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Paper No. : 12 Plant biotechnology Biotechnology and crop improvement

Module : 31 Detection, characterization and expression of transformants (Genetic markers, reporter genes, transgene stability and gene silencing)

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Description of Module	
Subject Name	Biotechnology
Paper Name	Plant biotechnology and crop improvement
Module Name/Title	Detection, characterization and expression of transformants (Genetic markers, reporter genes, transgene stability and gene silencing)
Module Id	31
Pre-requisites	
Objectives	
Keywords	

QUADRANT-I

Learning objectives

This module will help us to learn about the basic techniques for screening of transgenic plants with desired traits. To screen transgenic plants with particular trait, a researcher can use marker system which will help to know the presence or absence of gene target fused with screenable markers or selectable markers. Target gene stability approaches can be useful for stable transgene expression or passing on the desired trait from one generation to another without altering the structure or function of transgene. Gene silencing approach will help to learn about the basic causes and strategies for silencing of gene by three different modes (siRNA, miRNA and shRNA). Hope that this will help us to learn more about the topic and enhance our knowledge.

Marker Genes for Transgenic screening

To introduce desired trait/character of one plant into another plant, a researcher has to develop a stable method to monitor and detect plant transformation system. The gene(s) that are introduced into the plasmid with target gene are called as marker gene(s)/marker system. Marker genes that are used in plant transformation system are categorized into two groups.

1. Selectable marker gene
2. Reporter gene/ scorable marker gene/ screenable marker gene

Advantages of Marker genes in transgenic plants:

- Easy analysis
- It does not require DNA extraction, electrophoresis and autoradiography

Selectable Marker

For successful genetic transformation methods, selecting transformed cells is an important factor. This can be carried out by different selectable marker genes which are present in the plasmid with gene of interest/target gene. Integral part of plant transformation strategy involved selectable markers. These markers allow transformed cells to survive on media which contain toxic concentration of selecting agent whereas non transformed cells dies. These selectable markers belong to different categories viz. antibiotic antimetabolite and herbicide resistance genes, hormone biosynthetic genes and genes that confer resistance to lethal doses of amino acids or amino acid derivatives. The utility of particular resistance marker depends on:

1. Nature of selection agent
2. Resistance gene
3. Plant material

Selectable marker gene	**Substrate used for selection**	**Enzyme/ mode of action**	**References**
Antibiotic Resistance Markers			
Neomycin Phosphotransferase (*nptII*)	G418, Kanamycin, Neomycin, Paromycin	Neomycin phosphotransferase type II (NPTII)/ Detoxification	Beven *et al.* (1983)
Hygromycin Phosphotransferase (*hpt)*	Hygromycin B	Hygromycin phosphotransferase/ Detoxification	Elzen *et al*. (1985)
Gentamycin Acetyl Transferase (AAC-3)	Gentamycin	Gentamycin Acetyl Transferase/ Detoxification	Guerineau et al. (1990)
Streptomycin Phosphotransferase	Streptomycin	Streptomycin phosphotransferase/	Guerineau et al.

(spt)		Detoxification	(1990)
Antimetabolite Marker			
Dihydrofolate reductase (dhfr)	Methotraxate	Dihydrofolate reductase /Insensitive	Herrera-Esterella et al. (1983)
Herbicide Resistance Marker			
Phosphinothricin acetyltransferase (bar)	L-Phosphinothricin (PPT), bialaphos	Phosphinothricin acetyl transferase/ Detoxification	De Block et al. (1987)
5-Enolpyruvyl Shikimae 3-Phosphate (ESPS) synthase (aroA)	Glyphosate (Round up)	EPSP synthase/ Sensitive and Insensitive	Shah et al. (1986) Comai et al. (1985)
Acetolactate synthase mutant form (csr1-1)	Sulphonylurea, imidazolinones	Acetolactate synthase/ Insensitive	Haughn et al. (1988)
Bromoxynil nitrilase (bxn)	Bromoxynil	Nitrilase/Detoxification	Stalker et al. (1988)

Antibiotic Resistance Markers

1. **Neomycin phosphotransferase (nptII)**

- NPTII encodes an enzyme, aminoglycoside 3'phosphotransferase II (APH(3')-II), which has been derived from Tn5 transposon.
- NptII marker gene can inactivates various aminoglycoside antibiotics such as kanamycin, neomycin, geneticin (G418) and paromycin.
- Kanamycin is commonly used selective agent for transformants whose concentration ranging from 50 to 500 mg/L.

