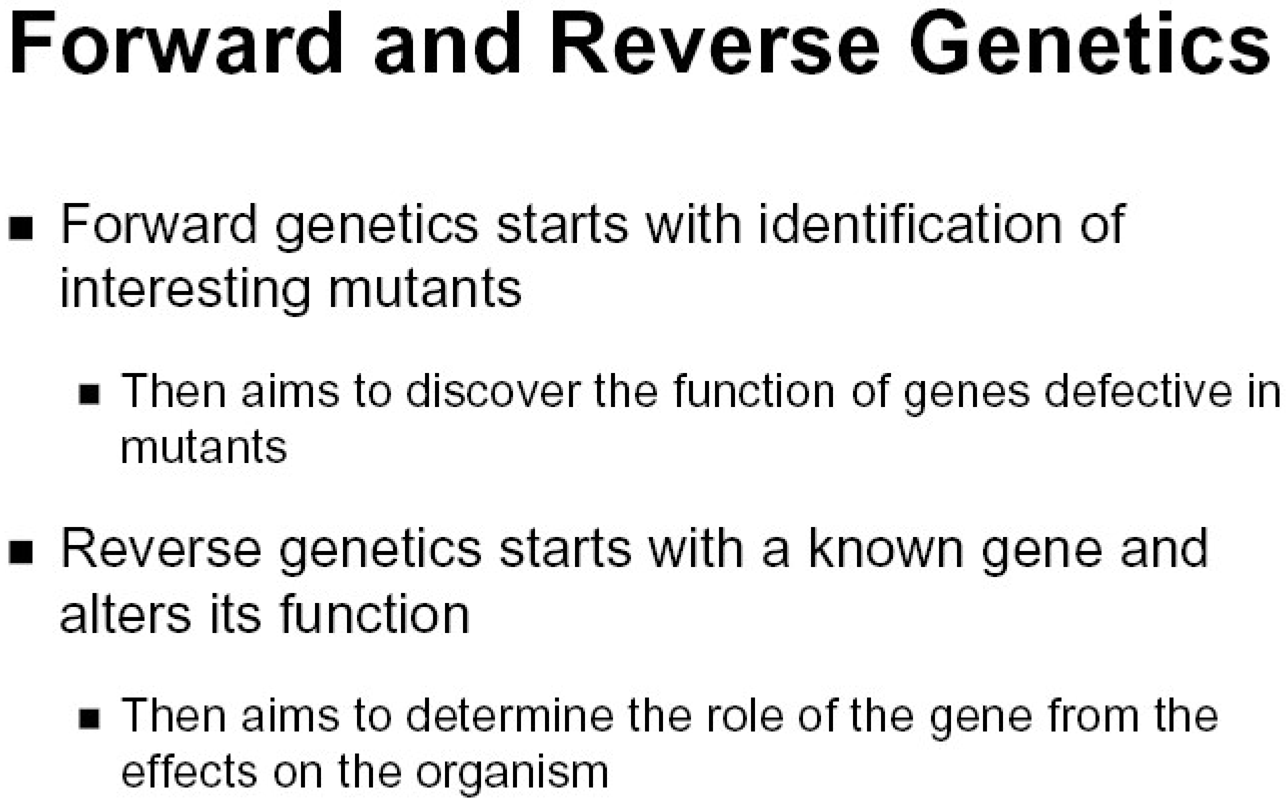
Lo studio della funzione dei geni



Forward and Reverse Genetics

- Forward genetics starts with identification of interesting mutants
  - Then aims to discover the function of genes defective in mutants
- Reverse genetics starts with a known gene and alters its function
  - Then aims to determine the role of the gene from the effects on the organism

Forward and reverse genetics

- Forward genetics
  : from phenotype to gene structure

Mutation → Phenotype → Gene

- Reverse genetics
  : from gene to phenotype

Gene → Disruption Mutation → Phenotype

Forward and Reverse Genetics

- Forward genetics starts with identification of interesting mutants
  - Then aims to discover the function of genes defective in mutants
- Reverse genetics starts with a known gene and alters its function
  - Then aims to determine the role of the gene from the effects on the organism

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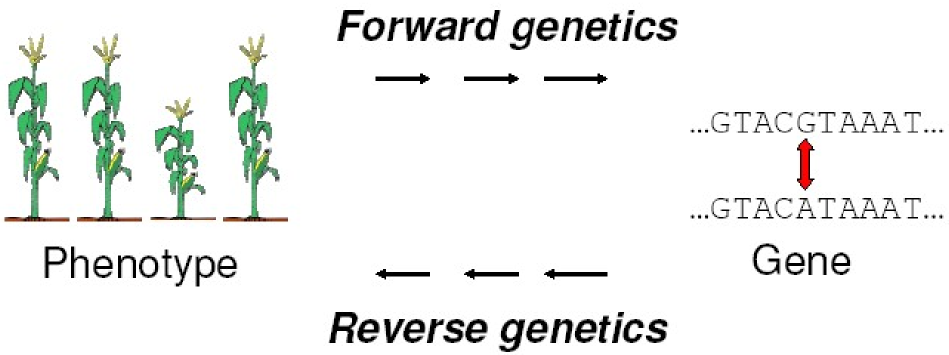
Forward *vs* Reverse



Forward genetics

→ → →

…GTACGTAAAT…

…GTACATAAAT…

Phenotype

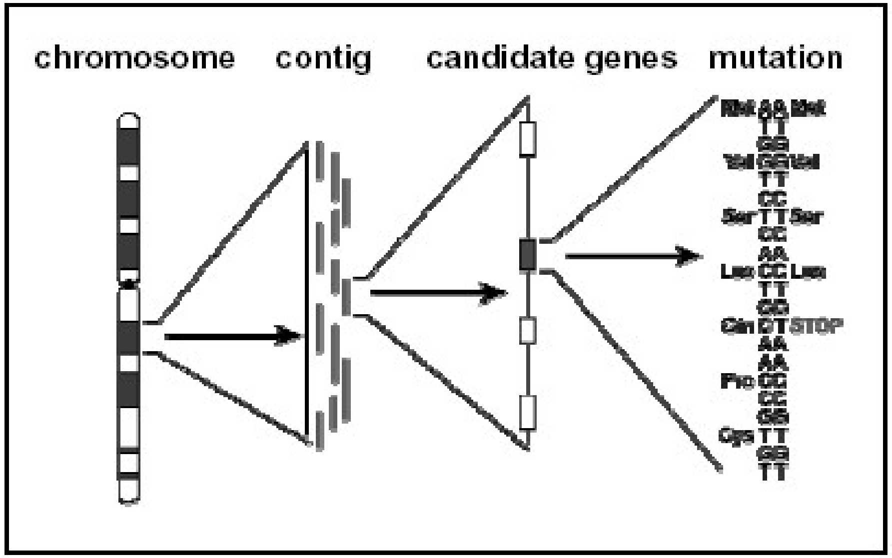
Gene

← ← ←

Reverse genetics

From Phenotype to Gene

- Once an interesting mutant is found and characterized, we want to find the gene in which the mutation occurred
- Positional cloning
  - First use genetic mapping
  - Then use chromosome walking



Buchanan et al: Cap 7 par. 7.1.5 , 7.2, 7.2.6

Basics of Forward Genetics

- Forward genetics usually starts with *mutagenesis* of an organism
    - Can use chemicals
        - e.g., ethyl methyl sulfonate (EMS)
        - often used in Arabidopsis
    - Can use radiation
        - e.g., X rays
    - Can use gene tagging
        - e.g., transposon tagging or T-DNA tagging
        - often used in Arabidopsis and maize
- Then *screen progeny* of mutagenized individuals for phenotypes of interest

Buchanan et al. 18.2.4 e Fig. 18.17

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:

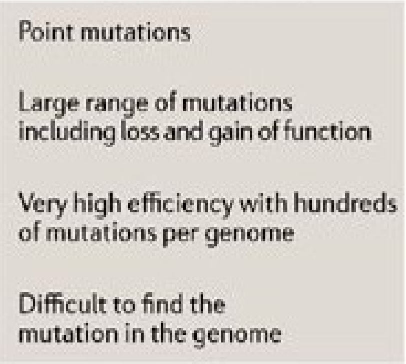
- How many plants can be reasonably screened?

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

Forward genetic

- Chemicals agents:

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



Point mutations

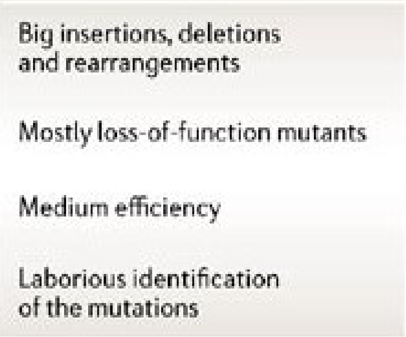
Large range of mutations including loss and gain of function

Very high efficiency with hundreds of mutations per genome

Difficult to find the mutation in the genome

- Physical agents:

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



Big insertions, deletions and rearrangements

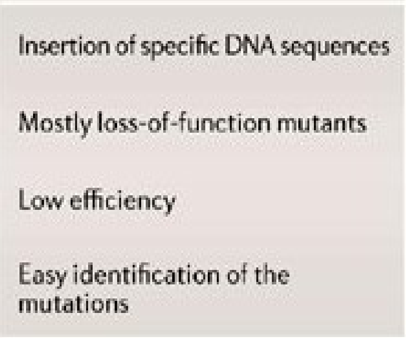
Mostly loss-of-function mutants

Medium efficiency

Laborious identification of the mutations

- Biological agents:

e.g., transposons and T-DNA



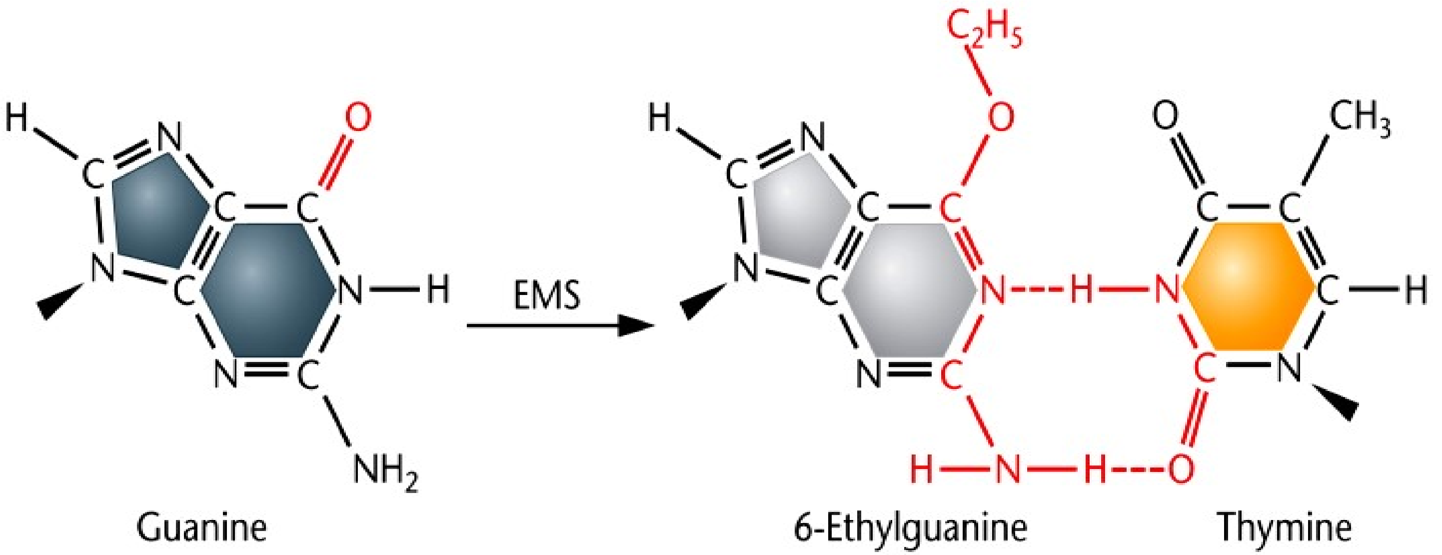
Insertion of specific DNA sequences

Mostly loss-of-function mutants

Low efficiency

Easy identification of the mutations

Chemical (alkylating) agents

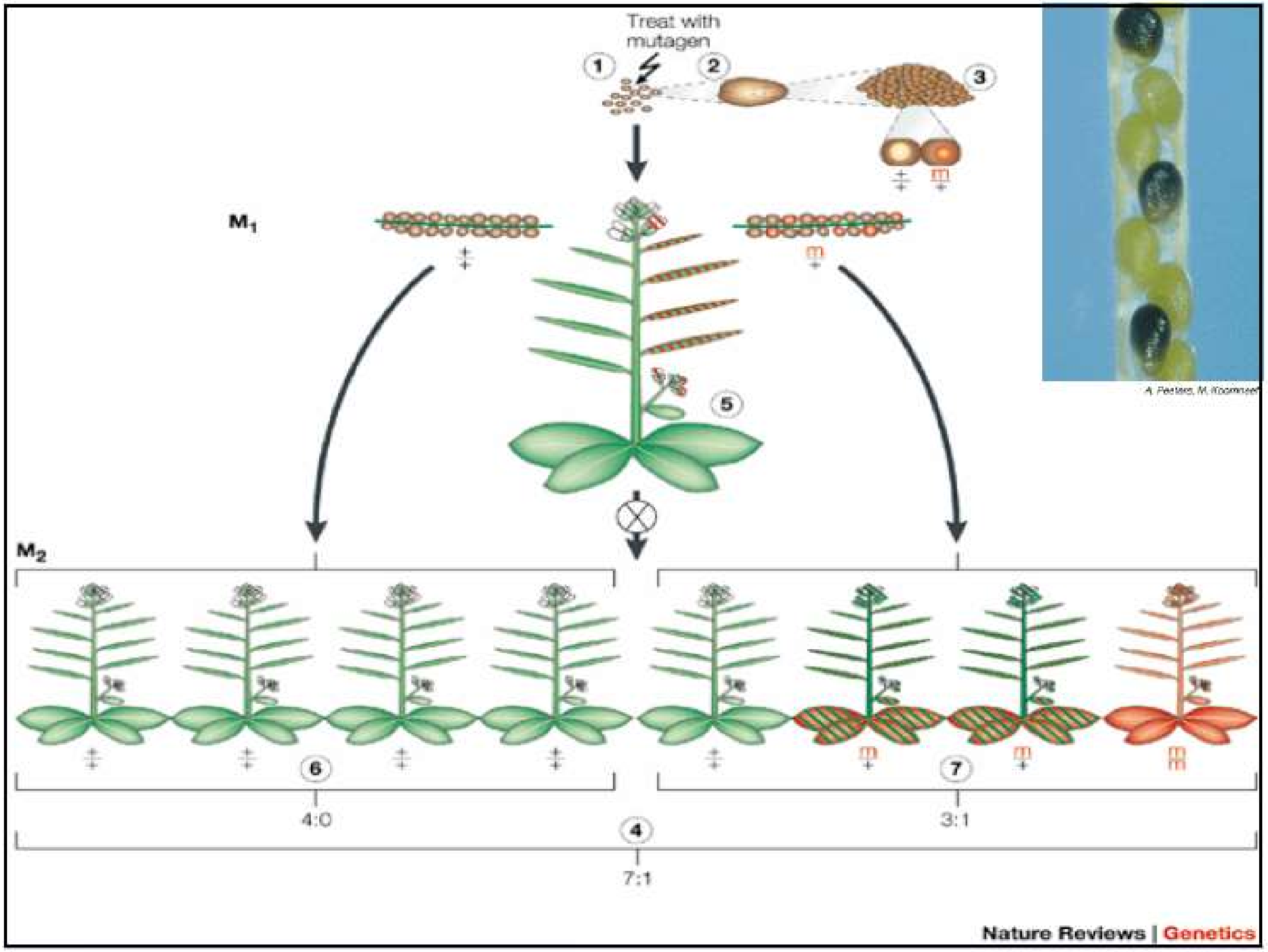


- EMS causes addition of chemical groups primarily to Gs.
- The extra groups can cause altered base pairing resulting in transitions.
- EMS can also cause depurination (complete loss of the base) which leaves gaps that cause misincorporation during replication.

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

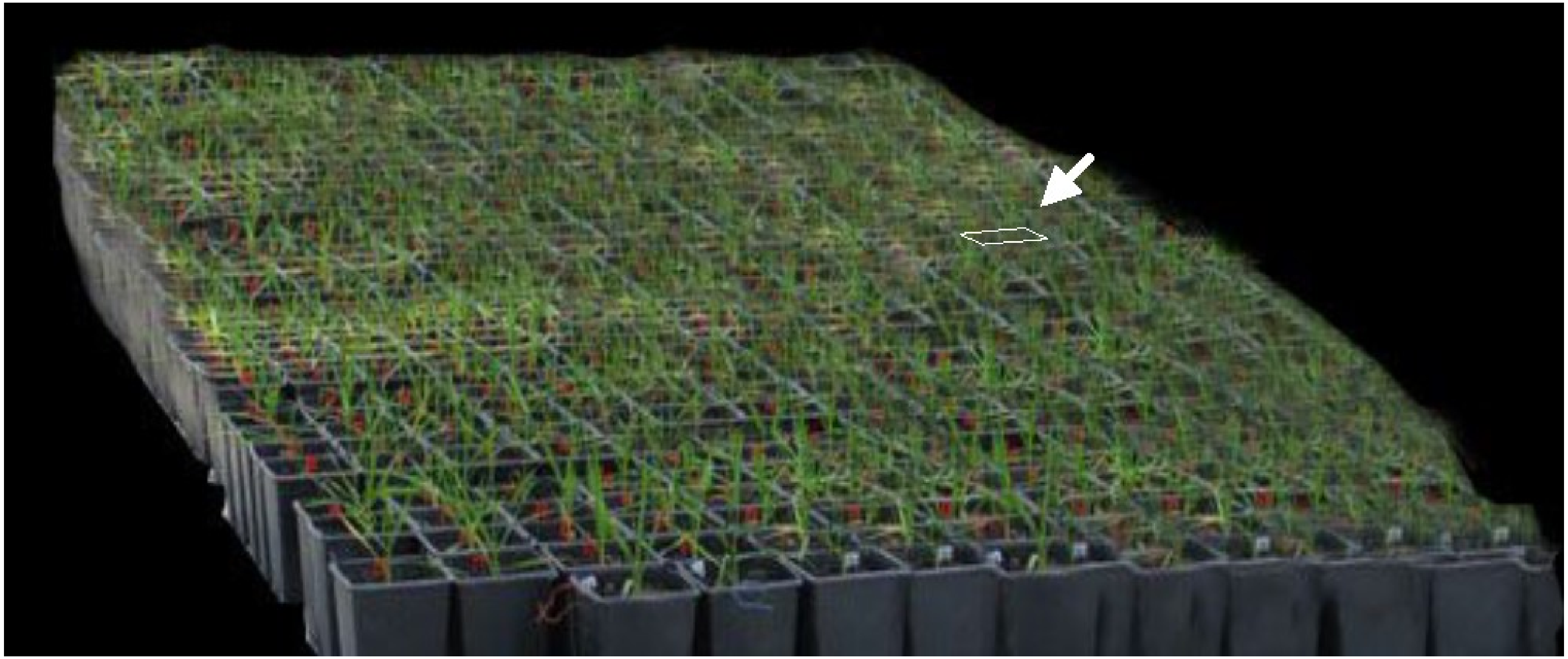


The targets of seed mutagenesis are the diploid cells of the fully developed embryo covered by the seed coat.
To assess the effectiveness of mutagenesis, it is crucial to know how many of the targeted cells will eventually contribute to the next generation. The functional germ line can be defined as 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

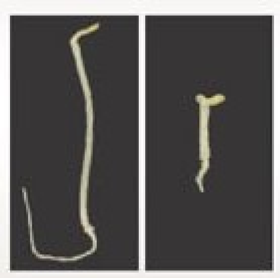
- Radiation ionize water and result in the formation of oxygen radicals such as superoxide ($O_2^{\cdot -}$) and hydroxyl radicals ($\cdot OH$),
- Radicals are highly reactive and can cause single and double strand breaks and base modifications,
- Single strand breaks are easily repaired but double strand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

Screening…

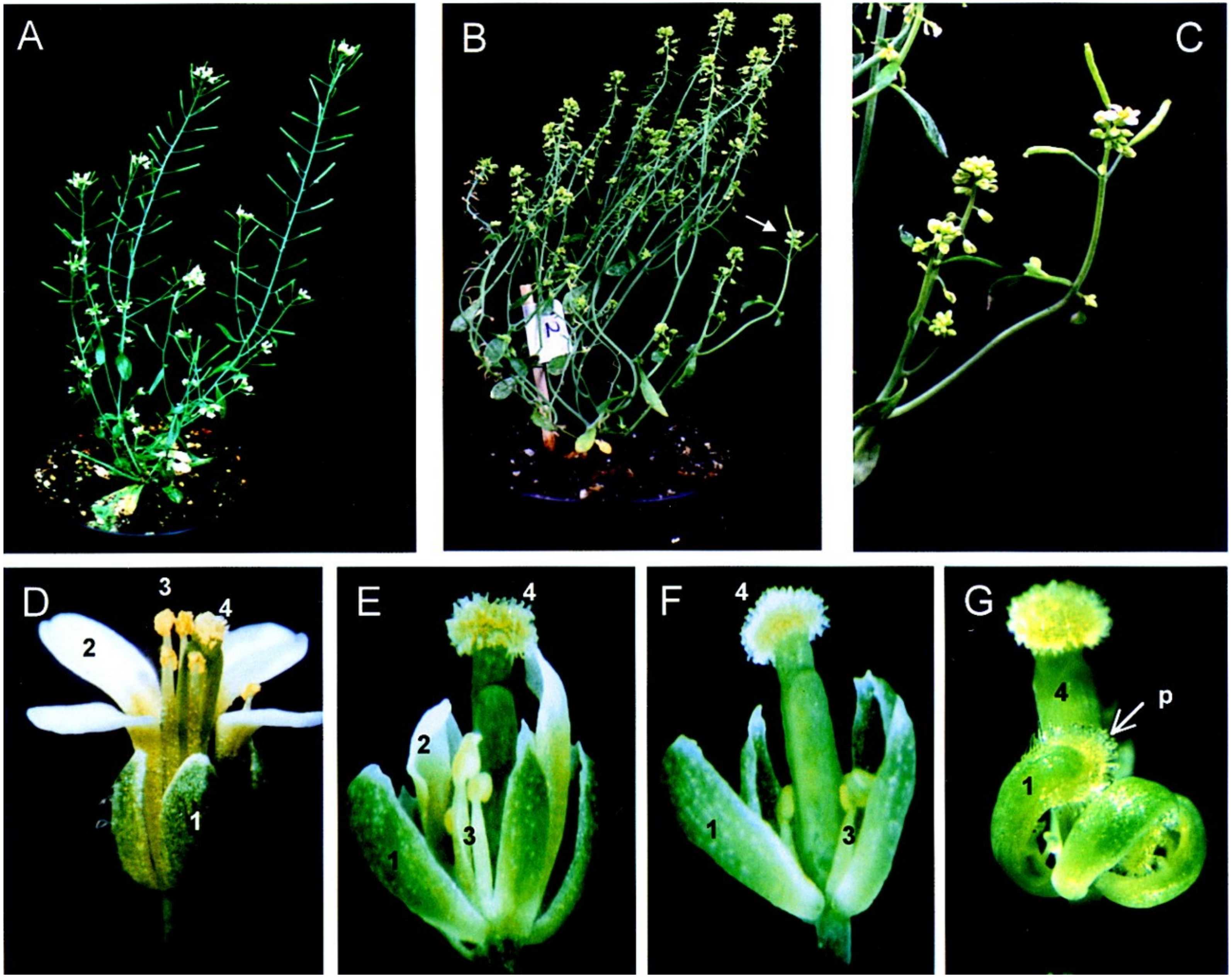


Forward genetic

You need a screenable phenotype



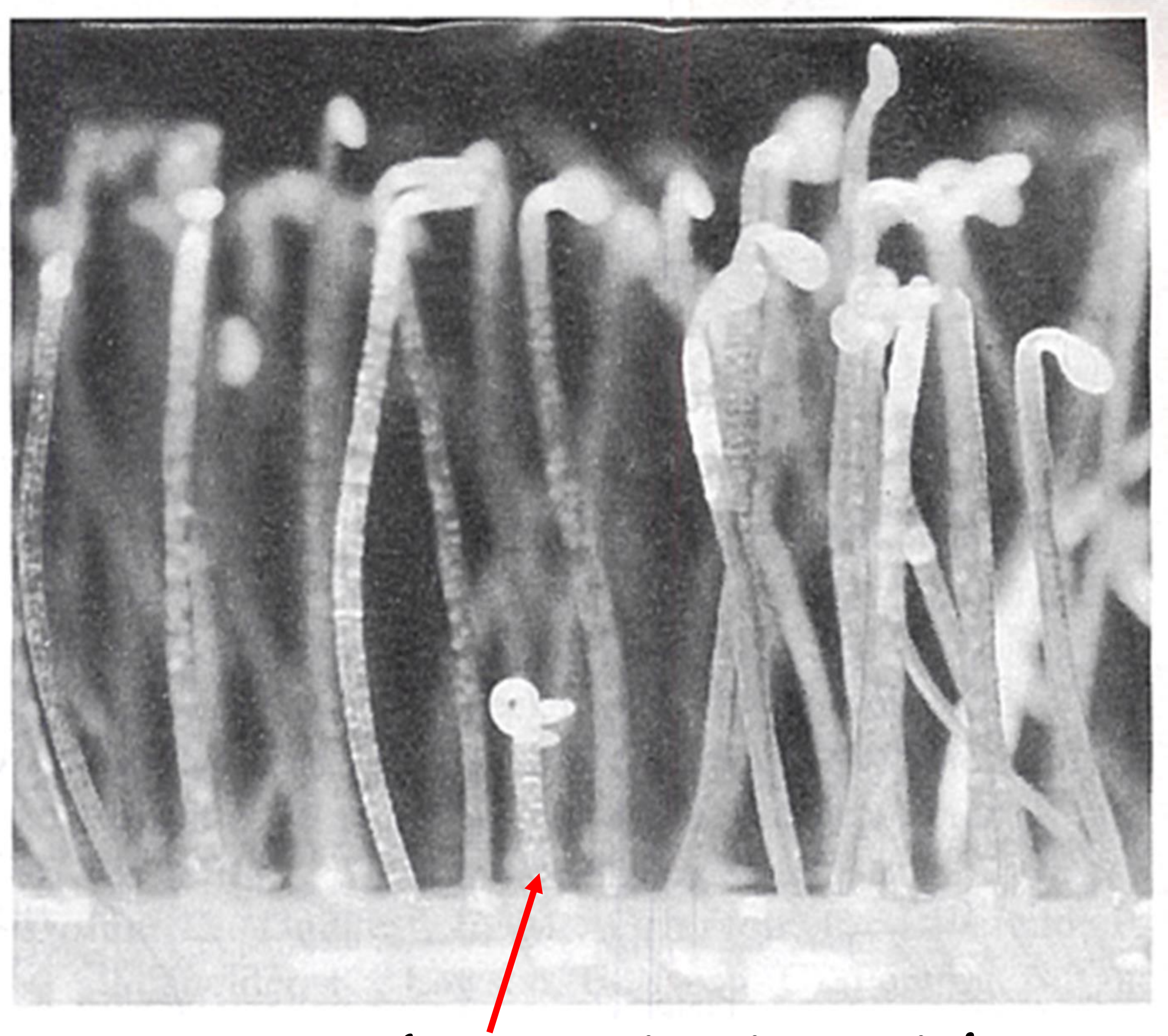
- Flower development
- Disease resistance
- Photomorphogenesis
- Responses to hormones etc.


