&gt;TM&lt;/sup&gt; Innovator)</em></td>
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<td><em>pat</em>–PAT, <em>neo</em>–NPTII</td>
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<td>HCN28</td>
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<td>MS1, RF1 → PGS1</td>
<td>Male sterility, fertility restoration, pollination control, glufosinate</td>
<td><em>neo</em>–NPTII, <em>bar</em>–PAT</td>
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<td>MS1, RF2 → PGS2</td>
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<td>MS8 X RF3</td>
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<td>OXY-235</td>
<td>Tolerance to herbicides bromoxynil and ioxynil</td>
<td><em>bxn</em>–nitrilase</td>
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<td>PHY 14, PHY35</td>
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<td><em>bar</em>–PAT</td>
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<td>PHY36</td>
<td>Male sterility, fertility restoration, phoshinothricin herbicide resistance</td>
<td><em>bar</em>–PAT</td>
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</tbody>
</table>
Conditional-positive selection systems using antibiotics

• Aminoglycoside-modifying enzymes

  — The aminoglycoside antibiotics include a number of molecules (e.g. kanamycin, neomycin, gentamicin derivative G418, paromomycin) that are very toxic to plant, animal and fungal cells.

  — Three major classes of aminoglycoside-modifying enzymes have been used to create selection systems for plants; they confer resistance through

    • ATP-dependent O-phosphorylation by phosphotransferases,

    • acetyl CoA-dependent N-acetylation by acetyltransferases, and

    • ATP-dependent O-adenylation by nucleotidyltransferases.
Aminoglycoside-O-phosphotransferases

• Neomycin phosphotransferase

  – Bacterial aminoglycoside 3’-phosphotransferase II, also known as neomycin phosphotransferase II (NPTII), was shown to be effective as a selectable marker in mammalian and yeast cells, therefore it was the first to be tested in plants.

  – Since that time it has become the most widely used selectable marker system in plants.

  – NPTII catalyses the ATP-dependent phosphorylation of the 3’-hydroxyl group of the amino-hexose portion of certain aminoglycosides including neomycin, kanamycin, geneticin (G418), and paramomycin.
Aminoglycoside-O-phosphotransferases

- **Hygromycin phosphotransferase**
  - **Hygromycin B** is an aminocyclitol antibiotic inhibitor of protein *synthesis* with a broad spectrum activity against prokaryotes and eukaryotes.
  - **Hygromycin B** is the *second most frequently used antibiotic* for selection after *kanamycin*.
  - In *plants*, the antibiotic is *very toxic*.
  - The *E. coli* gene *aphIV* (hph, hpt), coding for hygromycin B phosphotransferase, confers resistance on bacteria, fungi, animal cells and plant cells by *detoxifying hygromycin B* via an ATP-dependent phosphorylation of a 7”’-hydroxyl group.
Aminoglycoside-N-acetyltransferases.

- The aminoglycoside-N-acetyl transferases (AAC) are another class of aminoglycoside-modifying enzyme with potential to act as plant selectable marker genes.
- Two of these enzymes, AAC(3)-III and AAC(3)-IV, have been examined in petunia and Arabidopsis under the control of the 35S promoter and nos 3’ sequences.
- These enzymes acetylate gentamicin, kanamycin, tobramycin, neomycin and paromomycin.
- AAC(3)-IV additionally modifies apramycin and G418.
- Another enzyme that acetylates the 6’ amino group, aminoglycoside-6’-N-acetyltransferase (AAC(6’)) from Shigella sp., yielded efficient selection of transformed tobacco protoplasts on high levels of kanamycin. The gene, 6’ gat, under the control of the 35S promoter, is therefore a functional alternative to the nptII gene.
Aminoglycoside-O-nucleotidyltransferases.

- Aminoglycoside-O-nucleotidyltransferases represent the third class of enzymes that modify the aminoglycoside antibiotics that can be used as plant selectable marker genes.

- The bacterial **aadA gene** codes for the enzyme **aminoglycoside-3’’-adenyltransferase**.

- When driven by the 35S promoter, the aadA gene conferred resistance to *spectinomycin* and *streptomycin* in *N. tabacum*; however, the selection was for the contrast between green tissue and chlorotic tissue rather than for survival and growth.

- However, it is the **most widely used** selectable marker for plastid transformation.
Bleomycin resistance

• **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**.