- For selection of kanamycin resistant population, we can apply kanamycin solution of appropriate concentration locally or sprayed on soil grown plants.

2. **Hygromycin phosphotransferase (hpt)**

- Hpt gene was originally derived from E. coli which encodes hygromycin B (an aminocyclitol antibiotic) that can interfere with translation process and disturb protein synthesis
- This can be more toxic to cells and kills them faster as compared to kanamycin
- Hygromycin resistance can be checked by non-destructive callus induction test and segregation of hpt gene in progeny of transgenic plants can be scored by seed germination assay.

3. **Gentamycin Acetyltrasferase**
    - Gentamycin antibiotic selection is used in combination of three genes which encodes aminoglycoside-3-N-acetyltransferase (AAC3)
    - Three genes are AAC3-I, AAC3-III and AAC3-IV
4. **Streptomycin and spectinomycin resistance**
    - Two genes conferring resistance to streptomycin and spectinomycin resistance which are dominant genes used for plant transformation.
    - These two dominant genes are streptomycin phosphotransferase (*spt*) gene from Tn5 and aminoglycoside-3'-adenyltransferase gene (*aad*).
    - These markers are different from other transformation markers as they produce color changes
    - With this marker system, resistant cells obtain green color under appropriate conditions but sensitive cells get bleached without death of the cell.
5. **Methotrexate insensitive dihydrofolate reductase**
    - Methotrexate is an antimetabolite that restricts dihydrofolate reductase (DHFR) enzyme and thus interfere with DNA replication process.

- An experiment conducted with mutant mouse with *dhfr* gene encoding an enzyme with low affinity to methotrexate has been isolated
- *Dhfr* gene which is fused to CaMV promoter gives methotrexate resistant marker (which is used for plant transformation)

6. **Bar gene**
   - Bialaphos, phosphinothricin (PPT) and glufosinate ammonium are non-selective herbicide that stops glutamine synthase (GS) enzyme, which cause death of plant cells by accumulation of ammonia.
7. **EPSP synthase gene**
   - N-phosphonomethyl-glycine (Glyphosate) is a broad-spectrum herbicide, that attacks the photosynthesis process which is essential for plants
   - EPSP synthase (5-Enolpyruvyl-shikimate 3-phosphate) acts a competitive inhibitor for glyphosate
   - In plants, EPSP synthase is localized in chloroplast but maximum of EPSP synthase activity is encoded by nuclear genes

Types of selectable markers: There are 4 different types of selectable markers which are categorised as per their selection mode. These are-

- Dominant antibiotic resistance markers (Positive selection markers):
  - Spectinomycin, streprtomycin and kanamycin are dominant primary positive selection markers.
  - The aadA gene encodes aminoglycoside adenyl transferase (AAD) which provide resistance to aminoglycoside type antibiotics such as spectinomycin by inactivation of antibiotic.
  - Spectinomycin is used for chloroplast transformation as it is prokaryotic translational inhibitor and affects only chloroplast by showing little effect on plant cell.

- It is a non-lethal marker at cellular level, but is a selective marker at the plastid level
- We can differentiate between sensitive and non-sensitive cells on basis of color
- Sensitive cells get bleached whereas resistant cells remain green when grown on a selective medium
- AAD also inhibits streptomycin and was used to select transplastomic clones in tobacco.
- Neomycin phosphotransferase (*nptII*) gene which provides kanamycin resistance has also been used for chloroplast transformation

- Recessive selectable markers (Positive selection markers):
  - These markers are established by the genes that encodes antibiotic insensitive alleles of ribosomal RNA genes
  - Initially, vector used for transformation carried a plastid of 16S rRNA (*rrn16*) gene with point mutation that inhibits binding of spectinomycin or streptomycin to 16SrRNA.
  - The rrn16 target site mutations are recessive and were 100 fold less efficient that the currently used dominant *aadA* gene. These markers can be used with a limited set of mutant strains of plants
  - Other alternative approach for selectable markers are also being studied. BADH (betaine aldehyde dehydrogenase) which encode gene that produces an enzyme, that can convert toxic betaine aldehyde to non-toxic glycine betaine and serves as an effective selectable marker
- Secondary positive selection markers:
  - Genes that provide resistance to herbicides phosphinothricin (PPT) or glyphosate or to the antibiotic hygromycin (based on expression

of the bacterial hygromycin phosphotransferases) are the secondary markers

- Use of secondary selective markers is dose dependent, which are not suitable to select transplastomic clones when only a few ptDNA copies are transformed, but will confer a selective advantage when most genome copies are transformed.