A
B
2
C
D
3
4
2
1
E
4
2
3
1
F
4
1
3
G
4
p
1

Recettori dell'etilene sono stati clonati per isolamento di mutanti etilene -insensibili

(A)

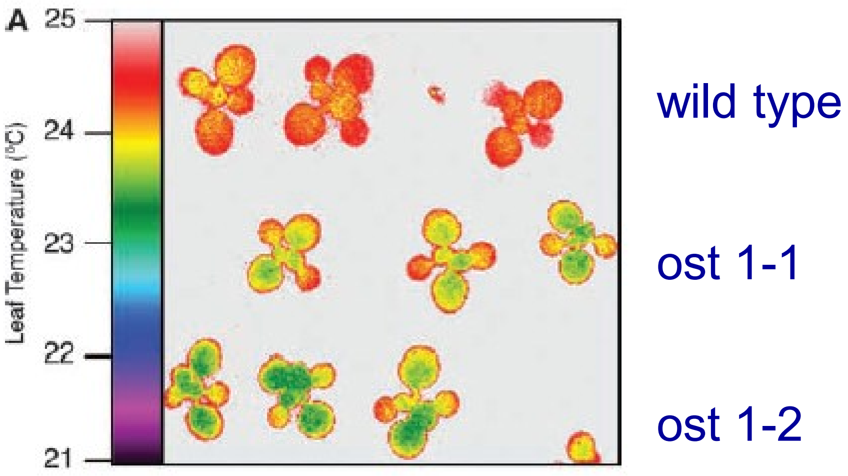
Isolamento di mutanti che presentano tripla risposta costitutiva (anche in assenza di etilene) ha permesso di identificare un regolatore negativo nella via di trasduzione

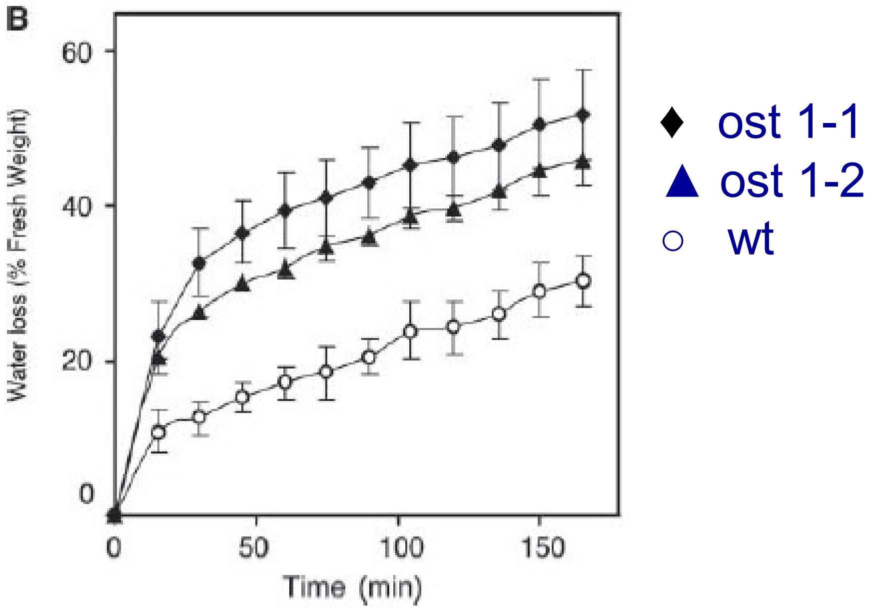


ctr1 : constitutive triple response

Un sistema per isolare mutanti (*ost1*)

- Popolazione mutagenizzata con etil metansulfonato
- Stress idrico
- Analisi mediante termografia infrarossa
- Selezione dei mutanti





I mutanti *ost1* sono difettivi nella regolazione della traspirazione sotto stress idrico

Drawbacks of Forward Genetics

- Breeding must be possible

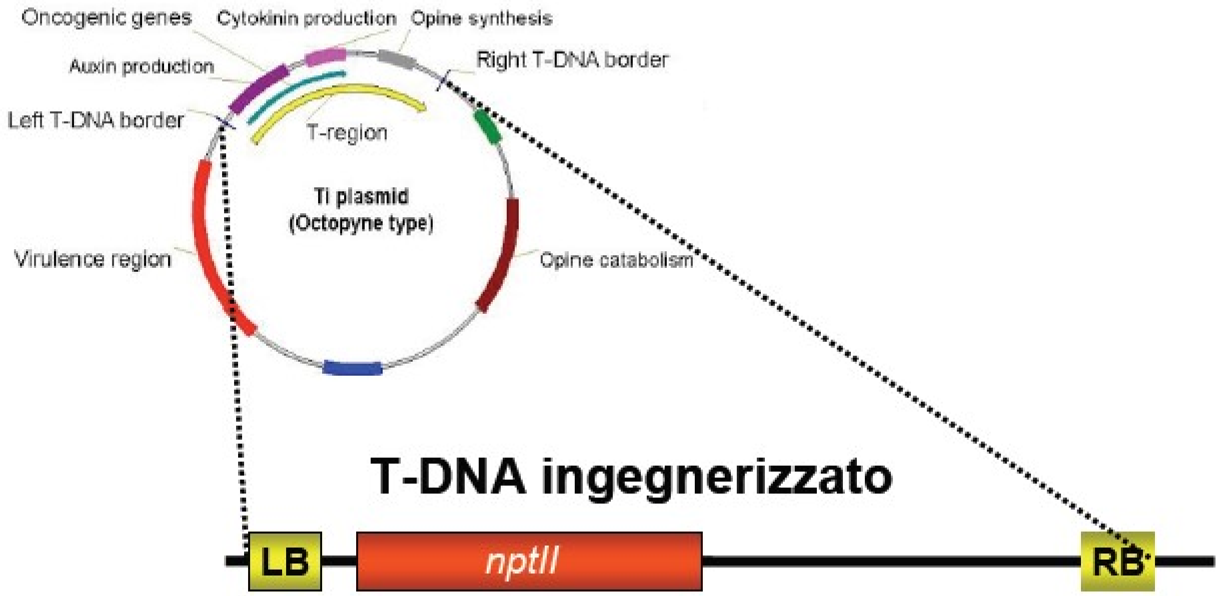
- You need a screenable phenotype
  - Easy with visible phenotypes
    - growth, color, development
    - not easy with metabolism
    - impossible for „essential genes“ (defects of which would be lethal)
- You need a dense genetic map and markers closely associated with the phenotype
  - Good for Arabidopsis, 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

Il T-DNA di *Agrobacterium* è stato utilizzato per generare collezioni di mutanti inserzionali

- Il T-DNA si integra in maniera **casuale** all'interno del genoma della cellula vegetale...

  .. permettendo la generazione di knock-outs

- L'integrazione è **stabile** per un buon numero di generazioni
- Inserzioni singole o più inserzioni (testa testa, testa coda o coda-coda)



Il “T-DNA” viene ingegnerizzato con:

Un marcatore per la selezione delle linee T/F (Kan)

Mutagenesis by insertion tagging

Principle: A DNA fragment with a known sequence (transposons, *A.tumefaciens* T-DNA) is allowed to randomly insert into the genome (it usually causes a recessive, loss of function mutation

Advantages:

- tags or marks the gene
- provides a powerful way to identify or fish the gene out

Disadvantages (like chemical and physical mutagenesis):

- cannot knock out essential genes,
- other redundant genes mask loss of disrupted gene
- may disrupt non-functional sub-region of 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

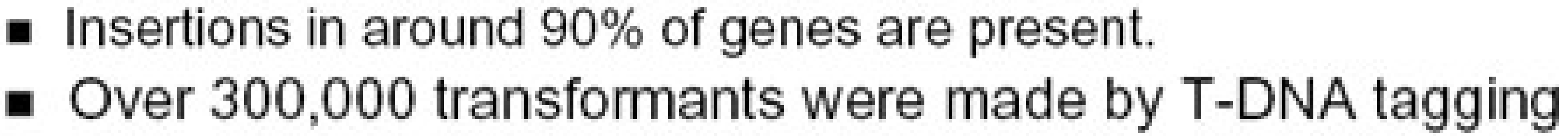
-Gene insertion is essentially random.

-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.

-

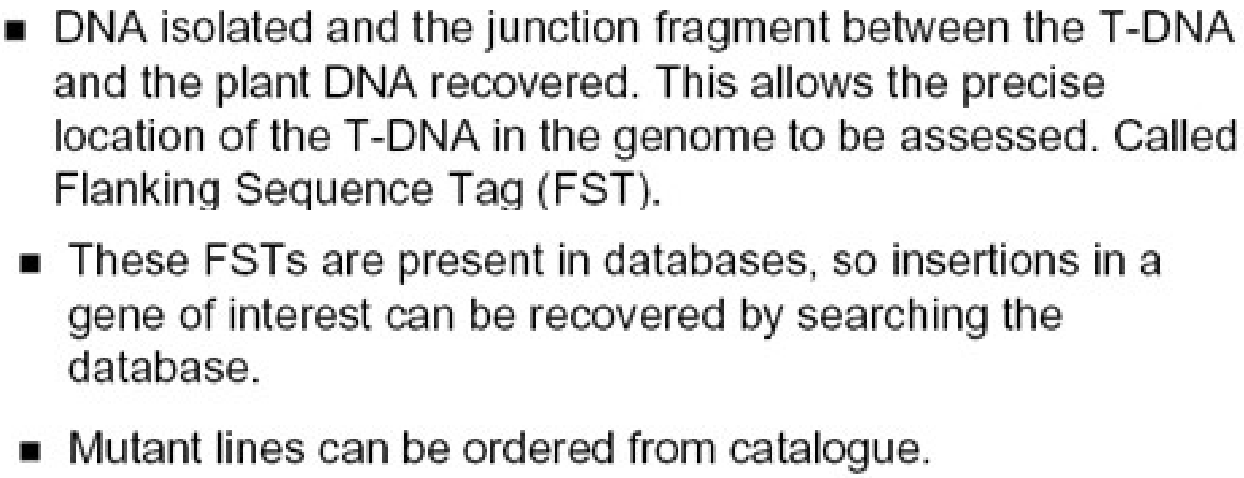


- Insertions in around 90% of genes are present.
- Over 300,000 transformants were made by T-DNA tagging

- 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)

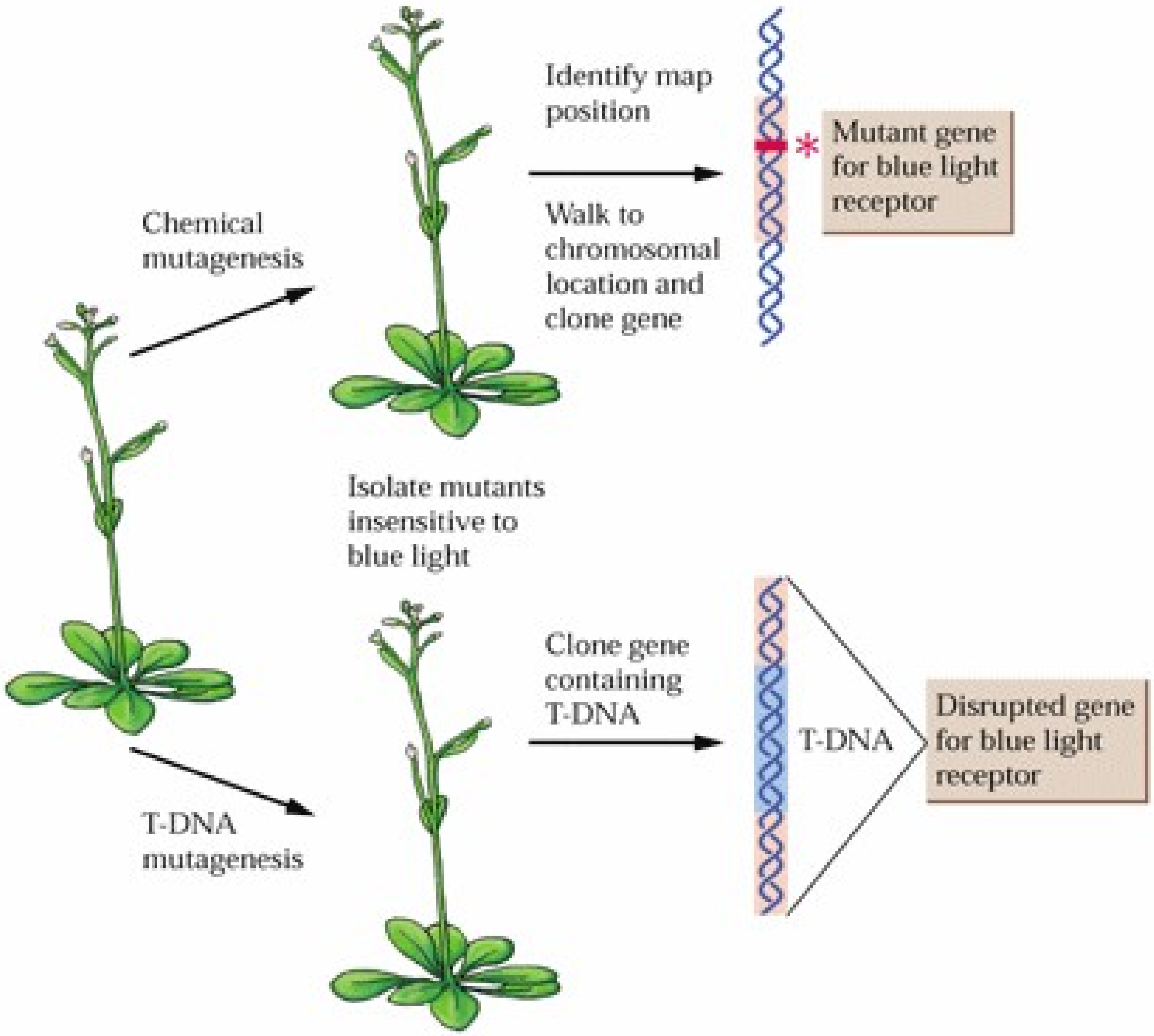


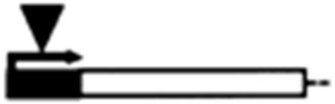
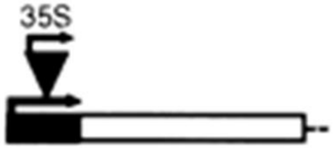
- DNA isolated and the junction fragment between the T-DNA and the plant DNA recovered. This allows the precise location of the T-DNA in the genome to be assessed. Called Flanking Sequence Tag (FST).
- These FSTs are present in databases, so insertions in a gene of interest can be recovered by searching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