• **Bleomycin interferes with tobacco plant regeneration through morphogenesis**.

• Two sources of resistance have been described for plants:
  – the resistance gene found on *E. coli* transposon *Tn5*, and
  – a chromosomal gene of *Streptoalloteichus hindustanus*.

• When expressed at high levels from the 35S promoter, both genes yield high levels of resistance to phleomycin and regeneration of tobacco plants.

• So far, this system does not appear to have been widely adopted.
Mutant dihydropteroate synthase

- A large number of **sulfonamides or sulfa drugs** exist as antimicrobial compounds that **inhibit the enzyme dihydropteroate synthase** (DHPS, E.C. 2.5.1.15).
- **DHPS catalyzes a rate limiting step for folic acid synthesis** in bacteria and plants.
- **Resistance** is encoded by **sul genes on bacterial R**.
- The resistance gene sull from plasmid R46 codes for a **mutant form of DHPS** that is resistant to inhibition by the sulfonamides.
- **To be effective in plants**, the enzyme must be **targeted to the chloroplast**.
- For example, cleavage of the **transit peptide sequence** of the pea ribulose bisphosphate carboxylase/oxygenase gene fused to the sull gene, results in the **deposition of the enzyme into the chloroplast stroma**.
- Effective selection and regeneration of tobacco were demonstrated when this construct was expressed using the 35S promoter.
- The **selection system differs** from the others described so far in that the mechanism is a mutation of the enzyme **resulting in resistance rather than detoxification of the antibiotic by the enzyme**.
Streptothricin acetyltransferase

- Streptothricins produced by *Streptomyces spp.*

- Are antimicrobial agents that consist of gulosamine, streptolidin and a peptide chain of 1–6 residues.

- Inhibit protein synthesis by binding to the ribosomal small subunit.

- *E. coli sat3* gene codes for an acetyl transferase activity that inactivates streptothricins.
Chloramphenicol acetyltransferase

- The **CAT gene controlled by the nos promoter** has also been **introduced in the tobacco chloroplast genome** by **Agrobacterium-mediated transformation** under selection with chloramphenicol.

- Selection on chloramphenicol was much **less efficient than selection on kanamycin** conferred by the nptII gene. The inefficiency has limited the use of the cat gene as a selectable marker.

- This enzyme is **no longer widely used** as a reporter gene.
<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Genes</th>
<th>Enzymes</th>
<th>Sources</th>
<th>Genome</th>
<th>References</th>
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<tbody>
<tr>
<td>Neomycin</td>
<td><em>neo</em>, <em>nptII</em></td>
<td>Neomycin</td>
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<td>Fraley et al., 1983</td>
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<td>Kanamycin</td>
<td><em>(aphA2)</em>,</td>
<td>Phosphotransferases</td>
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<td>Plastid</td>
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<td></td>
<td><em>aac4</em></td>
<td>transferases</td>
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<td>6′<em>gat</em></td>
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<td>Spectinomycin</td>
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<td>DeBlock et al., 1984</td>
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*References:
- Fraley et al., 1983
- Carrer et al., 1993
- Hayford et al., 1988
- Gossele et al., 1994
- Svab et al., 1990
- Svab and Maliga, 1993
- Maliga et al., 1988
- Waldron et al., 1985
- Hille et al., 1986
- Perez et al., 1989
- Guerineau et al., 1990
- Jelenska et al., 2000
- DeBlock et al., 1984
- DeBlock et al., 1985
Conditional-positive selection systems using herbicides

• The sources of genes used to achieve selection on herbicides range from **bacterial to plant in origin**.

• At least two mechanisms are employed to achieve resistance.

• One mechanism uses the resistance found in **natural isozymes or generated by enzyme mutagenesis**, and the second involves detoxification of the herbicide by metabolic processes.
Phosphinothricin N-acetyltransferase or bialophos resistance gene

• The **l-isomer of phosphinothricin** (PPT; glufosinate ammonium) is the **active ingredient** of several commercial broad spectrum herbicide formulations (e.g. Basta™, Ignite™, Liberty™).

• An analogue of l-glutamic acid, PPT is a **competitive inhibitor of glutamine synthetase** (GS) which is the only enzyme that can catalyse the assimilation of ammonia into glutamic acid in plants.

• **Inhibition of glutamine synthetase** ultimately results in the **accumulation of toxic ammonia levels resulting in plant cell death.**

• Two sources of resistance have been described.
  
