Gain-of-function mutants

(Identify function of plant genes)

1) **Many plant genes** in *Arabidopsis*, rice, and other plants **belong to gene families**, the characterization of gene functions by single-gene mutagenesis is not always possible.

2) Many mutants generated by single-gene disruption do not show clear phenotypes because of genetic redundancy.

A large proportion of genes in the ***Drosophila*** *melanogastor*, ***Caenorhabditis*** *elegans* and ***Saccharomyces cerevisiae*** genomes have no obvious loss-of-function phenotype. This phenomenon can be explained by **two mechanisms of compensation**.

The first is the **presence of duplicate genes**, where loss-of-function in one copy can be compensated by the other copy or copies.

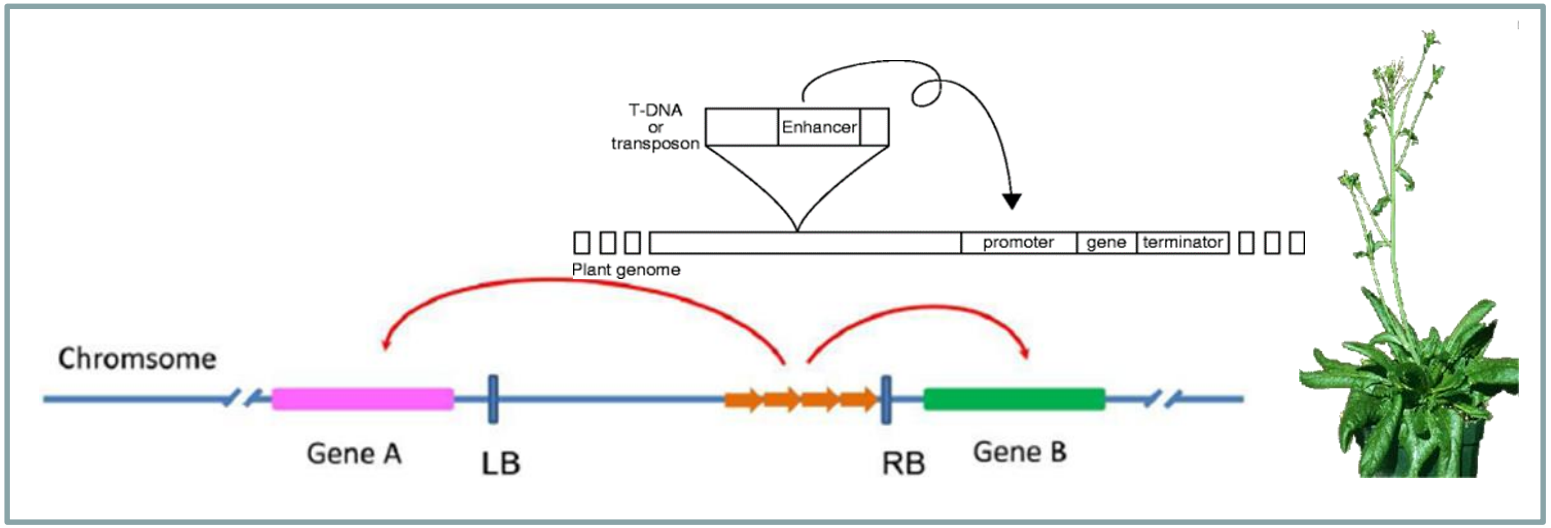
The second mechanism of compensation stems from the existence of **alternative metabolic pathways and regulatory networks**. Therefore, eukaryotic organisms have a significant genetic robustness against null mutations in a large number of genes. A significant limitation of classical loss-of-function screens to dissect genetic pathways is that they rarely identify such genes.

3) There is another tranche of genes that are also difficult to uncover by classical screens. Such as **genes encode products that function at multiple stages during the life cycle** of the organism, one of which is essential for early embryo or gametophyte development. **Loss-of-function mutations in these genes result in lethality**.

