VECTORS FOR PLANT TRANSFORMATION

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Introduction

Vector: a Latin word meaning "carrier".
In molecular biology vector is a vehicle used to transfer genetic material to a target cell. These vectors may include:

1. cloning vectors.

a. Plasmid
b. bacteriophage
c. Cosmid
d. Bacterial artificial chromosome.
e. Yeast artificial chromosome.

2. expression vectors.

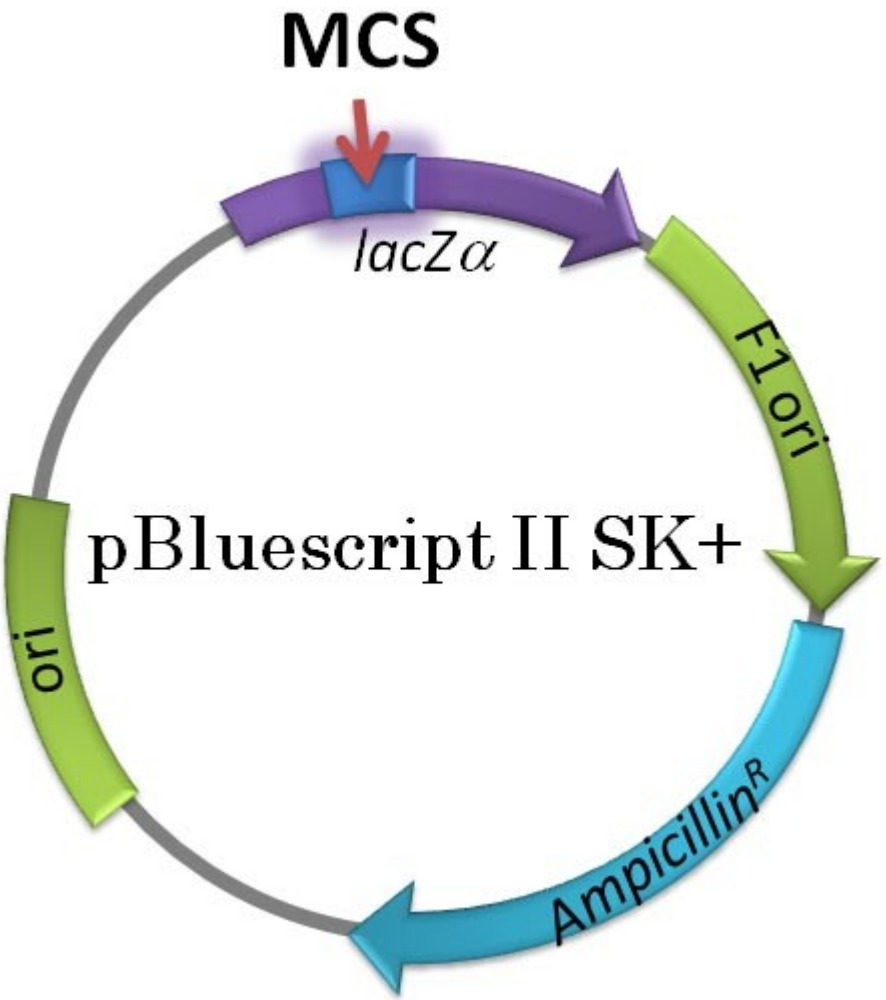
a. Prokaryotes expression vector
b. Eukaryotes expression vector

Desirable features of any plasmid vector

Cloning vectors should have the following properties:

- Be of a small size.
- Confer s selectable phenotype to the host cells.
- Contain single sites for a large no of restriction enzymes.
- Enable the identification of bacterial colonies containing recombinant plasmids.

A typical cloning vector used in molecular biology



Development of plant transformation vectors

Basic features of a vector for plant transformation

While considering the design of plant transformation vectors, several additional features should be considered:

- The plasmid must be able to replicate both in E.coli as well as in *Agrobacterium.*
- Additional selectable markers.
- Border sequences of tDNA.
- Promoters and terminators of plant origin.