  – Elevation of GS expression levels using strong promoters will confer resistance to PPT (Eckes et al., 1989) but this approach has not been adopted for commercial applications.

  – Secondly, bacterial acetyltransferases that confer resistance to bialophos (consisting of two l-alanine residues and PPT) have been used in plants to achieve resistance to herbicides that contain PPT.
Phosphinothricin N-acetyltransferase or bialophos resistance gene

- Two genes (pat and bar) encoding the enzyme phosphinothricin N-acetyltransferase (PAT) have been used to confer tolerance to l-PPT in transgenic plants.

- The bar (bialophos resistance) gene from *S. hygroscopicus* and the pat gene from *S. viridochromogenes* are 87% similar at the nucleotide level.

- PAT uses acetyl CoA as a cofactor to catalyze the acetylation of the free amino group of l-PPT. The acetylated form of l-PPT is unable to bind to and inactivate glutamine synthetase.
5-Enolpyruvylshikimate-3-phosphate synthase and glyphosate oxidase

- **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 plastid enzyme **5-enolpyruvylshikimate-3-phosphate synthase** (EPSP synthase, E.C. 2.5.1.19) which is essential in the shikimate pathway for the **biosynthesis of the aromatic amino acids**.
- A number of mechanisms for glyphosate resistance have been described.
  - **over expression of a petunia EPSP synthase** gene using the 35S promoter generated glyphosate tolerance in transformed petunia
  - **expression of mutant forms of the EPSP synthase** gene aroA from *Salmonella typhimurium* or *E. coli* targeted to chloroplasts, conferred glyphosate resistance to tobacco; a naturally-glyphosate-resistant EPSP synthase gene from the *A. tumefaciens* strain CP4 fused to the transit peptide sequence of Arabidopsis EPSP synthase for chloroplast targeting has conferred glyphosate resistance to several crop species.
  - **catabolism of glyphosate to glyoxylate and aminomethylphosphonic acid (AMPA)** by bacterial **glyphosate oxidoreductase (GOX)** targeted to the chloroplast has conferred glyphosate resistance to several different plants
Acetolactate synthase or acetohydroxyacid synthase

- **Acetolactate synthase**, also known as acetohydroxyacid synthase, is the target for several classes of herbicides including the sulfonylureas, imidazolinones, triazolopyrimidines and pyrimidinyl thiobenzoates.

- **ALS** is a regulatory enzyme in the biosynthetic pathway to branched-chain amino acids in chloroplasts and it is encoded by a limited number of nuclear genes depending on the plant species.

- ALS genes are amenable to mutation and yield mutant enzymes that are resistant to one or more of the herbicides that act on ALS.

- In general, herbicide resistant forms of ALS differ by only one or two amino acids from the native form.

- Selection for sulfonylurea and imidazolinone resistance is very efficient and was used to demonstrate targeted modifications of endogenous ALS from wild-type to herbicide resistance form using chimeric RNA/DNA oligonucleotides.

- It is therefore not surprising that mutant forms of plant ALS would act as effective selectable marker genes when combined with sulfonylurea or imidazolinone herbicides.
Bromoxynil nitrilase

• The oxynil herbicides, such as bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) and ioxynil (3,5-diiodo-4-hydroxybenzonitrile), are inhibitors of photosystem II electron transport that are active in many plants but not in monocots.

• A nitrilase enzyme (3,5-dibromo-4-hydroxybenzonitrile aminohydrolase, coded by the bnx gene from Klebsiella pneumoniae subspecies ozanaenae, hydrolyzes bromoxynil into 3,5-dibromo-4-dihydroxybenzoic acid and ammonia.
Toxic herbicides and selectable marker genes used for the conditional-positive selection of transgenic plants