Name	***Location***	***Result***
 *Knock-out*	coding region or promoter	null
 *Knock-down*	promoter or 3' UTR	reduced expression
 *Knock-on*	promoter	increased expression
 *Knock-about*	coding region	not null
 *Knock-knock*	coding region or promoter	multiple KOs in one plant
 *Knock-worst*	coding region or promoter	chromosomal rearrangement

Cellule di Agrobatterio contenenti il T-DNA ingegnerizzato vengono utilizzate per generare una popolazione di piante transgeniche (floral dip)

Agrobatterio

- semi (T1)

↓

- self impollinazione (T2)

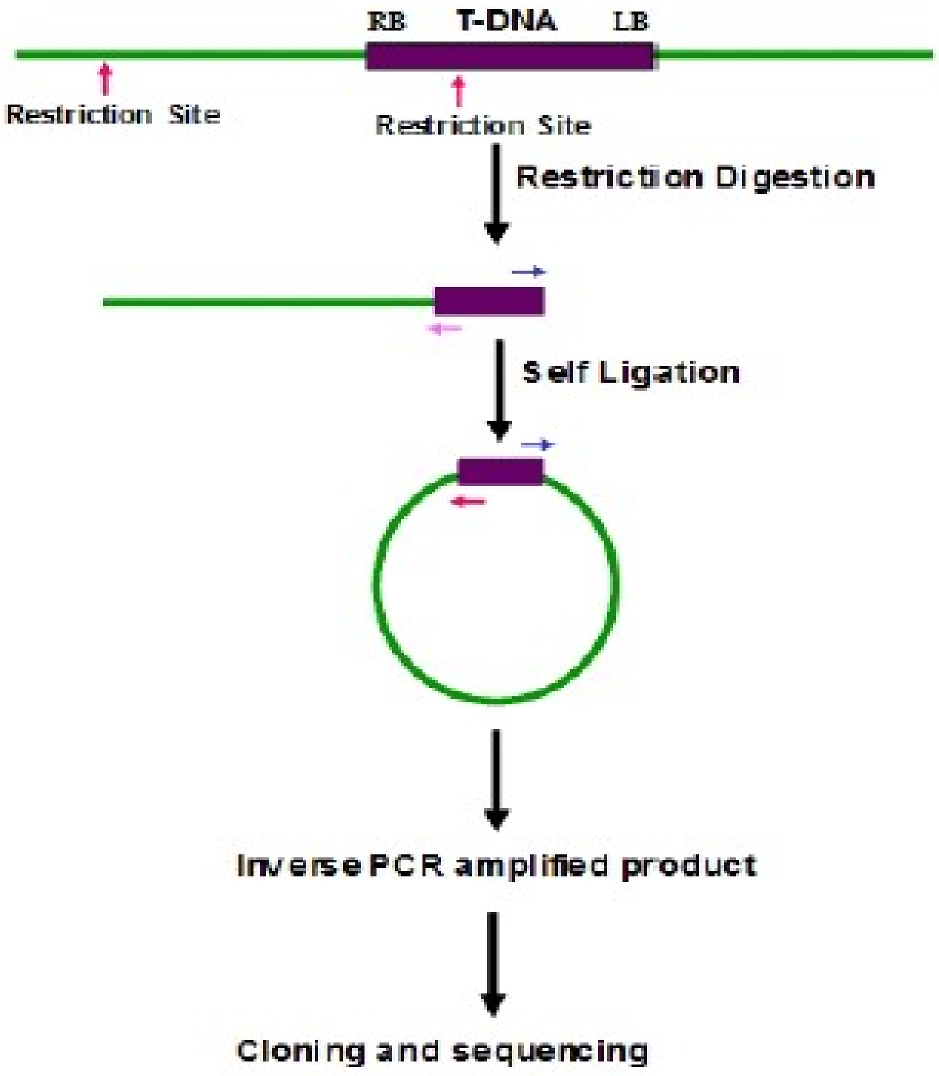
↓

- collezione di 40.000 linee indipendenti (T3)

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

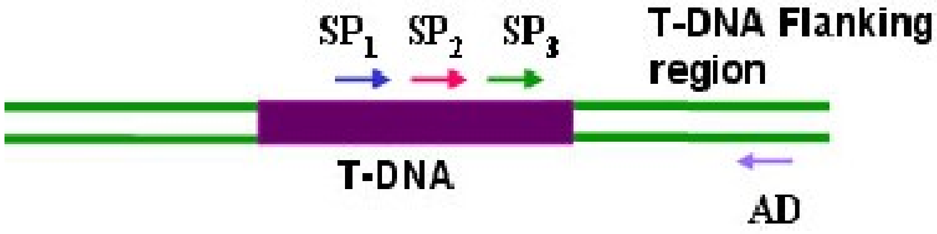
- Inverse PCR
- Thermal asymmetric interlaced (TAIL) PCR
- Plasmid rescue

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



Primary PCR: SP_1 and AD_1
Secondary PCR: SP_2 and AD_1
Tertiary PCR: SP_3 and AD_1

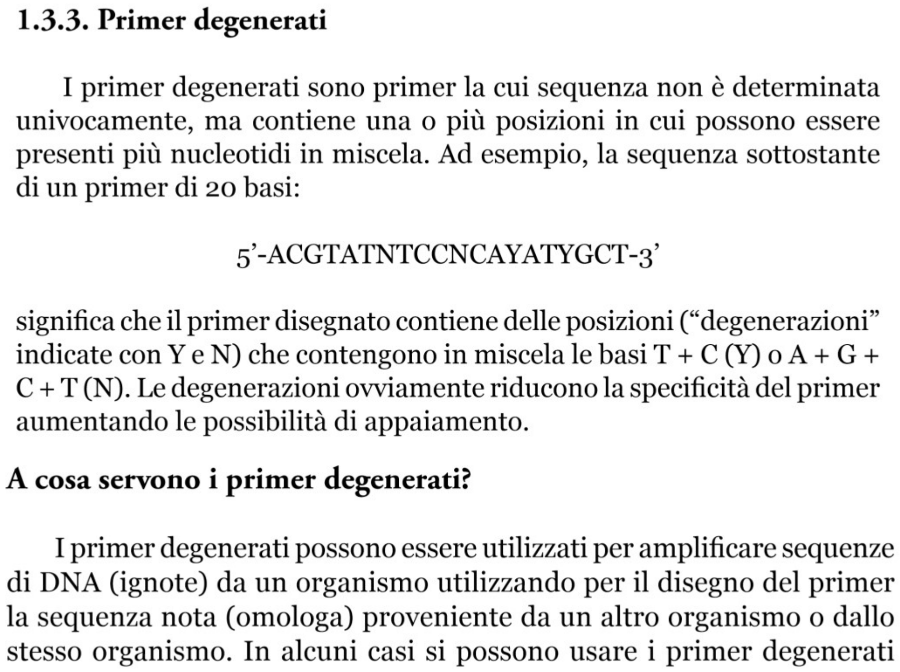
PCR amplified product

Cloning and sequencing

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'

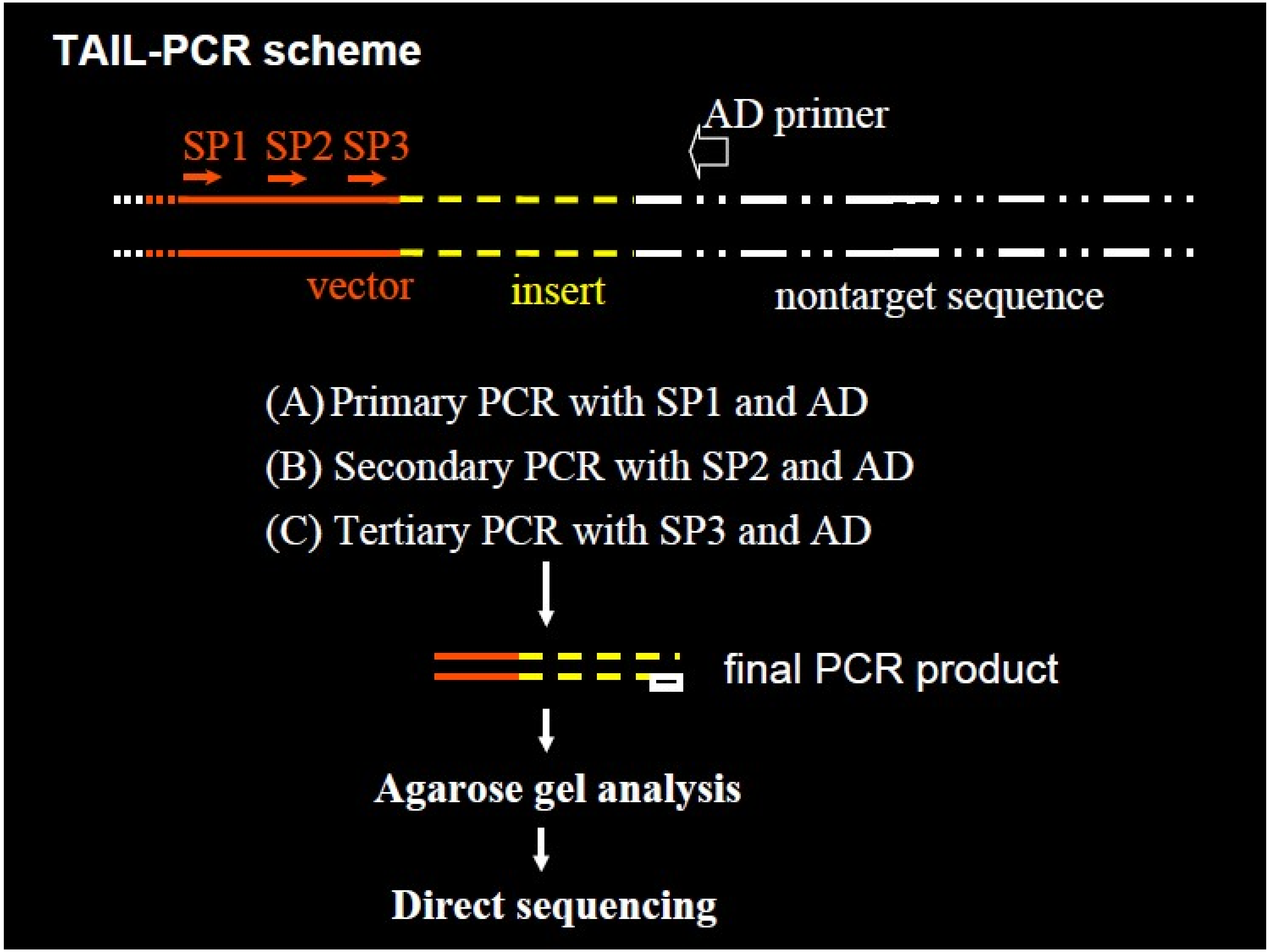
significa che il primer disegnato contiene delle posizioni ("degenerazioni" indicate con Y e N) che contengono in miscela le basi T + C (Y) o A + G + C + T (N). Le degenerazioni ovviamente riducono la specificità del primer aumentando le possibilità di appaiamento.

