Forward genetic

Select a biological process

Generate a mutant population

Screen a large number of mutagenised M2 plants

Identify the mutated gene

Forward genetic

***Mutant*:** An organism that differs from the "normal" or wild type by one or more changes in its DNA sequence

Generate or obtain a mutant population

Two major considerations in planning a mutant screen:

- How many plants can be reasonably screened?

- How many genes can mutate to cause the phenotype of interest?

Forward genetic

- Chemicals agents:

e.g., ethyl methyl sulfonate (EMS) or nitrosomethylurea (NMU)

- Physical agents:

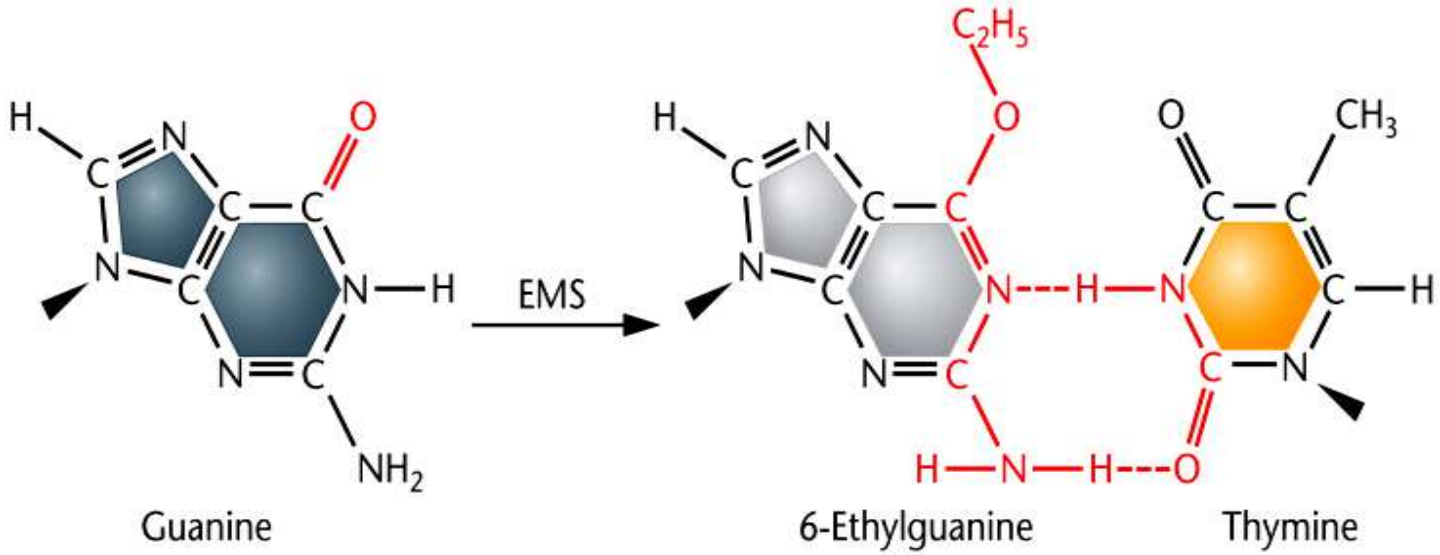
e.g., X-rays, fast neutrons and accelerated ions

- Biological agents:

e.g., transposons and T-DNA

Point mutations
Large range of mutations including loss and gain of function
Very high efficiency with hundreds of mutations per genome
Difficult to find the mutation in the genome
Big insertions, deletions and rearrangements
Mostly loss-of-function mutants
Medium efficiency
Laborious identification of the mutations
Insertion of specific DNA sequences
Mostly loss-of-function mutants
Low efficiency
Easy identification of the mutations

Chemical (alkylating) agents

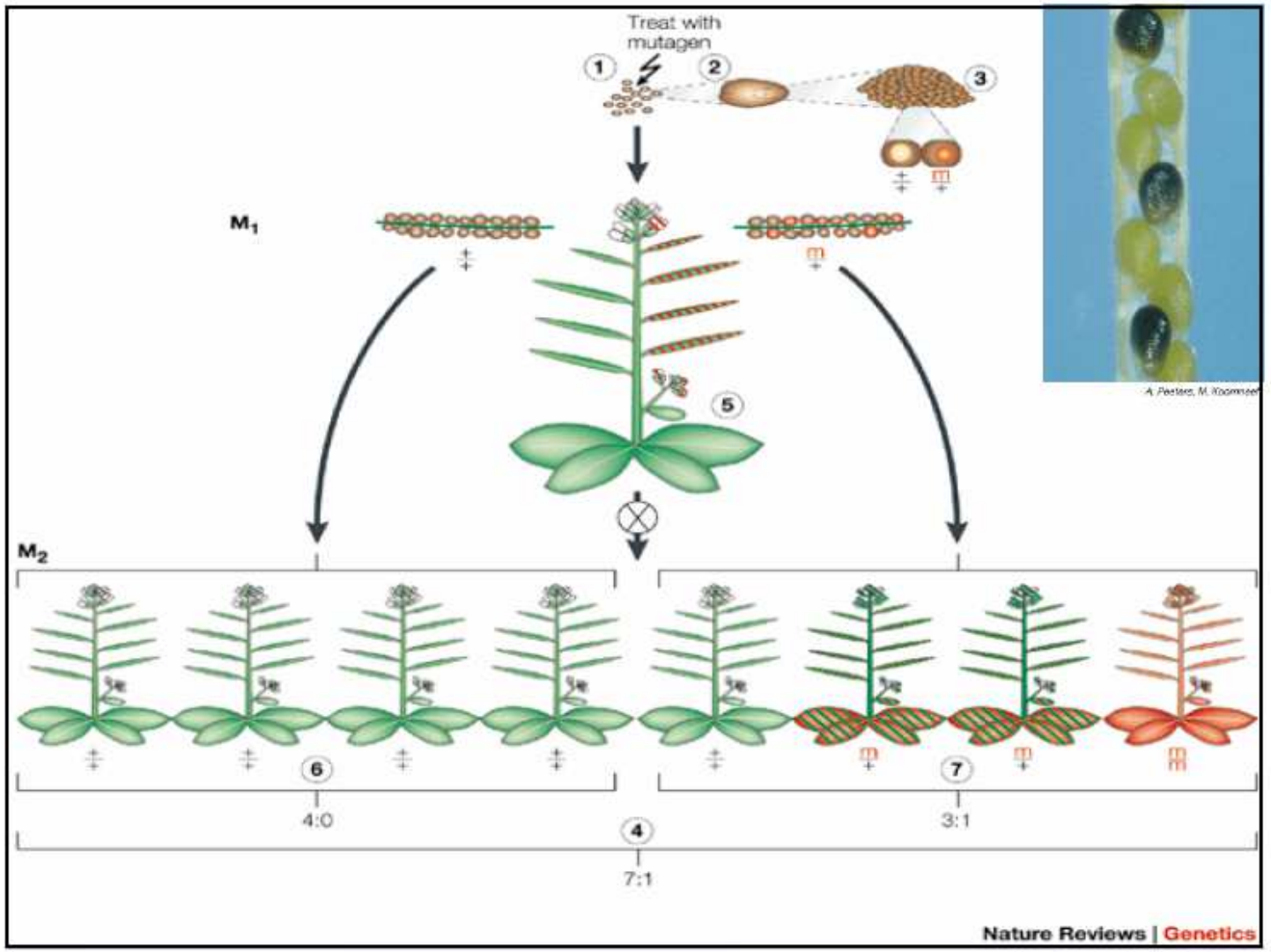


- EMS causes addition of chemical groups primarily to Gs.
- The extra groups can cause altered base pairing resulting in transitions.
- EMS can also cause depurination (complete loss of the base) which leaves gaps that cause misincorporation during replication.

Protocollo di mutagenesi EMS

Weight 0.2 gram seeds (10,000 seeds total) (20 microgram/seed).
Wash in 0.1% Tween 15'
Put into 15 ml ddH2O
Add 15-45 microliter (0.1% to 0.3%) EMS
Mix and incubate for 8-12 hrs (Rotating) in hood.
Remove the EMS (put EMS sol'n into 0.5M NaOH O/N, then dispose as regular waste)
Rinse the seeds once and then rinse them in 10 ml ddH2O 2-4 hrs.
Put all seeds into 100 ml 0.1% Agar (Autoclave agar, cool on ice, and let solidify for several hours first)
Sow the seeds using a P1000 pipetteman (1 ml per pot).

Vengono introdotte mutazione puntiformi con transizioni
GC-AT e AT-GC

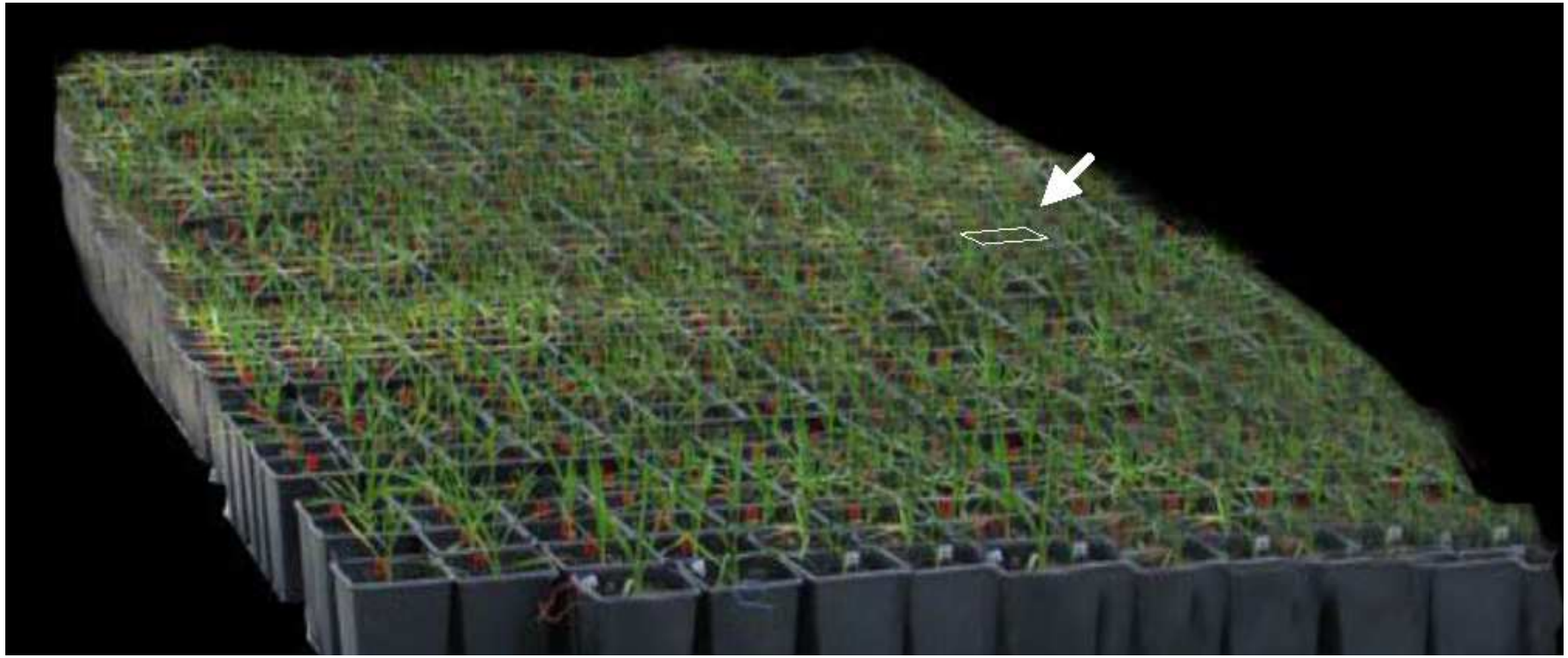


The targets of seed mutagenesis are the diploid cells of the fully developed embryo covered by the seed coat.
To assess the effectiveness of mutagenesis, it is crucial to know how many of the targeted cells will eventually contribute to the next generation. The functional germ line can be defined as the number of cells in the SAM of the embryo that will contribute to the seed output (the genetically effective cell number (GECN).
In *Arabidopsis*, recessive mutants segregate in a ratio of 7:1 in an M_2 population, therefore, GECN = 2. Because the functional germ line consists of two cells at the time of mutagenesis, the developing M_1 is chimeric and consists of two sectors, which might vary in size. Mutations segregate 4:0 or 3:1, depending on the sector from which the M_2 seeds derive.

Ionizing radiation

- Radiation ionize water and result in the formation of oxygen radicals such as superoxide ($O_2^{\cdot -}$) and hydroxyl radicals ($\cdot OH$),
- Radicals are highly reactive and can cause single and double strand breaks and base modifications,
- Single strand breaks are easily repaired but double strand breaks often result in deletions, insertions and translocations.

Screening…



Forward genetic

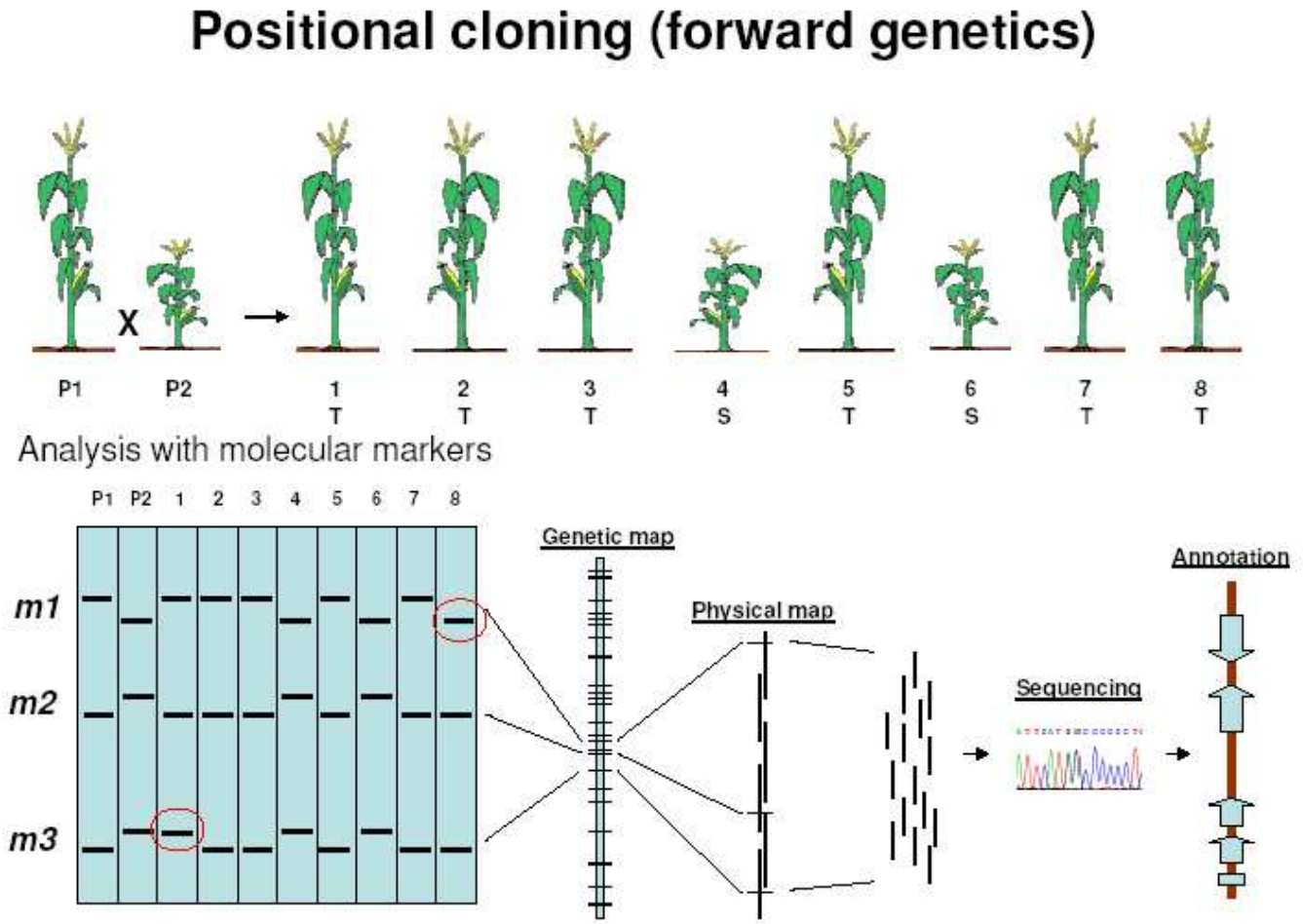
You need a screenable phenotype

- Flower development
- Disease resistance
- Photomorphogenesis
- Responses to hormones etc.

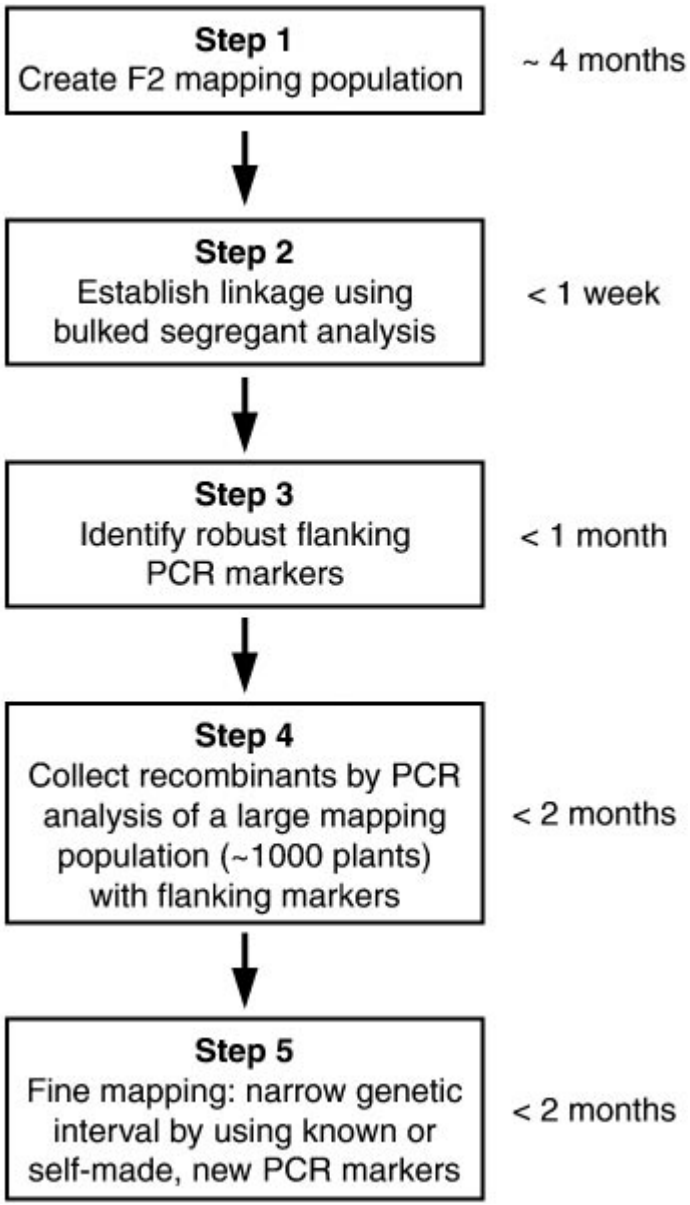
CLONAGGIO DI GENI BASATO SULLA MAPPATURA DI MUTAZIONI (MAP-BASED CLONING)

To isolate point mutations, deletions an translocation

Mapping will narrow down the genetic interval containing a mutation by successively excluding all other parts of the genome. Map positions are determined by measuring the recombination frequencies between the mutation and markers that lie in known position in the genome



MAP-BASED CLONING



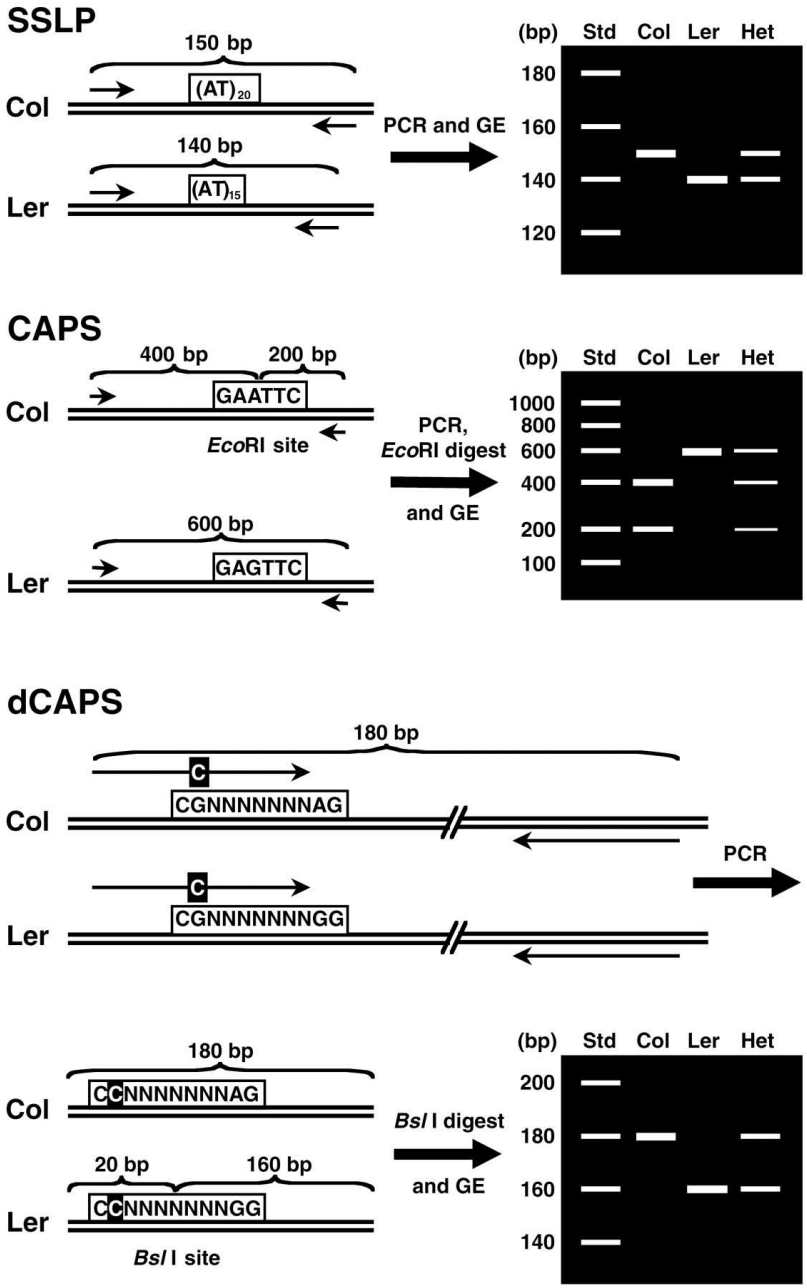
Mapping with a high resolution requires a high density of genetic markers

•Several Arabidopsis accessions, or ecotypes, are sufficiently divergent to support the design of molecular markers at this high density.
•The most commonly used combination for mapping purposes is Landsberg *erecta* X Columbia (Ler X Col).
•These two accessions have been estimated to differ in 4 to 11 positions every 1,000 bp (Chang et al., 1988_ Konieczny and Ausubel, 1993_Hardtke et al., 1996_).
•Most existing mutations, including mutations causing visible phenotypes that can be employed as genetic markers have been induced either in a Col or a Ler background.

Molecular markers commonly used for mapping (SSLP, CAPS and dCAPS)

•They are codominant (both chromosomes of a plant may be genotyped)
•They are PCR-based and can be analyzed on agarose gels (easy to use and inexpensive)
•Several methods for the extraction of plant DNA from small tissue samples have been described
•These methods are cheap and suitable for high throughput applications in microtiter plates.