Promoters and terminators

Major determinant of gene expression (level, location and timing) is the region upstream of the coding region, termed as promoter.

Any gene which has to be expressed must have promoter of plant origin.

As reporter genes and selectable marker genes are of bacterial origin, so they have to be supplied with a promoter that will drive their expression in plants.

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Transgenes also must have suitable Terminator sequences at their 3' terminus to cease the transcription at correct position.

Further considerations must be taken into account while selecting promoters:

- Promoter strength
- Tissue specificity
- Developmental regulation

1. Agrobacterium-derived promoter and terminator sequences

- The genes from the Ti plasmid that code for opine synthesis are widely used as promoters and terminators in plant transformation vectors.
- Although derived from bacterial genes, their presence on T-DNA meansthey are adapted to function in plants.

2. The 35S promoter

- Cauliflower mosaic virus 35S RNA gene.
- Most widely used.
- Expressed in all tissues of transgenic plants.
- High expression level in dicots, and low in monocots.
- Ideal for expression of selectable marker genes and in some cases reporter genes.
- Activity of 35S can be enhanced by inclusion of one or more copies of enhancer region.
- In monocots alternatives promoters are used, such as maize ubiquitin I promoter or rice actin promoter.

3. Tissue specific promoters

- Promoters that are used to derive expression in a specific tissue.
- In fact expression is tissue enhanced but not restricted to a given tissue.
- Such promoters are very useful.
- As the expression of any potentially harmful substance can be limited to tissues that are not consumed by animals or plants OR
- The genes involved in a specific process can be limited to tissue in which that process occurs.
- For example; banana TRX promoter and melon actin promoter (for fruit gene expression).

4. Inducible promoters

- Promoters that help to control the timing of transgene expression.
- Divided into three main categories:

    1. non plant-derived systems.

    2. plant-derived systems that respond to environmental signals, and

    3. plant-derived systems based on developmental control of gene expression.

- No one system is suited to all situations.

Selectable markers

- Selectable marker genes introduced during transformation allow transformed tissue to be selected, normally by conferring resistance to a toxic substance.
- Selection is based on inclusion of toxic substance into culture madia.
- A selectable marker gene on the vector confers resistance to the transformed plant against that toxin when expressed.

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- *nptII* most widely used selectable marker gene for kanamycin resistance.
- Some plants exhibit naturally a high resistance to the kanamycin (cereals), and
- Some species are too sensitive for it (soft fruits).
- So alternative selectable markers developed i.e bleomycin, hygromycin and spectinomycin.
- Some selectable markers genes that confer herbicide resistance to the transformed plants are also used e.g bialaphos.

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- Other selectable markers based on interesting principle of alternative carbon source utilization.
- Genes, from some bacteria that allow the use of mannose or xylose as carbon sources, have also been used as selectable markers.
- In addition reporter genes are also used as selectable markers.
- There is also a public concern about use of antibiotic and herbicide resistance selectable markers in transformed plants.

Reporter genes

- Reporter genes are used widely both for assessing gene expression by promoter analysis and as selectable markers.
- Ideally reporter genes should be:

1. easy to assay,
2. non-destructive assay system and
3. with no or low endogenous activity in plant to be trasformed.

1. β-Glucuronidase

GUS is most widely used reporter gene due to following advantages:

1. can be assayed sensitively by using easy, quick and non-radioactive methods.

2. give both quantitative (expression level) and qualitative (location of gene expression) data.

3. little or no endogenous activity.