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

Primer Design for TAIL-PCR

- Specific primer (SP)
  - Nested sequence specific primer complementary to vector sequence
  - High melting temperature, T_m=58-63^{o}C
- Arbitrary degenerate (AD) primer
  - Relatively shorter
  - Lower melting temperature, T_m =47-48^{o}C



TAIL-PCR scheme

(A) Primary PCR with SP1 and AD

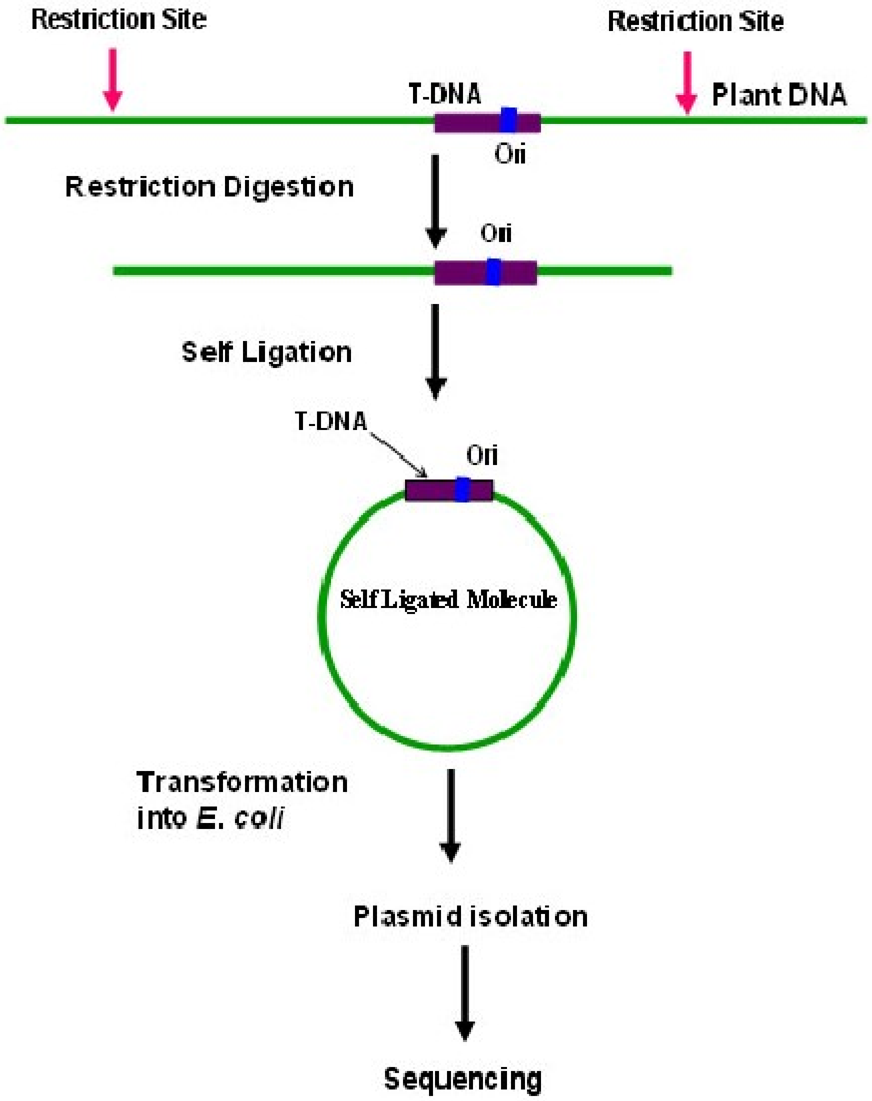
(B) Secondary PCR with SP2 and AD

(C) Tertiary PCR with SP3 and AD

Agarose gel analysis

Direct sequencing

Plasmid rescue

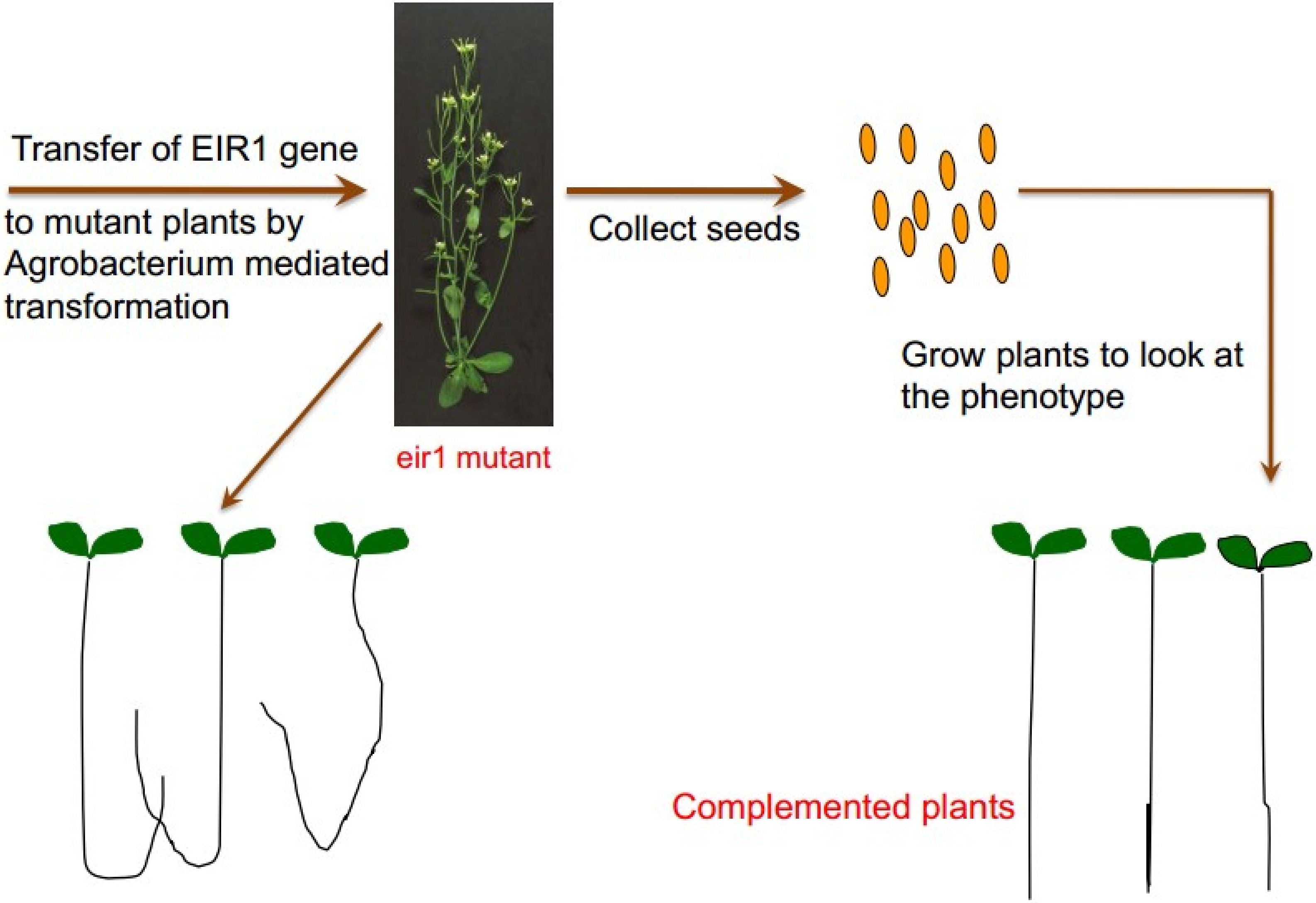


1) Restriction digestion of genomic DNA from mutant plant (preferably with an enzyme which does not cut within the T-DNA);

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



Transfer of EIR1 gene

to mutant plants by Agrobacterium mediated transformation

eir1 mutant

Collect seeds

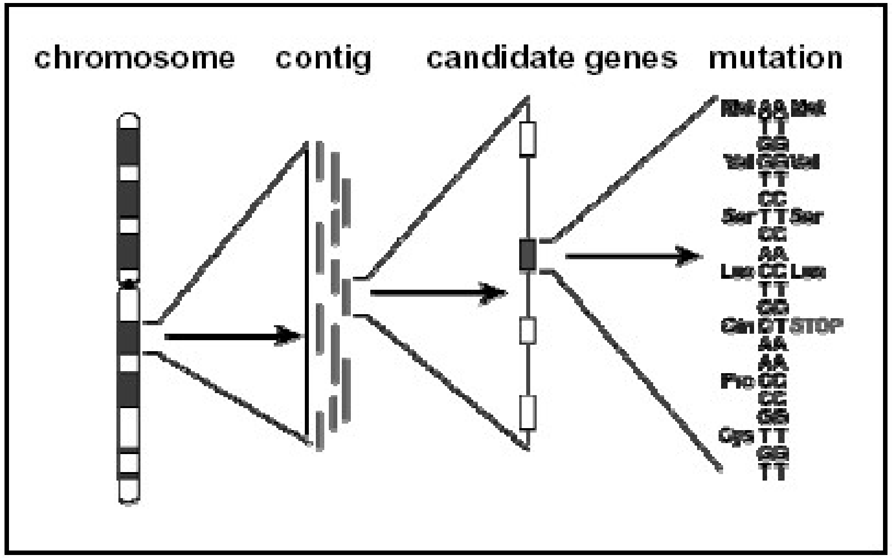
Grow plants to look at the phenotype

Complemented plants

From Phenotype to Gene

In case of chemical or physical mutagenesis

- Once an interesting mutant is found and characterized, we want to find the gene in which the mutation occurred
- Positional cloning
    - First use genetic mapping
    - Then use chromosome walking

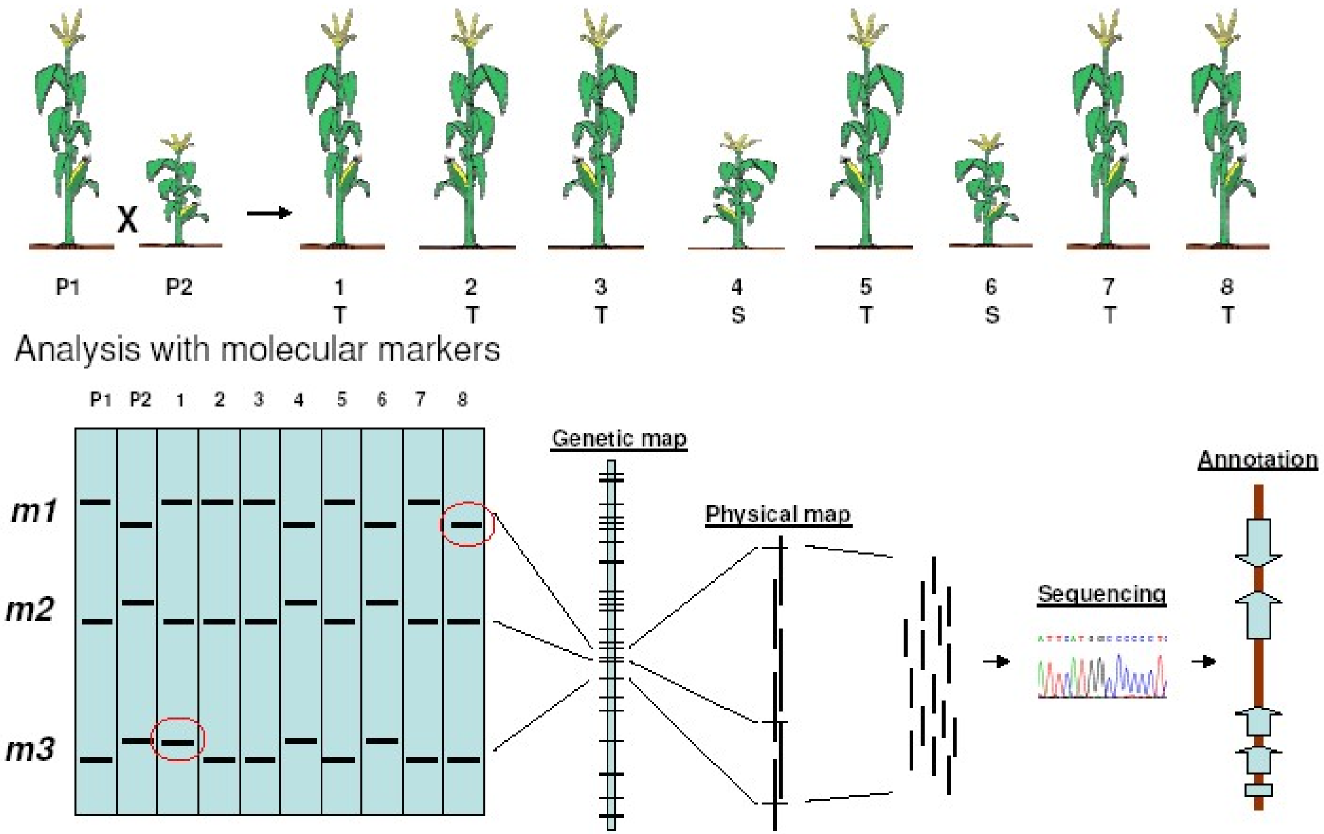


But now deep sequencing (it is becoming less and less expensive)

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

Positional cloning (forward genetics)



MAP-BASED CLONING

Step 1
Create F2 mapping population

~ 4 months

Step 2
Establish linkage using bulked segregant analysis

< 1 week

Step 3
Identify robust flanking PCR markers

< 1 month

Step 4
Collect recombinants by PCR analysis of a large mapping population (~1000 plants) with flanking markers

< 2 months

Step 5
Fine mapping: narrow genetic interval by using known or self-made, new PCR markers