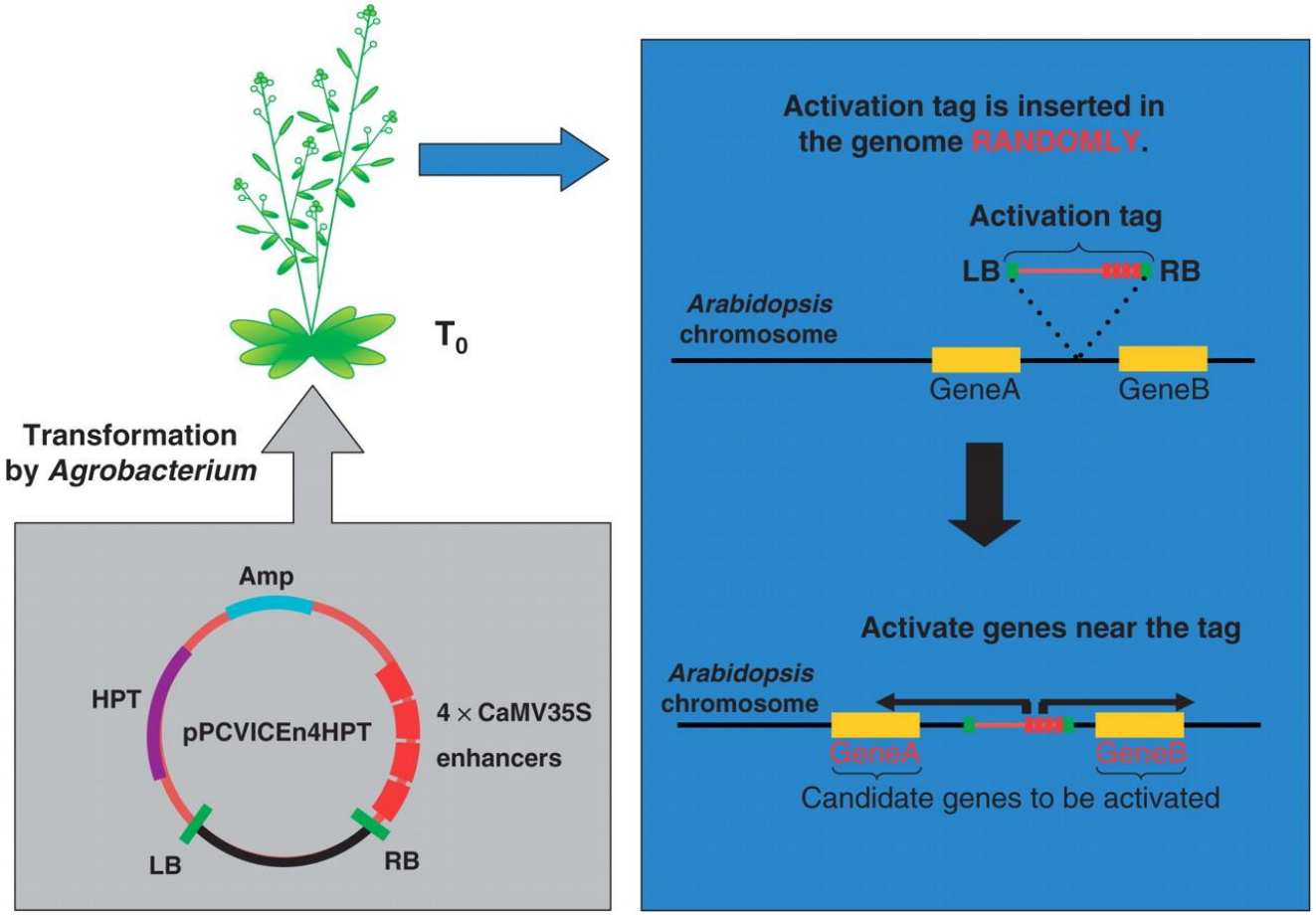
A number of approaches have emerged to help circumvent these potential problems. The **enhanced expression of genes providing gain-of-function phenotypes** has proved a productive strategy to identify gene function.

T-DNA activation tagging generate Gain-of-function mutant

- TANDEM repeats of ENHANCER sequence is introduced by T-DNA
- That enhances the transcription (expression) of neighboring genes on either side of the randomly integrated T- DNA tags, resulting in gain-of-function mutants