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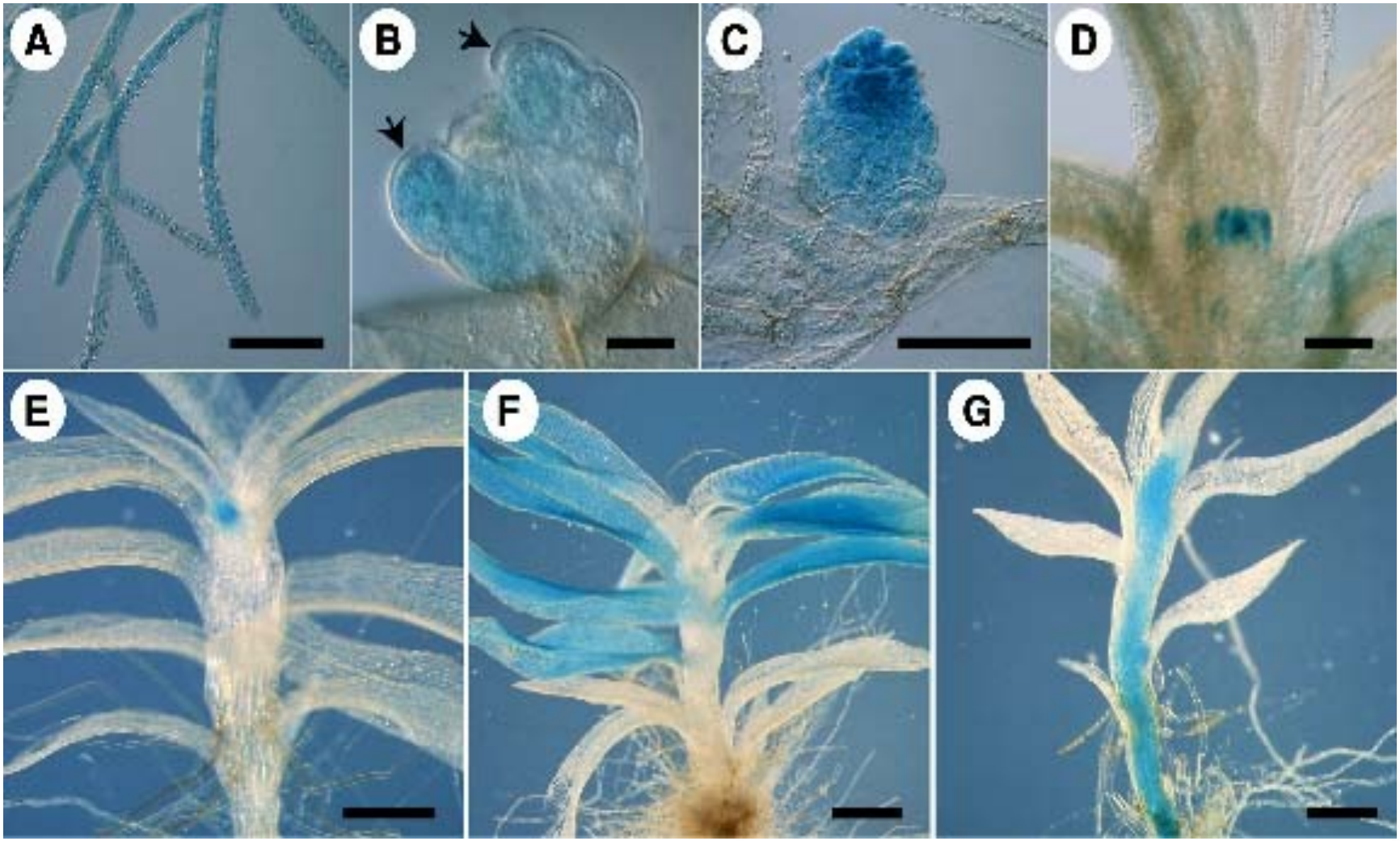
Principle

- B-glucuronidase is an enzyme isolated from E. coli, when incubated with some specific colorless or non-fluorescent substrates (different possible glucuronides), can transform them into colored or fluorescent products.
- The most common substrate for GUS histochemical staining is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc): the product of the reaction is in this case a clear blue color.
- Other common substrates are p-nitrophenyl β-D-glucuronide for the spectrophotometrical assay and 4-methylumbelliferyl-beta-D-glucuronide (MUG) for the fluorimetrical assay.