< 2 months

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

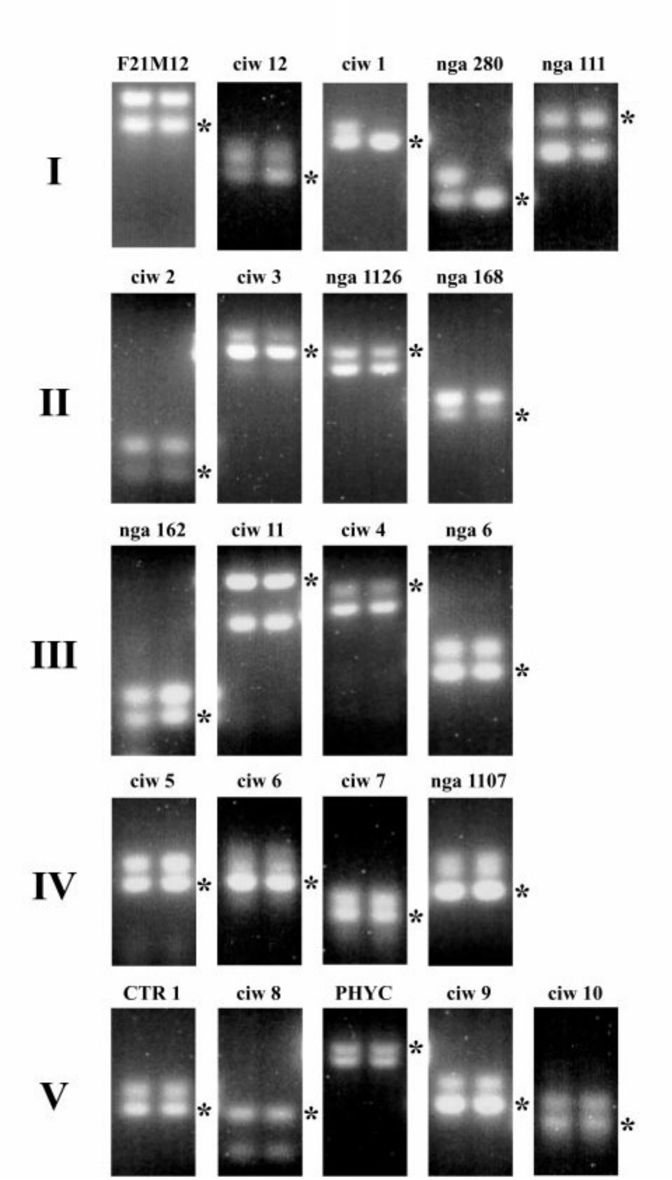
CAPS (cleaved amplified polymorphic sequences; Konieczny and Ausubel, 1993), CAPS marker exploits polymorphic restriction sites for mapping purposes

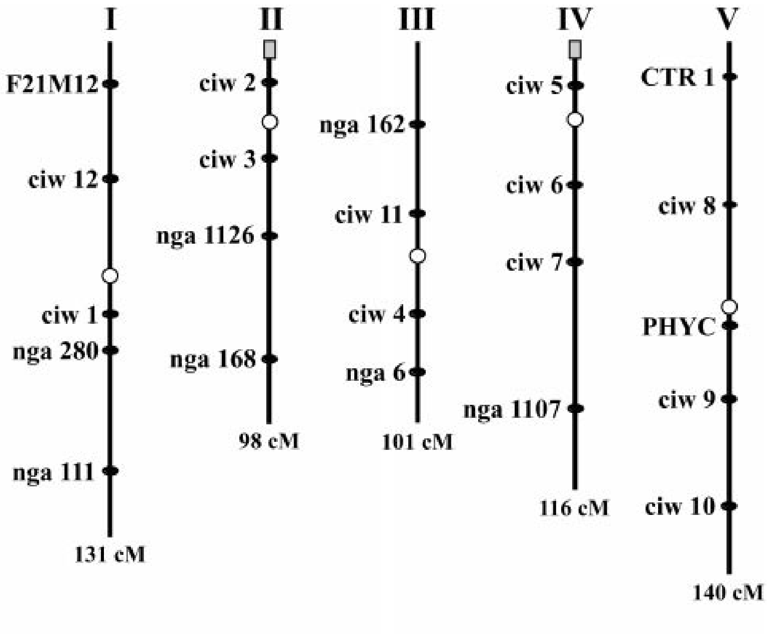
dCAPS (Michaels and Amasino, 1998; Neff et al., 1998), markers can exploit almost all single nucleotide changes for mapping purposes. To achieve this a mismatched PCR primer is designed next to the polymorphic position such that an artificial restriction site is created with the sequence variant of just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

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

BLUKED 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.

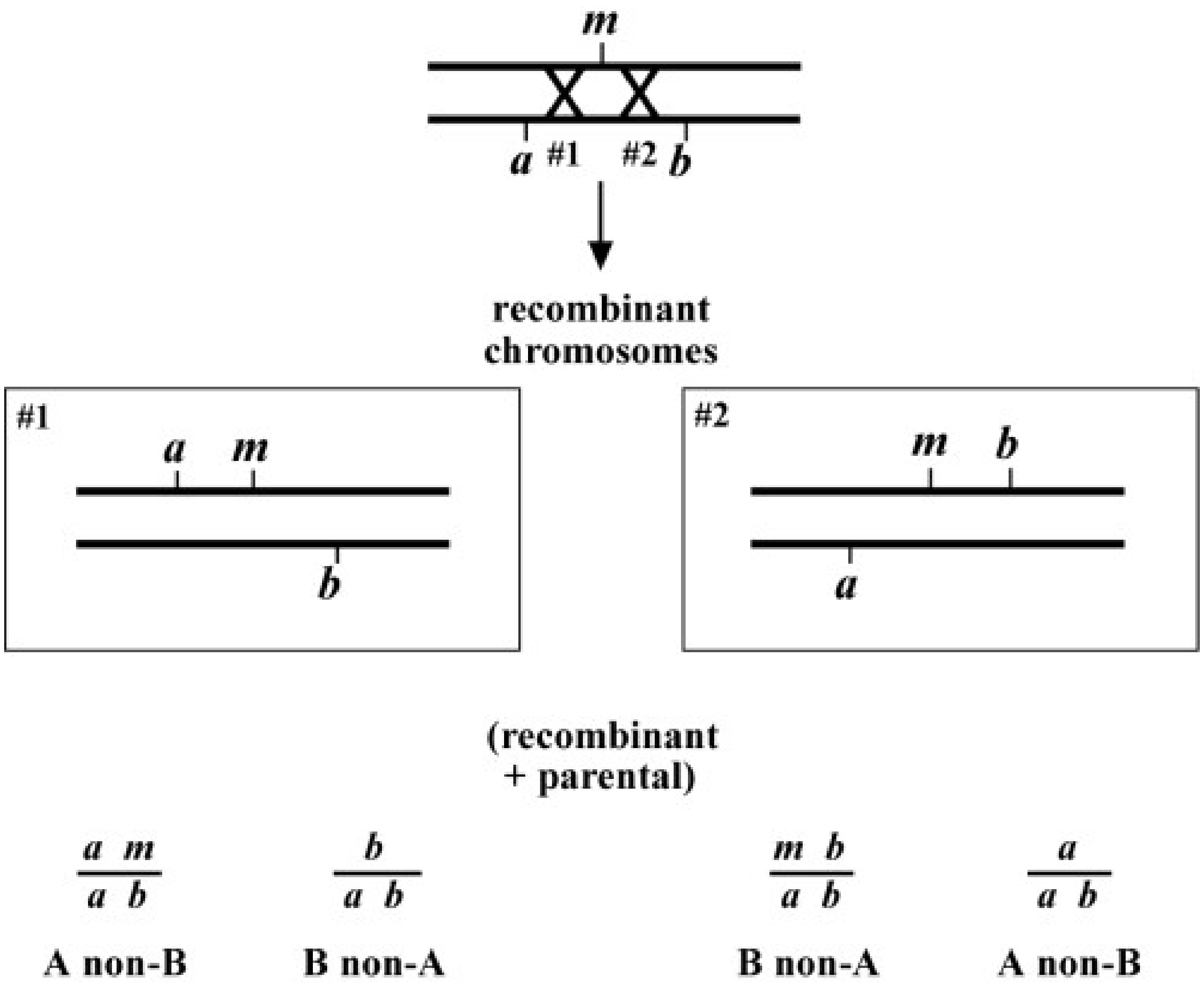
Even SNP mapping is usually preceded by three-point mapping with genetic markers.

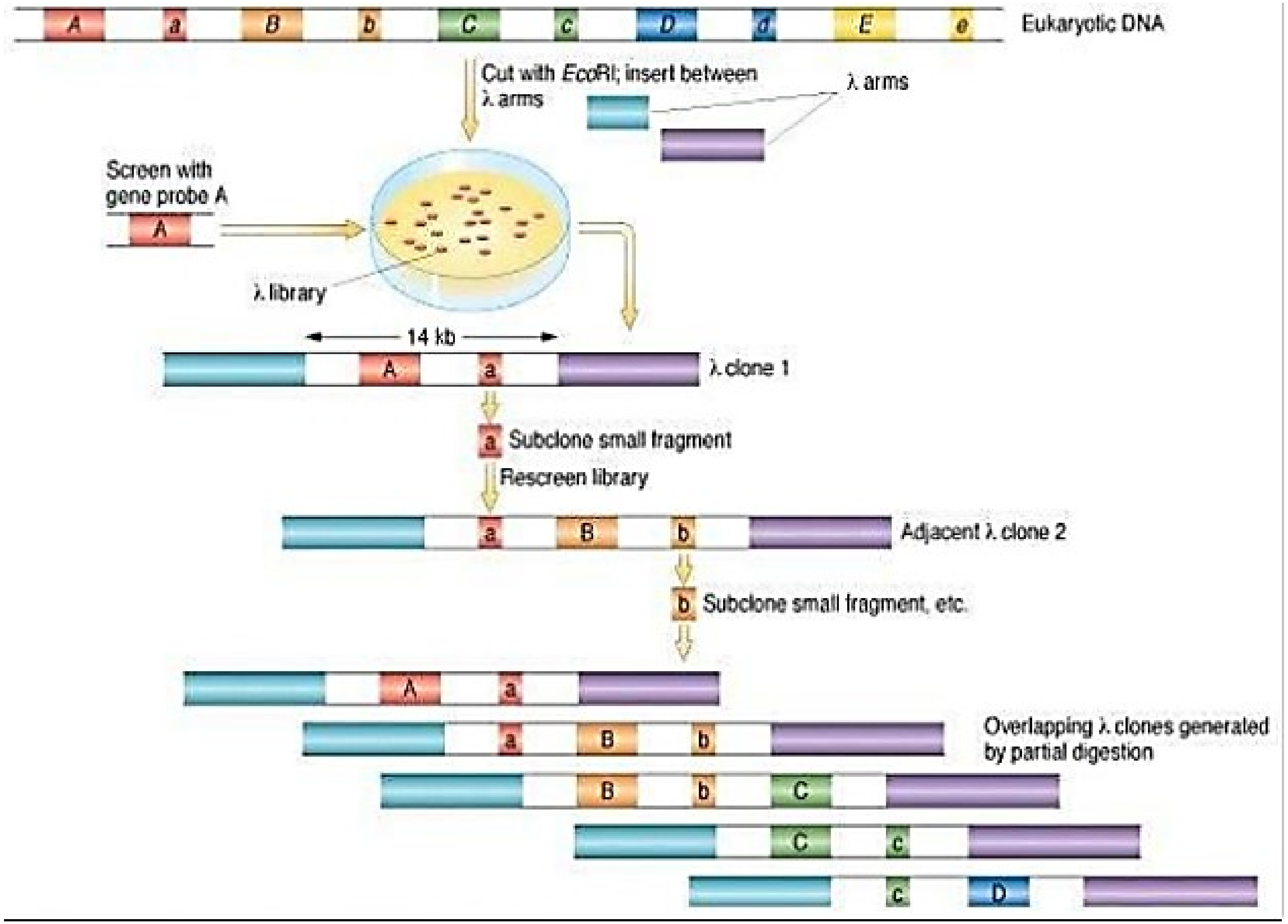
We cross our mutation (*m*) into a strain with two linked morphological markers (*a* and *b*) that are on the same chromosome as *m*, to generate the *m/a b* heterozygote.

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




A
a
B
b
C
c
D
d
E
e
Eukaryotic DNA
Cut with *Eco*RI; 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
B
b
C
C
c
c
D
Overlapping λ clones generated by partial digestion

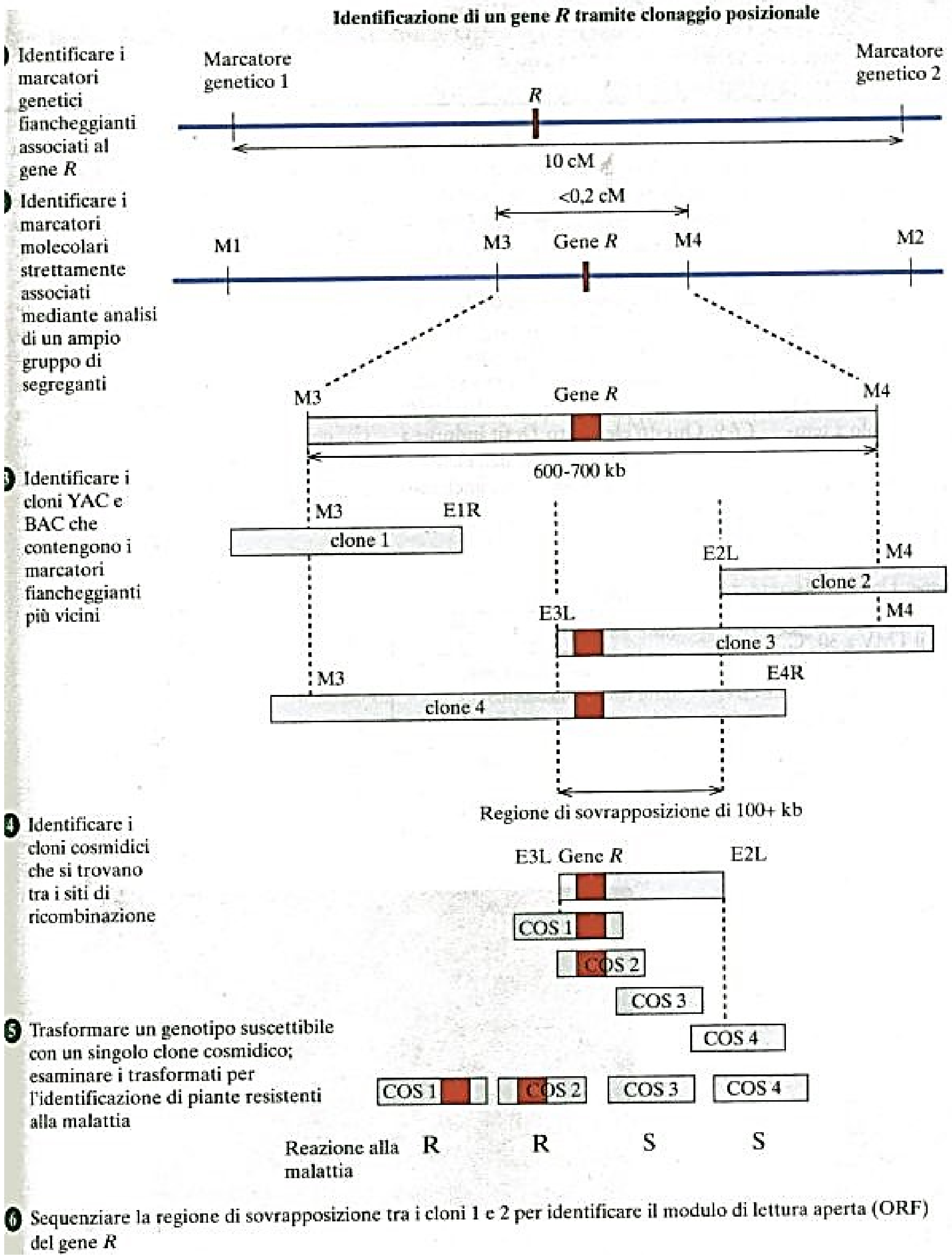


Figura 21.25 Identificazione di un gene *R* tramite clonaggio posizionale. R, resistente; S, suscettibile; YAC, cromosoma artificiale di lievito; BAC, cromosoma artificiale batterico.