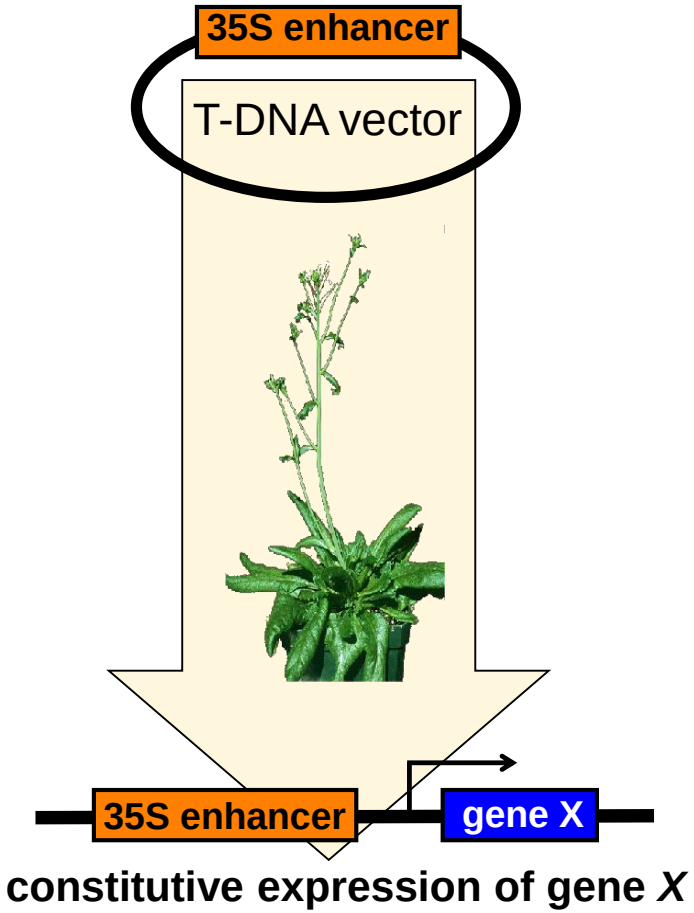


Method for generation of activation-tagging lines

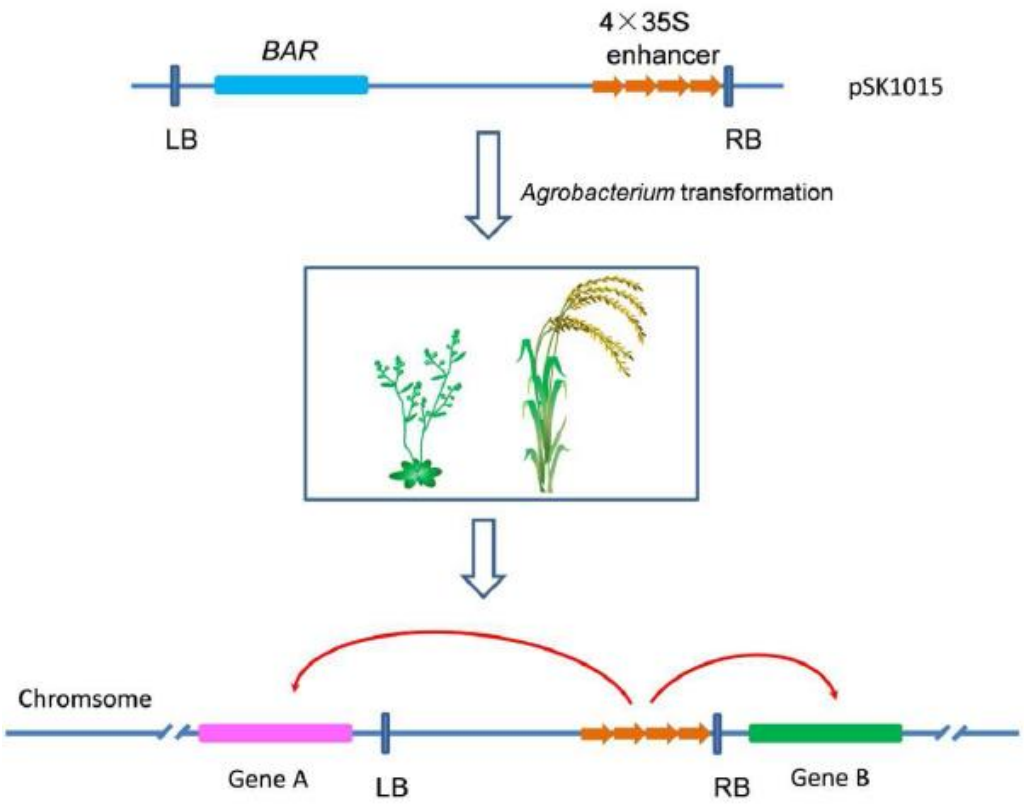


Activation tagging in *Arabidopsis*

- Strong constitutive viral promoter
  - CaMV 35S
- Inserted randomly
  - normally with T-DNA
- When inserts are near a gene, the following results occur:
  - Activation
  - Constitutive expression
- Can result in abnormal phenotype



Activation Tagging using T-DNA



Tetrameric CaMV 35S enhancer could mediate **transcriptional activation of nearby genes**.