GUS gene expression



http://www.nibb.ac.jp

2. Green Fluorescent Protein (GFP)

- Gfp gene is isolated from jellyfish *Aequorea victoria* which is a brightly luminescent organism.
- Gfp Easier to assay than GUS.
- Non-destructive.
- Can be use in such situations in which GUS cant be used in time course experiments.

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In order work efficiently gfp gene is modified:

1. A cryptic intron is removed.

2. Making a codon usage more “plant like” and

3. Prevent accumulation in the nucleoplasm.

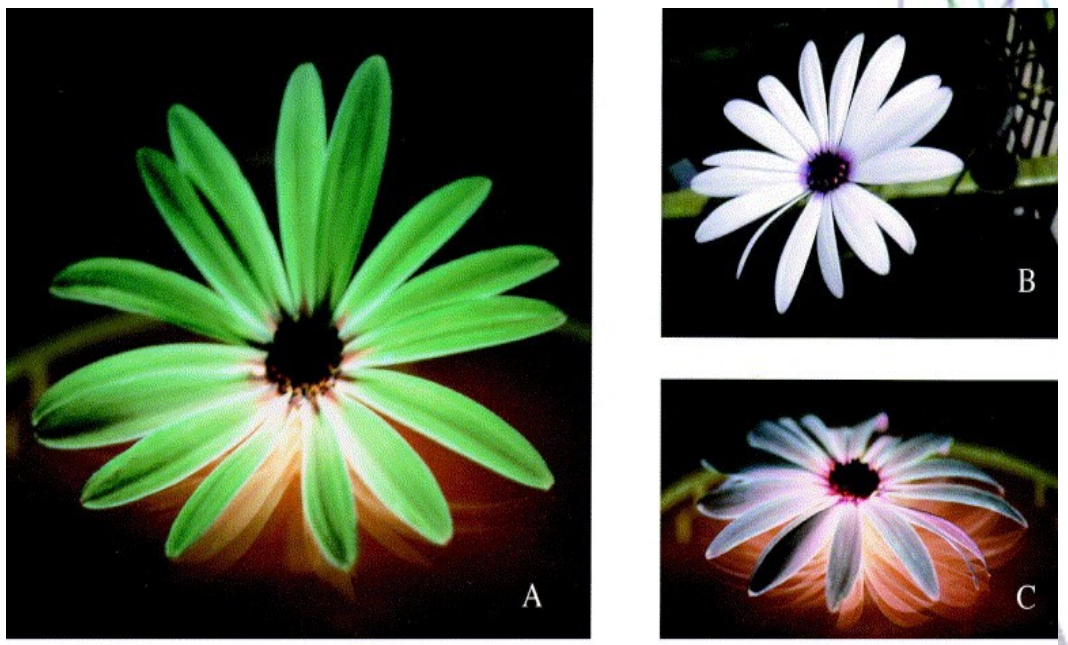


Fig. 4

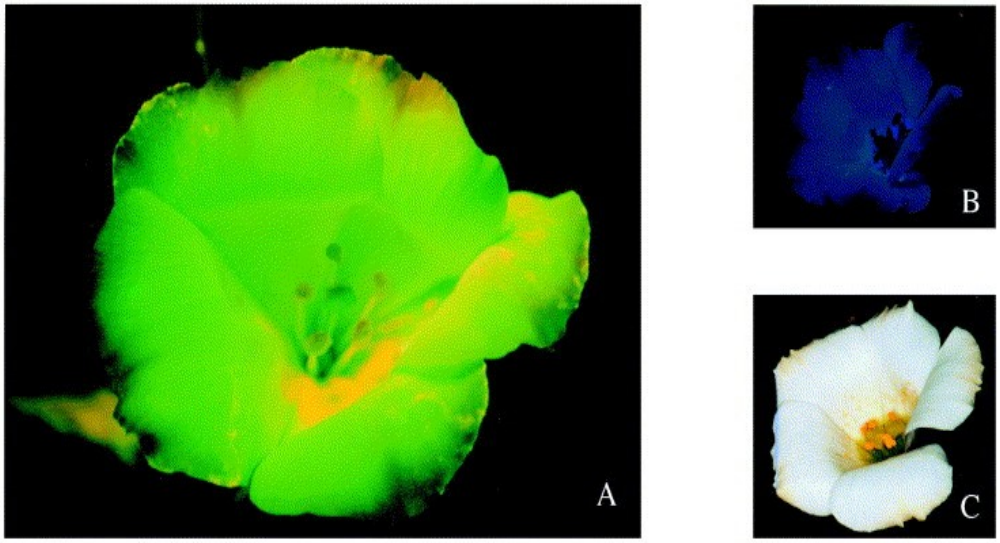


Fig. 5

3. Luciferase

- Luciferase gene (*luc*) is isolated from firefly.
- *luc* encodes an enzyme that catalyses D-luciferin in an ATP dependent fashion resulting light emission.
- Difficult assay but
- Useful for low level and highly localized expression.
- Bacterial luciferase genes (*luxA* & *luxB* from *vibrio harveyi*) are also used.
- They catalyse the oxidation of long chain fatty acids resulting in light emission.

4. chloramphenicol acetyltransferasea

- Most widely used as reporter gene in mammalian cells.
- Limited use in plants.
- Requires radioactive procedure.

Origins of replication

- Origin of replication_ from where replication starts.
- As both *E. coli* and *Agrobacterium* are gram-negative a single origin of replication with a broad host range can be used.
- Broad host range may cause low copy no. in *E.coli*.
- So two origins may also be used.
- But it results in larger vector and assossiated problems.