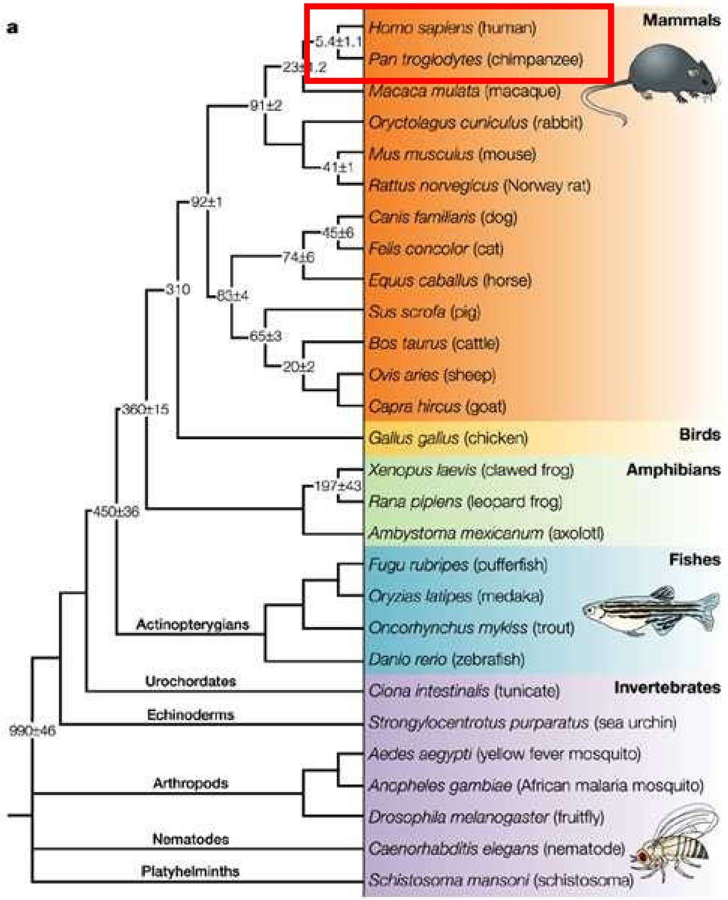
SINTENIA

- Dal greco = legati insieme
- Indica in genetica la presenza di due o più loci sullo stesso cromosoma
- Oggi il concetto è stato espanso per investigare l'**omeologia** (omologia residua tra cromosomi che in origine erano completamente omologhi)

Nella maggior parte delle piante, l’evoluzione delle porzioni di genoma, piccole ma essenziali, che codificano per i geni ha proceduto con tempi relativamente lenti

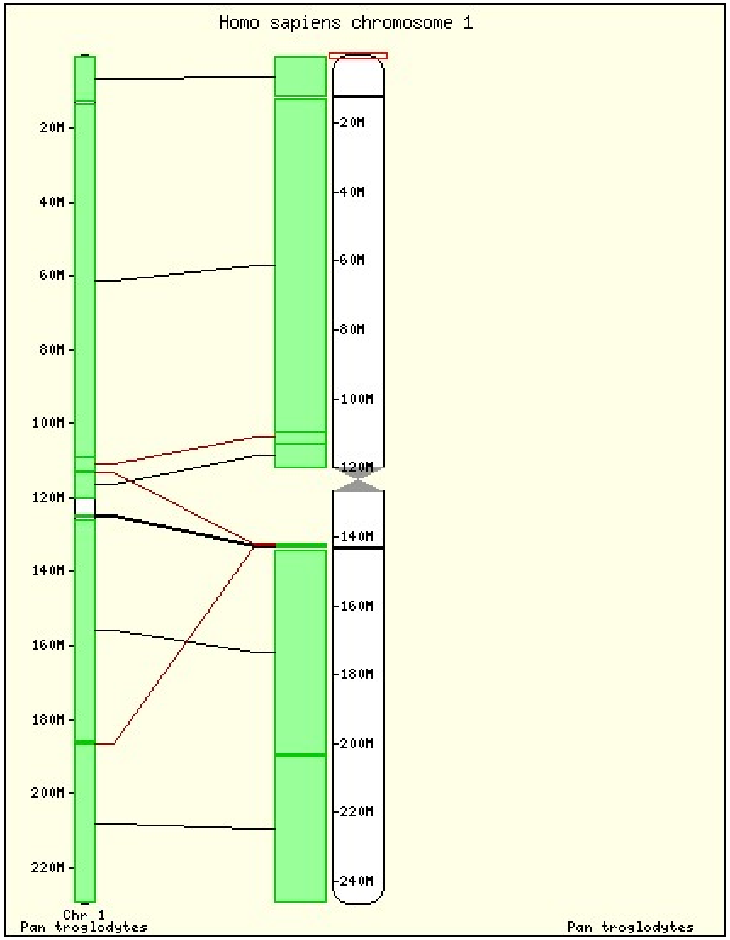
-> sequenze di DNA intrageniche e organizzazione dei geni lungo i cromosomi sono riconoscibili

Molti fattori, come duplicazioni cromosomiche o segmentali, mobilità di sequenze di DNA (es. trasposoni), delezioni e riarrangiamenti localizzati, si sono sovrapposti a tale lenta evoluzione, causando molte deviazioni dalla co-linearità



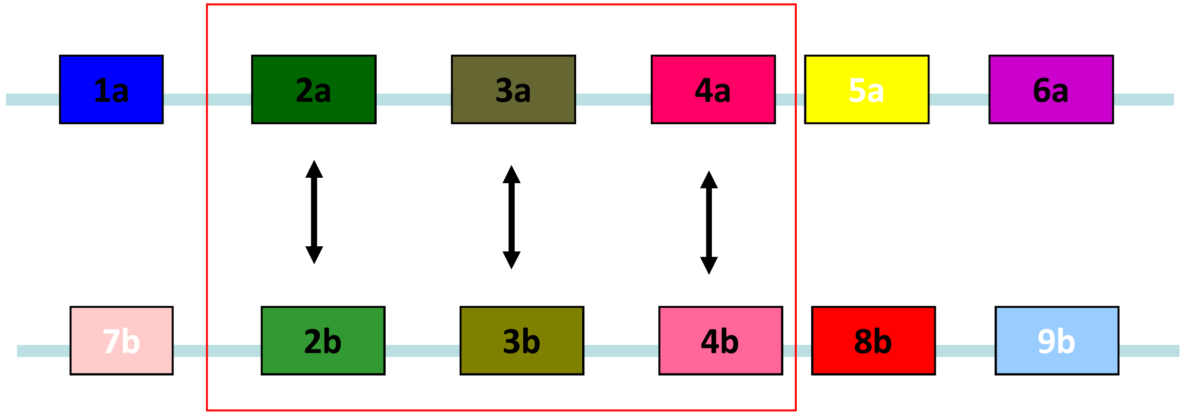
THE ORIGIN AND EVOLUTION OF MODEL ORGANISMS

Hedges, SB *Nature Reviews Genetics* **3**, 838 -849 (2002)



http://www.ensembl.org/

Blocchi di sintenia



Organismo A

Organismo B

SORGO (diploide) e CANNA DA ZUCCHERO (autopoliploide)

si sono separati circa 5 milioni di anni fa

-> alto grado di colinearità

-> potenziale uso del genoma di sorgo per identificare geni ortologhi nella canna da zucchero

Theor Appl Genet (2000) 101:962–969

C. Asnaghi · F. Paulet · C. Kaye · L. Grivet · M. Deu
J.C. Glaszmann · A. D'Hont

Application of synteny across Poaceae to determine the map location of a sugarcane rust resistance gene

Copyright © APS Press

Ruggine (*Puccinia melanocephela*) su canna da zucchero cv CP72-1210

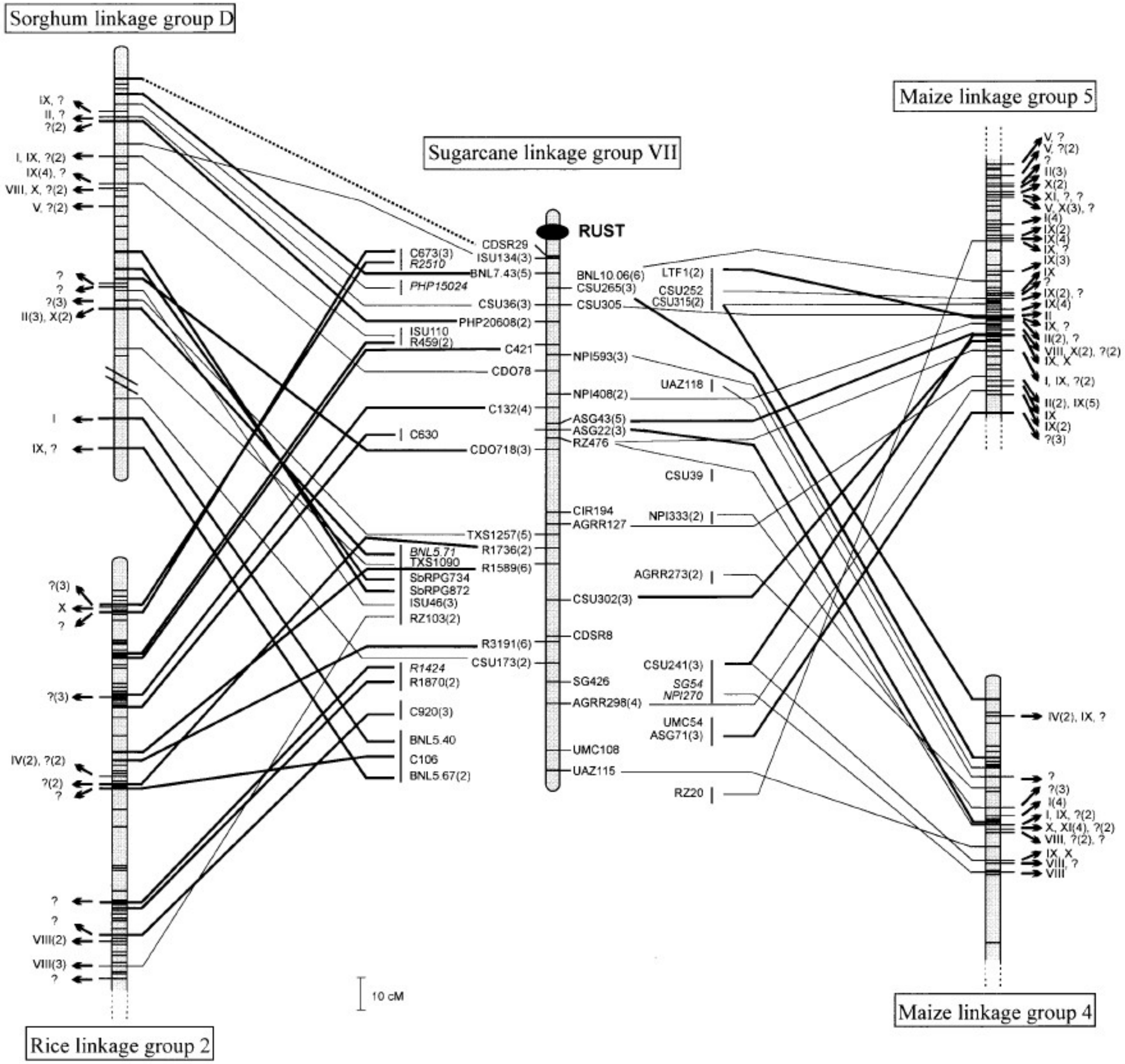
Può causare perdite fino al 40% del raccolto