Activation tagging is a gain-of-function method that generates transgenic plants by T-DNA vectors with tetrameric cauliflower mosaic virus (CaMV) 35S enhancers which can **lead to an enhancement expression of adjacent genes in the distance ranging between 0.4 to 3.6 kb from the insertion site**.

Differently from the action of the complete CaMV 35S promoter, CaMV 35S enhancers **can activate both the upstream and downstream gene transcription**.

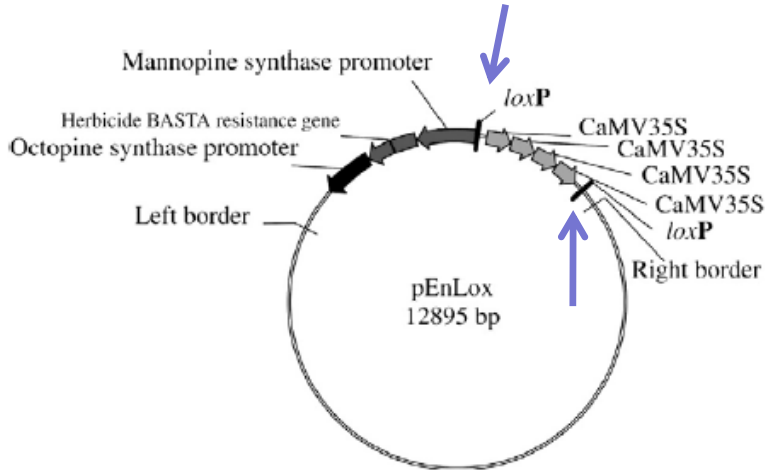
How would you know if the gain-of-function is due to enhancer induced activation of plant genes ?

Observed phenotypic changes could be due to

- activation of genes that had enhancer inserted within them OR
- overexpression of neighboring genes

Therefore, Reversion of mutation (plug-out) from mutant to wild-type and its comparison with Activation mutation (plug-in) can help in understanding gene function.

A new Activation tagging method (for reversion of Mutant to Wild type)

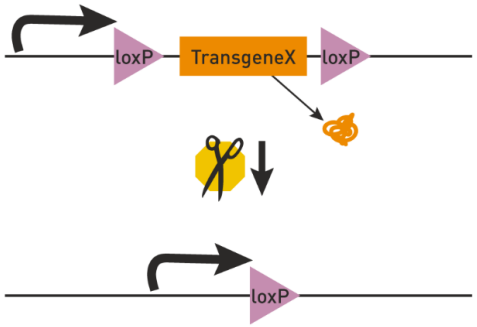


Vector pEnLox

Enhancer (consisting of 4 repeats of transcriptional enhancer domain from 35S RNA promoter from CaMV35S) is **flanked by loxP-sites on both sides**

Vector pCre

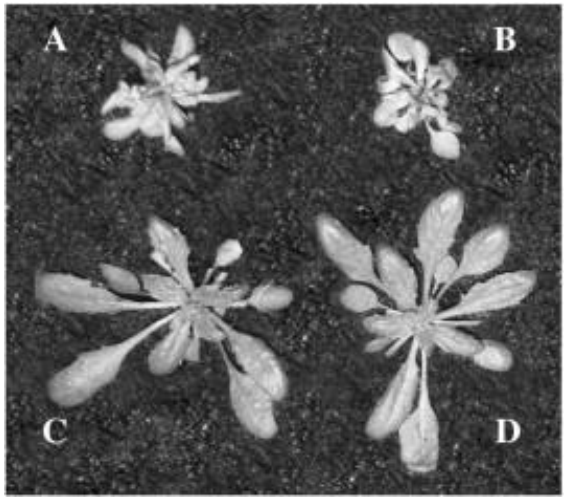
includes the ***cre recombinase*** gene which can remove the DNA sequence between two *lox P* sites



Vector pEnLox is intended to **induce insertion mutations** and **vector pCre** to obtain transgenic plants with expressed Cre recombinase genes.