<table>
<thead>
<tr>
<th>Herbicides</th>
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<th>Enzyme</th>
<th>Source</th>
<th>Genome</th>
<th>References</th>
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<tbody>
<tr>
<td>Phosphinothricin</td>
<td><em>pat, bar</em></td>
<td>Phosphinothricin acetyl transferase</td>
<td><em>Streptomyces hygroscopicus</em>, <em>Streptomyces viridochromogenes Tu494</em></td>
<td>Nuclear</td>
<td>DeBlock et al., 1989</td>
</tr>
<tr>
<td>Glyphosate</td>
<td><em>EPSP synthase</em></td>
<td>5-Enolpyruvylshikimate-3-phosphate synthase</td>
<td><em>Petunia hybrida</em>, <em>Zea mays</em></td>
<td>Nuclear</td>
<td>Zhou et al., 1995; Howe et al., 2002; Comai et al., 1988; della Cioppa et al., 1987; Barry et al., 1992; Barry et al., 1992</td>
</tr>
<tr>
<td></td>
<td><em>aroA</em></td>
<td></td>
<td><em>Salmonella typhimurium</em>, <em>Escherichia coli</em></td>
<td>Nuclear</td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>cp4 epsps</em></td>
<td>Glyphosate oxidodreductase</td>
<td><em>Agrobacterium tumefaciens</em></td>
<td></td>
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</tr>
<tr>
<td></td>
<td><em>gox</em></td>
<td></td>
<td><em>Ochrobactrum anthropi</em></td>
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<tr>
<td>Sulfonylureas</td>
<td><em>csr1-1</em></td>
<td>Acetolactate synthase</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Nuclear</td>
<td>Olszewski et al., 1988</td>
</tr>
<tr>
<td>Imidazolinones</td>
<td><em>csr1-2</em></td>
<td>Acetolactate synthase</td>
<td><em>Arabidopsis thaliana</em></td>
<td>Nuclear</td>
<td>Aragao et al., 2000</td>
</tr>
<tr>
<td>Oxnils</td>
<td><em>bnx</em></td>
<td>Bromoxynil nitrilase</td>
<td><em>Klebsiella pneumoniae</em> subspecies <em>ozanaenae</em></td>
<td>Nuclear</td>
<td>Freyssinet et al., 1996</td>
</tr>
<tr>
<td>Gabaculine</td>
<td><em>hemL</em></td>
<td>Glutamate-1-semialdehyde aminotransferase</td>
<td><em>Synechococcus PCC6301</em></td>
<td>Nuclear</td>
<td>Gough et al., 2001</td>
</tr>
<tr>
<td>Cyanamide</td>
<td><em>cah</em></td>
<td>Cyanamide hydratase</td>
<td><em>Myrothecium verrucaria</em></td>
<td>Nuclear</td>
<td>Damm, 1998; Weeks et al., 2000</td>
</tr>
</tbody>
</table>
Conditional-positive selection systems using toxic metabolic intermediates, analogues and drugs

• 2-Deoxyglucose-6-phosphate phosphatases
  – The glucose analogue, 2-deoxyglucose (2-DOG), is phosphorylated by hexokinase to form 2-DOG-6-phosphate.

  – 2-DOG-6-phosphate competes with glucose-6-phosphate causing cell death through the inhibition of glycolysis.

  – The yeast gene DOGR1, coding for 2-deoxyglucose-6-phosphate phosphatase, was placed under the control of the 35S promoter. Use of this construct as a selectable marker gene resulted in the selection of transgenic tobacco plants at lower efficiencies than with the nptII gene and the selection of transgenic potato with comparable efficiencies.
Dihydrofolate reductase

- Antifolate drugs, such as trimethoprim and methotrexate (Mtx), bind to the active site of the enzyme dihydrofolate reductase resulting in impaired protein, RNA and DNA biosynthesis and subsequently cell death.

- Plant cells are generally very sensitive to low levels of Mtx.

- Sources of resistant DHFR have been found in the bacterium *E. coli*, the fungus *Candida albicans* and mutant mammalian cells.
Conditional-positive selection systems using non-toxic metabolic intermediates

• This category differs significantly from the previously discussed systems in that the external substrates are basically inert until they are converted into molecules that provide the transformed plant cells with a growth advantage.

• This approach appears to yield generally higher transformation frequencies and seems to be broadly applicable across a range of plant species making it is an area of major interest for crop plants.
Xylose isomerase

- Plant cells from species such as tobacco, potato and tomato cannot use d-xylose as a sole carbon source.

- The enzyme xylose isomerase catalyzes the isomerization of xylose to d-xylulose, which can then be used as a carbon source.