- Negative selection markers:
  - Ability of identify loss-of-function of a conditionally toxic gene form the basis of negative selection
  - Bacterial cytosine deaminases (CD) enzyme encoded by the *codA* gene catalyses deamination of cytosine to uracil, enabling use of cytosine as sole of nitrogen and pyrimidine source
  - CD is present in prokaryotes and in many eukaryotic microorganisms, but is absent in higher plants
  - 5-Fluorocytosine is converted to 5-fluorouracil, which is toxic to cells. This is negative selection process has been utilized to identify seedlings on fluorocytosine-medium from which *codA* was removed by the CRE-*loxp* site-specific recombinase.

Reporter Gene Technology

A **reporter gene or marker** (also called as visual marker gene) creates a product that can be distinguished using a simple and often quantitative assay. Such marker genes are used for a variety of reasons eg. Confirming transformations, determining transformation efficiency and monitoring gene expression. Gene expression is measured by observing the RNA or protein products that are made. Reporter genes can be used to envisage and measure the efficiency of gene expression and to examine intracellular protein localization. The rate at which a gene is expressed depends on its upstream control sequences. The rate of expression of a gene can be observed by substituting its

protein-encoding portion with or blending it in the frame to a reporter gene expressing a protein whose presence can be easily determined.

The reporter genes used are mostly derived from bacterial genes coding for an enzyme that is readily sensed by the use of chromogenic or other substrates. A screening marker gene is functional only if an enzyme with analogous activity is not present in non-transformed cells. Many reporter genes are used and the most commonly genec1s are:

1. **LacZ gene of *E. coli*:** It acts as a reporter gene in the presence of X-gal because of its level of expressivity is readily quantitated by the intensity of the blue colour that is generated.
2. **Chloramphenicol acetyl transferases (CAT) encoding gene of *E. coli:*** CAT gene encoding the enzyme chloramphenicol acetyltransferase, which transfers acetyl groups from acetyl CoA onto the antibiotic chloramphenicol.
3. **Luciferase encoding gene of firefly, *Photinus pyralis*:** catalyses the oxidation of luciferin with emission of yellow-green light which can be detected easily even at low levels.
4. **GFP (Green Fluorescent Protein) encoding gene of bioluminescent jellyfish:** GFP is an auto fluorescent protein (238 aa residues) of bioluminescent jellyfish *Aequorea victoria*. In GFP, eleven strands make up a β-barrel and an α-helix runs through the centre. The chromophore Is located in the middle of the β-barrel. Amino acid residues 65-67 (Ser-Tyr-Gly) form the fluorescent chromophore, p-hydroxybenzylideneimidazoline. The chromophore of GFP is responsible for its fluorescence. It fluoresces with a peak wavelength of 508 nm (green light) when irradiated by UV or blue light (optimally 400nm).

GFP acts as an exclusive reporter and is used as a fusion tag for checking protein localization. GFP is used as a tag in a fusion protein, where it is

combined with the protein whose expression is to be stalked. The principle aim is to investigate the subcellular localization of the protein under examination. Genetic engineering can be used to generate vectors containing a GFP coding sequences into which a coding sequence for an unrevealed protein, A, can be cloned. Resulting GFP-A fusion construct can be transfected into appropriate target cells and expression of the GFP-A fusion protein can be examined to track the subcellular location of the protein.

Gene silencing

A natural phenomenon for defending host genome against the effects of transposable elements and viral infections in which expression of genes is regulated through modifications of RNA, DNA or histone proteins is called epigenetic silencing. Epigenetic silencing also acts as a modulator of expression of duplicated gene family members and as a silencer of transgenes.

A general process of interruption or suppression of transcription or translation of mRNA of the target gene by mechanism other than genetic modification is called as **Gene Silencing.** This topic is the hottest one in science at present and has been proclaimed to be the biggest breakthrough of year 2002, as per the journal Science. This mechanism is mediated by small RNA molecules known as siRNAs or miRNAs. siRNAs and miRNAs are produced by Dicer enzyme either from exogenously introduced dsRNAs or from endogenously transcribed MIR genes. These small molecules, siRNAs and miRNAs further activate RISC which inhibits transcription or translation of the target gene. Gene silencing maintains the genomic structure, differentiation and maintenance of stem cells and provides a promising treatment for disease like AIDS and cancer (if various limitations like problem of invitro delivery or off-target effects, associated with its application as a curing tool, are overcome).