A new activation-tagging method

(to know the recapitulation process of phenotype resulted from the enhancer of T-DNA insertion)



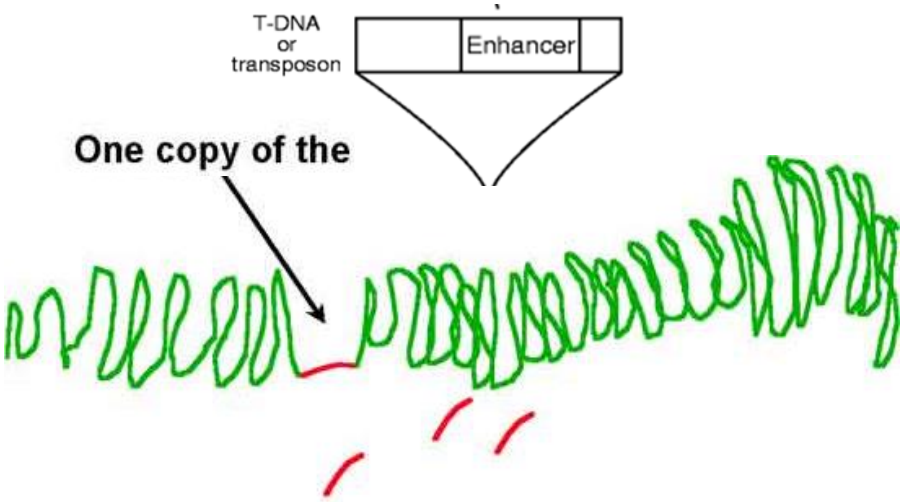
The **activation-tagging lines** containing the pEnLOX (named as **E-lines**)

the **helper lines containing pCre** (named as **C-lines**)

By crossing the E-lines with the C-lines, the CaMV 35S enhancers can be removed from the chromosome coming from E-lines and thus the **reversion from mutant phenotypes to the wild-type phenotype** may be detected **in the next generation**.

This system is to effectively identify genes that are NOT expressed in wild-type plants under normal environmental conditions.

Limitations of T-DNA use
for Activation Tagging



T-DNA insertions are often complex, and characterization of **multiple inverted** or **tandem copies** or **truncated T-DNA inserts** often makes molecular analysis difficult.

To circumvent this problem, several researchers have established the **SINGLE copy transposon tagging system**.

Activation tagging using the maize transposon system, **Enhancer-inhibitor (En-1) or Enhancer/Suppressor mutator (En/Spm)**, generated a population of about 8,300 independent stable activation-tagging lines

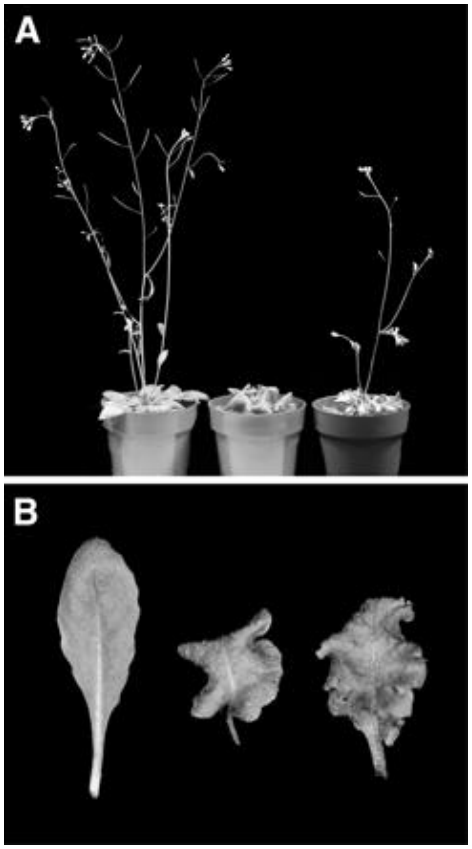
Activation tagging in *Arabidopsis*

- Examples of **mutant phenotypes** found in activation-tagging screen
- **Activation-tagging** carried out by Detlef Weigel's lab at the **Salk Institute**, **many different abnormal phenotypes** were observed for *Arabidopsis*.
- Among the genes that were activated were **Flowering Locus T (FT)**, which

  controls flowering time, and

  genes that control plant growth and

  leaf shape.



Limitations of Activation tagging method

Activation-tagging system is useful to construct a **large-scale gain-of-function mutant population**,

but this system has some disadvantages because the effects of inserted transcriptional enhancers in the genome are **subject to changes in (1) more than one gene located near the enhancers**.