- The efficiency of selection was much greater than for the nptII gene and the regeneration of shoots was significantly faster.
<table>
<thead>
<tr>
<th>Drugs and analogues</th>
<th>Genes</th>
<th>Enzymes</th>
<th>Sources</th>
<th>Genome</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>2-Deoxyglucose</td>
<td>DOGR1</td>
<td>2-Deoxyglucose-6-phosphate phosphatase</td>
<td>Saccharomyces cerevisiae</td>
<td>Nuclear</td>
<td>Kunze et al., 2001</td>
</tr>
<tr>
<td>Betaine aldehyde</td>
<td>BADH</td>
<td>Betaine aldehyde dehydrogenase</td>
<td>Spinacia oleracea</td>
<td>Nuclear, plastid</td>
<td>Ursin, 1996; Daniell et al., 2001</td>
</tr>
<tr>
<td>S-Aminoethyl L-cysteine (AEC)</td>
<td>DHPS</td>
<td>Dihydropicolinate synthase, Octopine synthase</td>
<td>Escherichia coli, Agrobacterium tumefaciens</td>
<td>Nuclear</td>
<td>Perl et al., 1993; Koziel et al., 1984; Goddijn et al., 1993</td>
</tr>
<tr>
<td>4-Methyltryptophan (4-mT)</td>
<td>ocs</td>
<td>TDC</td>
<td>Catharanthus roseus</td>
<td>Nuclear</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>DHFR</td>
<td>Dihydrofolate reductase</td>
<td>Escherichia coli mouse</td>
<td>Nuclear</td>
<td>Herrera-Estrella et al., 1983; Eichholtz et al., 1987; Irdani et al., 1998</td>
</tr>
</tbody>
</table>

Non-toxic agents and enzymes used for the conditional-positive selection of transgenic plants

<table>
<thead>
<tr>
<th>Non-toxic agents</th>
<th>Genes</th>
<th>Enzymes</th>
<th>Sources</th>
<th>Genome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Xylose</td>
<td>xylA</td>
<td>Xylose isomerase</td>
<td>Streptomyces rubignosus, Thermoanaerobacterium sulfurogenes</td>
<td>Nuclear</td>
<td>Haldrup et al., 1998a; Haldrup et al., 1998b</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>manA</td>
<td>Phosphomannose isomerase</td>
<td>Escherichia coli</td>
<td>Nuclear</td>
<td>Joersbo et al., 1998</td>
</tr>
<tr>
<td>Benzyladenine-N-3-glucuronide</td>
<td>uidA (gusA)</td>
<td>β-Glucuronidase</td>
<td>Escherichia coli</td>
<td>Nuclear</td>
<td>Joersbo and Okkels, 1996</td>
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</table>
Non-selectable marker gene systems—
Reporter genes/Scorable marker

<table>
<thead>
<tr>
<th>External substrates</th>
<th>Genes</th>
<th>Enzymes</th>
<th>Sources</th>
<th>Genomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ONPG, X-gal</td>
<td>lacZ</td>
<td>β-Galactosidase</td>
<td><em>Escherichia coli</em></td>
<td>Nuclear</td>
</tr>
<tr>
<td>MUG, X-gluc</td>
<td><em>uidA</em> (<em>gusA</em>)</td>
<td>β-Glucuronidase</td>
<td><em>Escherichia coli,</em> <em>Bacillus sp.</em></td>
<td>Nuclear, Plastid</td>
</tr>
<tr>
<td>Luciferin</td>
<td><em>Luc</em></td>
<td>Luciferase</td>
<td><em>Photinus pyralis</em></td>
<td>Nuclear</td>
</tr>
<tr>
<td>Decanal</td>
<td><em>luxA</em>, <em>B luxF</em></td>
<td></td>
<td><em>Vibrio harveyi</em></td>
<td></td>
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<td>None</td>
<td><em>gfp</em></td>
<td>Green flourescent, protein (GFP)</td>
<td><em>Aequorea victoria</em></td>
<td>Nuclear, plastid</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>Phytoene synthase</td>
<td><em>Erwinia herbicola</em></td>
<td>Nuclear</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>Anthocyanin pathway regulatory factors</td>
<td>Maize</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>Thaumatin II</td>
<td><em>Thaumatococcus danielli</em> Benth</td>
<td>Nuclear</td>
</tr>
<tr>
<td>Oxalic acid</td>
<td></td>
<td>Oxalate oxidase (OxO)</td>
<td>Wheat</td>
<td>Nuclear</td>
</tr>
</tbody>
</table>
β-Galactosidase

• The bacterial enzyme β-galactosidase, which is coded by the E. coli lacZ gene, has been a useful marker gene in many cell systems because it can be easily assayed and can form N-terminal translational fusions with other proteins.