Molecular markers commonly used for mapping



SSLP (simple sequence length polymorphisms) markers exploit the variability of short repetitive sequences for mapping purposes

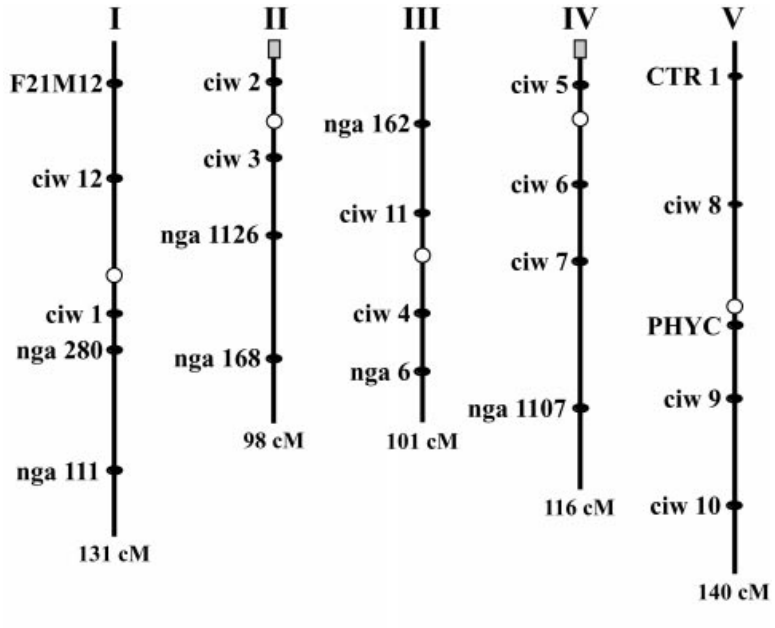
CAPS (cleaved amplified polymorphic sequences; Konieczny and Ausubel, 1993), CAPS marker exploits polymorphic restriction sites for mapping purposes

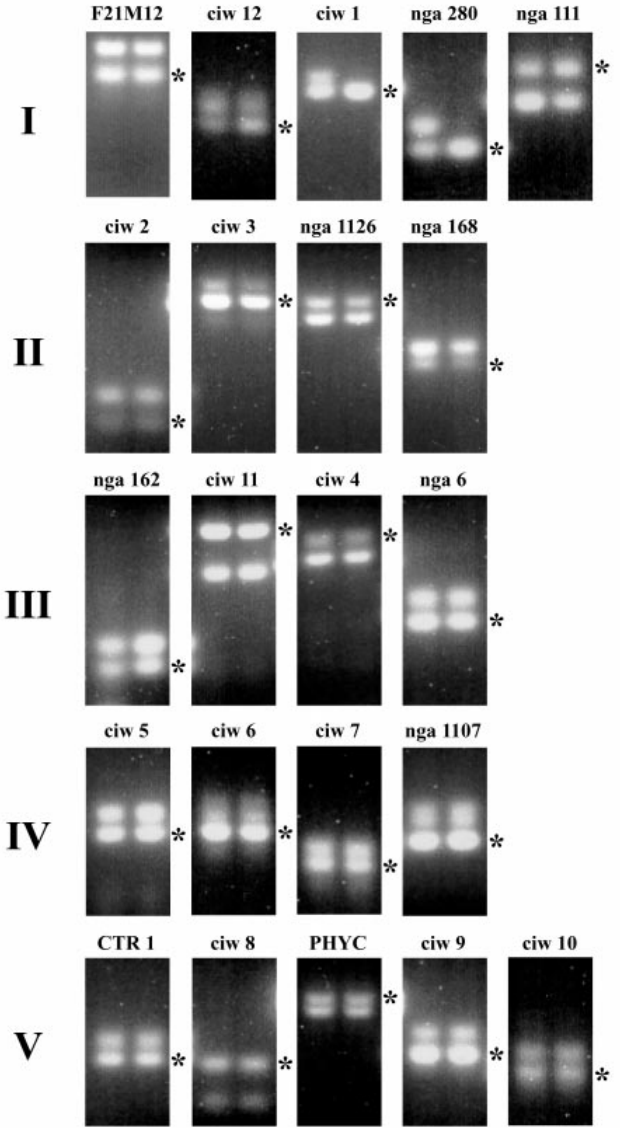
dCAPS (Michaels and Amasino, 1998; Neff et al., 1998), markers can exploit almost all single nucleotide changes for mapping purposes. To achieve this a mismatched PCR primer is designed next to the polymorphic position such that an artificial restriction site is created with the sequence variant of just one accession.

Lukowitz W. et.al. Plant Physiol. 2010:123:795-806

BULKED SEGREGANT ANALYSIS

- Su pool di DNA da diversi individui mutanti della popolazione segregante F2
- Controllo: DNA di individui F1 (eterozigoti in tutti i loci)





Three-point mapping

Once you have assigned your mutation to a chromosome, three-point mapping is almost always an obligatory step in the process of cloning our mutants.

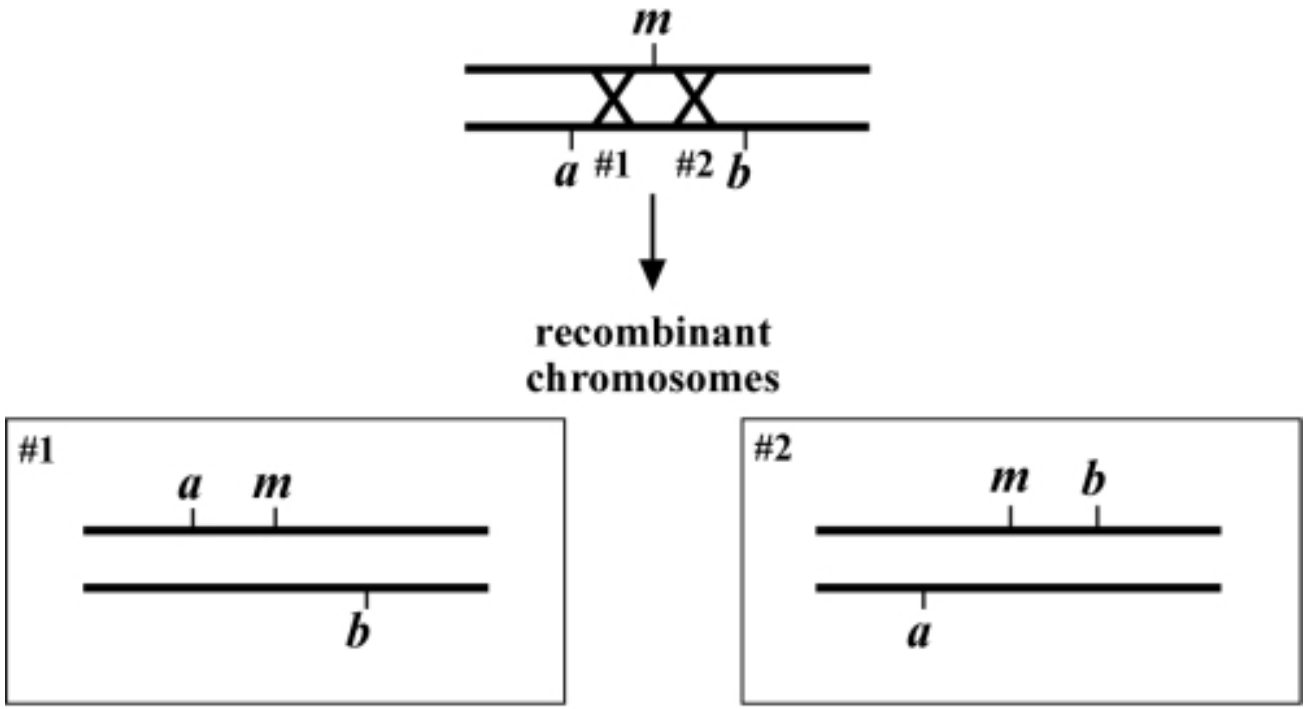
Even SNP mapping is usually preceded by three-point mapping with genetic markers.

We cross our mutation (*m*) into a strain with two linked morphological markers (*a* and *b*) that are on the same chromosome as *m*, to generate the *m/a b* heterozygote.

We then isolate and follow two classes of recombinant progeny; those that display the **A** phenotype only (**A non-B** recombinants) and those that display only the **B** phenotype only (**B non-A** recombinants). By seeing which of these two classes produce the mutant phenotype (**M**) and by scoring the percentages for each, we can determine whether our mutation lies to the left, to the right, or between our set of markers.

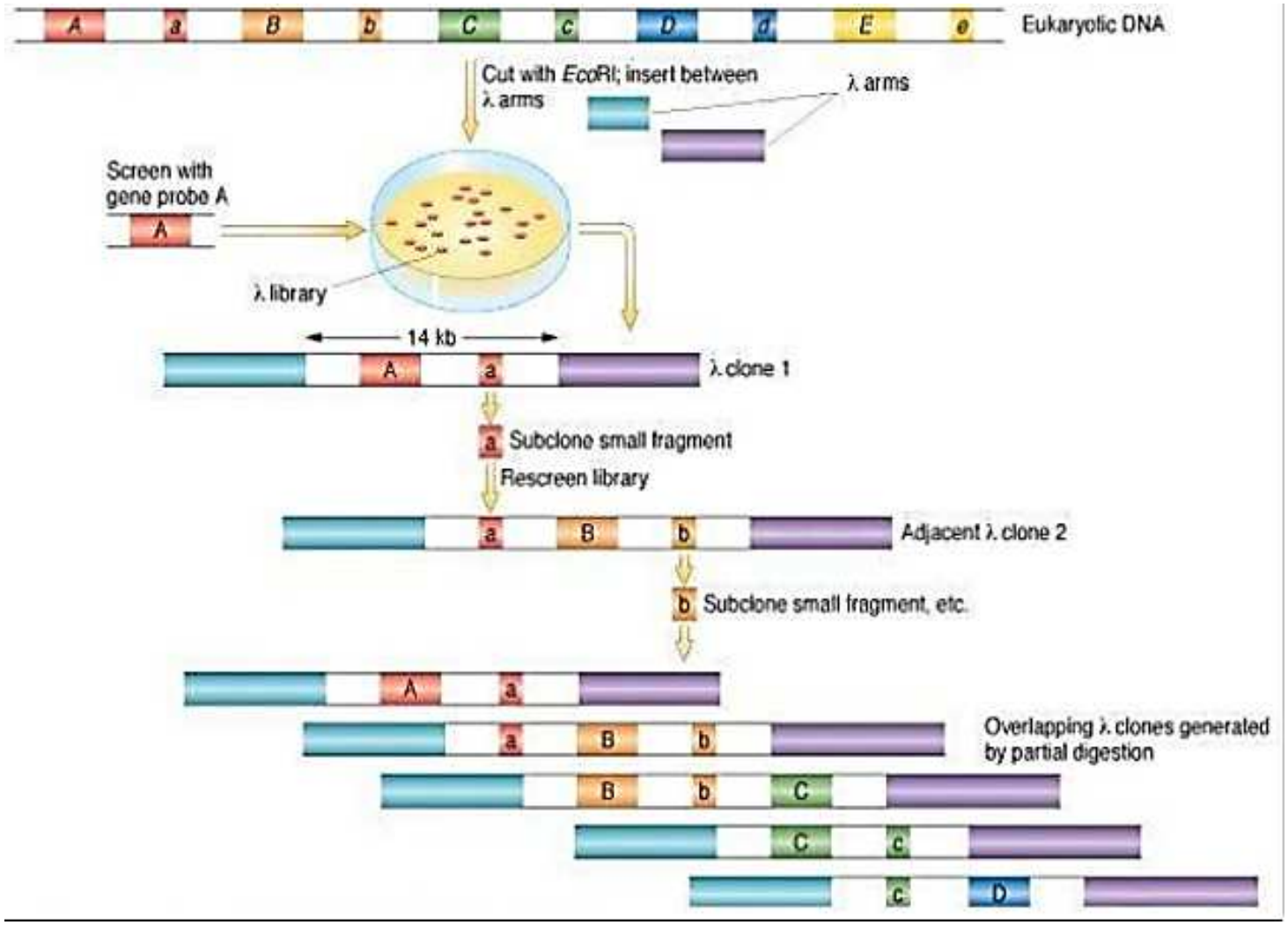
In the case where the mutation lies in between, we may then determine the approximate distance from each marker.

Three-point mapping



(recombinant
+ parental)

$\frac{a\ m}{a\ b}$	$\frac{b}{a\ b}$	$\frac{m\ b}{a\ b}$	$\frac{a}{a\ b}$
A non-B	B non-A	B non-A	A non-B


A
a
B
b
C
c
D
d
E
e
Eukaryotic DNA
Cut with EcoRI; insert between λ arms
λ arms
Screen with gene probe A
A
λ library
14 kb
A
a
λ clone 1
a
Subclone small fragment
Rescreen library
a
B
b
Adjacent λ clone 2
b
Subclone small fragment, etc.
A
a
a
B
b
Overlapping λ clones generated by partial digestion
B
b
C
C
c
c
D

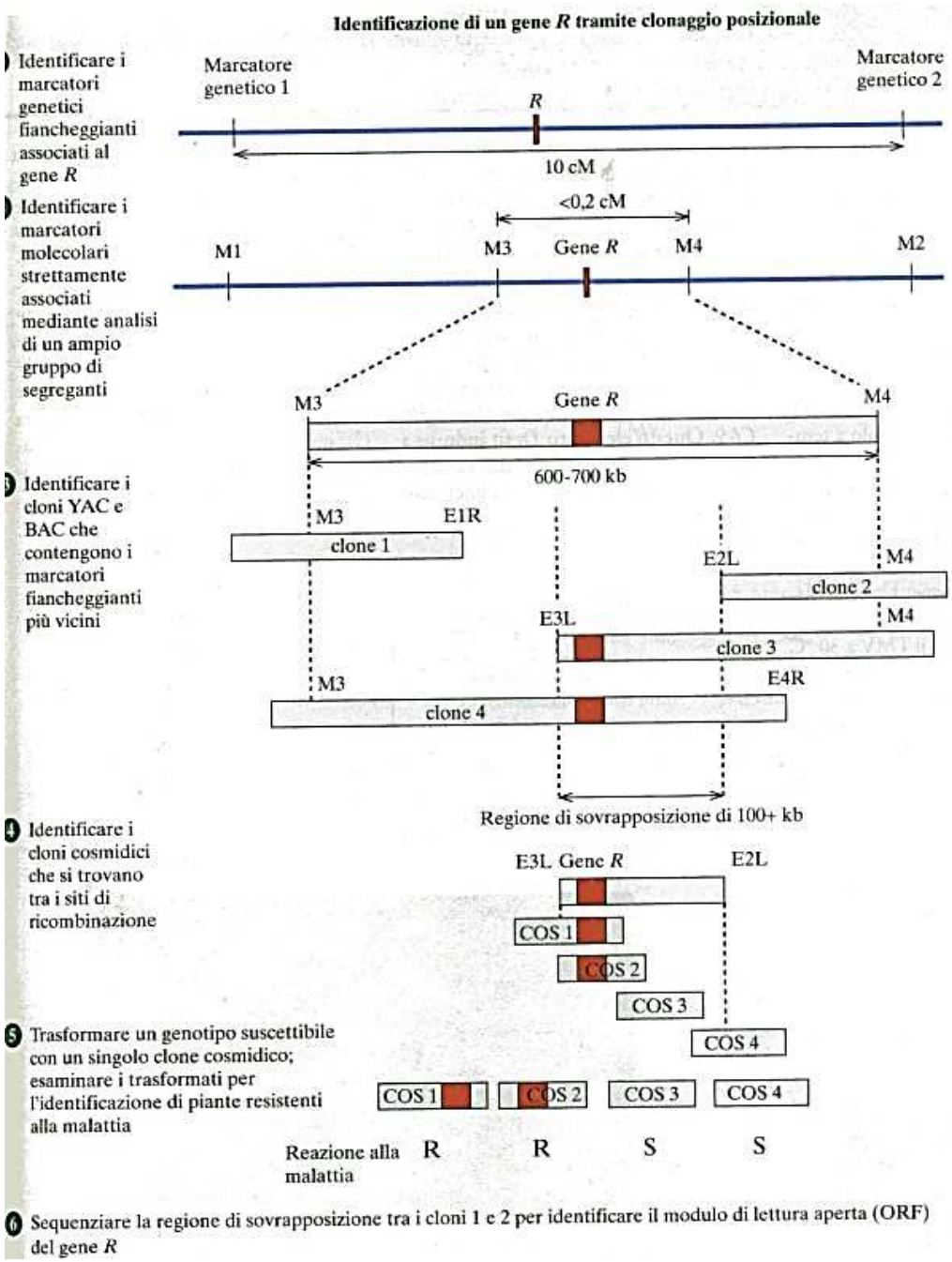


Figura 21.25 Identificazione di un gene *R* tramite clonaggio posizionale. R, resistente; S, suscettibile; YAC, cromosoma artificiale di lievito; BAC, cromosoma artificiale batterico.

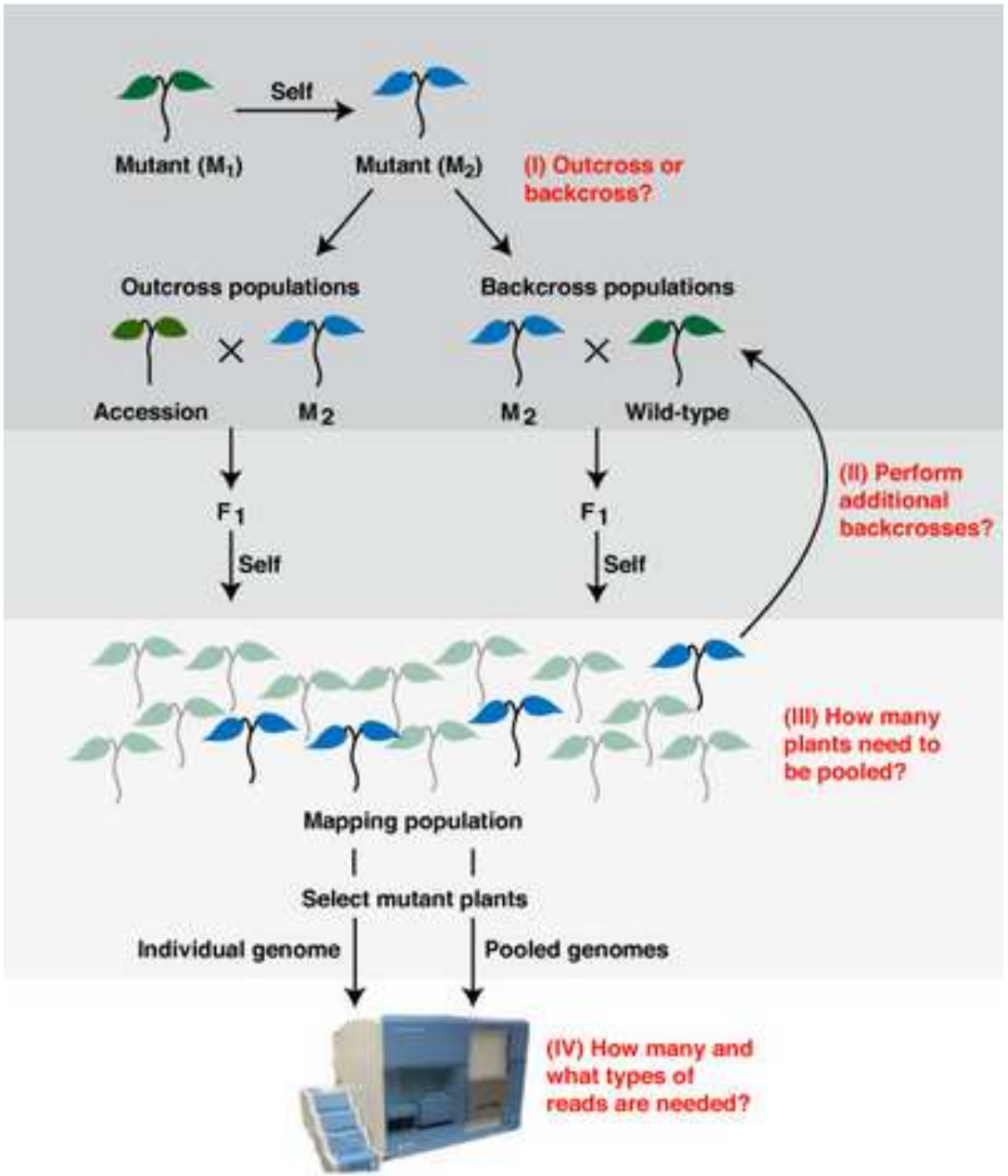
Mutation mapping by deep sequencing

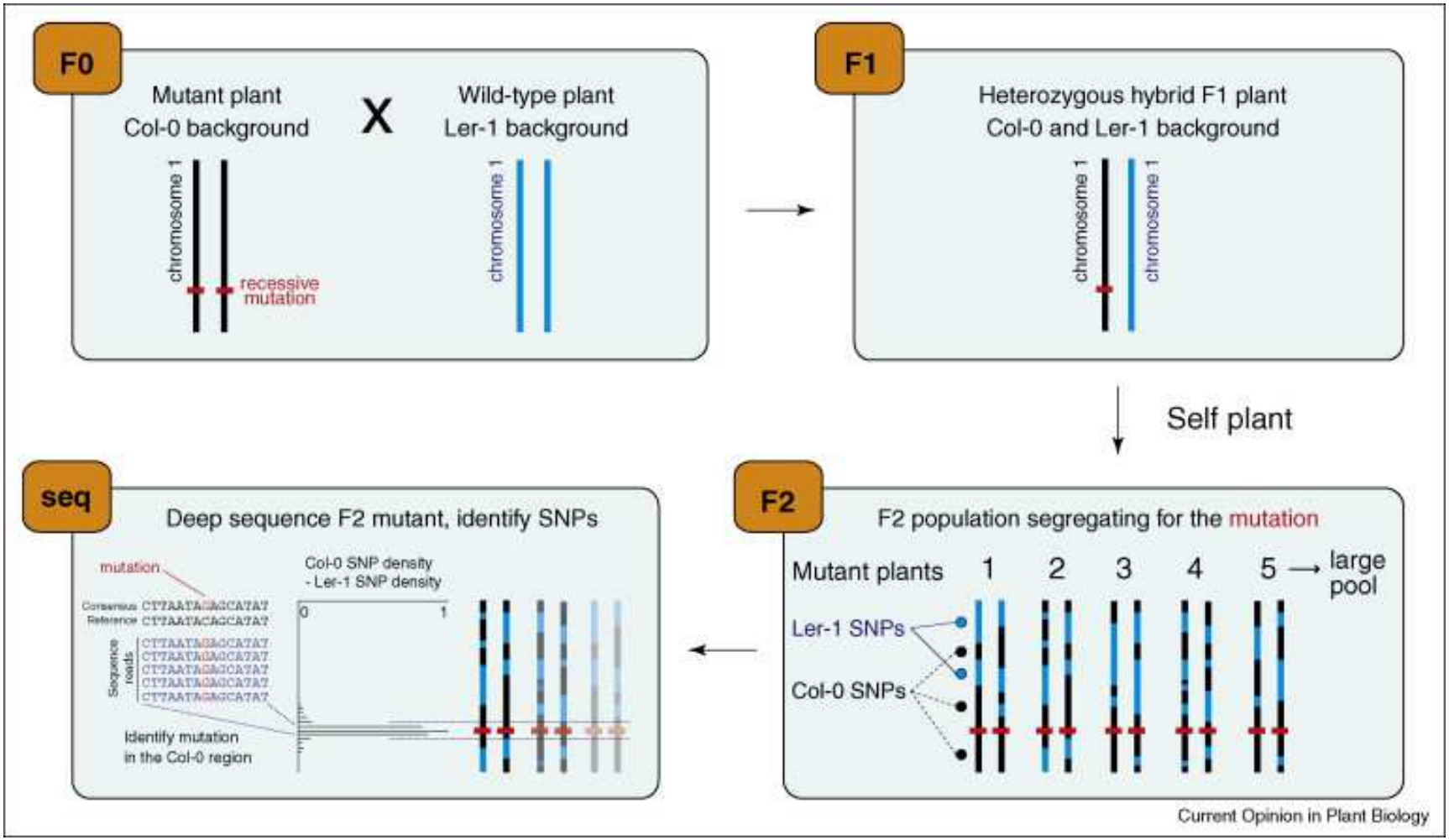
Forward genetic screens have proven extremely powerful in *Arabidopsis thaliana* for assigning genes to specific biological pathways .

The success of this approach is, in part, due to the highly accurate sequence of its compact genome, facile genetics, and extensive collection of mapping markers.

However, identifying the causative mutation commonly takes several months to years after generating a mapping population, so approaches to expedite this step will be highly valuable.

Deep sequencing of a pool of F2 individuals containing only mutant plants from a mapping population enables rapid mapping of enables rapid mapping of the mutation.


Self
Mutant (M1)
Mutant (M2)
(I) Outcross or backcross?
Outcross populations
Backcross populations
Accession
M2
M2
Wild-type
F1
Self
F1
Self
(II) Perform additional backcrosses?
(III) How many plants need to be pooled?
Mapping population
Select mutant plants
Individual genome
Pooled genomes
(IV) How many and what types of reads are needed?



Identification of mutations by deep sequencing. A plant with Col-0 background that harbors a recessive mutation leading to a mutant phenotype is crossed to a wild-type Ler-1 plant. The heterozygous F1 hybrid plant is allowed to self-fertilize to produce a large pool of F2 plants that are segregating for the mutation. A large number of F2 plants that display the mutant phenotype are pooled and their gDNA subjected to deep sequencing. The density of single nucleotide polymorphisms (SNPs) inherent in the Ler-1 strain is subtracted from the density of SNPs indicative of the Col-0 background, identifying a discrete region on the chromosome in which only Col-0 marker SNPs are present. The deep sequencing data in this interval are then scoured for the potential causative mutation.

Table 1

Suggestions for the design of mapping-by-sequencing experiments

	Outcross populations	Backcross populations	Direct sequencing	Deep candidate resequencing (dCARE)
Generation	F_2	BC_1F_2	$BC_{1\text{-}3}F_2$[a]	n/a
Mutants (n)	Approximately 150	Approximately 50	1	As many as possible
Optimal coverage	>25	Approximately 50	>25	n/a
Sequencing type	Paired-end	Paired-end	Paired-end	Single-end

[a]Depending on mutation rate.

James *et al. Genome Biology* 2013 **14**:R61 doi:10.1186/gb-2013-14-6-r61

Drawbacks of Forward Genetics

- Breeding must be possible

- You need a screenable phenotype
  - Easy with visible phenotypes
    - growth, color, development
    - not easy with metabolism
    - impossible for „essential genes“ (defects of which would be lethal)
- You need a dense genetic map and markers closely associated with the phenotype
  - Good for Arabidopsis, Maize, Rice
  - Not established for many tropical plants, hops (*Humulus lupuls*) and other speciality crops
- Access to genomic library (YAC, BAC) required for cloning the gene

Insertion tagging

[signature tagged mutagenesis (STM)]

-The advantage of insertion tagging over traditional forms of mutagenesis is that the interrupted gene becomes”tagged” with the insertion elements, hence the strategy is sometimes termed Signature-tagged mutagenesis (STM) .