CaMV 35S enhancer can influence the expression of genes up to **(2) several kb from the insertion site**, thereby causing difficulties in the identification of genes responsible for the observed mutant phenotypes.

FOX hunting system

a novel gain-of-function approach for plant phenome analysis

adding an extra copy of a single gene external to the chromosomes

For the past few years, fl-cDNA clones of several plant species have been collected. The Arabidopsis fl-cDNA population termed '**RIKEN Arabidopsis full-length (RAFL) cDNA clones**' was collected and sequenced.

Using these fl-cDNA resources a **novel gain-of-function-type approach**, the **FOX hunting system**, was developed.

In this system fl-cDNAs are randomly expressed in Arabidopsis plants and these transformed lines are termed **FOX lines**.

Using FOX to hunt for gene function

Researchers develop a new technique for understanding genomes

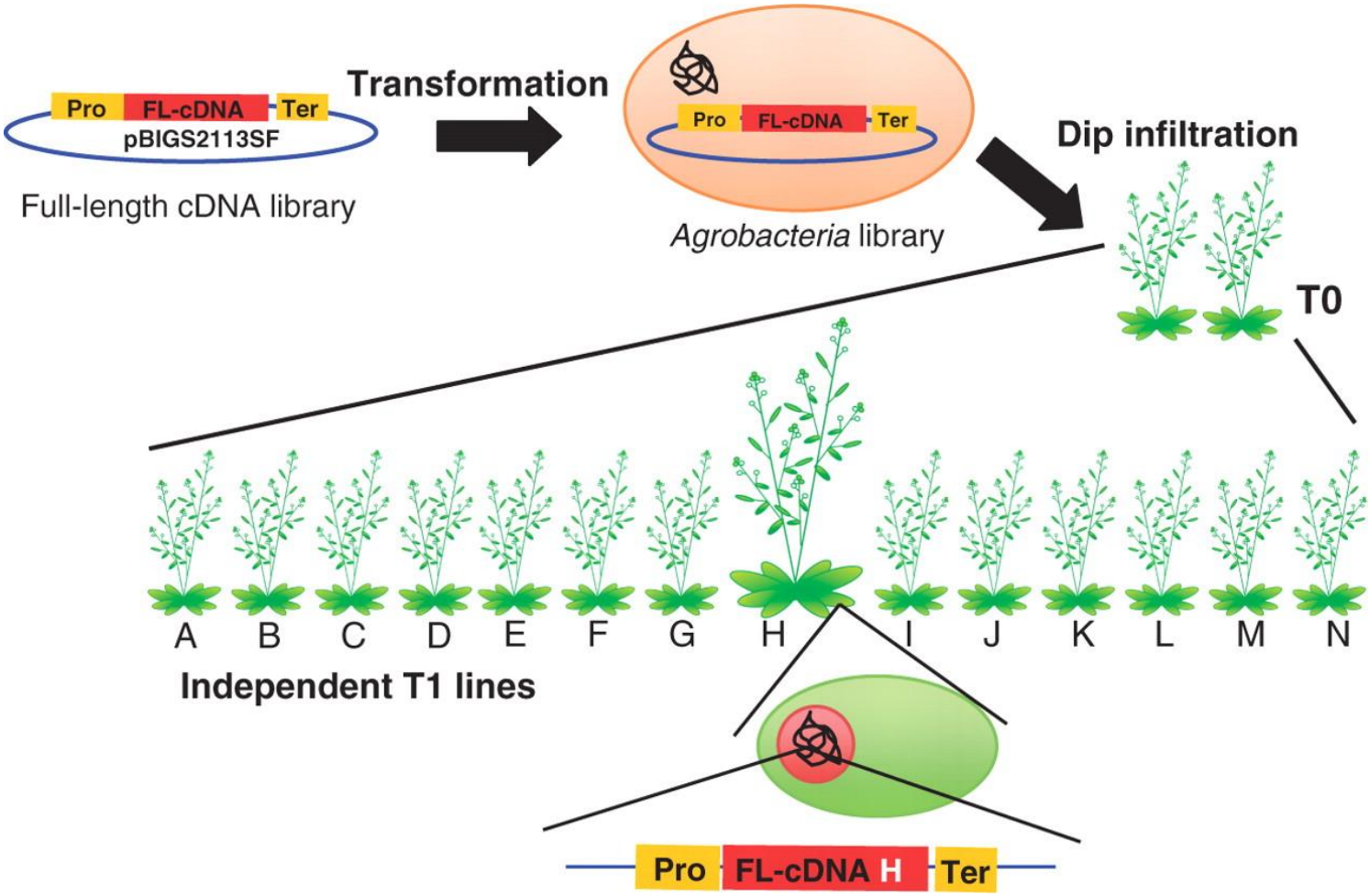
Where **knockout mutation of genes results in a marginal differences from wild type** as a result of gene redundancy, it would not be possible to isolate the mutants by conventional T-DNA insertion mutagenesis.