Co-intigrative and binary vectors

A. Co-intigrative vectors:

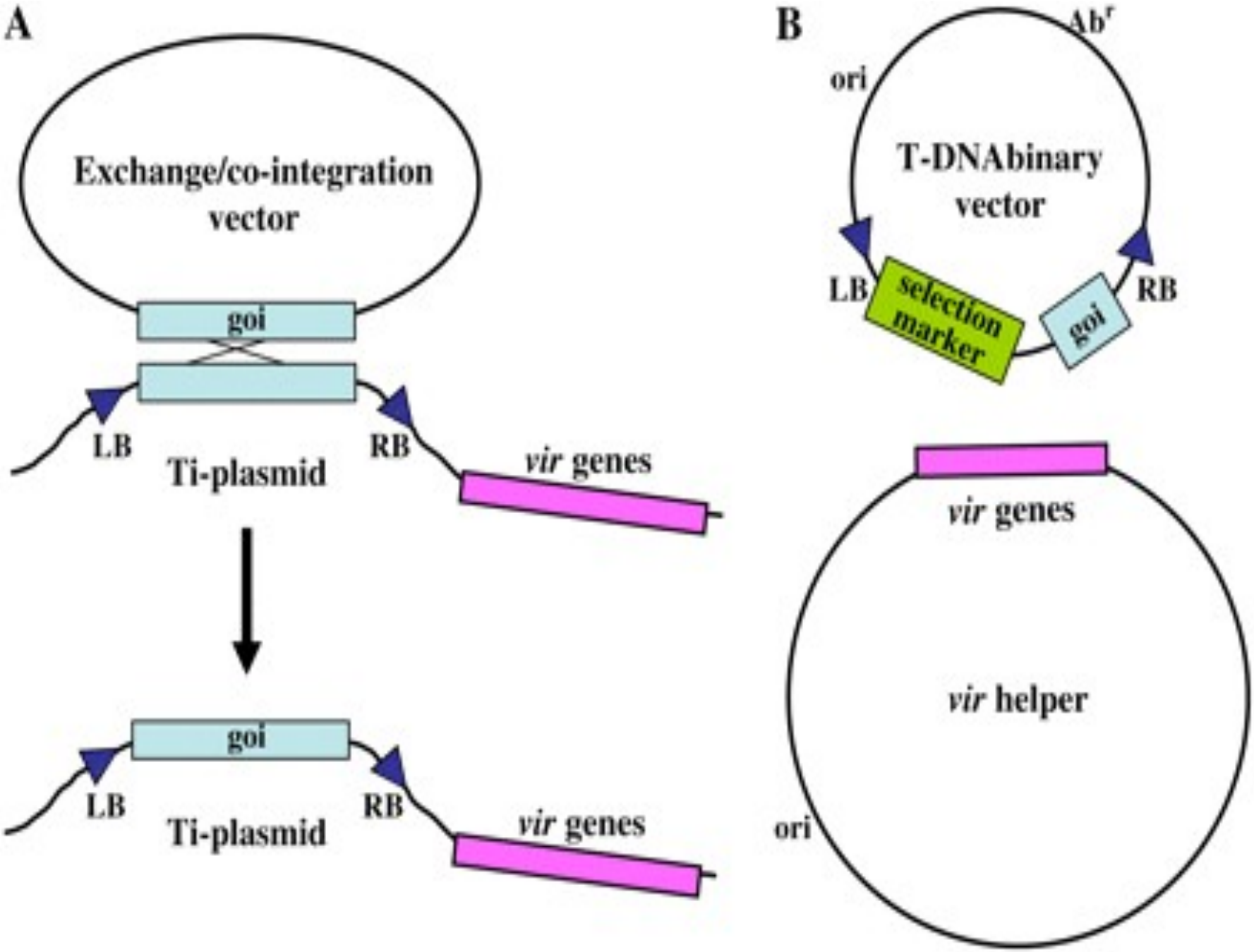
- The DNA to be integrated is cloned first into an intermediate vector and recombination within the bacterium is used to clone the sequences.
- Less common.
- Easily manupulated.

B. Binary vectors

In binary vectors transfer apparattus and T-DNA are present on separate plasmids.

Only border sequences are present with T-DNA.

These vectors are relatively small.



Optimization

There are several aspects of vector design that influence the efficiency of transgene expression.

1. Arrangement of genes in a vector.
2. Trasgene copy no.
3. Transgene position.
4. Transgene features.

1. Arrangement of genes in a vector.

- There are few simple rules about vector design:
- If more than one gene, use different promoters and terminators _ otherwise gene silencing.
- Multiple genes on one vector should not be immidiately adjacent to each other.
- Multiple genes should be in same direction.
- This avoids adjacent inverted repeats that cause plasmid instability in bacteria and incraesed gene silencing in plants.

2. transgene copy number

- Multiple copy no of trasgene can be incorporated in target plant genome.
- More often multiple copy no _ High levels of transgene expression.
- But not always.

3. Transgene position

- Transgene intigration into plant genome is a random event.
- And it have a marked effect on transgene experession level.
- Higher plants do not have homologous recombination system that allows transgene to be targeted to aparticular region of genome.
- Alternatively, bacteriophage or yeast site specific recombinases have used with some success.

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- Another approach is the inclusion of matrix-attachment regions.
- These are AT rich regions thought to be involve in maintaining chromatin in an open structure allowing gene expression.
- It provides position independent expression.

3. Transgene features

- Heterologous genes from non plant species tend to be expressed poorly in plants.
- Genes from different origins have different G+C content.
- It is commonly seen that A+T rich transggenes interfere with mRNA processing leading to little or no transgene expression.
- And it may recognize as foreign.
- High A+T content often results in presence of AUUUA sequences, destablizing mRNA.
- Crypic introns may also be present.