• Although some plants have background galactosidase activity, experiments with tobacco and sunflower showed that ectopic enzyme activity could be measured with the synthetic substrate O-nitro-phenyl--d-galacto pyranoside (ONPG) and tissues that express the enzyme will stain with 5-bromo-4-chloro-3-indoyl--d-galactosylpyranoside (X-Gal).

• The lac Z gene is therefore a conditional non-selectable marker gene. The protein does not appear to be toxic to plant cells.

• It has not been widely adopted.
β-Glucuronidase

- The bacterial enzyme β-glucuronidase, which is coded by the *E. coli* uidA (gusA) gene is the most widely used reporter in plants.
- The enzyme utilizes the external substrates 4-methyl umbelliferyl glucuronide (MUG) for measurements of specific activity and 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) for histological localization.
- It is therefore a **conditional non-selectable marker gene**.
- A useful feature of GUS is that it can be fused with other proteins.
- GUS is rapidly degraded under conditions found in the stomach.
- Humans and animals are continuously exposed to GUS from bacteria residing in their intestinal tracts and from non-transgenic food sources without harmful effects; therefore, the low level of GUS protein from genetically modified plants is not a concern with regard to toxicity or allergenicity.
- GUS expression was used as a reporter to help detect transformation events in tissue culture during the production of a number of plant lines approved for commercialization.
Advantages of β-Glucuronidase

• β-Glucuronidase assays are very sensitive.

• Quantitative estimation of the enzyme can be done by fluorometric method (using substrate 4-methylumbelliferryl P-D-glucuronide which is hydrolysed to 4-methylumbelliferone).

• Qualitative data on the enzyme can be obtained by histochemical means (enzyme localization can be detected by chromogenic substance such as substrate X-gluc).

• No need to extract and identify DNA.
Luciferase

• As a reporter, offers several advantages including the capability of monitoring gene expression patterns non-destructively in real time with great sensitivity.
• The firefly (*Photinus pyralis*) luciferase catalyzes the ATP-dependent oxidative decarboxylation of luciferin. After the reaction occurs the luciferase is inactive until the oxyluciferin is released from the enzyme complex.
• This is a slow process and the LUC half life is very short; thus, it is believed that LUC activity more accurately reflects transcriptional activity than some other reporter genes that are more stable and accumulate over time.
Green fluorescent protein

- The green fluorescent protein (GFP) from jellyfish (*Aequorea victoria*) has become a powerful reporter gene to complement selectable marker genes and can be used to select for transformed material alone.
- The great advantage of GFP as a non-conditional reporter is the direct visualization of GFP in living tissue 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.
- GFP has not been extensively used as a reporter for studies in the regulation of gene expression or the study of regulatory elements; however, it has been a very useful tag for monitoring intracellular location and transport when fused to other proteins of interest.
Phytoene synthase

- The bacterial gene coding for phytoene synthase from *Erwinia herbicola* can act as a non-conditional reporter gene by altering the carotenoid biosynthetic pathway in chloroplasts so that coloured carotenoids accumulate.

- The coloured tissues expressing the reporter gene can then be manually removed and cultured to generate transgenic plants.

- Phytoene synthase catalyses the synthesis of phytoene from geranylgeranyl pyrophosphate and phytoene is a precursor of lycopene, the carotenoid that imparts the red colour to tomato.

- *E. herbicola* phytoene synthase targeted to the chloroplast, generated transgenic orange callus as a visual marker for transgenic tissue at about 50% efficiency and may be used to monitor transgenic plants.
Marker-free strategies

• There is a growing concern among the public regarding the use of antibiotic or herbicide resistance genes as selectable markers of plant transformation:
  – The products of some marker genes may be toxic or allergic.
  – The antibiotic resistance might be transferred to pathogenic microorganisms in the soil.
  – There is a possibility of creation of super weeds that are resistant to normally used herbicides.
  – A transgenic plant with selectable marker genes cannot be transformed again by using the same selectable markers.

• In light of the apprehensions listed above, the public is concerned about the safety of transgenic technology, particularly related to the selectable marker genes (antibiotic/herbicide resistance genes).
• There are fears about the safety of consumption of foodstuffs derived from genetically engineered plants.
• This is despite the fact that so far none of the marker genes have been shown to adversely affect human, animal or environmental safety.
Marker-free strategies

• For commercialization of transgenic plants it would simplify the regulatory process and improve consumer acceptance to remove gene sequences that are not serving a purpose in the final plant variety.