Steps:

1. T-DNA or transposon tagging.
2. Techniques can be used to obtained the flanking DNA.
3. The sequence of the flanking DNA can then be used to interrogate sequence databases, allowing the tagged gene to be associated with its “parent” genomic clone phenotype.
4. If insertion also generates a mutant phenotype, the gene in the database can then be ascribed a tentative function.

Mutagenesis by T-DNA tagging

-Gene insertion is essentially random.

-It affects only the gene, where it incorporated (within the gene space).

- Disadvantage is the tendency to generate complex, multicopy integration patterns and sometimes deletions and gene rearrangements of surrounding genes.

-As *Arabidopsis* is a gene dense plant- having small introns, little intergenic space and therefore, about 80 % genome is thought to represent genome.

-
  - Insertions in around 90% of genes are present.
  - Over 300,000 transformants were made by T-DNA tagging

- As T-DNA is not a transposon, it has no ability to 'jump' following integration…….therefore, having advantage of generating stable insertions

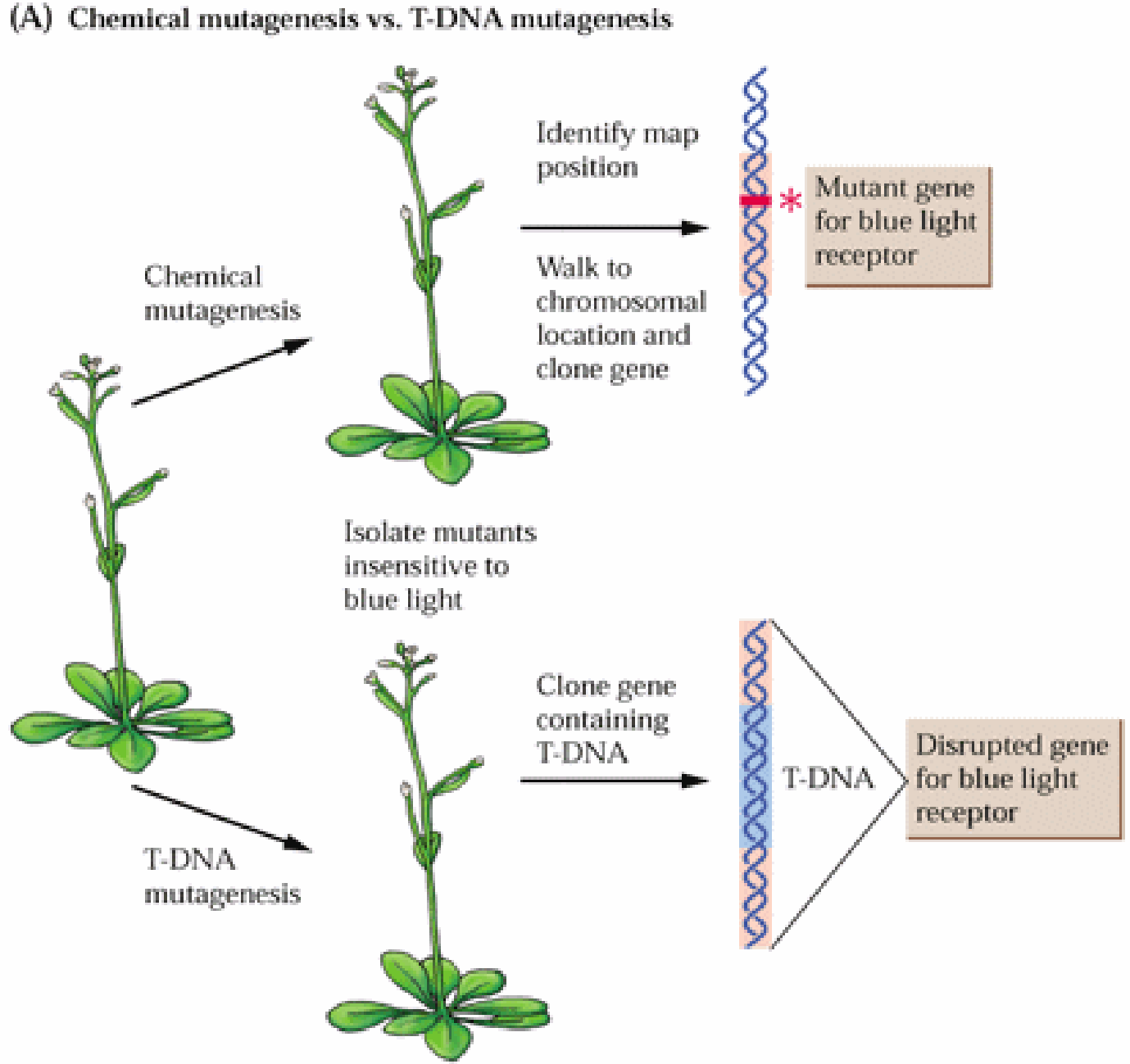
.

Flanking sequences **have been** characterized. Mutants can be used for reverse genetics (see ahead)

- DNA isolated and the junction fragment between the T-DNA and the plant DNA recovered. This allows the precise location of the T-DNA in the genome to be assessed. Called Flanking Sequence Tag (FST).
- These FSTs are present in databases, so insertions in a gene of interest can be recovered by searching the database.
- Mutant lines can be ordered from catalogue.

T-DNA insertion lines

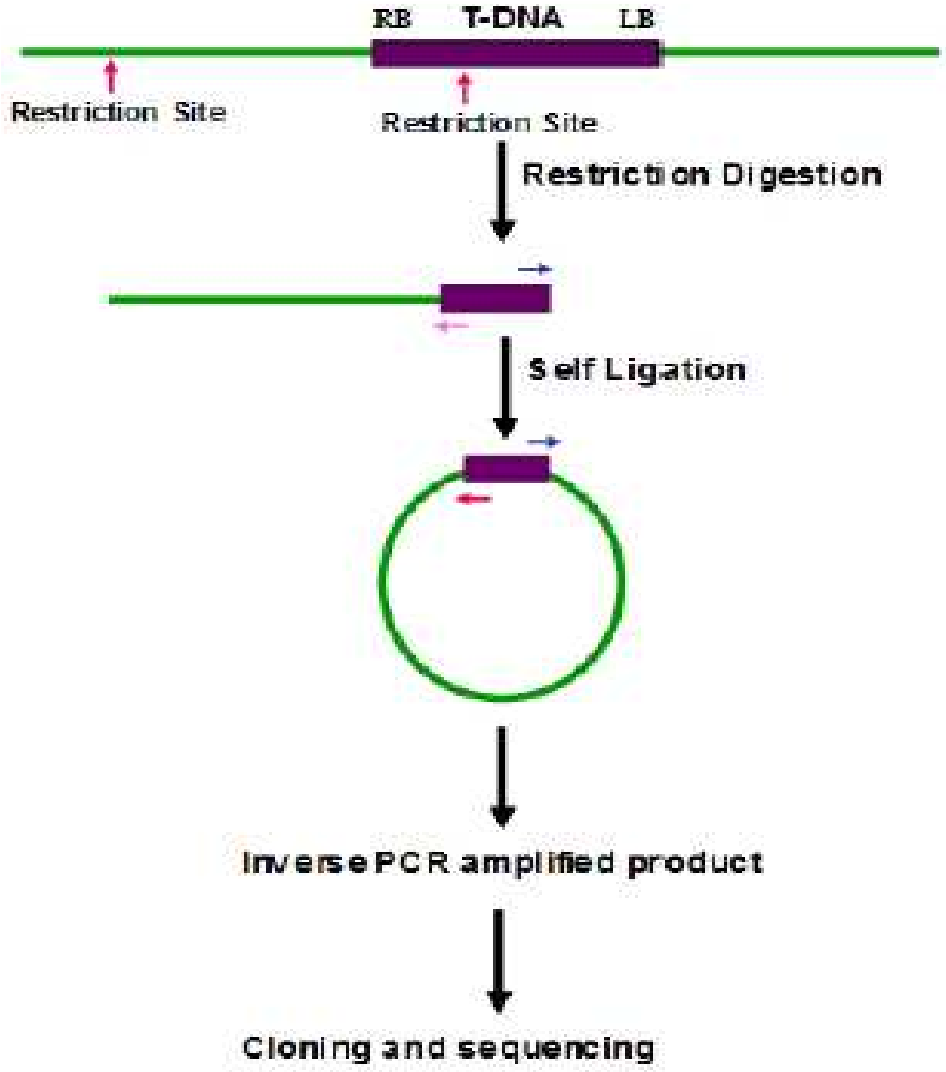
- Average number of independent insert 1.5 per diploid genome
- 57% of the transformants containing single insert
- 25% of the transformants containing double insert
- Bias observed against integration events in exons and introns in favour of promoters, 5' and 3' UTRs
- Number of integrations decrease from chromosome arms towards centromers


(A) Chemical mutagenesis vs. T-DNA mutagenesis
Chemical mutagenesis
Identify map position
Walk to chromosomal location and clone gene
Mutant gene for blue light receptor
Isolate mutants insensitive to blue light
T-DNA mutagenesis
Clone gene containing T-DNA
T-DNA
Disrupted gene for blue light receptor

Identification of sequences flanking T-DNA (= mutated gene)

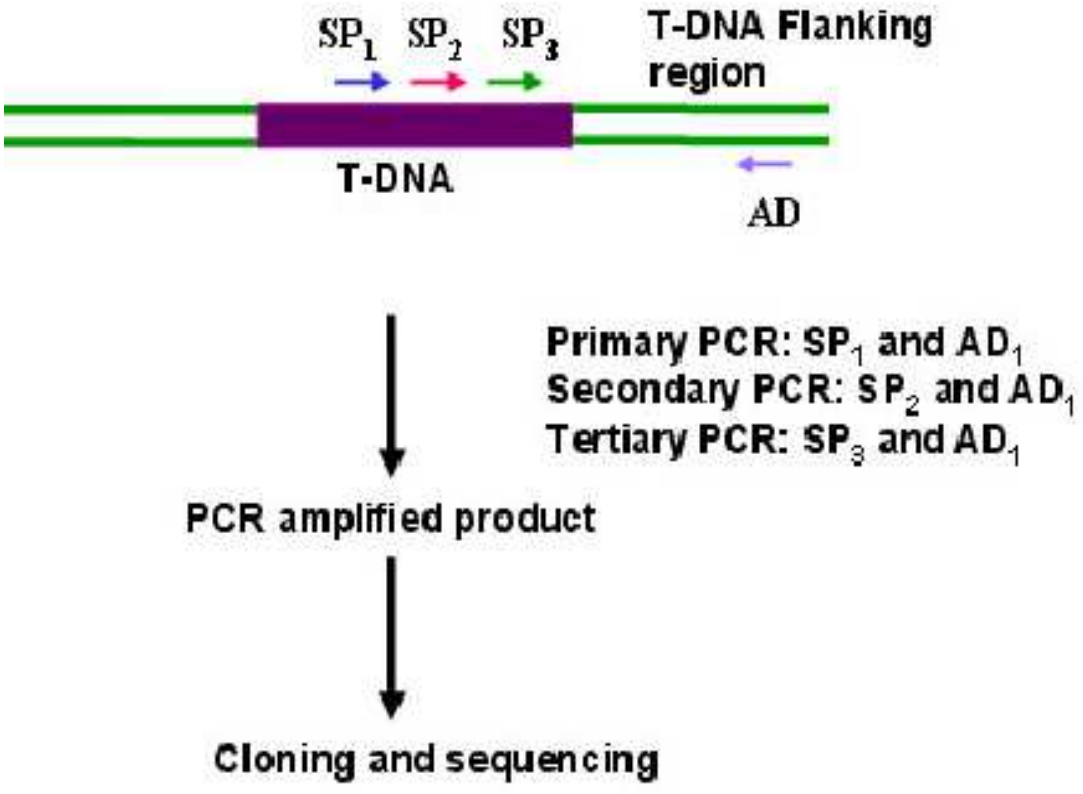
- Inverse PCR
- Thermal asymmetric interlaced (TAIL) PCR
- Plasmid rescue

Inverse PCR



1) Restriction digestion of genomic DNA from mutant plant (preferably single cut within the T-DNA);
2) self-ligation;
3) circularized ligation products are used for PCR amplification using appropriate primers from the T-DNA region.

Thermal asymmetric interlaced (TAIL) PCR



Three consecutive rounds of PCR, performed with a set of three nested T-DNA specific primers (SP1, SP2 and SP3) and a small, arbitrary primer.

AD is arbitrary degenerate primer indicated by purple arrow.

Arbitrary or degenerate primer

1.3.3. Primer degenerati

I primer degenerati sono primer la cui sequenza non è determinata univocamente, ma contiene una o più posizioni in cui possono essere presenti più nucleotidi in miscela. Ad esempio, la sequenza sottostante di un primer di 20 basi:

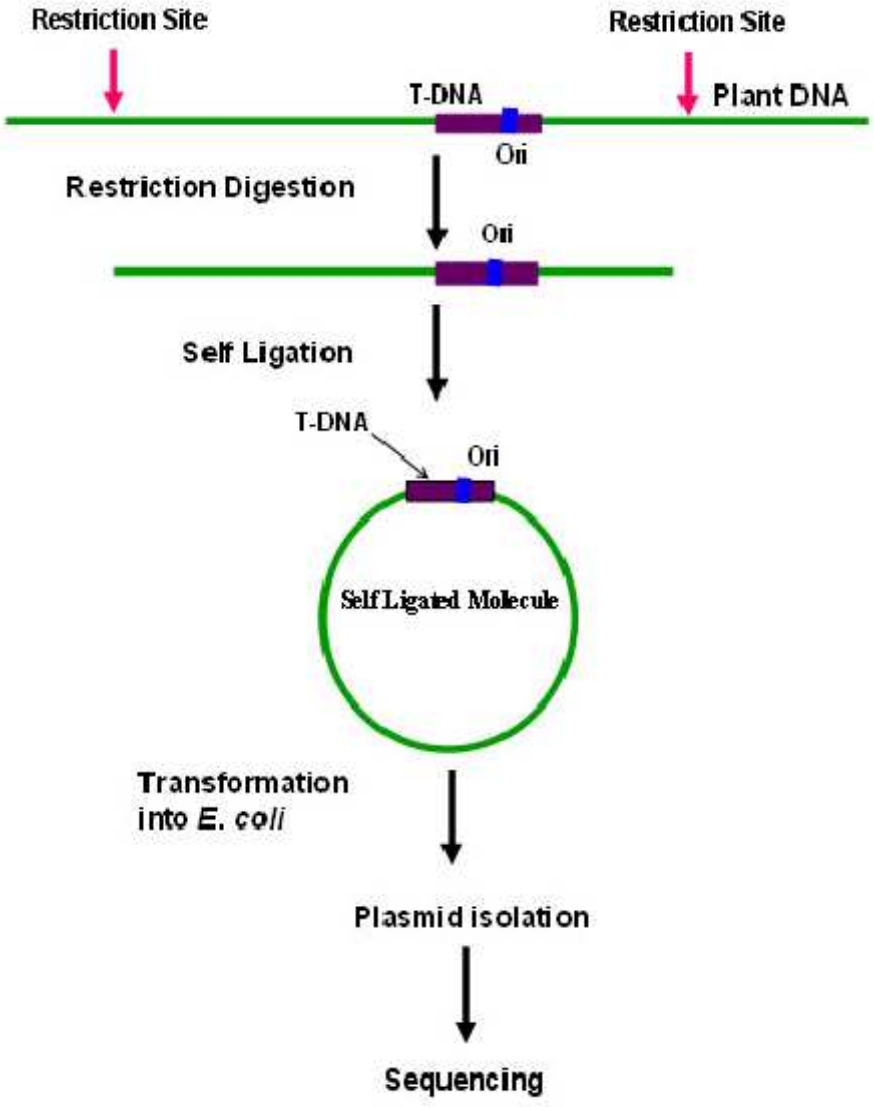
5'-ACGTATNTCCNCAYATYGCT-3'

significa che il primer disegnato contiene delle posizioni ("degenerazioni" indicate con Y e N) che contengono in miscela le basi T + C (Y) o A + G + C + T (N). Le degenerazioni ovviamente riducono la specificità del primer aumentando le possibilità di appaiamento.

A cosa servono i primer degenerati?

I primer degenerati possono essere utilizzati per amplificare sequenze di DNA (ignote) da un organismo utilizzando per il disegno del primer la sequenza nota (omologa) proveniente da un altro organismo o dallo stesso organismo. In alcuni casi si possono usare i primer degenerati

Plasmid rescue

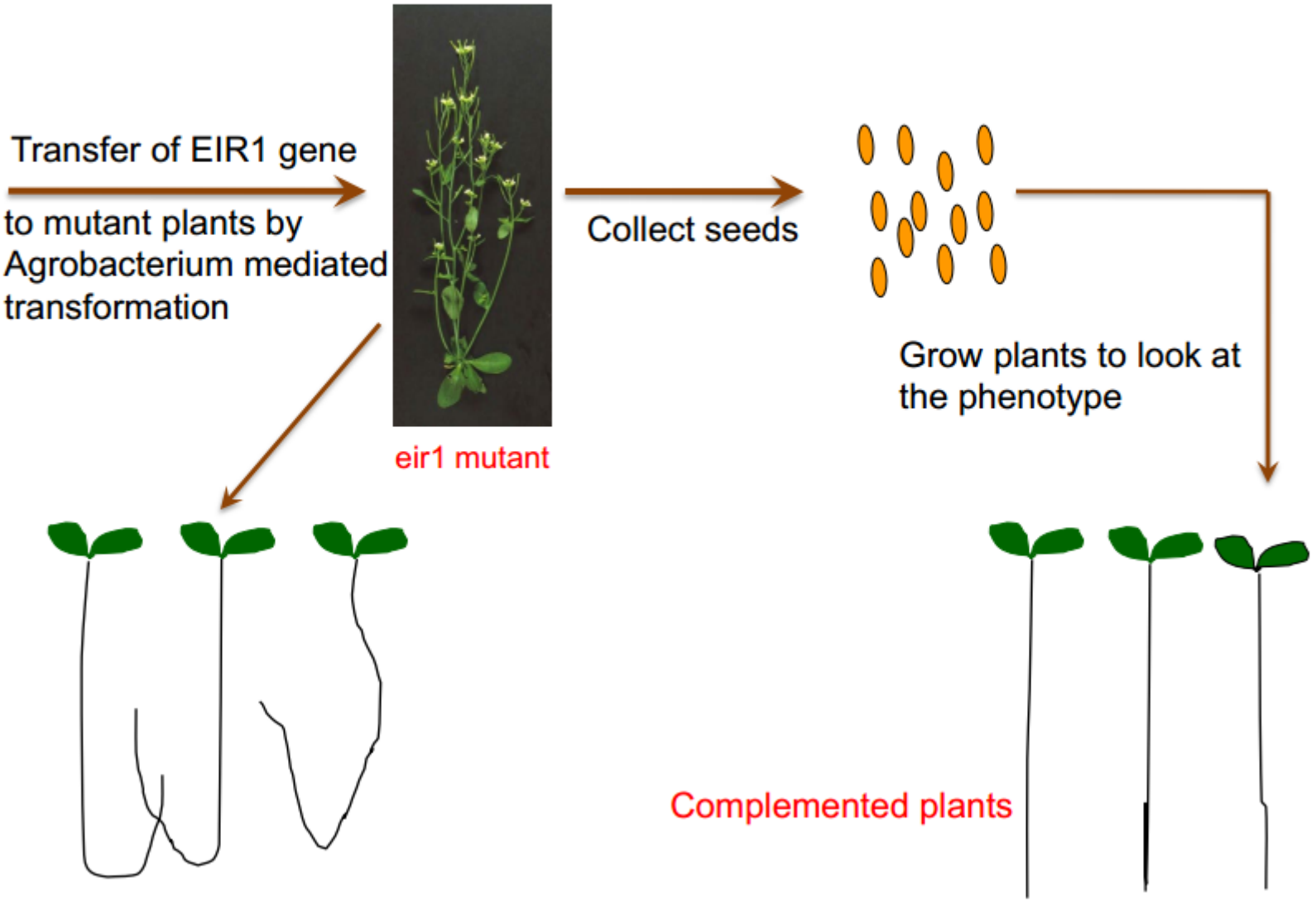


1) Restriction digestion of genomic DNA from mutant plant (preferably with an enzyme which does not cut within the T-DNA);

2) self ligation;

3) transformation of *E. coli* with the self-ligated fragments

Final step: to validate the gene function we need to restore the mutant phenotype

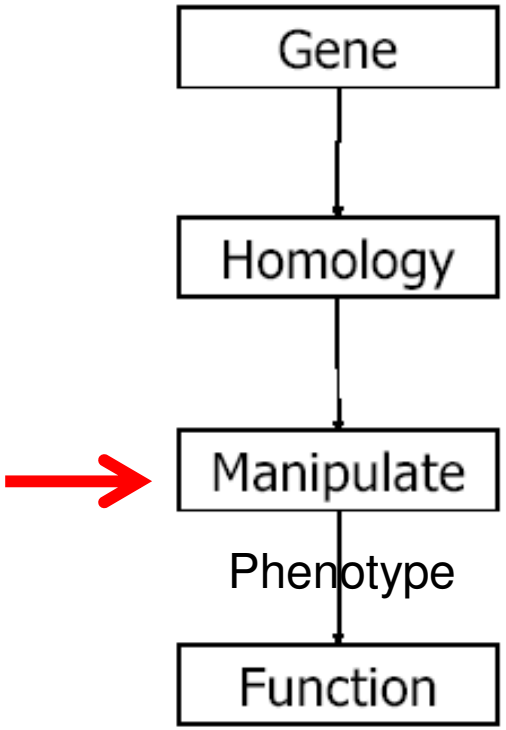


Mutant collections

Submitted by	Background	Selectable marker	Promoter/enhancer/reporter gene	Population size	References
SALK	Columbia-0	Kanamycin	---	145589	Alonso et al. 2003
GABI-Kat[2]	Columbia-0	Sulfadiazine	---	59455	Rosso et al. 2003
Czaba Koncz		Hygromycin	---	300	Szabados et al. 2002
Syngenta	C24	Hygromycin	GUS	1250	Sessions et al. 2002
INRA-Versailles	Ws, (Wassil-evskija)	Basta	GUS	1480	Balzergue et al. 2001
Le Clere and Bartel	Columbia	Basta	CaMV35S-cDNA	33100	LeClere and Bartel, 2001
Haseloff	C24	Kanamycin	GAL4-GFP	8000	Kiegle et al. 2000
Weigel	Columbia	Basta	Multimerised CaMV35S enhancers	>20000	Weigel et al. 2000
Sussman and Amasino[3]	Ws-2	Kanamycin	Ap2::GUS	37800	Sussman et al. 2000
Jack	Columbia	Kanamycin	CaMV-GUS	11370	Campisi et al. 1999
Ehrhardt	Col-2 (CS907)	Basta	CaMV 35S -GFP	108	Cutler et al. 2000

GENETICA INVERSA

‘Reverse Genetics’



- Know a gene
  - genome sequence, EST etc
- and want to discover function
- Manipulate the gene
  - and do as for forward genetics

BUT

- Need to know roughly what to look for in phenotype (guess by homology)
  - But no idea of function of 30% of Arabidopsis genes
- Need gene not to have function covered by another gene
  - Multigene families (37% of Arabidopsis genes in families of >5 genes)

MANIPULATION OF THE EXPRESSION OF YOUR PLANT GENE FOR REVERSE GENETICS

- Gene disruption (Knock-out)
- Gene Knock-down

  (you mostly buy mutants)

- Gene silencing (TARGETED) (Hairpin-loop, artificial miRNAs)
- Gene OVEREXPRESSION (35S promoter or inducible promoters)
- Genome editing

  (you generate mutants)

- Gene overexpression or silencing in specific tissues (ex. GAL4-UAS)

  (buy +generate)

Reverse genetics

Enabling tools for reverse genetics:

- Collections of mutations that reside in almost every gene

- Methods to rapidly knock out or knock or silence or overexpress genes of interest

How to choose a gene:

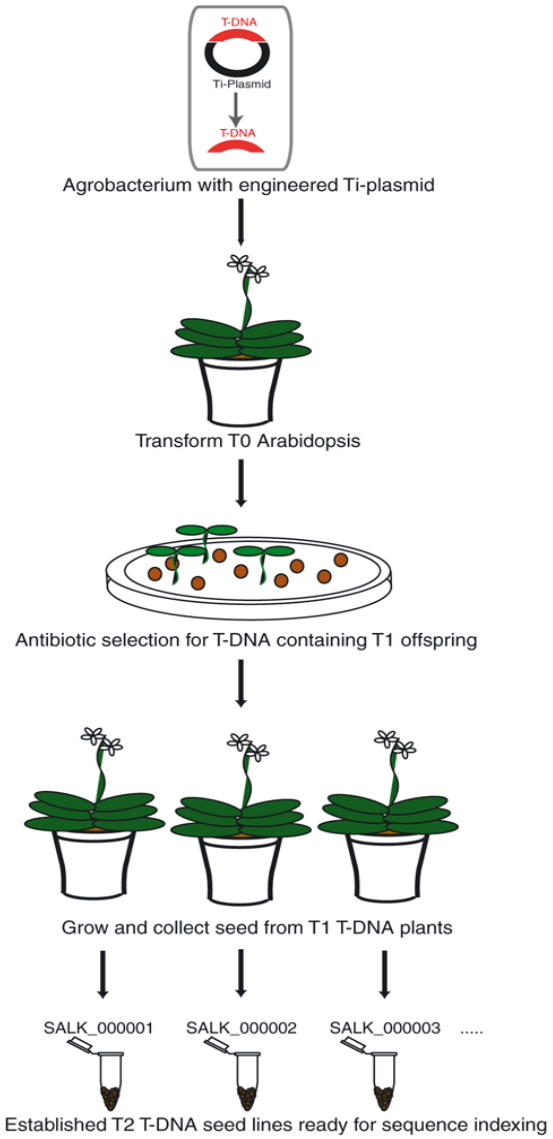
- Your favorite gene family or gene function

- Candidate genes from other genomics approaches associated with your favorite biological process

- Candidate genes from other organisms associated with your favorite biological process

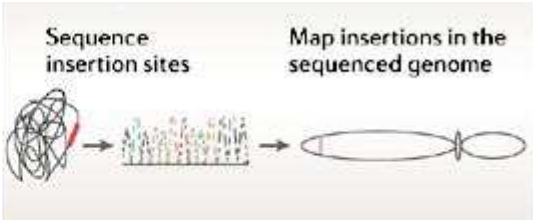
T-DNA insertional mutant collection for reverse genetics (commercially available)

Collection of mutants



Identification of insertion sites (TAIL PCR, Inverse PCR, plasmid rescue)

Catalogued mutants



Commercially available

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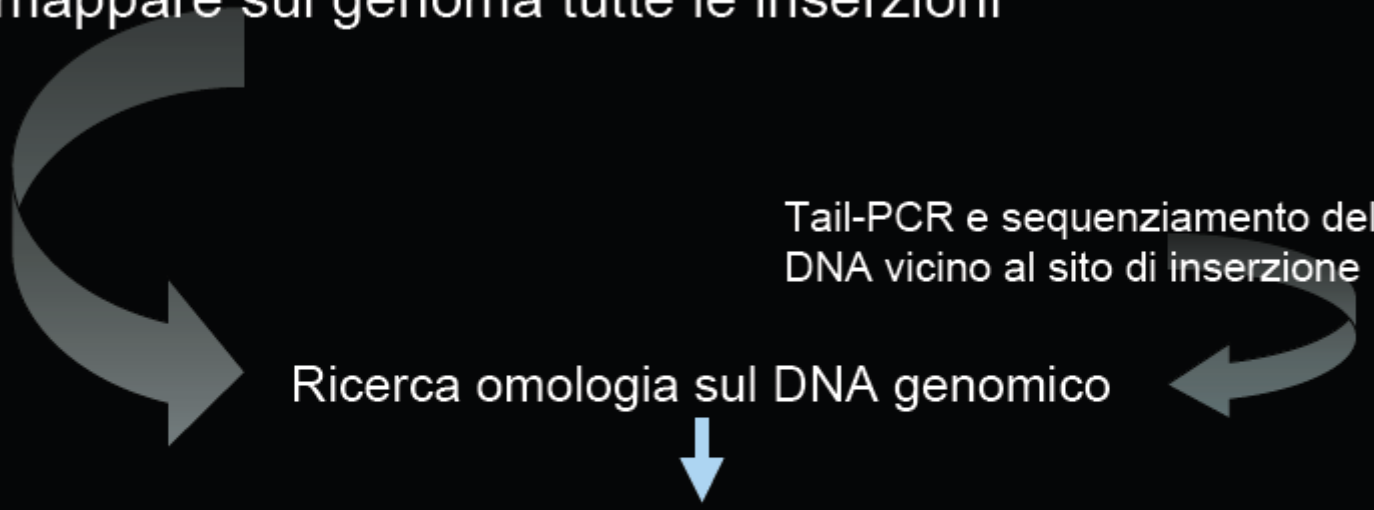
Ehrhardt	Col-2 (CS907)	Basta	CaMV 35S -GFP	108	Cutler et al. 2000

La disponibilità della sequenza del genoma di *Arabidopsis* ha permesso di mappare sul genoma tutte le inserzioni



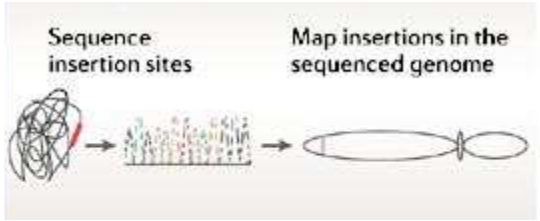
Organizzazione in banche dati

Per Arabidopsis il SALK raccoglie i risultati di più collezioni di mutanti (SAIL, Wisc) e la distribuzione è gratuita

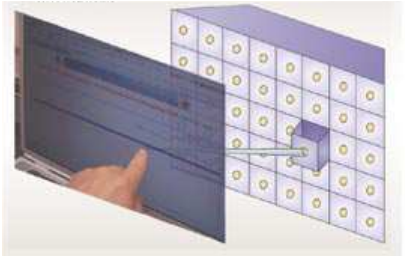
http://signal.salk.edu/tabout.html

Then, what do **you** have to do?

Catalogued mutants



Select on the Internet and buy mutants in the gene or genes of interest



Molecular genotyping

T-DNA Express: Arabidopsis Gene Mapping Tool (May. 7, 2008)

1. Search : [?]

Type: Gene Query: At5g42350

Chr: chr1 Posn:

Display: Graphy Search Clear

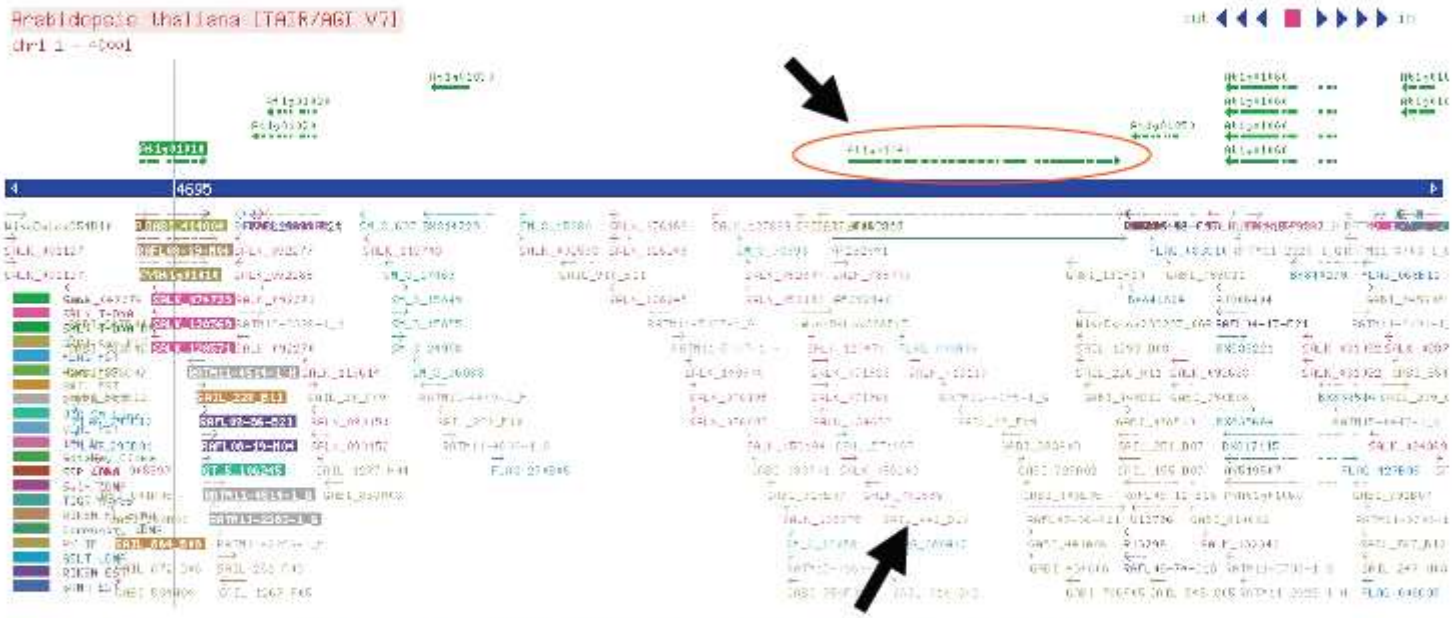
2. iSect Tool : [?] Please Select one

3. Data Source :

a. Data Source, Detail and Summary.
b. Gene Expression Atlas Data Source.
c. Data Release Policy.
d. FAQs.

SIGnAL
Salk Institute Genomic Analysis Laboratory
Funded by the National Science Foundation

SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (Apr. 22, 2007)



Please note: We are using AGI/TAIR V7 pseudo-molecules & annotation (Apr-06-2007).

http://signal.salk.edu/cgi-bin/tdnaexpress

Ordino il mutante !!

NASC - European Arabidopsis Stock Centre Home Page - Windows Internet Explorer

http://arabidopsis.info/

screen capture

File Modifica Visualizza Preferiti Strumenti ?

Collegamenti HotMail gratuita Personalizza collegamenti Personalizzazione collegamenti Windows WindowsMedia

NASC - European Arabidopsis Stock Centre ...

Pagina Strumenti

Arabidopsis.info

NASC

European Arabidopsis Stock Centre

Seed and information resources.

Stock Catalogue
Search Catalogue
Browse Catalogue
The Wiki
About plant ontology
How to Order
Ordering FAQ
Stock Overview
Price Information
Check Order Progress
Seed Donation Form
MTA FAQ
Transcriptomics
Microarray Homepage
Apply for Service

Stock Catalogue

NASC now maintains over 300,000 accessions of *Arabidopsis thaliana* representing over half a million genotypes

Search Catalogue

Browse Catalogue

Browse by Ontology

How to link to NASC stocks
How to reference NASC services
The great annotation experiment:
WeedsWorldWiki
Donate Seed

News

Latest Additions to the Stock Catalogue:

GABI-Kat lines - donated by Bernd Weisshaar
JIC Bancroft RI lines - donated by Ian Bancroft.

Current News:

NSF fund $50M iPlant Co-operative at Arizona
Also see: webcast (choose archive button: top-right)
International Arabidopsis Meeting (ICAR) 2008 - Montreal
MASC report 2007 available
WeedsWorld Wiki - review that stock.
Weed for Thought
- a Dutch Ph.D. thesis considering NASC and TAIR.

Transcriptomics

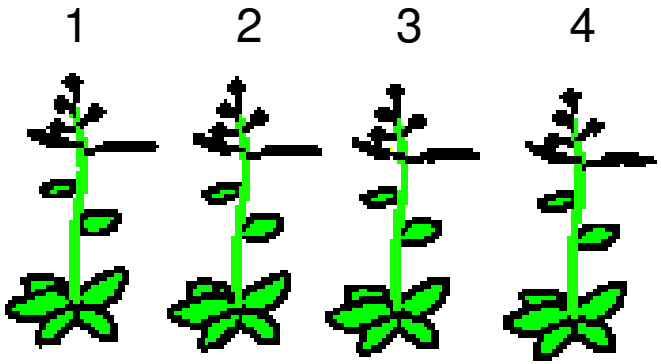
NASCarrays processes Affymetrix

Genomics

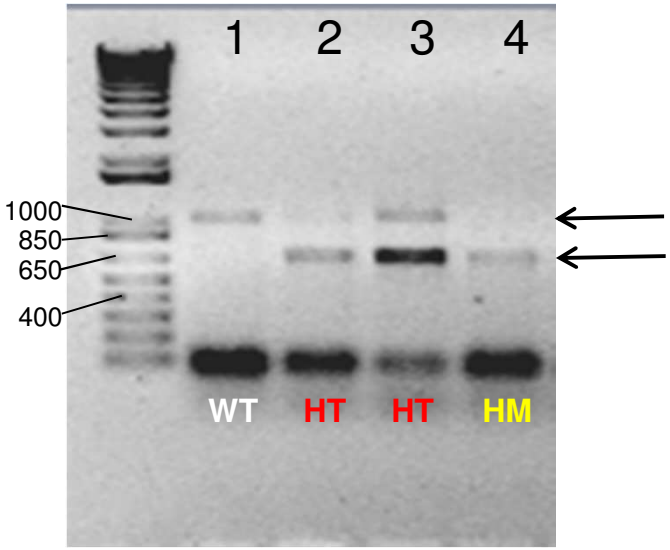
NASC's well established genomic

Internet 100%

Mutant genotyping

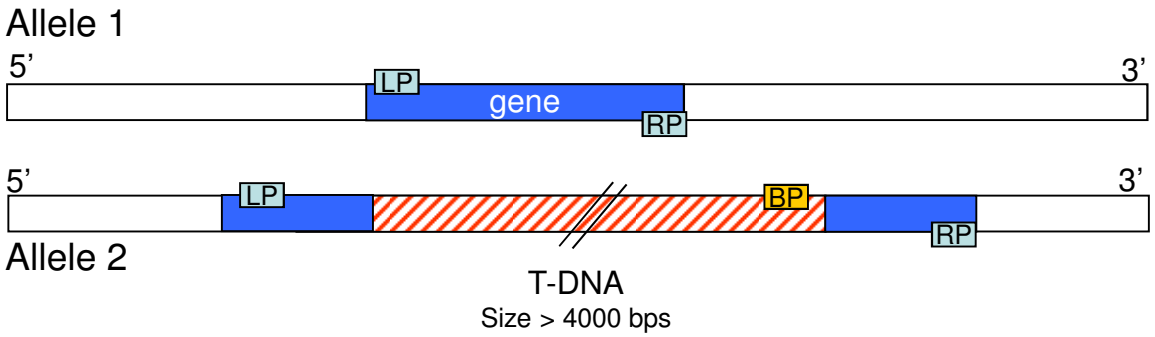


Genomic extraction



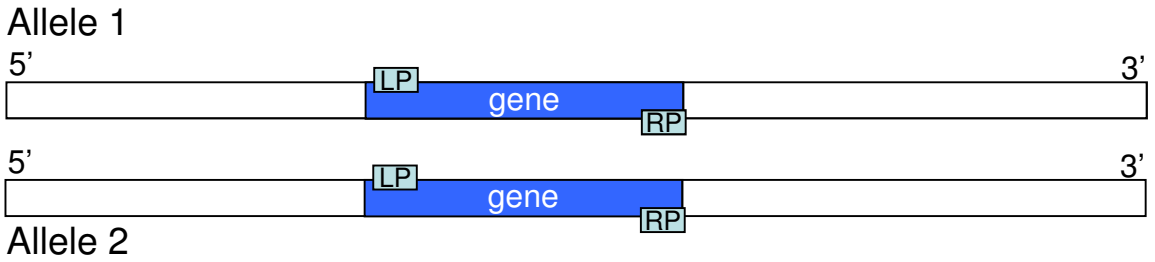
PCR

Mutant genotyping

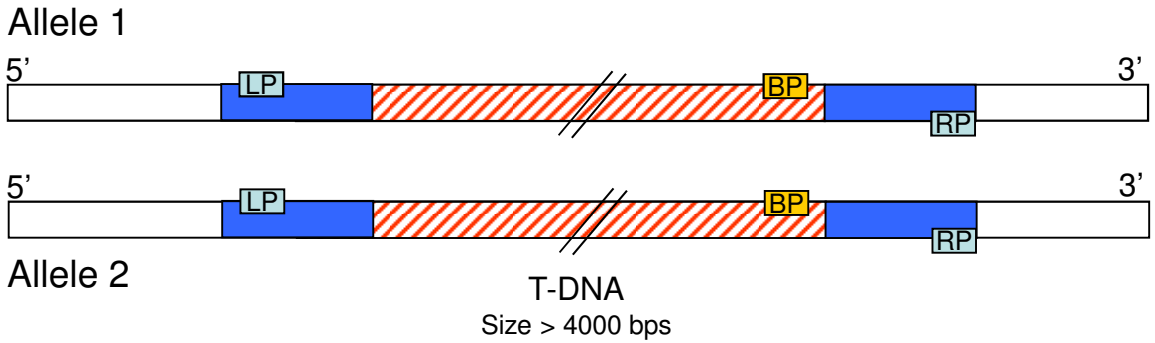


PCR primer pairs

LP	+	RP	Product
LP	+	BP	No product
BP	+	RP	Product

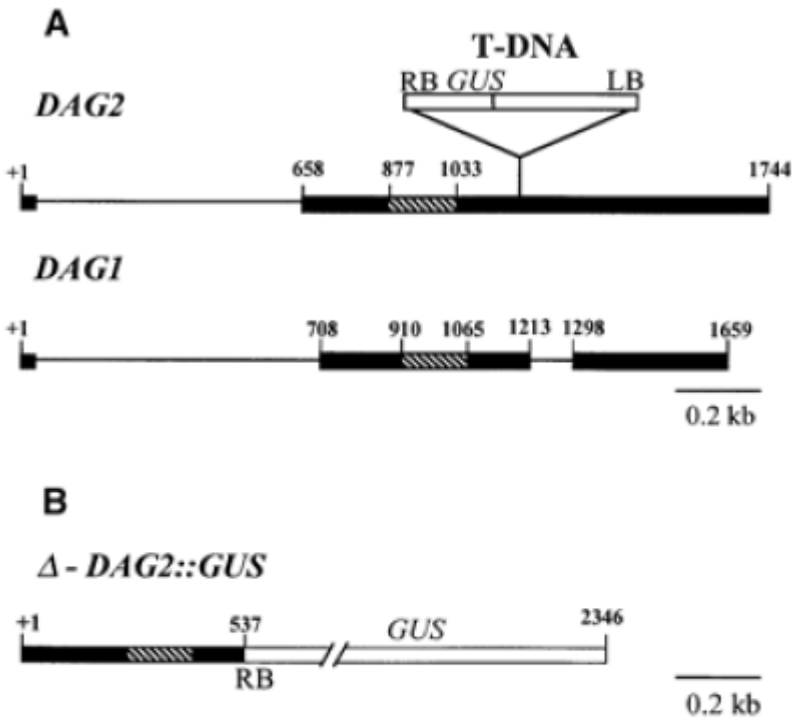


LP	+	RP	Product
LP	+	BP	No product
BP	+	RP	No product

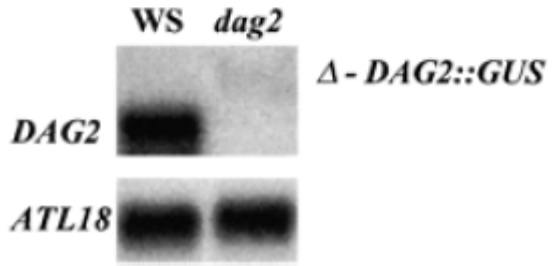


LP	+	RP	No product
LP	+	BP	No product
BP	+	RP	Product

Schematicamente i mutanti inserzionali possono essere rappresentati come nei disegni a fianco



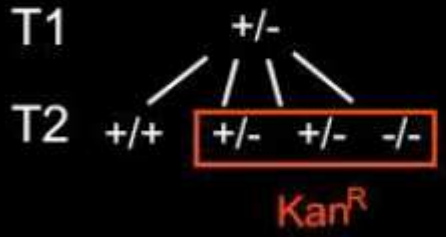
Se il gene è distrutto non si dovrebbe avere la presenza del trascritto



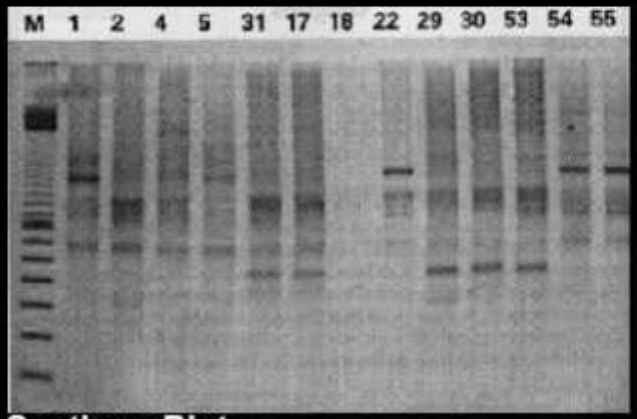
La T/F con *Agrobacterium* ha una efficienza media di 1,5 inserzioni di T-DNA per linea

Più inserzioni nella mia linea?

- in loci differenti
  Rapporti di segregazione di linee eterozigoti sulla resistenza e Southern
- nello stesso locus
  Southern



- = Allele selvatico
- = T-DNA



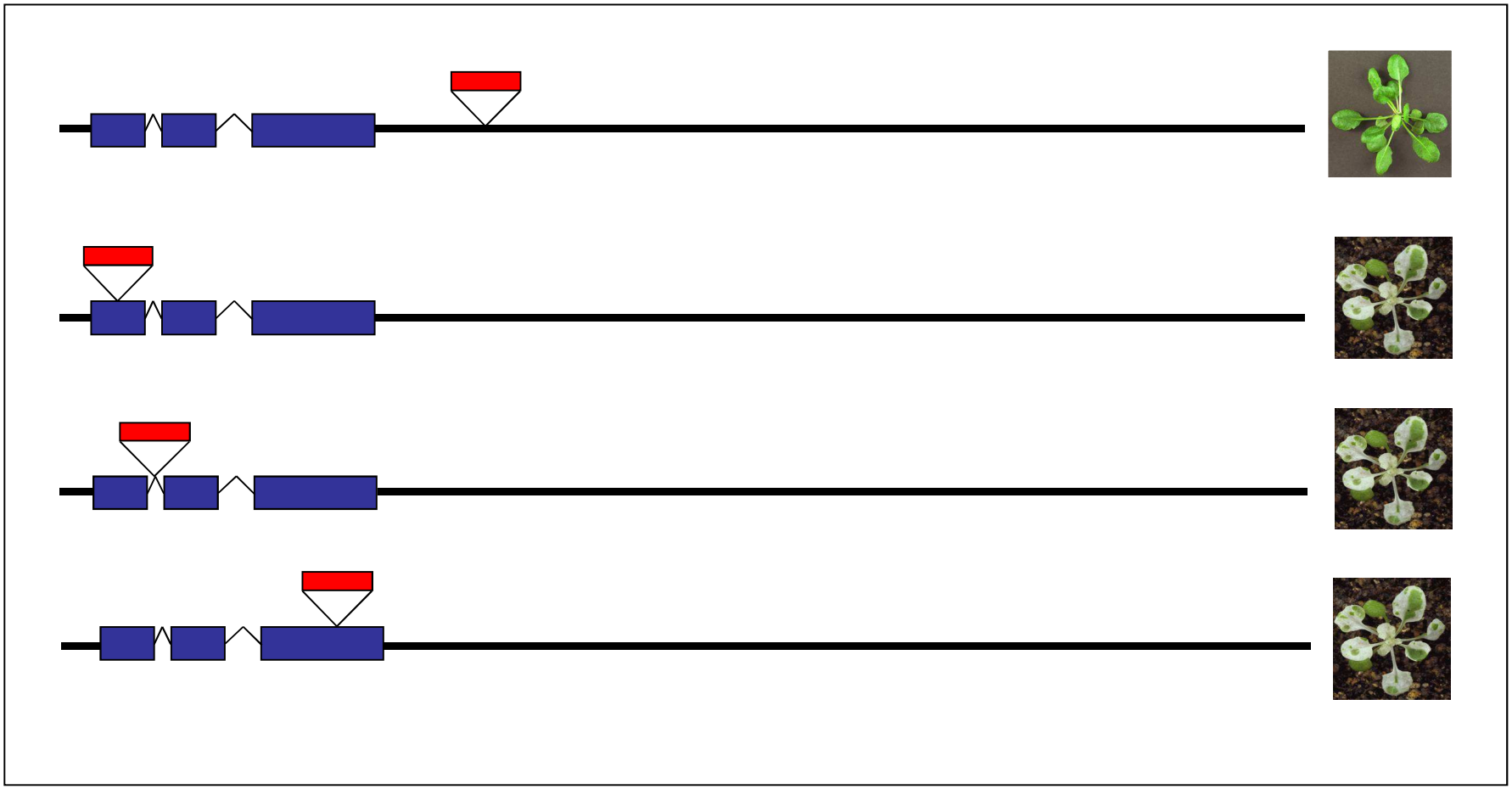
Southern Blot

→ Analisi del pattern di bande

Se ci sono più inserzioni in loci differenti si incrocia la linea mutante con la rispettiva linea selvatica (reincrocio)

Finchè non trovo un rapporto di segregazione di 3:1

How to chose the mutant of interest (you should have at least two alleles)



Select homozygous and propagate

Back crossing

IN general, but expecially for pleiotropic mutants, it is necessary to determine whether the phenotypes result from same or from two different mutations. This can be determined by multiple round of backcrossing or by co-segregation analysis
Backcross of the mutant to wt is important to clean up the mutant.

Theoretically, each back cross removes half of the unlinked secondary mutations.