Causes of Gene Silencing: Gene silencing can occur at transcriptional or translational level which could be partial or complete. Major cause of gene silencing is an increase in methylation pattern after the entry of DNA into recipient genome, which leads to inactivation of gene(s). There are many causes of gene silencing which are as follows:

1. DNA methylation
2. Homology dependent gene silencing
3. Suppression by antisense genes
4. Silencing by RNA interference
5. Position effect
6. Increased copy number

DNA methylation: *de novo* methylation of transgenes can be occurred by different methods which are as follows-

- **Methylation of DNA by DNA-DNA pairing:** When two or more transgene copies integrate as concatemer and DNA coiling occurs, then these copies of transgenes come in front of each other as homologous sequences. Due to their mutual suppressing effect there is increase in methylation pattern that leads to suppression of genes.
- **Recognition of transgenes:** transgenes get integrated properly but due to older age of transgene(s) or certain environmental stresses the transgene get hyper methylated and inactivated.
- **Insertion within hyper methylated genomic regions:** When a transgene is get inserted into hyper methylated genomic region, there may be spreading of hyper methylation pattern that leads to inactivation of transgenes.

Homology dependent gene silencing: Homologous sequences not only affect the stability of transgene expression but that activity of endogenous genes could

be altered after insertion of transgene into genome. The homology dependent silencing of gene involves different ways which are as follows-

- **Homologous transgenes inactivation:** Retransformation of transgenic plants with construct that are partially homologous with integrated transgene. In the presence of second transgene construct, the primary construct becomes hyper methylated within the promoter region.
- **Paramutation:** interaction of homologous alleles of plants that leads to heritable epigenetic effects. Paramutation phenomenon is the combination of two homologous alleles that are different in their methylation state.
- **Co-suppression:** Endogenous gene expression can be restricted by introducing homologous sense construct which is capable of transcribing mRNA of same orientation as transcript gene. In co-suppression, there is suppression of transgene and homologous resident gene (both) or inactivation of either or both.

Suppression of antisense genes: Major cause of gene silencing is the antisense technology. Antisense RNA may block RNA production along different pathways. Antisense gene may result in reduced levels of target mRNA and can potentially form a double stranded structure with complementary mRNAs. Antisense RNA may interfere with the following processes-

- Antisense RNA may affect the target gene directly in the nucleus, then inhibiting mRNA synthesis (at transcriptional level)
- Antisense RNA technology may block the processing of mRNAs by masking sequences recognised by splicing and the polyadenylation system. Antisense RNA may disturb the normal transport of mRNAs out of the nucleus by forming a hybrid with their target mRNAs and disturbs the regular flow of transcripts
- Most of antisense RNAs complementary to ribosome binding site have been shown to inhibit translation initiation.

- Antisense RNA stops accumulation of target mRNA. Antisense and sense RNA form a double RNA intermediate that is degraded quickly by dsRNA specific ribonucleases.

Silencing by RNA interference: RNAi (RNA interference) is a process in which the introduction of dsRNA endogenously synthesized or exogenously applied into diverse range of organisms and cell types that causes degradation of complementary mRNA.

Position effect: Inactivation of gene(s) can occur through another mode which provides an explanation that when a transgene gets involved into the genome through improper region, i.e., hypermethylated, heterochromatic, telomeric, compositionally different genomic region, then due to suppressing effect of adjacent region or environment, it gets inactivated.

Increased copy number: A relation between number of integrated copies and frequency of inactivation is well elaborated for copies arranged in cis position. Decrease of copy number inside a locus was shown to increase gene expression or decrease the suppressing effect. This is because of two reasons: i. duplication of promoter or ii. Addition of truncated transgene coding region, that can cause decreased expression. Increased copy number may be because of direct gene transfer methods as there is no control on copy number.

The silencing of genes could be achieved by following ways:

1. **Drugs:** these bind to target protein and causes protein inhibition.
2. **RNase H-independent oligonucleotides:** these oligo deoxyribonucleotides hybridize to target mRNA and causes inhibition of translation of protein.
3. **RNase H-dependent oligonucleotides:** these hybridize to target mRNA and mediates its degradation by RNase H.

4. **Ribozymes and DNA enzymes:** these catalyse cleavage of mRNA and hence causes its degradation.
5. **siRNA and miRNA:** these hybridize to target mRNA by antisense strand and guide it to endoribonuclease enzymes, thereby causing its degradation or inhibition of translation.