However, activation-tagged mutagenesis does have some disadvantages. One is that the effect of gene activation by a transcriptional enhancer is **not restricted to one gene** and in some cases transcription of several genes is increased, resulting in a complex phenotype.

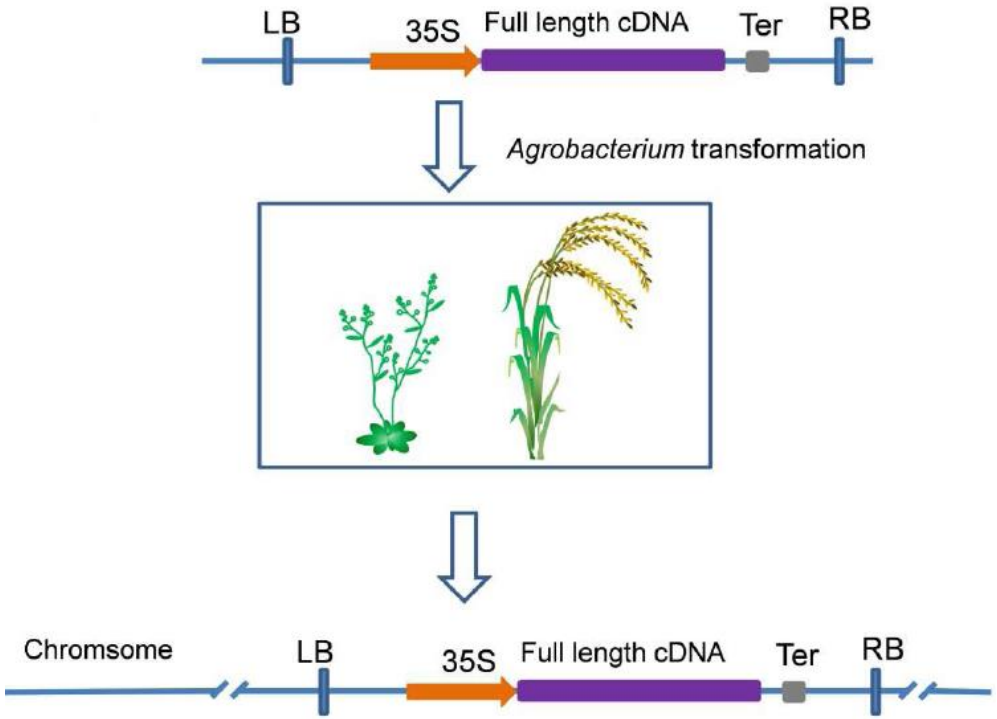
To circumvent this problem, and for systematic analysis of gain-of-function mutations, alternative gain-of-function mutant lines can be generated by overexpression of full-length cDNAs.

10000 independent Arabidopsis full-length cDNAs into an expression vector under the control of the cauliflower mosaic virus *35S* (CaMV *35S*) promoter. This cDNA expression library was introduced into Arabidopsis by *in planta* transformation.

Method for the generation of FOX lines



FOX hunting system for systematic understanding of gene function



by generation of transgenic plants (also named FOX lines) expressed the fl-cDNA derived from the **same species or heterologous host**

FOX hunting system

FOX hunting system (full-length cDNA overexpressor gene hunting system) for the ectopic expression of plant genes by using full-length cDNAs (fl-cDNAs) to systematically produce gain-of-function mutants.