• For scientific purposes, eliminating the marker genes from the final plant would permit the use of experimental marker genes that have not undergone extensive biosafety evaluations or that may generate negative pleiotropic effects in the plants.
1. Co-transformation and segregation of marker genes

• Co-transformation with separate plasmids in one or two agrobacterium strains.
  – When compared to methods that produce plants where the marker gene is linked to the gene of interest, this method requires about a four-fold greater production of transgenic lines to recover a comparable number of marker-free plants.

• Co-transformation with single plasmids carrying multiple T-DNA regions
  – In maize, co-transformation with an octopine strain carrying a binary vector with two T-DNAs yielded co-transformation frequencies of 93% for the bar and GUS genes in the R0 generation. 64% of the R1 progeny segregated as bar-free plants expressing GUS. This contrasted dramatically with the 11.7% co-transformation frequency with mixed Agrobacterium strains.
2. Transposon-mediated repositioning of genes

• Tranposition-mediated repositioning of the gene of interest
  – This technology relies on crossing plants to segregate the gene of interest from the marker gene and the transposase; therefore, this technology is of limited use in plants that are vegetatively propagated or have a long reproductive cycle.
  – This technology also has limitations for pyramiding multiple genes because introduction of the transposase in subsequent rounds of transformation and marker gene removal may result in the transposition of the first transgene into another chromosomal location.

• Tranposition-mediated elimination of the selectable marker gene
  – An alternative strategy for exploiting the Ac/Ds system is to transpose the genes coding for the selectable marker and the transposase from the T-DNA leaving only the gene of interest in the inserted copy of the T-DNA.
3. Intrachromosomal homologous recombination to remove selectable marker genes

- Studies on the use of homologous recombination to eliminate selectable marker genes after insertion are few and presently poorly understood.
- This strategy is not always associated with homologous recombination and larger deletions may occur as a result of illegitimate recombination.
4. Site-specific recombinase-mediated excision of marker genes

- **Cre–lox**
  - The Cre–lox system from bacteriophage P1 was the first of the recombination systems shown to be effective in the generation of marker-free plants.
  - The T-DNA vector carrying the gene of interest was constructed with lox sites flanking the hpt selectable marker gene and inserted into tobacco.
  - The Cre recombinase was then introduced by a second round of transformation to achieve precise excision of the marker gene.
A significant refinement of the strategy was developed using the β-estradiol-inducible promoter system in which an artificial transcription factor, XVE was constructed for use in plants with its target promoter.

In this system, the gene of interest was separated from its promoter by a fragment containing the genes coding for the XVE transcription factor, the nptII selectable marker and the Cre recombinase (under the control of the inducible promoter) surrounded by lox sites.

Transformation of Arabidopsis was achieved by selection for kanamycin resistance. Subsequent induction with -estradiol resulted in the excision of the complete induction system along with the Cre recombinase and selectable marker genes. The final product was the reconstituted gene of interest, in this case GFP.
FLP–FRT

• The FLP–FRT system derived from the *Saccharomyces cerevisiae* 2μ plasmid has also been tested in plants.

• In tobacco and Arabidopsis, plants transformed with the FLP recombinase were crossed with plants transformed with T-DNA in which the GUS coding region is separated from the 35S promoter by a hpt gene bracketed by FRT sites. This resulted in excision of the hpt gene and activation of the GUS gene in all cases.
Assignment

• What is the difference between Marker and Reporter genes? [3]
• What problem arises during positive selection using toxic substrates? [1]
• Write in detail about various types of selectable marker used for selection of transformed plants. [7.5]
• Write in detail about various types of reporter genes used for selection of transformed plants. [7.5]
• Write about strategies on how to create marker free transformants. [7.5]
• What are the problems associated with presence of marker in the transformants? [3]
• What do you understand by positive and negative selection of transgenic tissues? [3]
• What is the purpose of negative selection of transgenic plants? [1]
• What are the features of an ideal marker gene? [3]
• What do you understand by conditional and non-conditional selection systems? Explain with examples. [3]
• Describe the various strategies with examples by which a gene can act as a positive selectable marker gene. [7.5]