The probability that a particular unlinked mutation remains after n rounds of back crossing is $(1/2)^n$

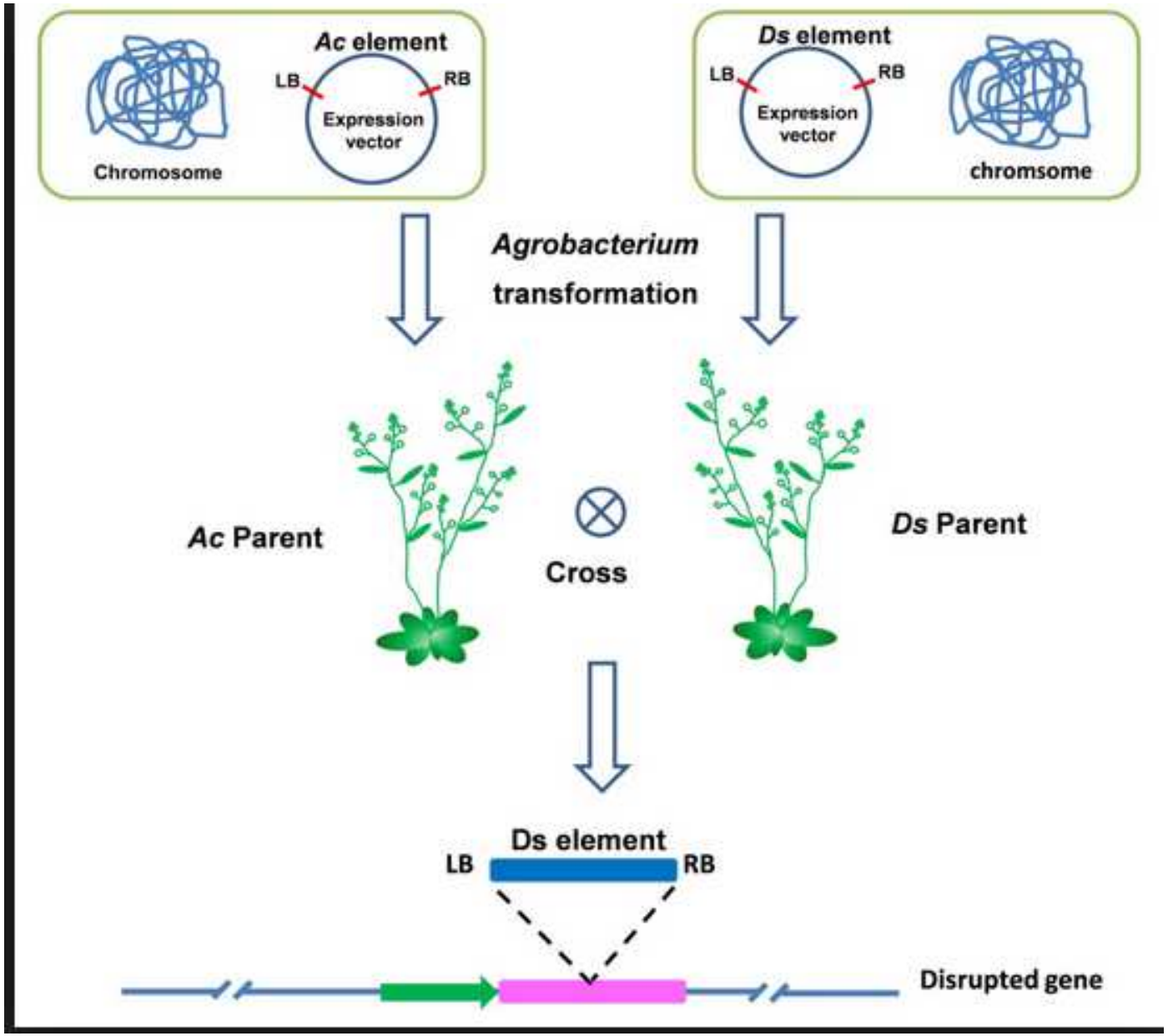
Analyze phenotype

Constraints on use of knockouts

Genes may have no obvious loss-of-function phenotype due to:

- presence of duplicate genes
- existence of alternative metabolic pathways and regulatory networks
- lethality

TRASPOSONI ED ACTIVATION TAGGING


Ac element
LB
RB
Expression vector
Chromosome
Ds element
LB
RB
Expression vector
chromsome
Agrobacterium transformation
Ac Parent
Cross
Ds Parent
Ds element
LB
RB
Disrupted gene

Mutagenesis by transposon tagging

- Two maize-derived transposons are used
  - » Ac/Ds Transposon
  - » (En1 Transposon)

- Ds is the transposon (does not encode a transposase)
- *Ac* is an immobile element encoding the transposase

- Ds transposon is maintained immobile because Ac (encoding transposase) is maintained in a separate plant line

- To mobilize the Ds trasposon you cross the plants and obtain an F1

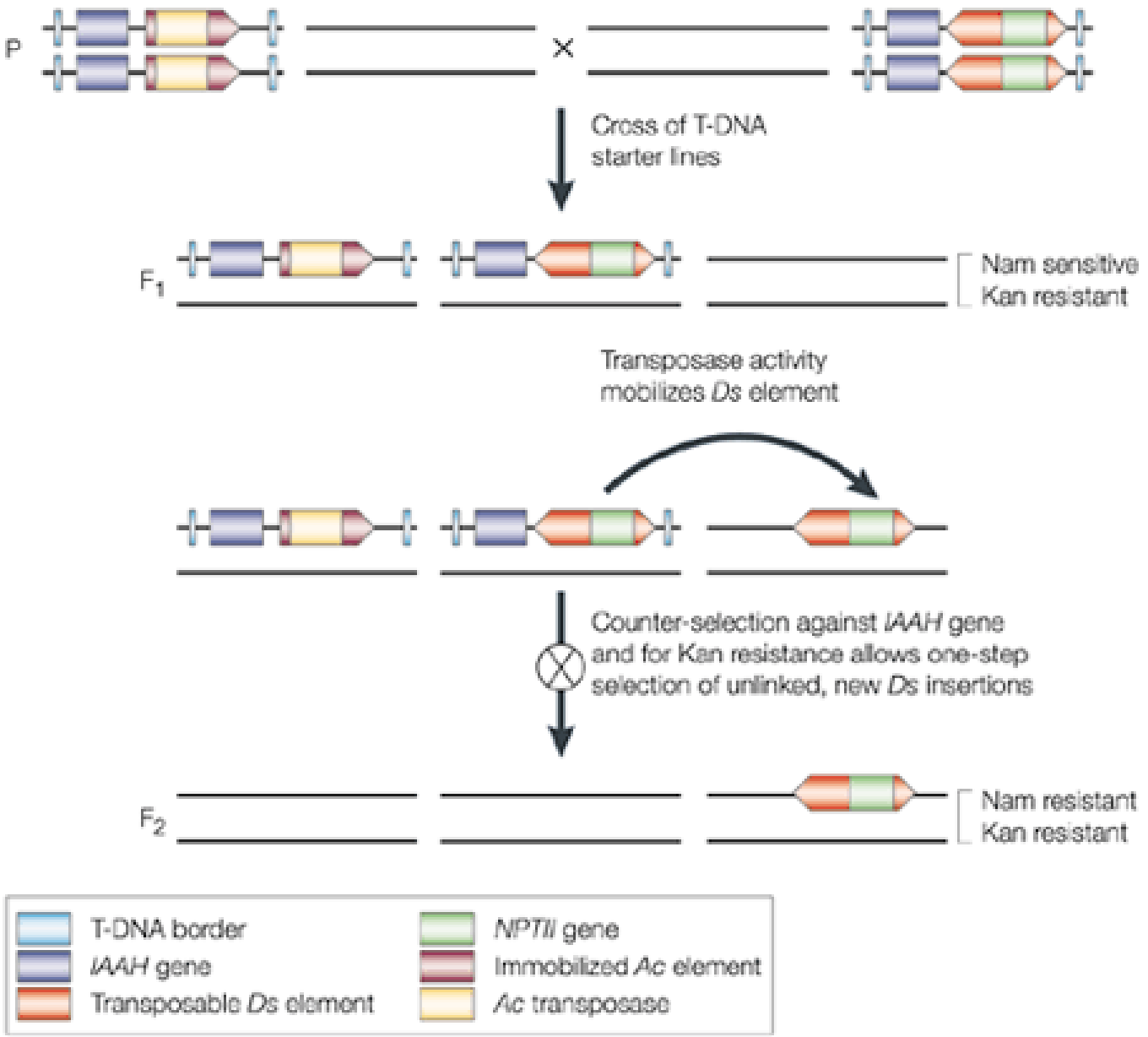
- Eventually you segregate out Ac to have stable lines

Transposon tagging

Ac-Ds system: contain 11 bp terminal inverted repeat (TIR), create 8 bp target site
duplication. The 4.5 kb *Ac* element codes for 3.5 kb mRNA for transposase.

En-Spm system: contain a 13 bp TIR and create a 3 bp target site duplication. *En*
element is 8.3 kb long and contains 2 alternately spliced gene products, *Tnp*A and
*Tnp*D. Both are required for transposition.

Robertson's mutator (*Mu*)/ *Mu*DR: Very mobile, short (1.5 or 1.7 kb) in maize. Cause high rate of mutation, somatic instability. *MuDR* encodes the MURA transposase required for *Mu* transposition and MURB, a helper protein implicated in insertion. All *Mu* elements share 215-bp terminal inverted repeat (TIR) sequences and the mobile *Mu* elements contain a highly conserved 32-bp MURA transposase binding site. Characteristic 9-bp host sequence duplications are generated during *MuDR/Mu* germinal insertion.


P
×
Cross of T-DNA
starter lines
F1
Nam sensitive
Kan resistant
Transposase activity
mobilizes Ds element
Counter-selection against IAAH gene
and for Kan resistance allows one-step
selection of unlinked, new Ds insertions
F2
Nam resistant
Kan resistant
T-DNA border
IAAH gene
Transposable Ds element
NPTII gene
Immobilized Ac element
Ac transposase

Constraints on use of knockouts

Genes may have no obvious loss-of-function phenotype due to:

- presence of duplicate genes
- existence of alternative metabolic pathways and regulatory networks
- lethality

- Gene function studies can be performed also on the basis of expression patterns or **gain-of-function** phenotypes. These screens are particularly useful for genes that have redundant and multiple functions during development.
- Before 1992 there was no technique to isolate gain-of-function mutants.

- **Activation tagging technology** was originally developed by Rick Walden at the Max Planck Institute.
- This technology has made the production of gain-of-function mutants possible.

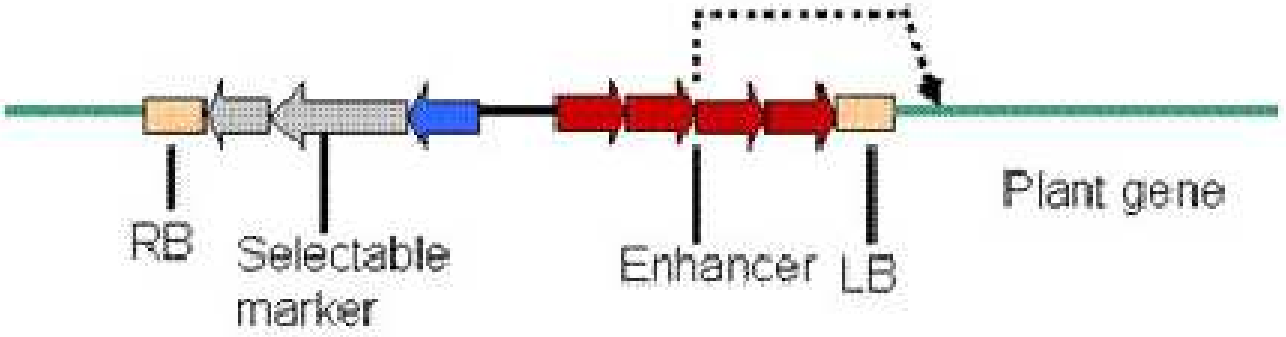
1) Generation of gain of function mutants by activation tagging

-In this technique instead loss of function in response to insertion element, gain of function takes place.

-The insertion element (T-DNA or transposon) carries an enhancer (or a a strong outward-facing promoter) and if, it integrates adjacent to an endogenous gene, the gene will be activated by the enhancer (or the promoter).

-This type of tagging causes over expression or ectopic expression of endogenous gene.

Activation TAG lines



The vector contains multimerized cauliflower Mosaic Virus (CaMV) 35S enhancer that can function in either orientation and can cause transcriptional activation of nearby genes resulting in dominant gain of function mutations.

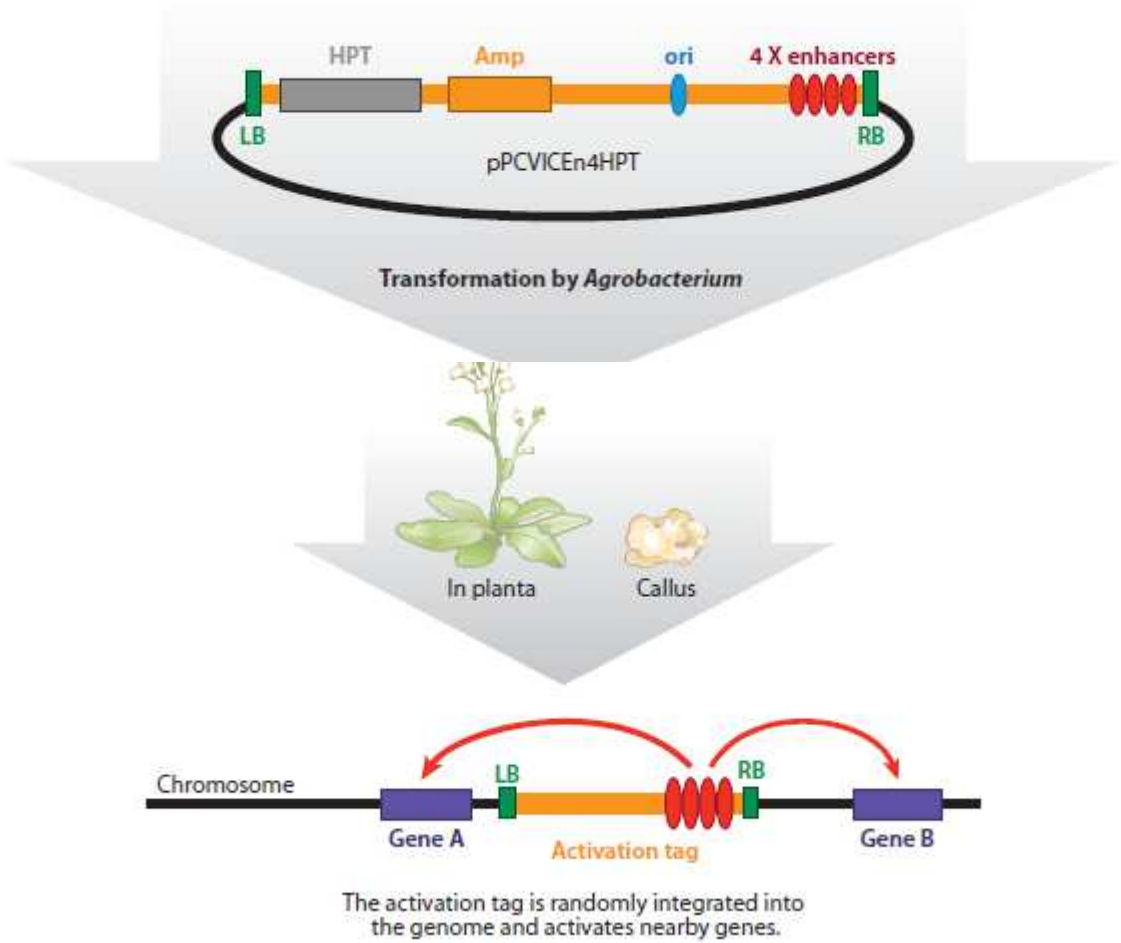


Figure 1

Overview of the activation-tagging system. The T-DNA region of the activation-tagging vector, pPCVICEn4HPT, contains tetrameric *CaMV 35S* enhancers (4 X enhancers), left (LB) and right border (RB) sequences, the hygromycin resistance gene (HPT) for the plant selection marker, the ampicillin resistance gene (Amp) for bacterial selection, and the replication origin (ori) for stable maintenance in *E. coli*. Amp and ori are necessary for plasmid rescue. Plants and callus can be transformed by the activation-tagging vector through *Agrobacterium*. T-DNA (the activation tag) is randomly inserted into the plant chromosome. *CaMV 35S* enhancers in the activation tag activate the nearby genes (Genes A and B) in the activation-tagged mutant.

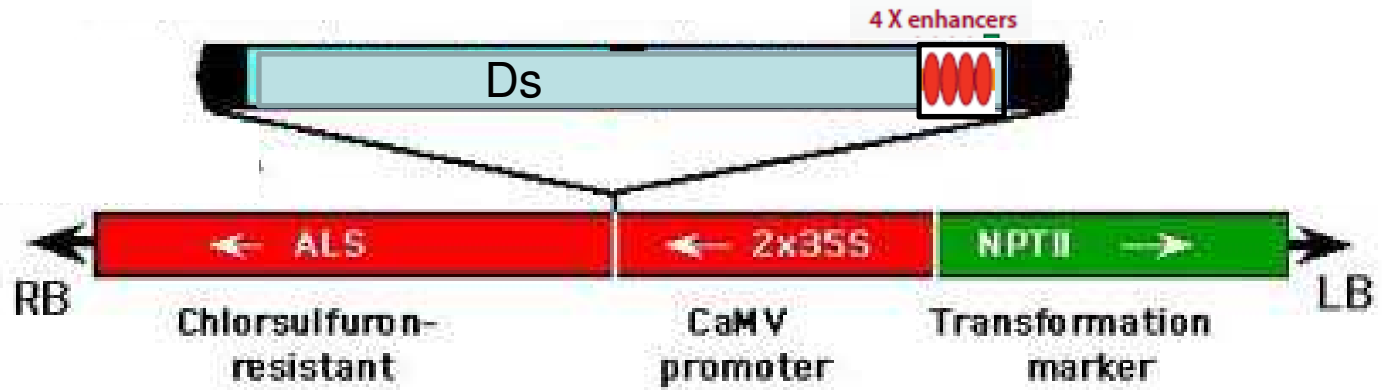
Transposon-based Activation Tagging

- Two transposons used:
  - Ac/Ds Transposon
  - En1 Transposon

Construct for transposon-based Activation Tagging

Figure 1: Structure of *Ac*- and *Ds*-(T-DNAs

a. Transposon T-DNAs



b. Transposase T-DNAs

Bam*Ac*

Conclusion

- Using Activation tagging numerous genes that play a vital role in plant growth and development are being isolated and maintained.
- This method is extensively used for cloning several genes in all on-going plant genomics research.

2) Gain-of-function mutants generated by ectopic expression of cDNAs Driven by a Constitutive Promoter

Arabidopsis lines that overexpresses random cDNAs driven by the *CaMV 35S* promoter.
More than 30,000 *Arabidopsis* transgenic plants have been generated

Problem cDNA may not be full length

(risk of dominant-negative effects due to truncated proteins)


d
1
2
3
4
5
6

Overexpression of your gene of interest to look at the phenotype is also a reverse genetics approach

Usually the gene is placed under the CaMV 35S promoter

Inducible promoter

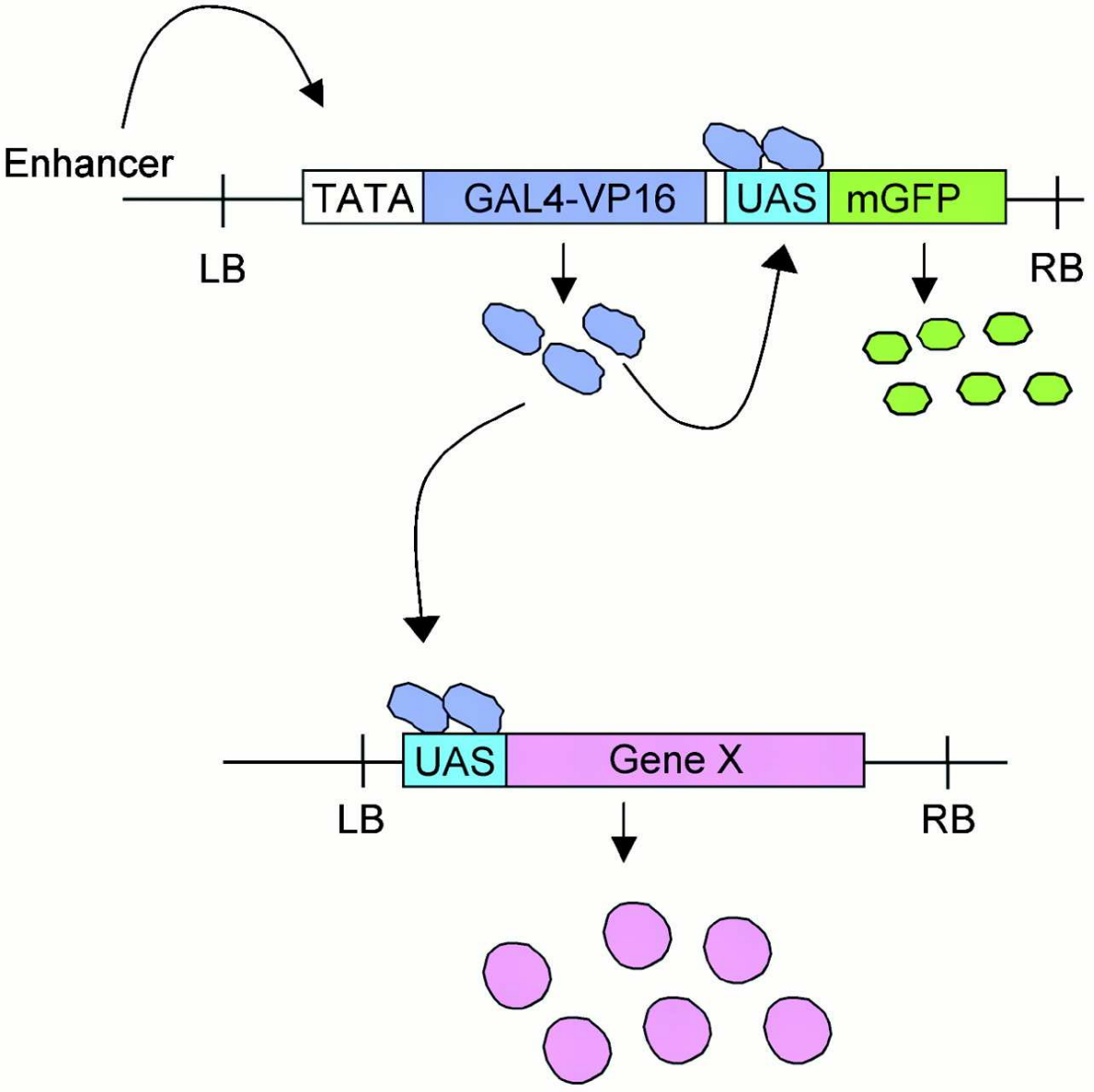
Dominant phenotype

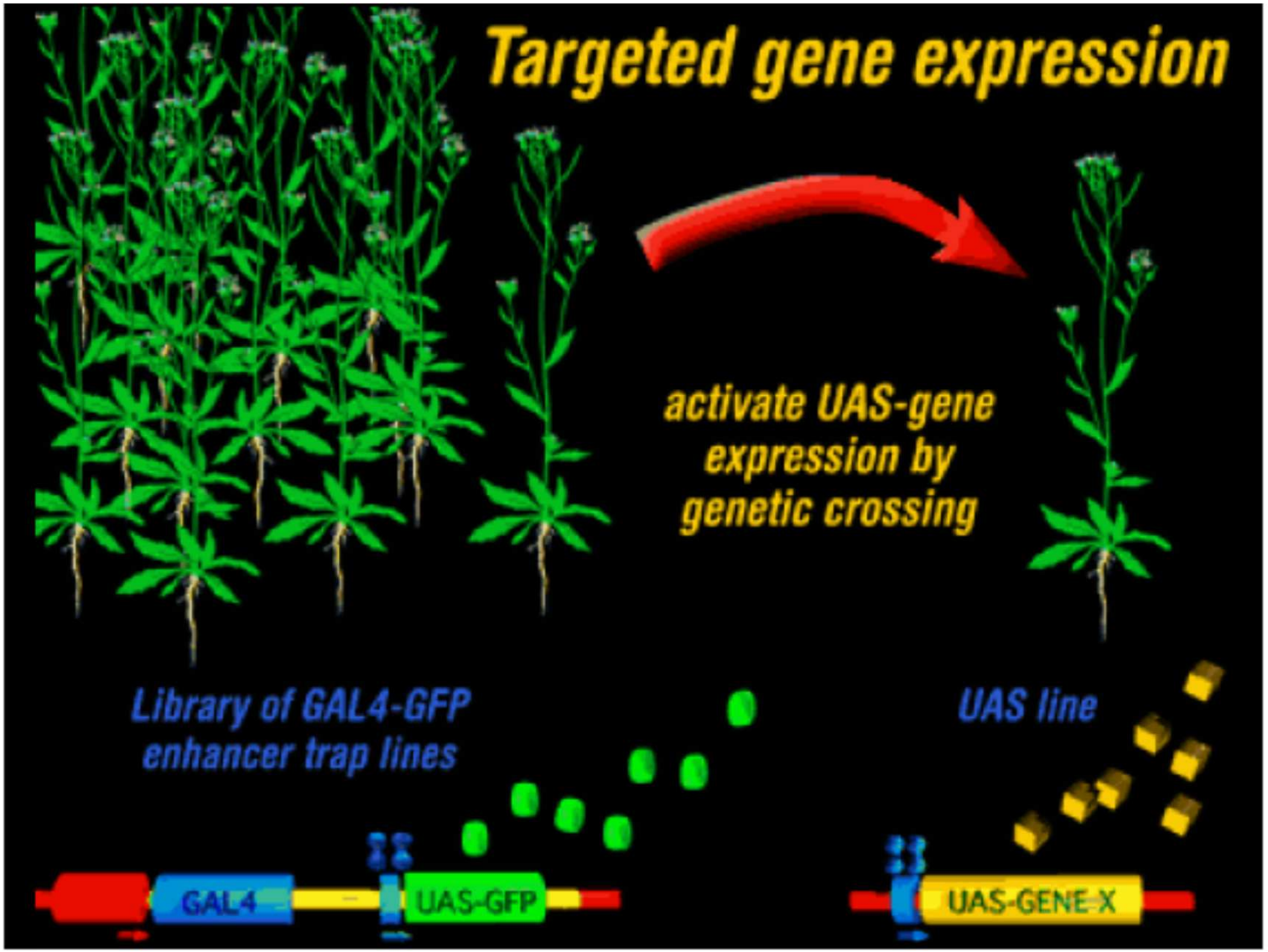
Espressione ectopica in specifici tessuti per generare fenotipi dominanti