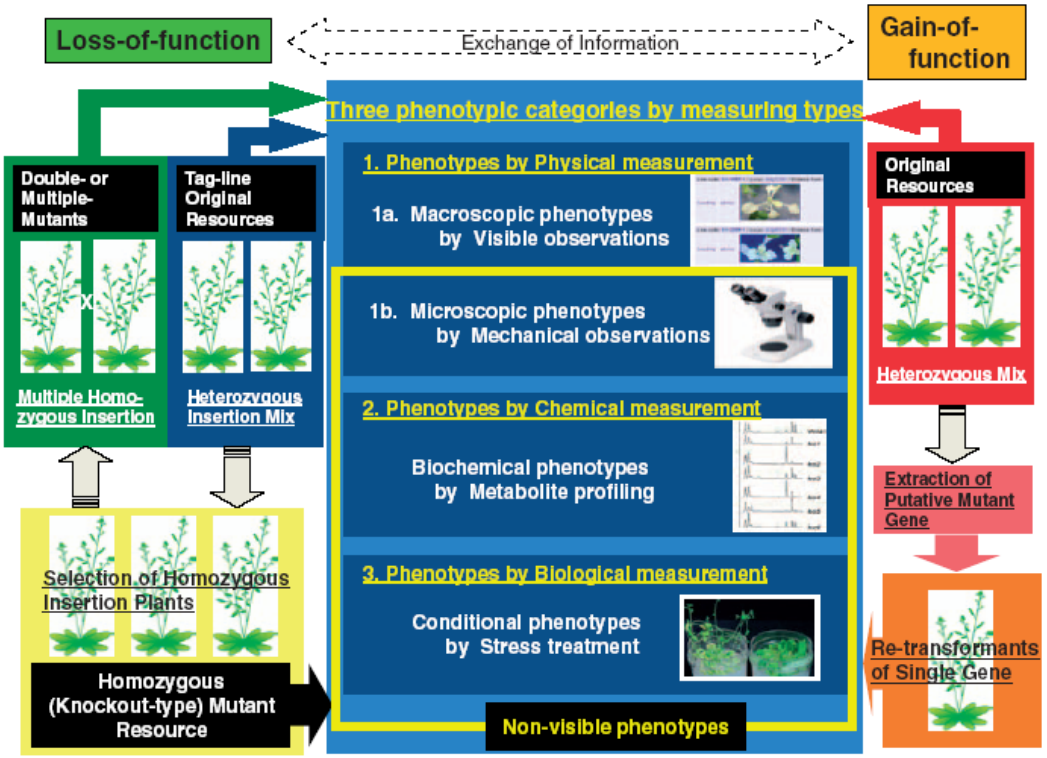
Transgenic Arabidopsis mutant lines were generated that overexpress Arabidopsis fl-cDNAs. These transgenic lines (named FOX lines), express one to two Arabidopsis fl-cDNAs under the control of *CaMV 35S* promoter. Various morphological and physiological mutants were generated from these FOX lines.

This technology has also been applied to rice to generate **FOX rice lines** ectopically **expressing rice fl-cDNAs under the control of the maize ubiquitin promoter**.

Although analysis of gene function has focused on Arabidopsis and rice as model plants, **many other plants produce unique chemicals or metabolites** synthesized by unique enzymes (proteins), **new phyto-chemical library**.

A similar approach through the FOX hunting system has been applied to **isolate salt stress tolerance genes using cDNA of salt cress** (*Thellungiella halophila*), a close relative of Arabidopsis.

Application of mutant resources to phenome analysis



Three phenotypic categories,

- physical,
- chemical and
- biological measurements

are used to search for various traits for total phenome analysis.

Phenotypes described by **Physical measurement** consist of macroscopic phenotypes detected by visible observations and microscopic phenotypes detected by observations using specialized equipment.

Phenotypes categorized by **Chemical measurement** are biochemical phenotypes, for example metabolite profiling.

Phenotypes observed through **Biological measurement** are conditional phenotypes revealed by environmental changes or stress treatments.

Application of mutant resources to phenome analysis

Of the **mutant resources** available, the **tag-line original resources**,

which are usually **heterozygous for the insertion**, are amenable to visible observation but often not to non-visible observation.

Plants **homozygous for the insertion** can be selected from the original tag-line resources and will be a novel resource of **homozygous (knockout-type) mutants**, which are **useful to detect non-visible phenotypes**. In addition, homozygous insertion lines will be the **starting material to produce multiple homozygous insertion mutants**, which can be applied to phenotyping again.

The original **gain-of-function resources, which are heterozygous**, can be analyzed in all three phenotypic categories. To confirm the phenotype of an isolated mutant, the candidate gene must be isolated and re-transformed. Re-transformants of the single gene can then be phenotyped.