Cellular components of Gene silencing:

- miRNAs (MicroRNAs)
- siRNAs (Small Interference RNAs)
- Dicer enzyme
- RISC (RNA-induced silencing complex)
- Histones
- Chromatin and heterochromatin
- Transposons

Levels of Gene Silencing:

1. **Post –transcriptional Gene silencing (PTGS):** A known level of gene silencing in RNAi mechanism. It causes silencing by hybridizing to mRNA of the target gene to which siRNA shows perfect complementarity.
2. **Transcriptional Gene silencing (TGS):** It causes gene silencing by:
    a. DNA methylation
    b. Heterochromatin formation
    c. Programmed DNA elimination

RNA Interference (RNAi Effect)

The term RNA interference is phenomenon in which sequence specific RNA degradation process that is triggered by the formation of double stranded RNA (dsRNA) that can be introduced by virus or transgene. RNAi is a molecular

mechanism in which dsRNA interefere with the expression of particular gene that shares a homologus sequence with dsRNA. dsRNA activates the specific degradation of homologus RNAs only withinthe region of identity with the dsRNA. The dsRNA can be either, miRNA (microRNA) or siRNA (small interference RNA).

Features of RNAi

- Double stranded RNA rather than single-stranded antisense RNA is the interfering agent.
- High degree of specific gene silencing witth less effort
- Highly potent and effective, only a few dsRNA molecules per cell are required for effective interference.
- Silencing can be introduced in different developmental stages
- Systemic silencing
- Avoid problems with deformities caused by a knocked out gene in early stages which could mask desired observations.
- Silencing effects passed through generations

miRNA (MicroRNA)

dsRNA molecules which originate endogenously from MIR genes are called miRNAs. These molecules originate from single stranded RNA. miRNAs which are 21-24 bp duplexes that are normally incompletely base paired and form partial duplexes within the 3'-untranslated region (UTR) of targeted transcripts via an association with RISC. Their functional roles vary depending on the organism, but in mammals the primary mechanism of miRNA action to inhibit mRNA translation. These clumped miRNAs are expressed polycistronically and are processed through following steps:

- Generation of 70 nucleotides precursor microRNAs from the longer transcripts (termed pri-miRNAs)

- Processing of pre-miRNAs into mature miRNAs

miRNA biogenesis

Mature miRNAs derived from two processing events driven by sequential cleavage by the RNase III enzymes- Drosha and Dicer. miRNAs are transcribed by RNA polymerase II, producing primary miRNAs (pri-miRNAs). These are long, capped and polyadenylated, similar to the production of mRNAs from protein encoding genes. These pri-miRNAs are then subjected to processing by microprecursorcomplex, composed of Droshaa enzyme and its associated binding partner, Pasha (also known as DGCR8), which causes excision of 65-75bp sstem-loop structure called as precursor miRNA (pre-miRNA). Pre-miRNA are then recognised and transported from nucleus to cytoplasm via Ran-GTP dependent nuclear transmembrane protein, Exportin 5, where they are by action of Dicer with 2 nucleotide long 3'overhangs, are quickly unwounded by helicase enzyme and a single mature strand is produced which is asymmetrically incorporated into RNA-induced silencing complex (RISC). Where they can act by translational repression by a cleavage incompetent RISC or mRNA degradation by a cleavage competent, slicer-containing RISC.

Small interference RNA (siRNA)

siRNAs are exogenous dsRNA from viruses, transgenes or transposons i.e; they originate from dsRNA. These results from the Dicer enzyme cutting up a larger fragment of perfectly complimentary double stranded RNA. siRNAs are small molecules of 19-24 bp long with symmetric 2 nucleotides at 3' end. These molecules are slightly shorter than miRNAs. siRNA fragments then bind to the nuclease complex RISC and serve as a guide to recognition of complementary base pairing on target mRNA, ultimately bringing its degradation.

Structure of siRNA and miRNA

siRNA is slightly shorter than miRNA. siRNA is about 21-25 nucleotide long and have 5' phosphates and 3' hydroxyl groups and 2 to 3 nucleotide overhangs on the 3' ends. The 5' phosphate is essential for their activity. One of the strand is known as guide strand/ antisense strand while the other is known as passenger strand/ sense strand. siRNA is unwound by RISC activity and guide strand is left to bind to target mRNA while the passenger strand is degraded.