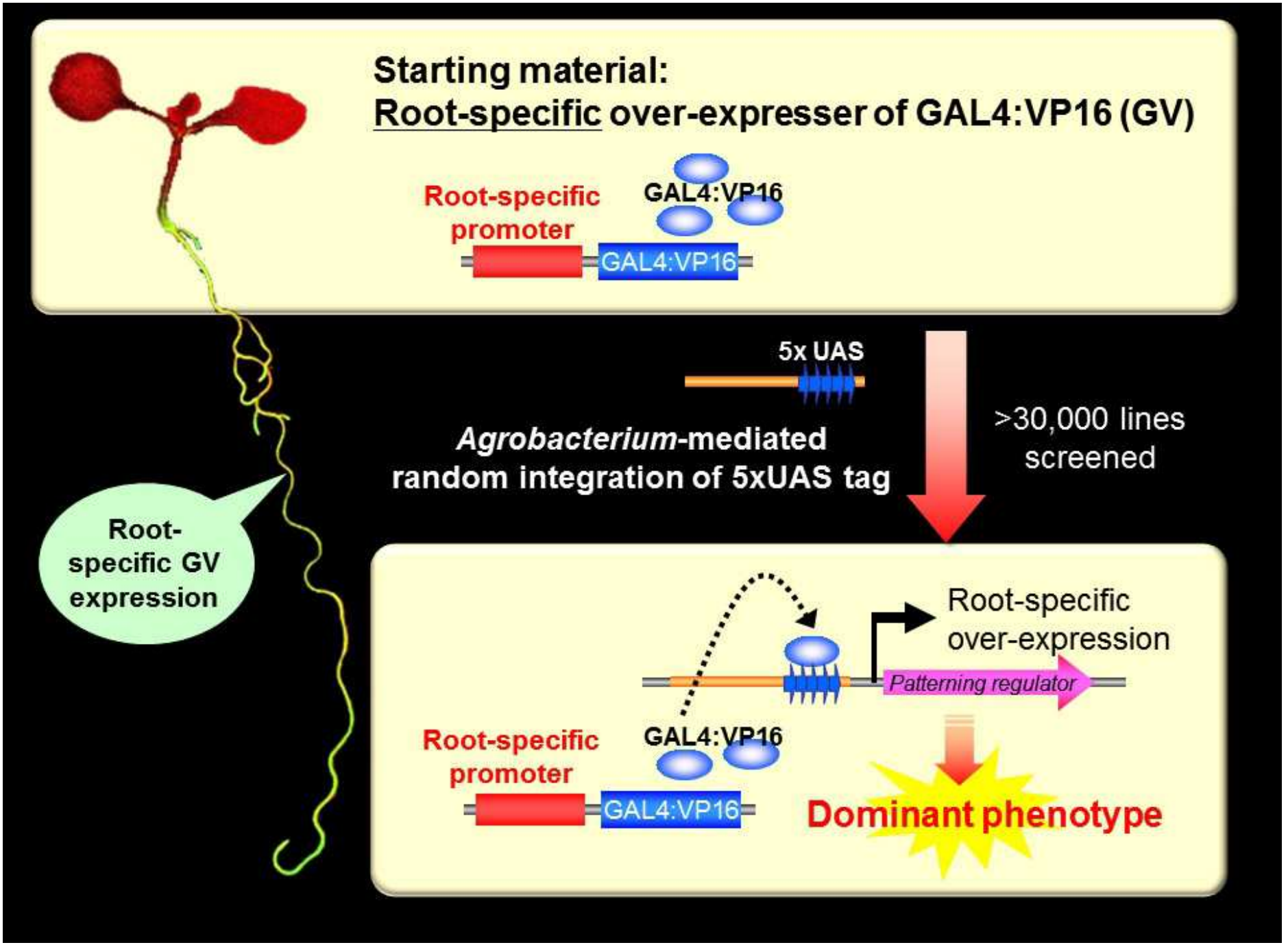
Sistema GAL4-UAS

- protocollo per attivare selettivamente l'espressione di un gene X in modo tessuto o cellula-specifico
- studio dell'effeto dovuto all'espressione ectopica di un gene X
- attivatore GAL4 e gene X sono clonati in linee diverse → si possono studiare anche i fenotipi letali
- le 2 linee vengono incrociate e il gene X verrà espresso solo in quelle cellule dove è espresso GAL4 → studio dell'effetto dell'espressione del gene X durante lo sviluppo

SISTEMA GAL4-UAS

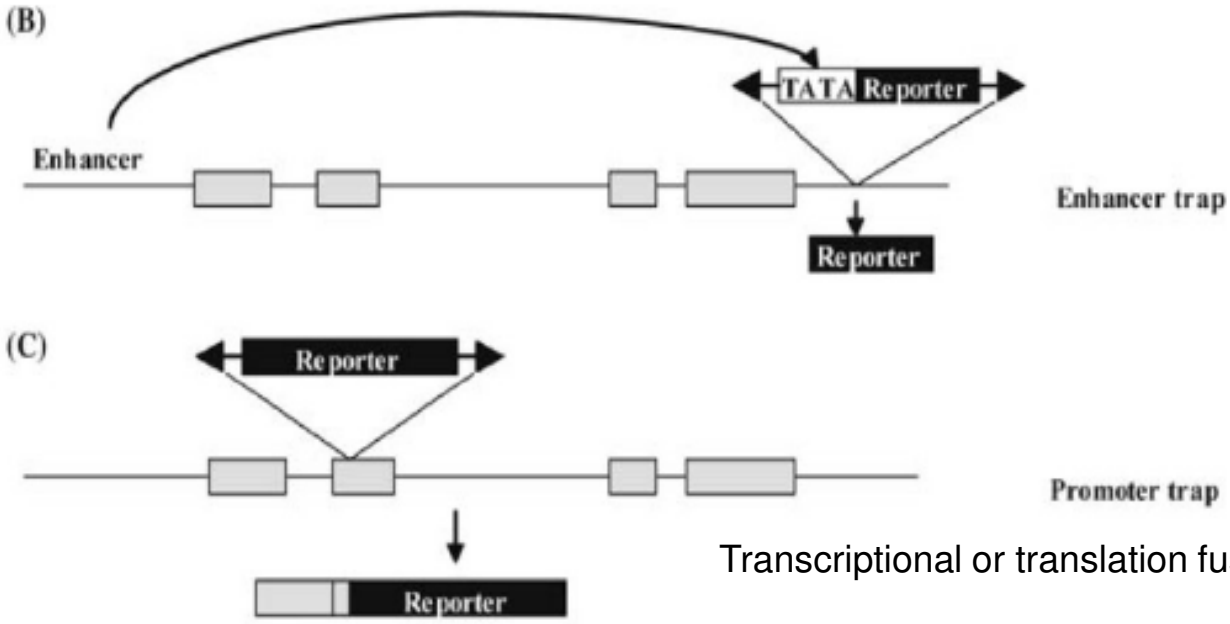



Targeted gene expression
activate UAS-gene expression by genetic crossing
Library of GAL4-GFP enhancer trap lines
UAS line
GAL4
UAS-GFP
UAS-GENE-X


Starting material:
Root-specific over-expresser of GAL4:VP16 (GV)
Root-specific promoter
GAL4:VP16
GAL4:VP16
5x UAS
Agrobacterium-mediated random integration of 5xUAS tag
>30,000 lines screened
Root-specific GV expression
Root-specific over-expression
Patterning regulator
Root-specific promoter
GAL4:VP16
GAL4:VP16
Dominant phenotype

Promoter/ enhancer trapping

To find genes and corresponding regulatory sequences with specific expression patterns



Transcriptional or translation fusions are possible

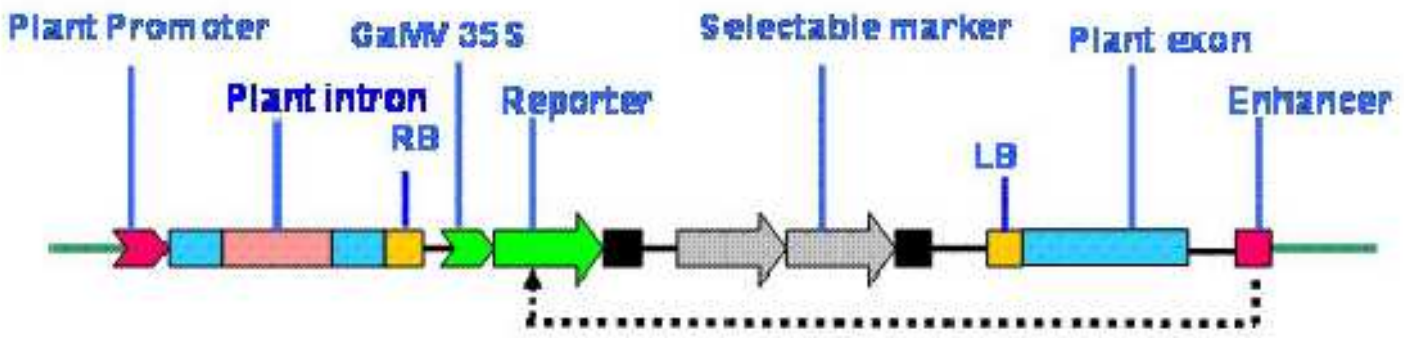
An et al., 2005 PMB 59, 111

Enhancer/promoter trap vectors

-In these vectors, the insertion element contains a visible marker gene such as *Gus A* (Encoding *β- glucuronidase)* or GFP promoterless or downstream of a minimal promoter (that requires an enhancer for activity).

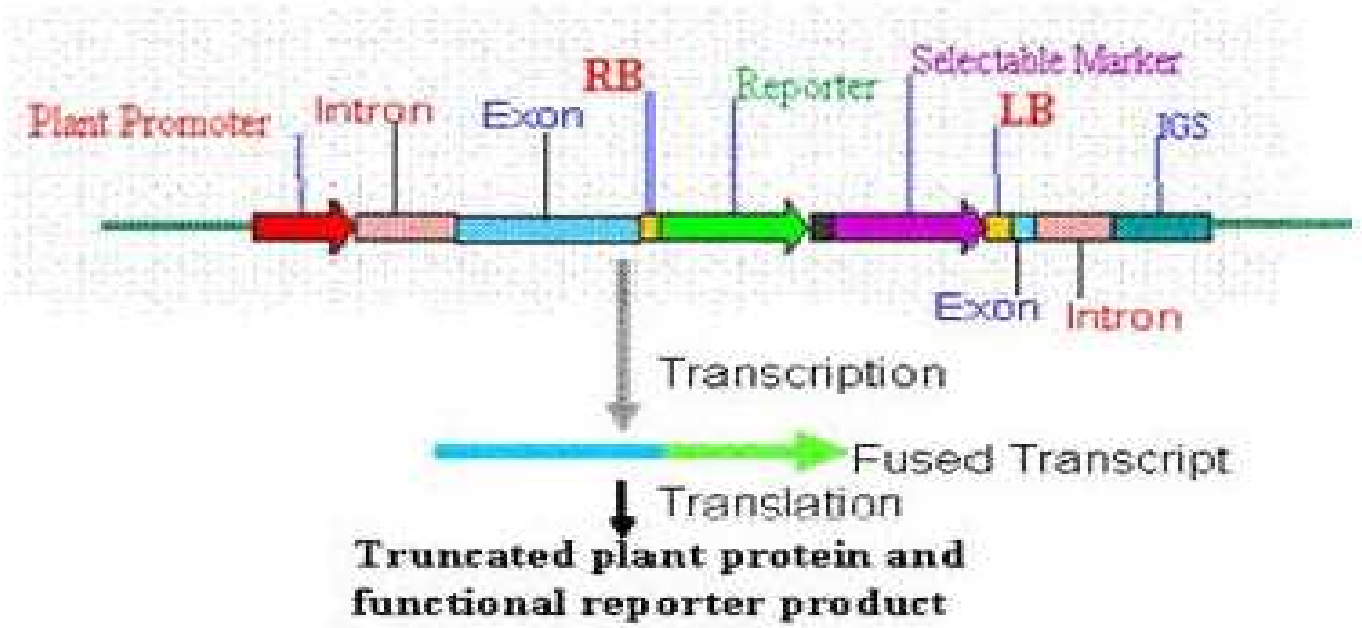
Note- The marker gene will be expressed only when the element insert in proximity of a transcriptional unit (minimal promoter construct) or within the transcription unit of a gene generating either a transcriptional or an in-frame translational fusion (promoterless construct).

Enhancer Trap lines



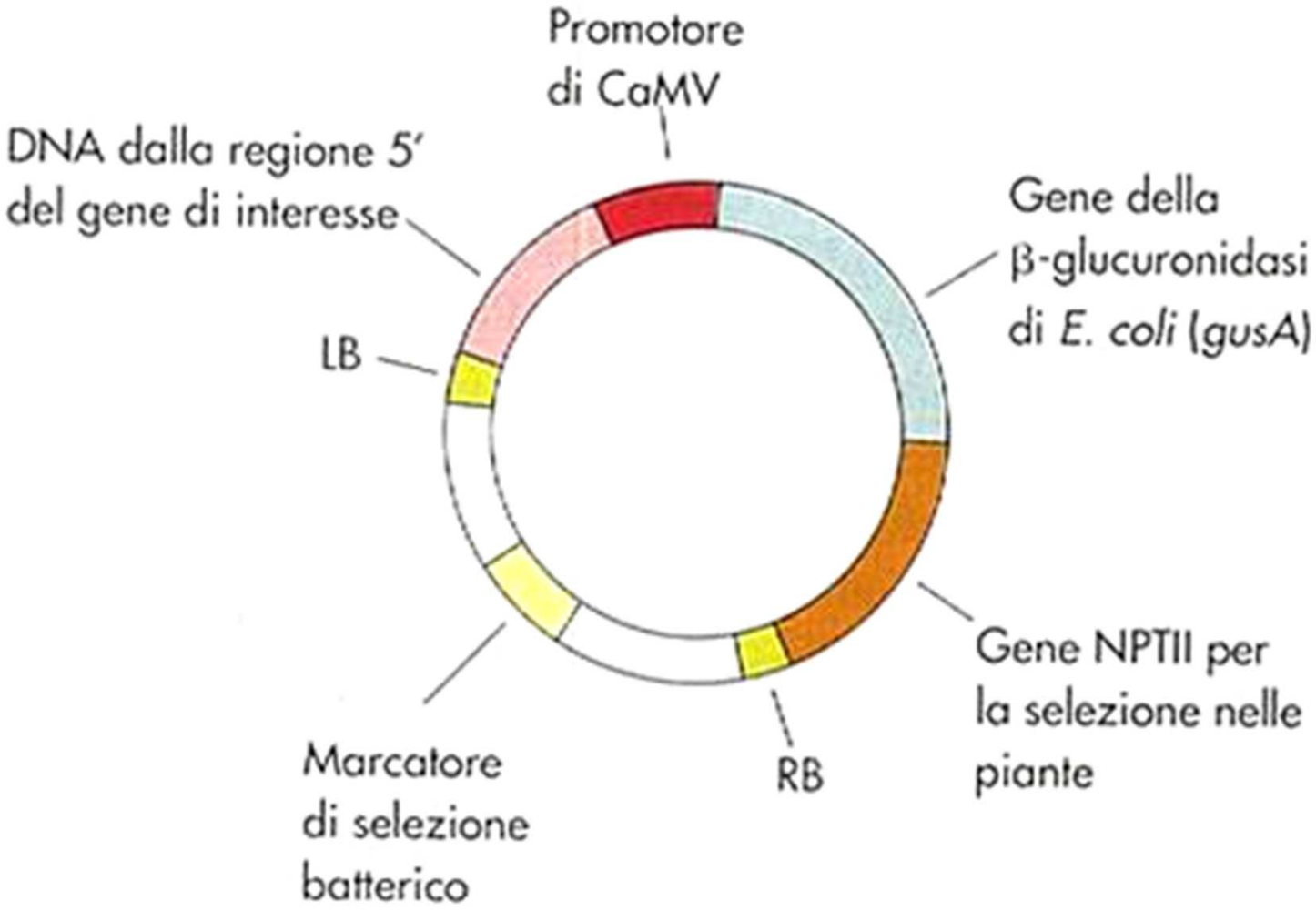
The minimal promoter of the reporter gene is activated by a chromosomal enhancer element, resulting in over-expression of the reporter gene.

Promoter Trap lines



The promoterless reporter gene can be expressed when insertion of a promoter trap vector occurs in an exon so as to result in a transcriptional fusion and disruption of native gene product.

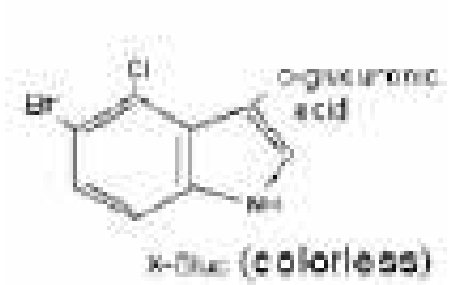
Analisi dell'espressione genica mediante l'uso di geni reporter



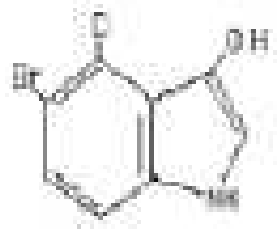
GUS

- GUS or β-glucuronidase is an *E.coli* K12 enzyme encoded by the *gusA* gene
- Classically used in plants
- Oxidative dimerization is required for formation of the insoluble blue pigment