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- Even transgene transcribed efficiently it may not be translated efficiently probably due to:
- Architecture of translation initiation codon or
- The presence of codons that are infrequently used in plants.
- Consensus sequences flancking the ATG initiation codons are different in plants and other species.
- So plant specific sequences inclusion before initiation codon can improve the translatability.

Clean-gene technology

- New technologies are being developed to reduce the possible environmental impact and increase and increase the acceptability of transgenic plants.
- Hence, the transformation technology where selectable marker is no longer present in the field-grown crops _ so called clean-gene technology.
- To develop crops that contain no selectable marker following approaches are available:

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1. Simply insert transgene without using a selectable marker. PCR based selection. Too expensive and labour-intevsive.

2. Insert gene of interest and selectable marker on separate T-DNA molecules. Most effective approach is to use binary vector containing several T-DNA regions that are integrated unlinked in plant genome.

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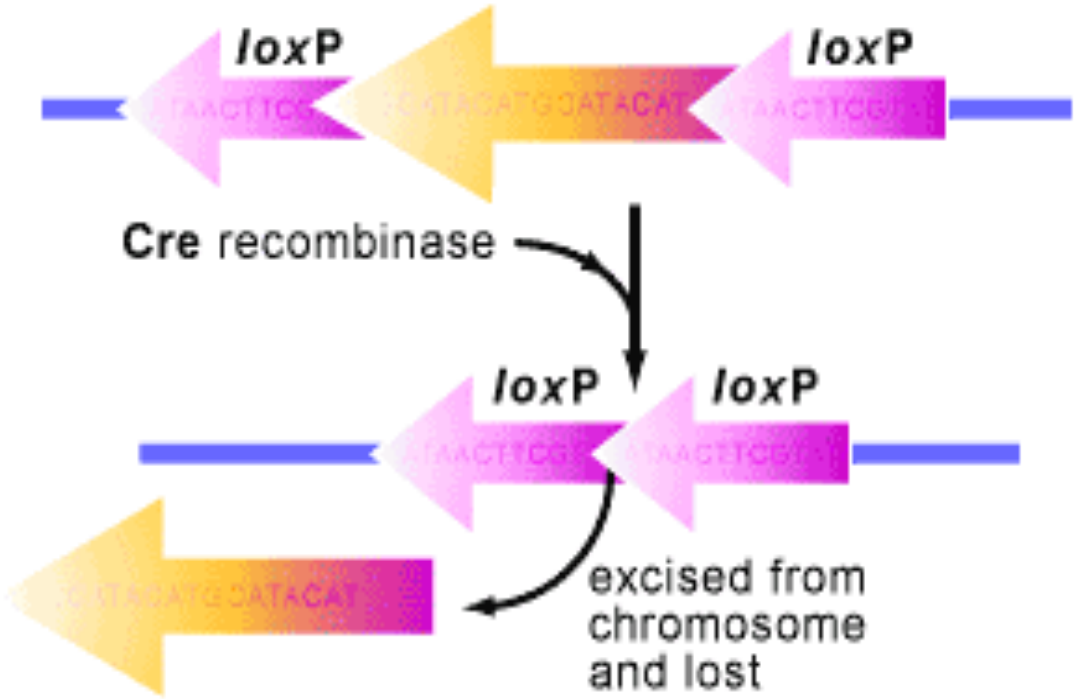
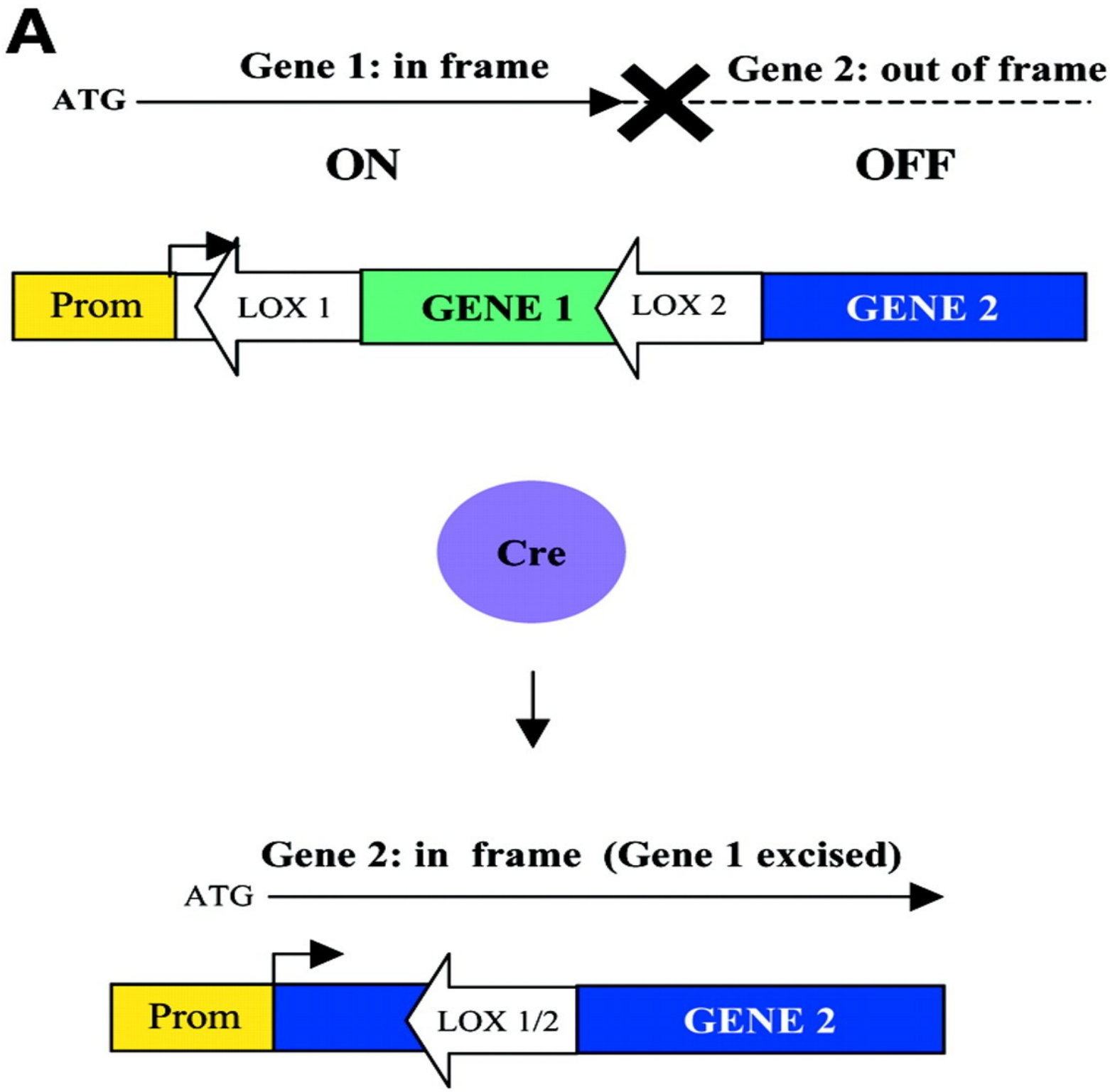
3. Another approach is to excise the selectable marker from plant genome. This can be done by using site specific recombinase systems e.g *cre-lox* system. Vectors in which the selectable marker gene is placed between recognition sites for a site specific recombinase to transform plants. Subsequently, the recombinase is expressed resulting in selectable marker gene excision from plant genome.

Cre-loxp system





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4. Recently, vectors have been demonstrated in which selectable marker gene was flancked by that apparently considerably increase the frequency of intrachromosomal recombination leading to excision of marker gene.