Mechanism of gene silencing

- dsRNA is chopped into short interference RNA (siRNAs) by using Dicer
- siRNA-Dicer complex recruits' additional components to form an RNA-induced silencing complex (RISC). The siRNA unwinds
- the unwound siRNA base pairs with complementary mRNA, thus guiding the RNAi machinery to the target mRNA.
- The target mRNA is effectively cleaved and subsequently degraded which results in gene silencing.

Transcriptional gene silencing

siRNAs work not only at post transcriptional stage but also leave their indelible marks on the genome to repress the gene transcription activity or selectively remove portions of the genomes, especially of protozoans. siRNAs yield three different biochemical products with the chromatin DNA: DNA methylation (as revealed mostly in plant system), heterochromatin formation and programmed elimination of DNA. A major source of transcriptional gene silencing was supposed to be DNA methylation and mechanistically transcriptional gene silencing had been viewed different from PTGS. The discoveries in epigenetics stream have ignited a revolution and showed the developments to differentiate between these two pathways.

Significance and applications of gene silencing

1. **Biological function**

a. **Immunity:** It is known to provide antiviral immune response. In plants, where it may also avoid self-propagation by transposons.

b. **Downregulation of genes:** miRNA or siRNA are most important to conquer translational activity of genes and also regulates the development, especially in timing of morphogenesis and the maintenance of undistinguishable or incompletely differentiated cell types eg. Stem cells.

c. Upregulation of genes: siRNA or miRNA (RNA molecules that are complementary part of a promoter) can increase gene transcription. Part of RNA activation mechanism for how siRNA or miRNA upregulates gene is known: dicer and argonaute proteins are involved, and there is histone demethylation.

2. Technical application

a. **Gene knockdown:** RNAi pathway is often exploited in experimental biology to study the function of genes in cell culture and in vivo in model organisms. Using RNAi mechanism, researchers can origin a drastic decrease in the expression of a targeted gene. Studying the effects of lowered gene expression can show the physiological role of the gene product.

b. **Functional genomics:** *C. elegans* is particularly useful for RNAi research for two reasons: first, the effects of the gene silencing are generally transmissible, and secondly, because delivery of the dsRNA is extremely simple, by oral delivery.

c. **Medicine:** It is difficult to introduce the long dsRNA strands into mammalian cells due to interferon response, the use of siRNA imitators has been more successful. Among the first applications to reach clinical trials were in treatment of macular degeneration and respiratory syncytial virus.

d. **Biotechnology:** RNAi has been used for applications in biotechnology, particularly in the engineering of food plants that yield lower levels of natural plant toxins. Such techniques take benefit of the stable and heritable RNAi phenotype in plant stock.

Challenges to overcome

Applications of RNAi for biological and clinical uses provides a number of serious challenges like *invivo* delivery of the dsRNA, off-target effects and antagonism with cellular RNAi components.

Target stability of gene

To get stable expression and inheritance of transgenes in genetically transformed plants, the following methods should be considered:

- Gene silencing is observed frequently when there is integration of complex inserts into genome. So, it is good to choose integration of single insert of transferred gene without duplication in the form of tandem array or inverted repeats and containing single unique elements. This can be achieved by using vector mediated gene transfer method, which generally introduce one copy (two copies rarely) in the cell.
- Irrespective of exact mechanism homology at DNA or RNA levels seems to stimulate silencing events. If homology can be avoided or length and degree of homology should be controlled by interrupting perfect homology with mismatch or intron sequences
- Integration of unmethylated, single copy sequences of plant genome may increase the chances for continuously stable expression as the structure of integrated DNA itself, the environment of insert may affect the stability of gene expression
- After transmission of gene to next generation, gene silencing may become evident. In some cases, transgene expression reduced

continuously over subsequent generations. So, continuous monitoring of expression levels in progeny of even established transgenic lines might be a precaution against unexpected epigenetic modifications.

It is important to arrange for gene product to appear in correct sub-cellular location. It may be approved to have nuclear encoded genes that are involved in functions such as detoxification or resistance switched on all the time in some situations. However, it is desirable to ensure the expression of foreign gene at particular developmental stage such as flowering, grain filling, during seed development, tuber formation, fruit ripening or in response to environmental signals. Once the gene is integrated into the genome, DNA is usually stably maintained.

Summary

Now-a-days, gene silencing, marker system and target gene stability are important approaches that are used by researchers to developing transgenic plants with desired traits. By using these approaches, one can plan their experiment to screen transgenic plants by using different marker system (scorable or screenable) or to know the function of gene or genes, gene silencing is helpful.