GUS



GUS

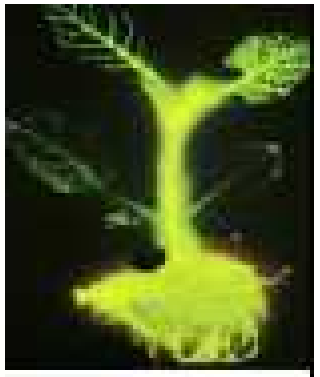


Glucuronic acid

Oxidation dimerisation

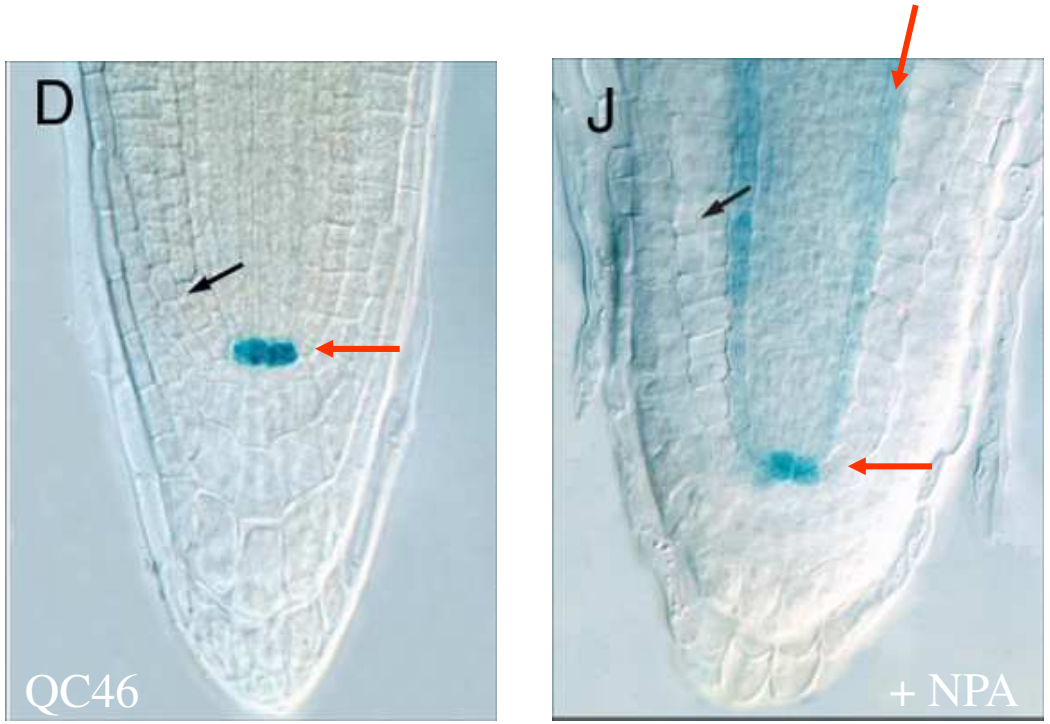
Insoluble, blue product

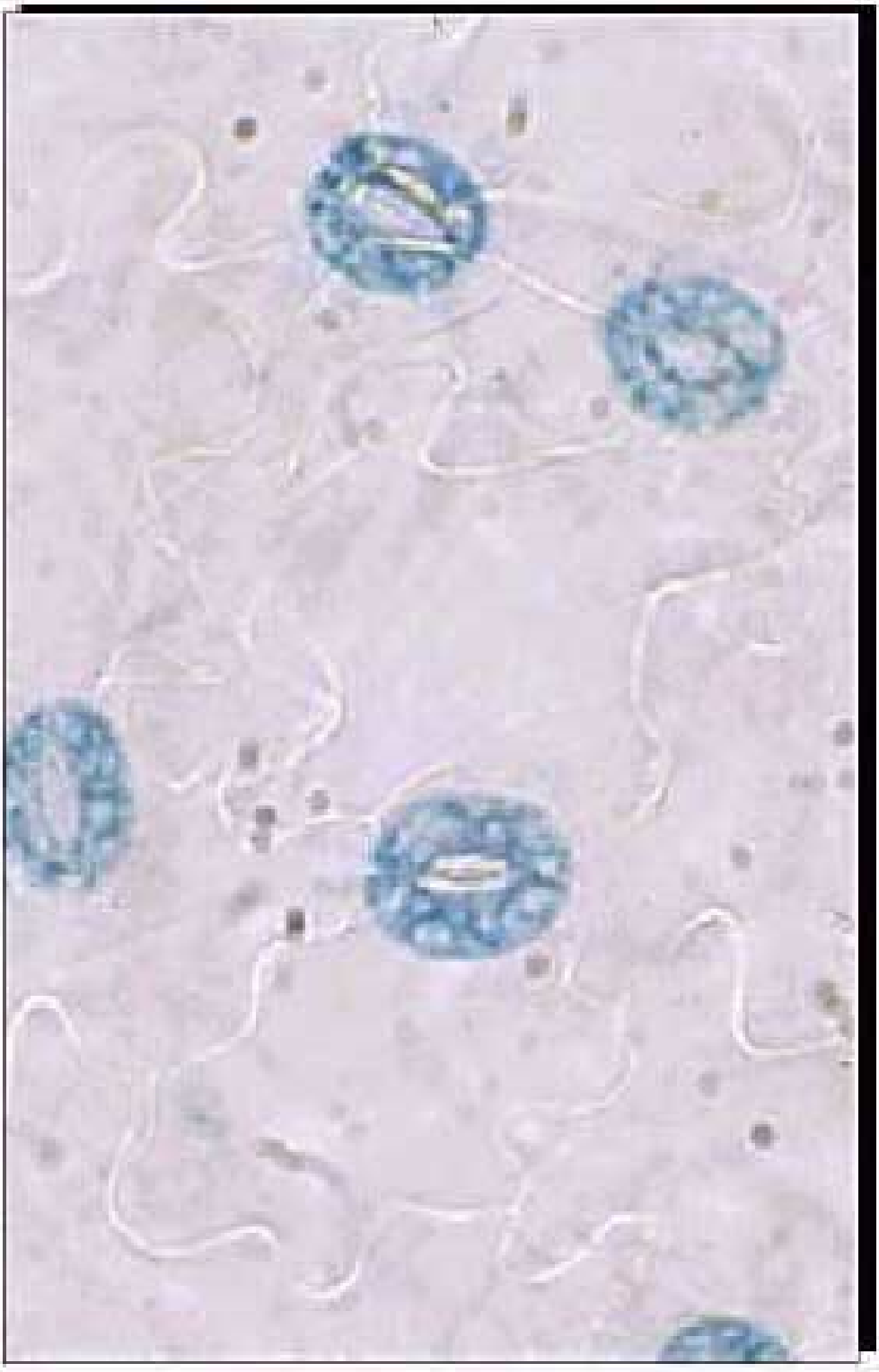
Luciferase

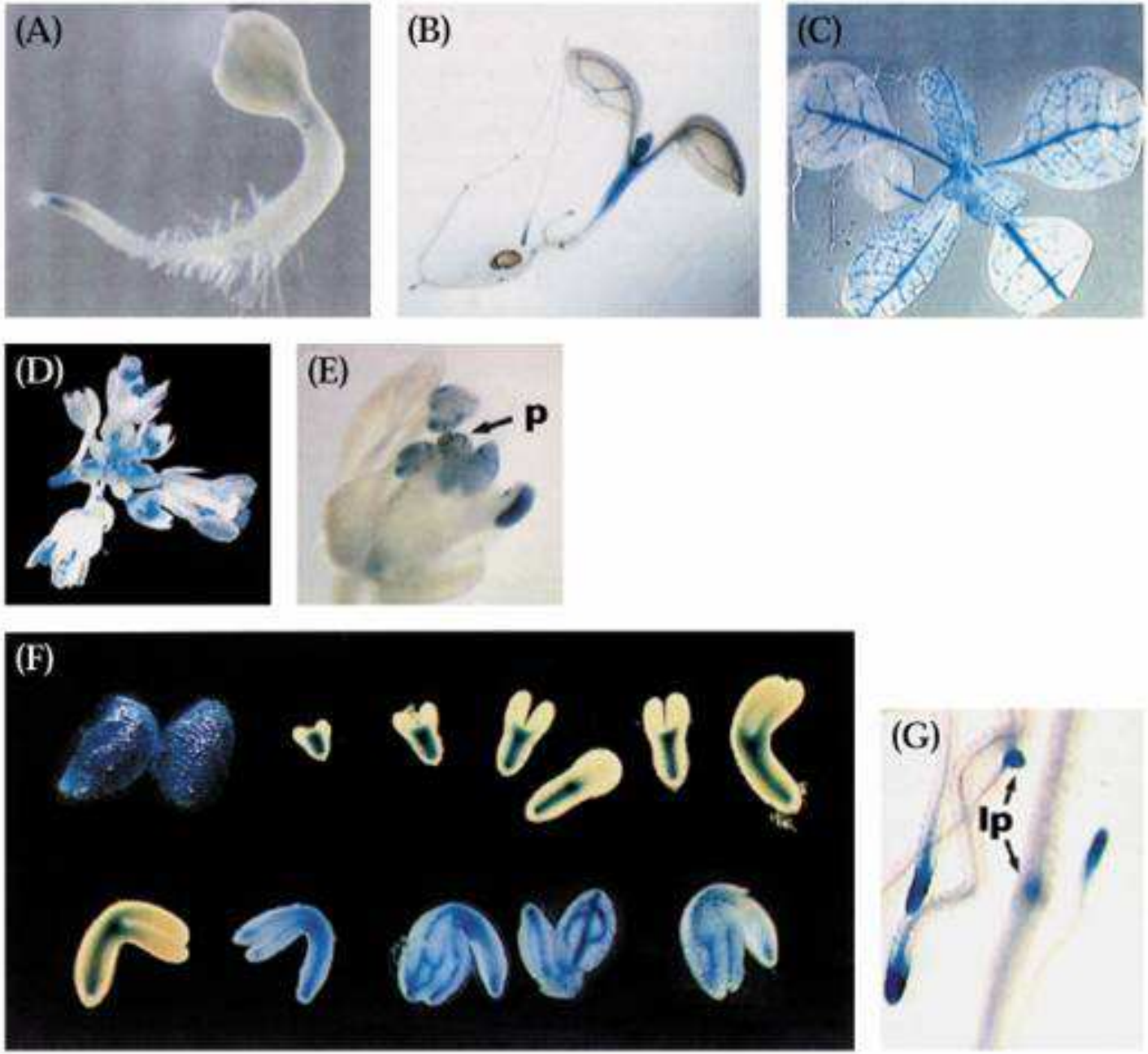


- Initially cloned from North American Firefly
- Enzymatic reaction emits yellow-green light (560nm)
- Requires ATP, Mg^{2+} O_2 and luciferin
- Usually detected using a luminometer

Beta-glucuronidasi batterica (GUS)






(A)
(B)
(C)
(D)
(E)
P
(F)
(G)
lp

GFP – Green Fluorescent Protein

-1962: Osamu Shimomura et al.

-*Aequorea victoria*,

-238 Aminoacids

-26.9 kDa

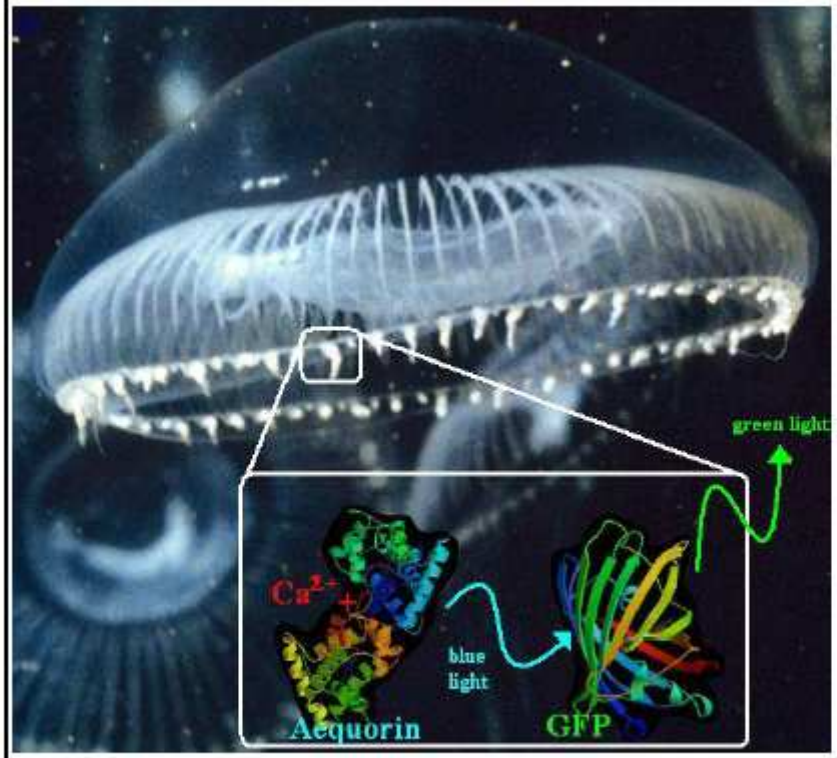
-Fusion-protein Marker since 1994: Doug Prasher et al.

-Unique quarternary structure for fluorescence

XFPs originate from reefs

Coral – *Zoanthus*

Jellyfish - *Aequorea*

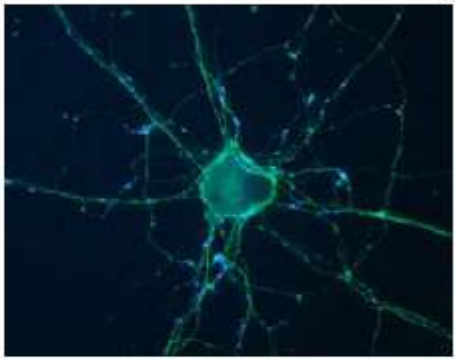


GFP found in Aequorea victoria – a jellyfish.

Jellyfish actually glow blue because of aequorin.

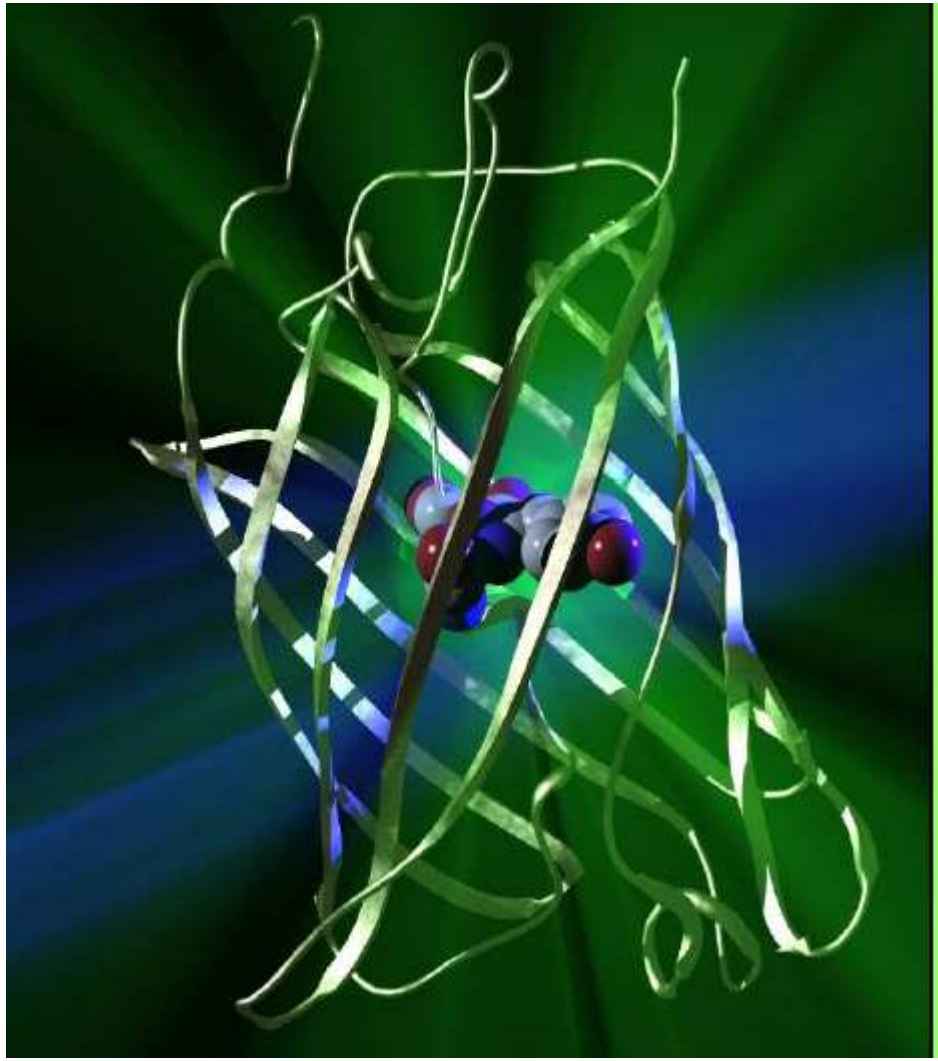
Calcium ions bind aequorin, which then emits blue light. This blue light is absorbed by GFP which then emits green light.

GFP in use



www.physiology.wisc.edu/ chapman.html

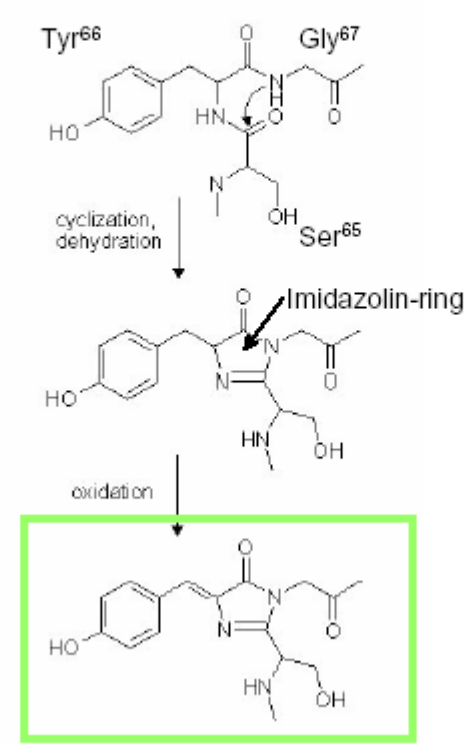
- can be used to tag proteins within a cell or visualize specific cell types in an organism
- Fluorescence lasts ~10mins when illuminated by 450-490nm (340-440nm get some photobleaching)



3D-Structure of GFP

- "Paint in a can"
- Each monomer composed of a central helix surrounded by an eleven stranded cylinder of anti-parallel beta-sheets (shields fluorophore from solvent)
- Cylinder has a diameter of about 30A and is about 40A long
- Fluorophore is located on the central helix

The Fluorophore – Protein Maturation



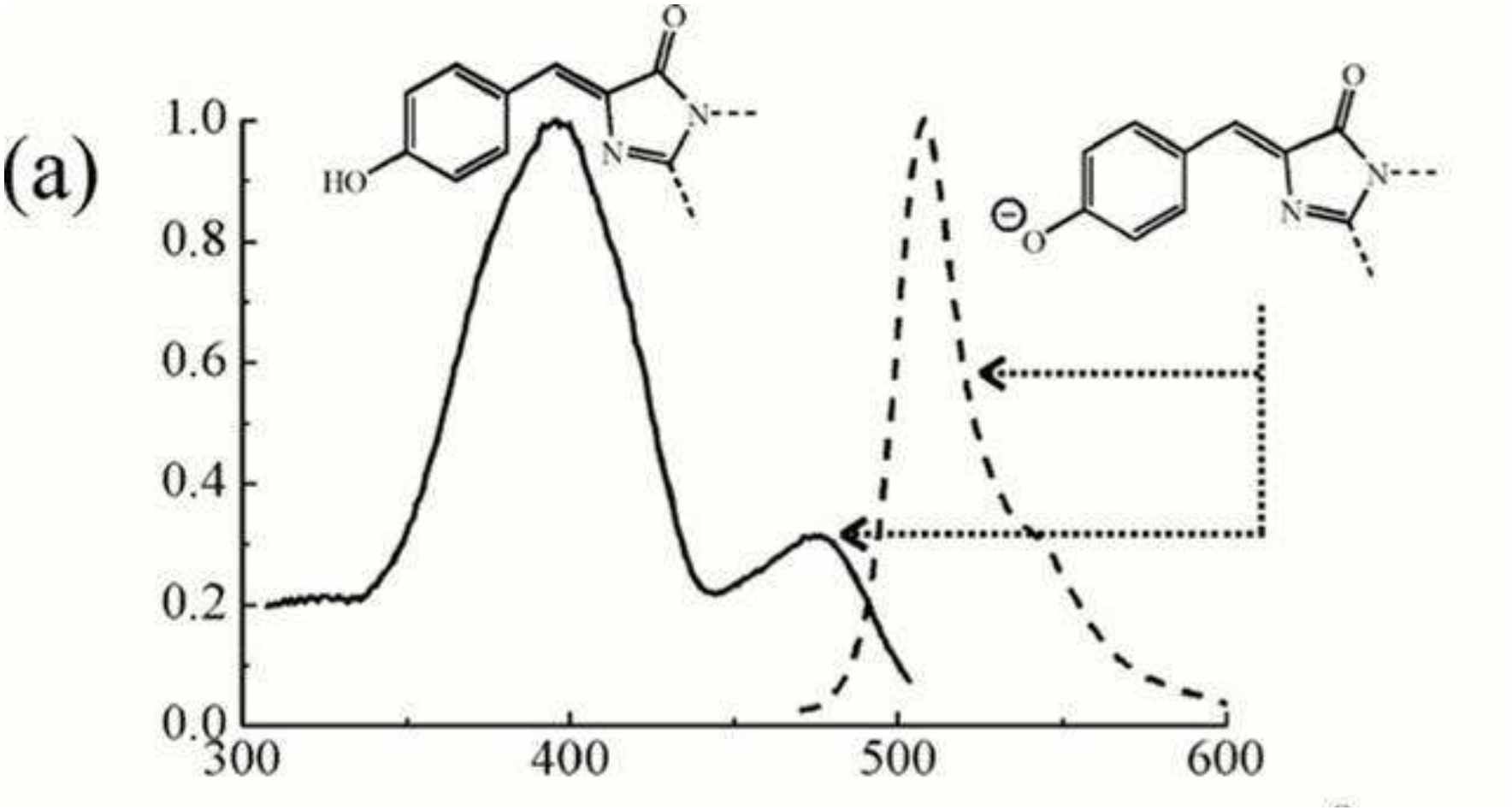
- 3AS: Serine65, Tyrosine66, Glycine67
- 2Step maturation:

  1)Cyclization, dehydration: formation of Imidazolin-ring

  2)Oxidation: Extension of conjugated pi-electron system (reversible with reducing agents)

→ Environment matters:

- Oxidizing Environment
- Thermostable but temperature sensitive: Lower temperature →better protein folding.
- Long maturation time (up to 6h)



395nm 475nm

506nm

XFP Mutants

Single AS substitutions make the difference:

-Wavelength shifts for excitation 395nm wtGFP → 488nm EGFP

-Wavelength shifts for emission: RFP, CFP, YFP… → better reporter systems

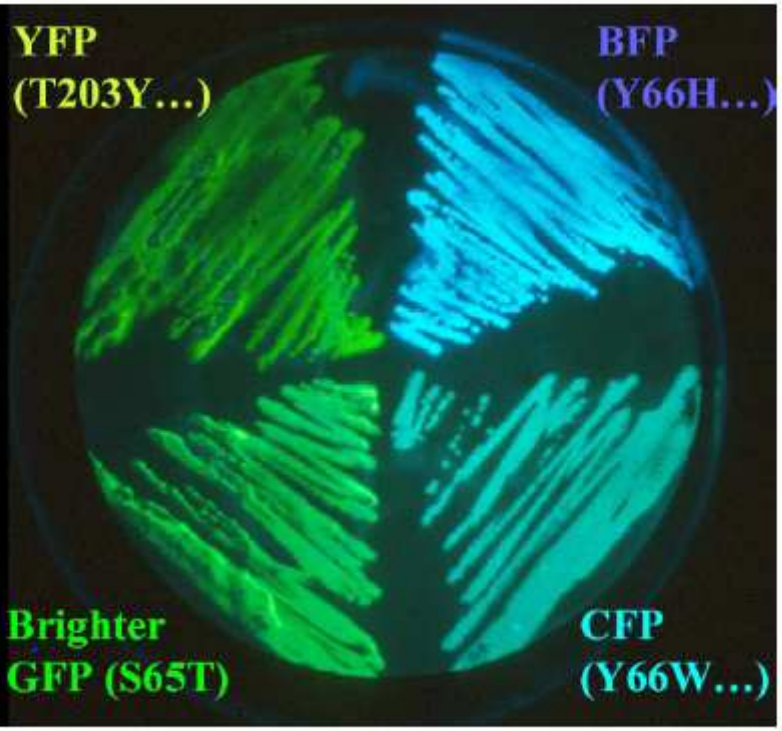
-Enhanced expression in mammalian cells

-Human codon optimization for expression in mammalian cell-lines

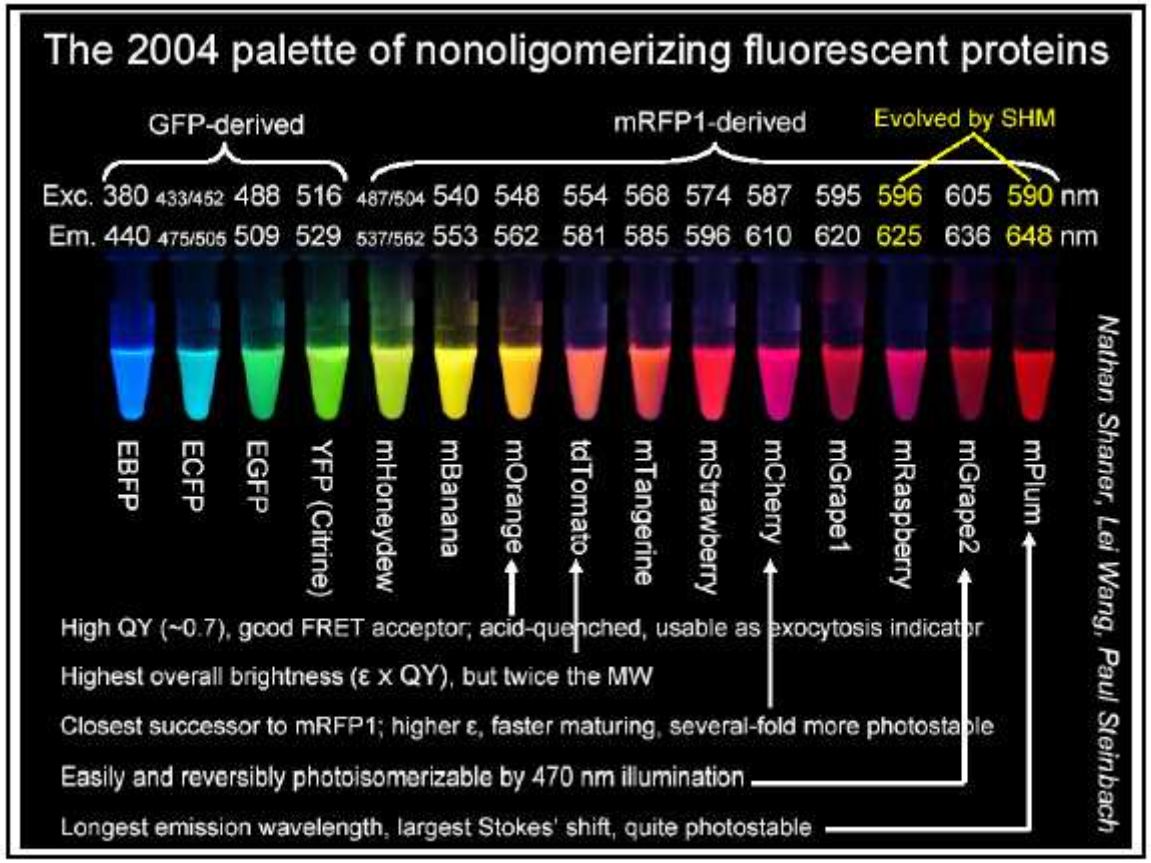
-Enhanced fluorescence: GFP-Ser65Thr → 4-6 fold

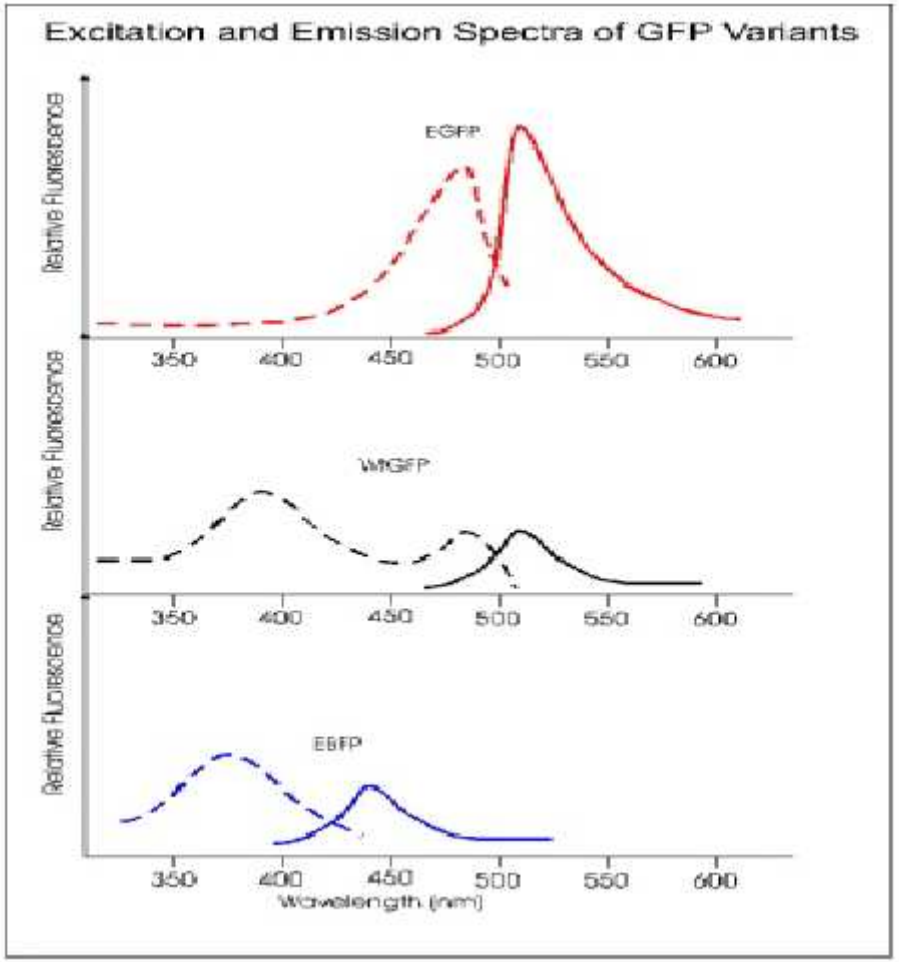
EGFP-Phe64Leu → up to 35 fold

4 colors of GFP mutants expressed in *E. coli*

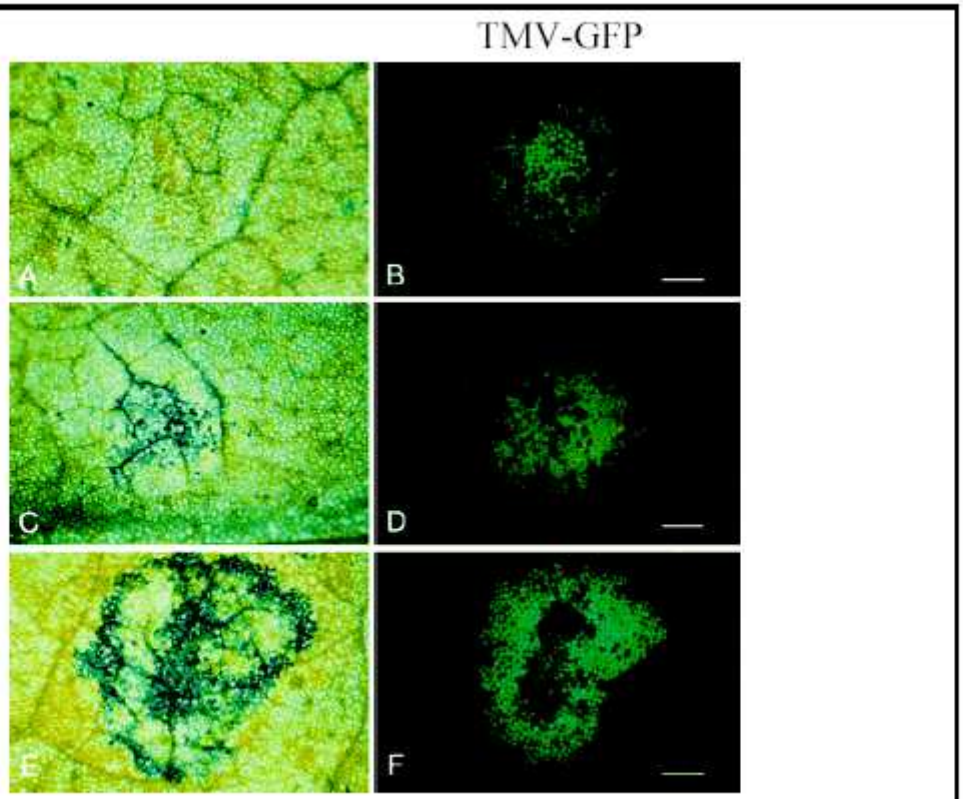


R. Heim, A. Cubitt


The 2004 palette of nonoligomerizing fluorescent proteins
GFP-derived
mRFP1-derived
Evolved by SHM
Exc. 380 433/452 488 516 487/504 540 548 554 568 574 587 595 596 605 590 nm
Em. 440 475/505 509 529 537/562 553 562 581 585 596 610 620 625 636 648 nm
EBFP
ECFP
EGFP
YFP (Citrine)
mHoneydew
mBanana
mOrange
tdTomato
mTangerine
mStrawberry
mCherry
mGrape1
mRaspberry
mGrape2
mPlum
High QY (~0.7), good FRET acceptor; acid-quenched, usable as exocytosis indicator
Highest overall brightness (ε x QY), but twice the MW
Closest successor to mRFP1; higher ε, faster maturing, several-fold more photostable
Easily and reversibly photoisomerizable by 470 nm illumination
Longest emission wavelength, largest Stokes' shift, quite photostable
Nathan Shaner, Lei Wang, Paul Steinbach


Excitation and Emission Spectra of GFP Variants
Relative Fluorescence
EGFP
350
400
450
500
550
600
Relative Fluorescence
WtGFP
350
400
450
500
550
600
Relative Fluorescence
EBFP
350
400
450
500
550
600
Wavelength (nm)

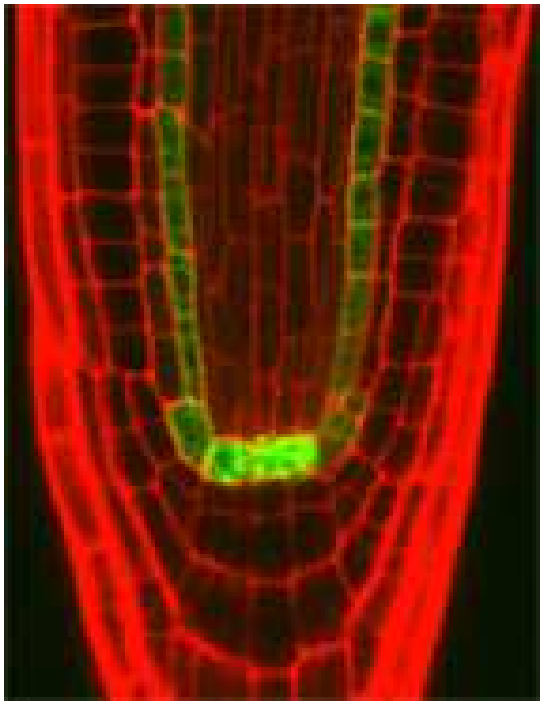

MICE EXPRESSING GFP
Courtesy of Advanced Cell Technology



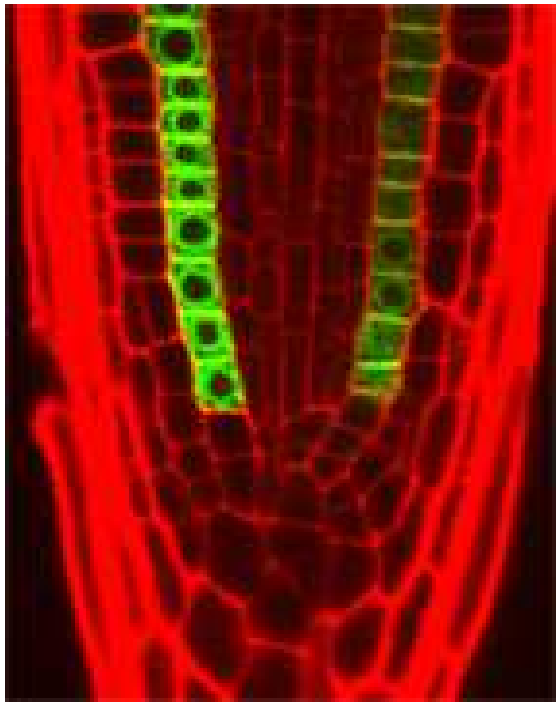
Plant Physiol. August 2000, Vol. 123, pp. 1375-1386

Proteina verde fluorescente ("Green fluorescent protein" o GFP

clonata da una medusa

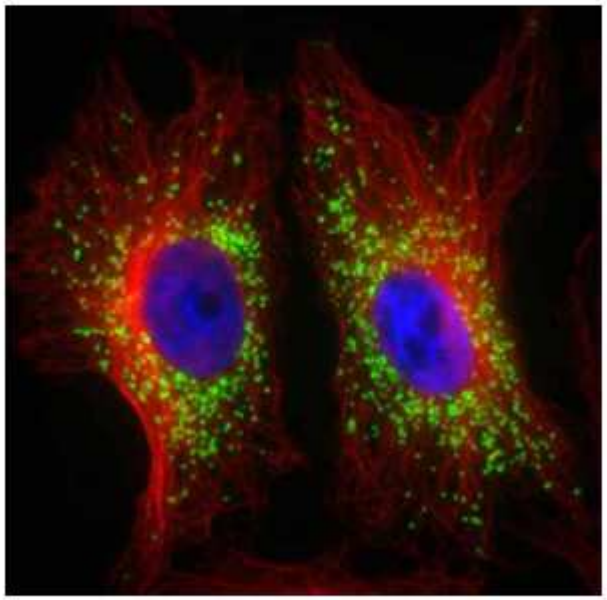


wt / pSCR::GFP



scr-1 / pSCR::GFP

Confocal Microscopy



- XFP Tagged Proteins
- Specific antibodies for XFPs
- Excitation filter at 488nm
- Emission filter at various wavelenth
- FRET (fluorescence resonance energy transfer)
- Precise protein localization?
- Organic solvents such as: methanol, ethanol, acetone do not preserve XFPs
- fixing with paraformaldehyde

Importance of GFP

- Before GFP, molecules used to visualize in vivo needed co-factors (β-gal, firefly, bacterial luciferase)
- Since GFP is autocatalytic, no need for co-factors
- Now GFP is widely used for in vivo studies

(confocal microscopy)

Seminars on Signal
Transduction

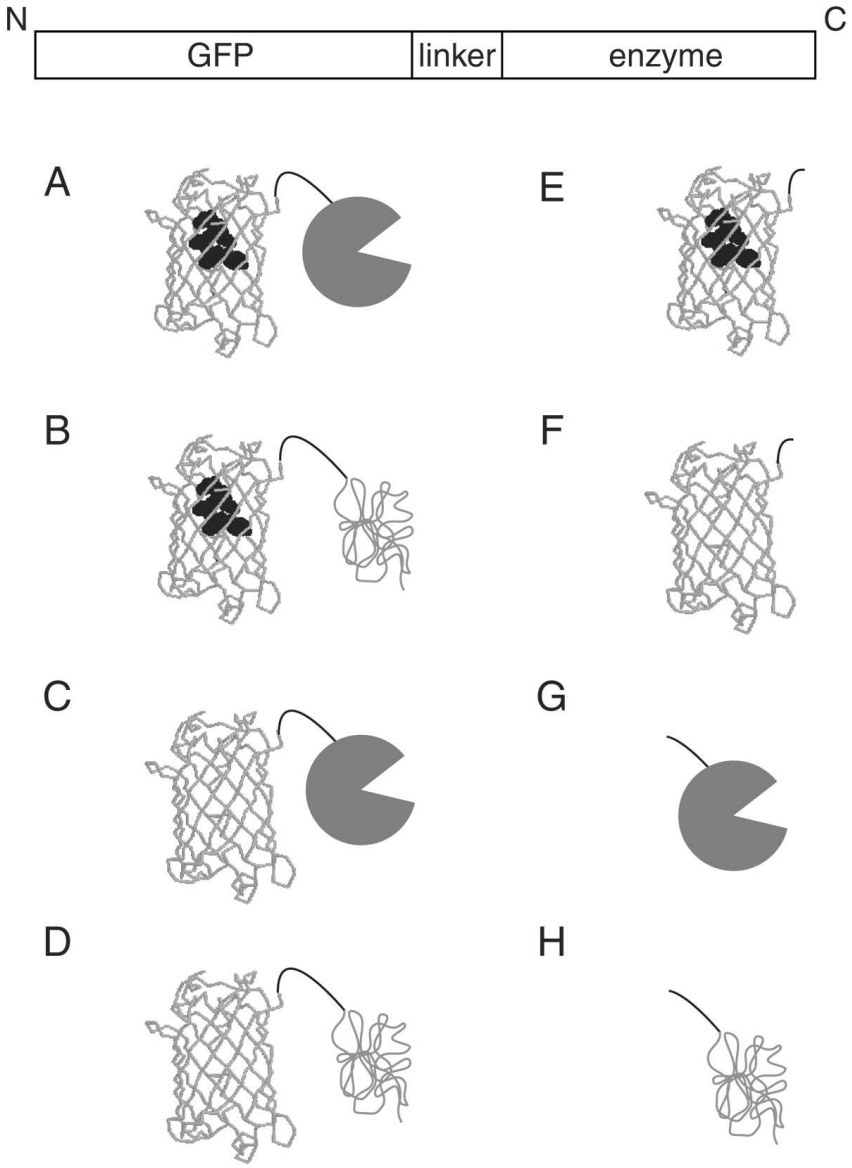


Figure 4. Some Possible Products inside Cells When a Chimeric Protein GFP Enzyme Is Expressed

The primary structure of the chimeric protein is at the top of the figure.

(A) The integrity of the chimeric protein is perfect.

(B) GFP is fluorescent, but the enzyme is unfolded.

(C) GFP is not fluorescent, but the enzyme is functional.

(D) GFP is not fluorescent, and the enzyme is not functional.

(E–H) Possible proteolyzed products when the linker is cleaved.

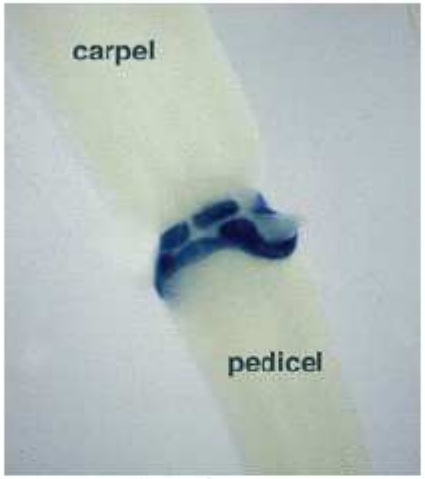
Enhancer trap in *Arabidopsis*

La tecnica dell' enhancer trap permette inoltre di identificare nuovi geni basandosi sul loro pattern di espressione.
L'analisi di linee enhancer trap di *Arabidopsis* si basa sulla selezione di linee che mostrano uno specifico pattern d'espressione e non su fenotipi mutati.
Un vantaggio di questa tecnica rispetto all'analisi di mutanti consiste nel fatto che anche se l'inserzione del T-DNA (enhancer trap) avviene in un gene essenziale la pianta sarà in quasi tutti i casi vitale essendoci nella generazione T_1 una condizione di emizigosi.
La possibilità di disporre di linee di *Arabidopsis* enhancer trap permette anche di ottenere, mediante incroci, piante in cui si può far esprimere un determinato gene in un limitato numero di cellule o tessuto.

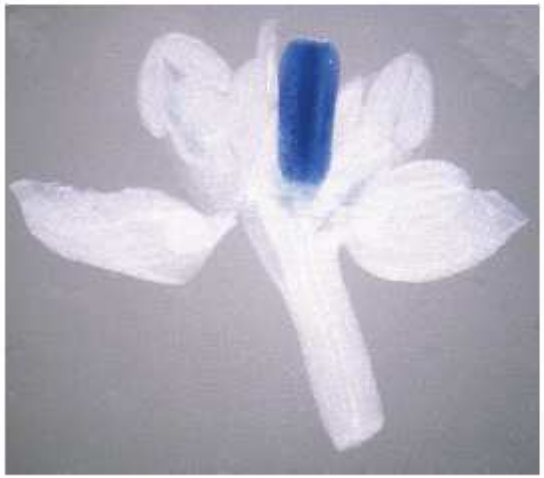
Enhancer trap (GUS) in *Arabidopsis*

Selection of the desired mutants is based on the expression profile of the reporter gene

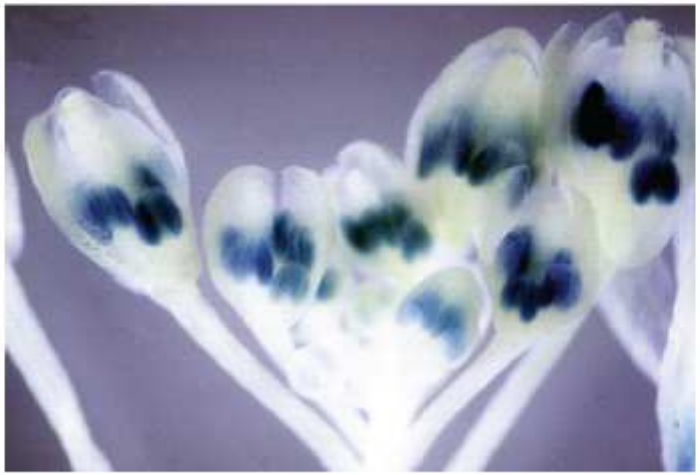
Espressione diffusa



Zona di abscissione



Carpello specifica



Antera specifica

Petalo specifica

Enhancer trap (GUS) in *Arabidopsis*

- Primo progetto di enhancer trap su larga scala
- sono state generate più di 11,000 linee enhancer trap.
- semi di queste linee sono disponibili per la distribuzione sia al "Arabidopsis Biological Resource Center" (**ABRC**) presso l'università dell'Ohio sia al "Nottingham Arabidopsis Stock Center" (**NASC**).
- Il T-DNA con cui le piante sono state trasformate contiene il promotore minimale -60CaMV fuso al gene per la β-glucuronidasi (GUS).
- dopo l'integrazione, elementi enhancer che si trovano sul DNA genomico adiacente al T-DNA provocano un aumento della in trascrizione a partire dal promotore minimale e si osserva una aumento del livello dell'espressione di GUS
- l'espressione di GUS risulta spesso essere cellula-, tessuto- o organo-specifica come specificato dall'enhancer. Questa espressione può essere visualizzata mediante la colorazione per il GUS usando il substrato cromogenico X-gluc
- 31% delle linee mostra colorazione a livello delle infiorescenze

Limitations

- gene trap strategy selects for insertions into genes -> is very useful in plants with large amount of non-genic DNA

- Dependency on ‘in frame’ insertions -> two-third of all “hits” on genes were not recognized

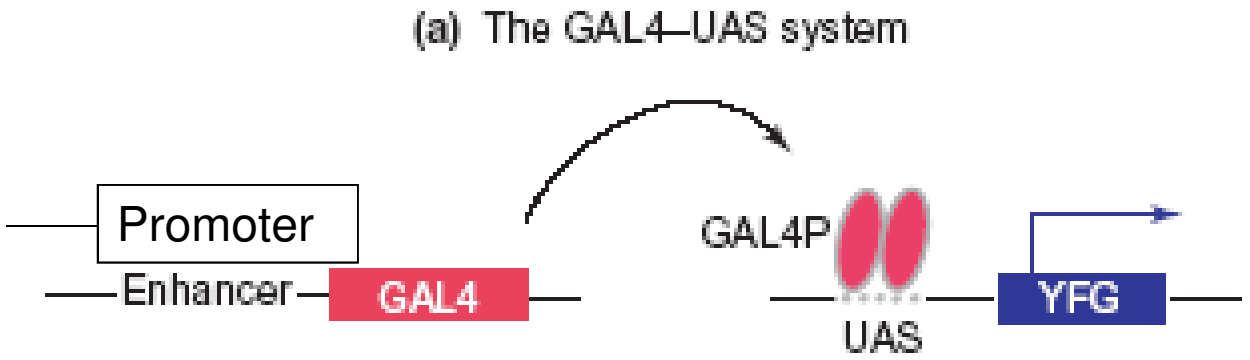
- expression of marker relies on the transcriptional activity of the surrounding gene, and therefore, non-expressed genes are not detected

Solution

-The use of ribosome entry sites (IRES) bypasses the need for in-frame design, it greatly increases the hit rate of gene traps.

A bipartite system for promoter/enhancer trapping

The bipartite GAL4-UAS system



- GAL4 is a transcriptional activator from yeast that recognizes a DNA sequence called the UAS (upstream activating sequence)

- The UAS to which Gal4 binds is CGG-N_{11}-CCG, where N can be any base

- We can use this to control expression of YFG in a tissue specific manner by using enhancer elements specific for the tissue we are interested in

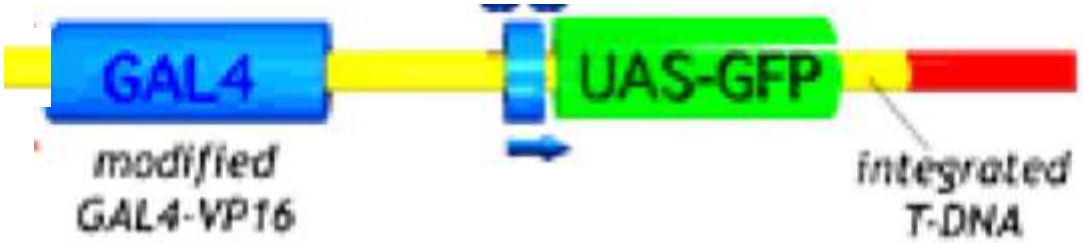
GAL4-VP16 is an unusually potent transcriptional activator.

Formed by fusing a DNA-binding fragment of the yeast activator GAL4 to a highly acidic portion of the herpes simplex virus protein VP16 (ref. 11; also called Vmw65).

VP16 activates transcription of immediate early viral genes by using its amino-terminal sequences to attach to one or more host-encoded proteins that recognise DNA sequences in their promoters.

The hybrid protein (GAL4-VP16) activates transcription unusually efficiently in mammalian cells when bound close to, or at large distances from the gene.

A collection of trangenic plants have been obtained carrying T-DNA insertions containing both sequences for GAL4-based promoter/enhancer trapping and UAS-GFP



Mutants have been characterized for expression profile and made available [useful for reverse genetic approaches (see ahead)]

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How to order
Order by code
View selection
Order selection
Fees
FAQ / Help

Your Details
Create/Update

New charging system

All single stocks are charged at £2.25 each with a fee of £8.50 per order

Haseloff GAL4-GFP Enhancer-trap lines

These lines have been produced, and donated, by Dr. Jim Haseloff, MRC Laboratory of Molecular Biology, Hills Road Cambridge, UK.

Ordering lines

Four sets of GAL4-GFP lines are now available. The sets correspond to those mentioned on Jim Haseloff's web site and fall into the following categories.

- Set 1 and 1a Lines *pre-screened for root expression* by Jim Haseloff and Sarah Hodge.
- Set KS Lines *pre-screened for shoot and floral expression* by Kirby Siemering and Marion Bauch.
- Set M Lines *prescreened for shoot and floral expression* by Marion Bauch.

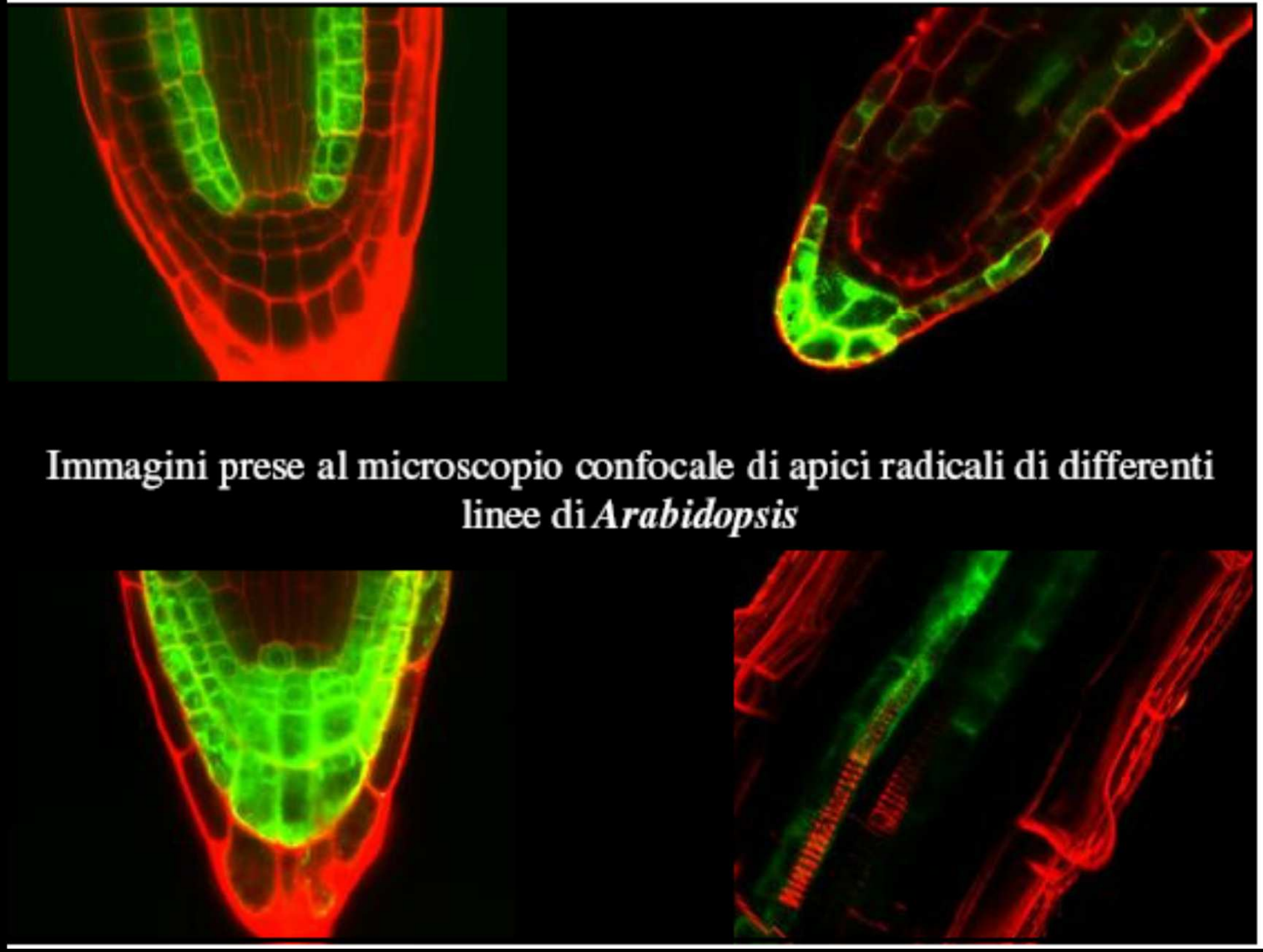
Individual lines may also be ordered, to do so follow the details link in the table below.

For detailed descriptions of these lines, and information relating to these lines, please refer to Jim Haseloffs' web page.

- About the GFP lines

Category	Stock No.	Price £	
GAL4-GFP Enhancer trap lines - set 1 (116 lines) details	N9089	122	☐
GAL4-GFP Enhancer trap lines - set 1a (29 lines) details	N9340	33	☐
GAL4-GFP Enhancer trap lines - set KS (31 lines) details	N9341	36	☐
GAL4-GFP Enhancer trap lines - set M (70 lines) details	N9342	82	☐

Add to Selection



Immagini prese al microscopio confocale di apici radicali di differenti linee di *Arabidopsis*