La cultivar R570 possiede un gene di resistenza

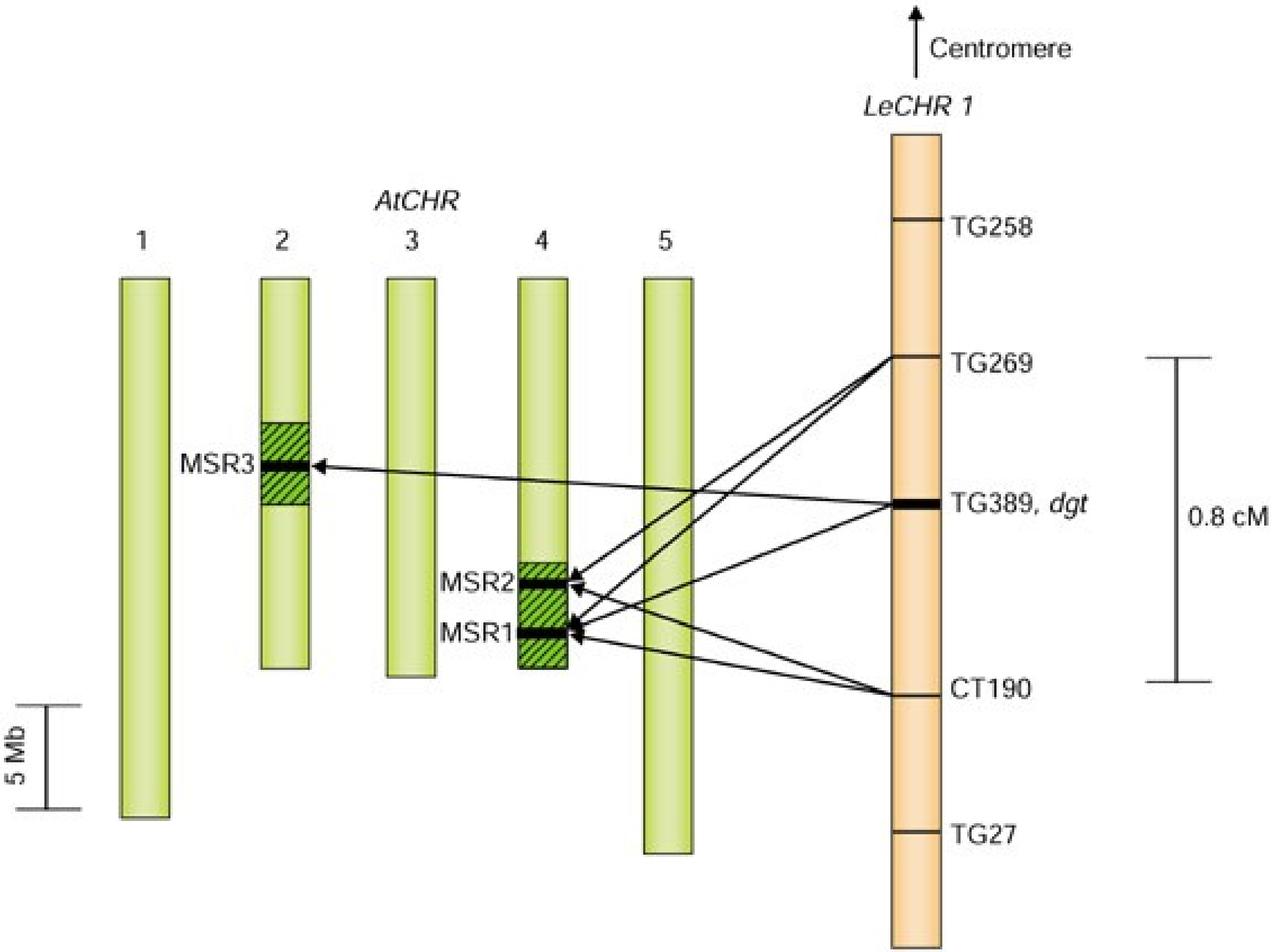
Table 1 Distribution of the 279 markers mapped on R570 linkage groups and locus coincidence with sorghum, maize and rice homoeologous segments

Linkage group[a]	Number of markers/linkage group	Number of probes involved	Locus coincidence with homoeologous regions			
			Sorghum D	Maize 4	Maize 5	Rice 2
I	11	5	2	2	2	–
II	12	6	2	–	4	–
IV	4	2	–	1	–	1
V	4	4	1	–	3	–
VII	122	53	17	11	14	14
VIII	10	7	1	3	1	2
IX	36	18	4	3	13	–
X	13	8	2	2	4	1
XI	5	2	–	1	1	–
Total	**217**		**29**	**23**	**42**	**18**
L	32	9				
U	30	34				
Total	**279**					

[a] Roman numbers indicate sugarcane linkage groups (in accordance with Grivet et al. 1996); L indicates co-segregation groups yet unassigned to any defined linkage group; U indicates markers yet unlinked. The nomenclature of sorghum linkage groups is that of Pereira et al. (1994)


Sorghum linkage group D
IX, ?
II, ?
?(2)
I, IX, ?(2)
IX(4), ?
VIII, X, ?(2)
V, ?(2)
?
?
?(3)
II(3), X(2)
I
IX, ?
Sugarcane linkage group VII
RUST
CDSR29
ISU134(3)
BNL7.43(5)
CSU36(3)
PHP20608(2)
C421
CDO78
C132(4)
CDO718(3)
TXS1257(5)
R1736(2)
R1589(6)
R3191(6)
CSU173(2)
C673(3)
R2510
PHP15024
ISU110
R459(2)
C630
BNL5.71
TXS1090
SbRPG734
SbRPG872
ISU46(3)
RZ103(2)
R1424
R1870(2)
C920(3)
BNL5.40
C106
BNL5.67(2)
BNL10.06(6)
CSU265(3)
CSU305
NPI593(3)
NPI408(2)
ASG43(5)
ASG22(3)
RZ476
CIR194
AGRR127
CSU302(3)
CDSR8
SG426
AGRR298(4)
UMC108
UAZ115
LTF1(2)
CSU252
CSU315(2)
UAZ118
CSU39
NPI333(2)
AGRR273(2)
CSU241(3)
SG54
NPI270
UMC54
ASG71(3)
RZ20
Maize linkage group 5
V, ?
V, ?(2)
?
II(3)
X(2)
XI, ?, ?
V, X(3), ?
I(4)
IX(2)
IX(4)
IX, ?
IX(3)
IX
?
IX(2), ?
IX(4)
II
IX, ?
II(2), ?
VIII, X(2), ?(2)
IX, X
I, IX, ?(2)
II(2), IX(5)
IX
IX(2)
?(3)
Rice linkage group 2
?(3)
X
?
?(3)
IV(2), ?(2)
?(2)
?
?
?
VIII(2)
VIII(3)
?
10 cM
Maize linkage group 4
IV(2), IX, ?
?
?(3)
I(4)
I, IX, ?(2)
X, XI(4), ?(2)
VIII, ?(2), ?
IX, X
VIII, ?
VIII

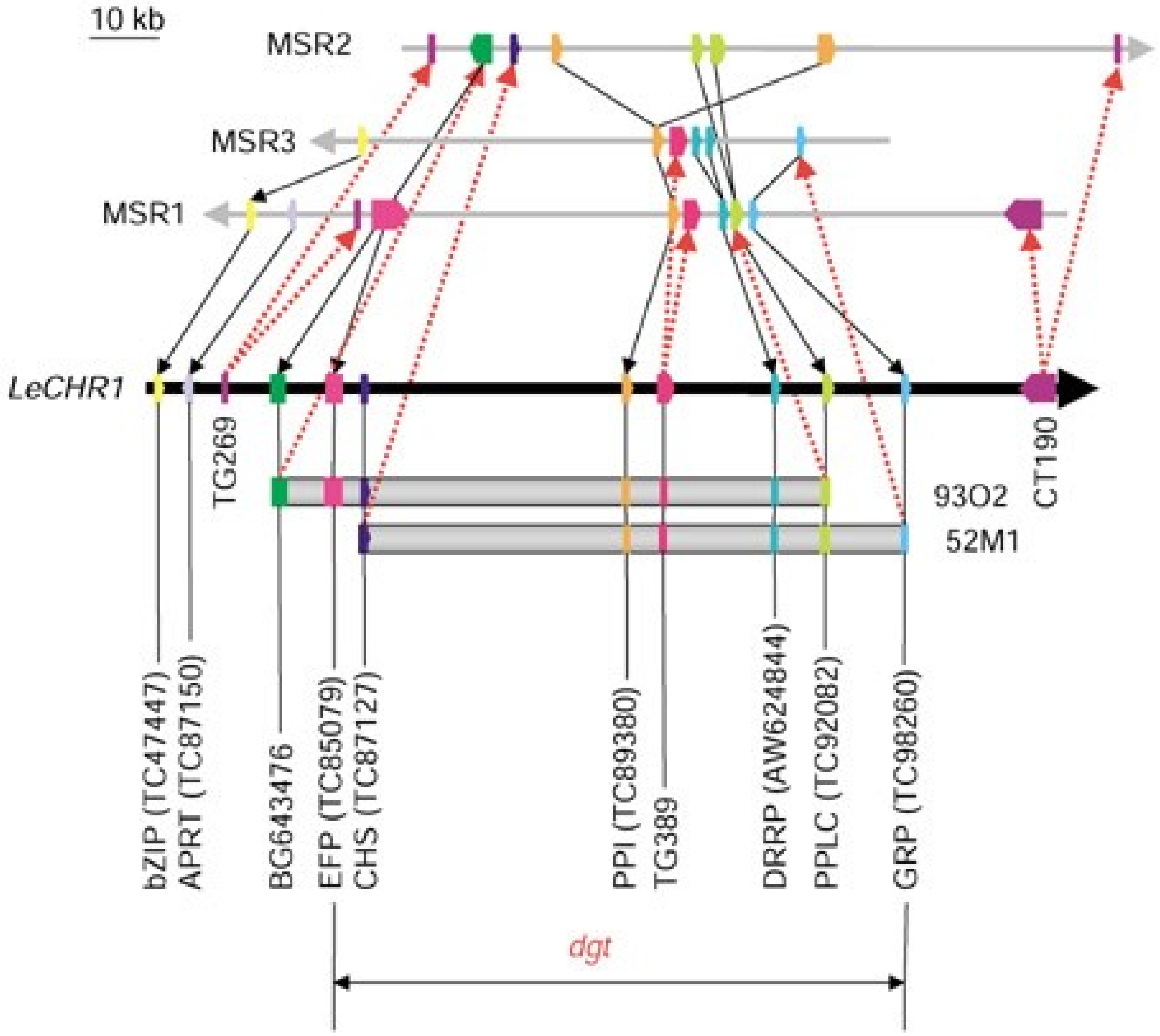
Regioni microsinteniche tra arabidopsis e pomodoro

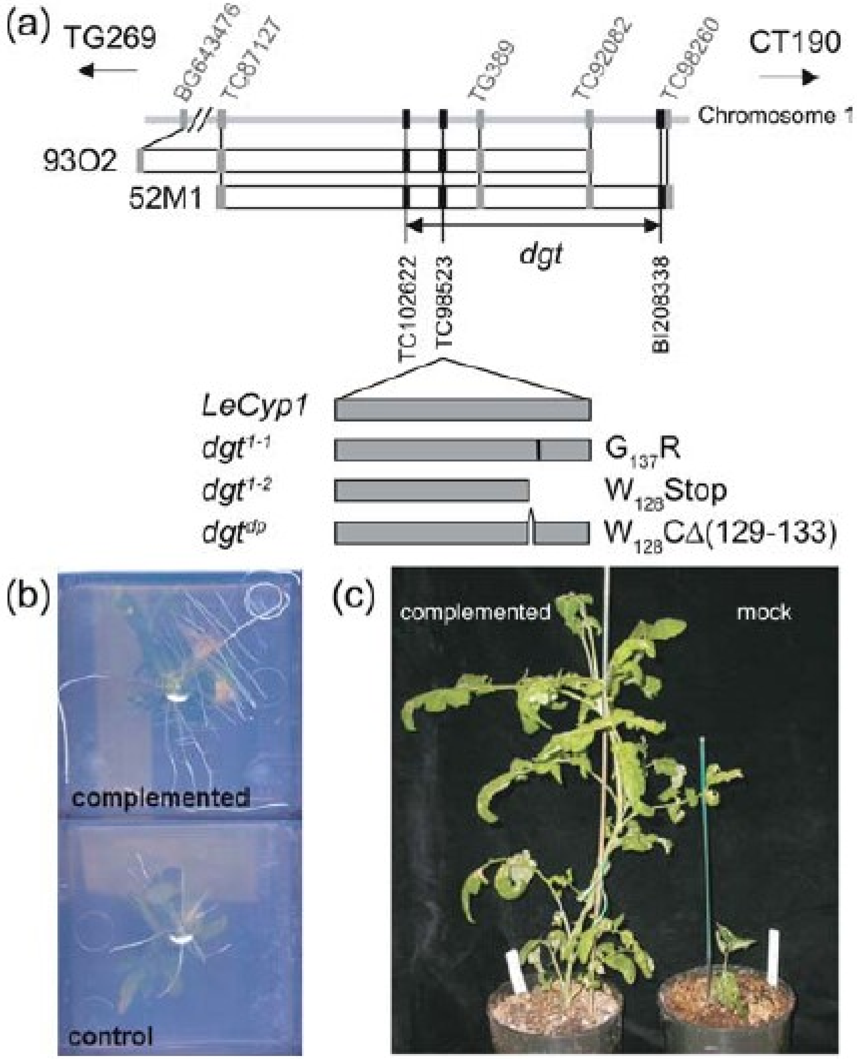


Il mutante *diageotropica* (*dgt*) di pomodoro

- **Ridotta sensibilità all'auxina**

Mappatura del locus *dgt* di pomodoro sulla base della microsintenia con arabidopsis




(a)
TG269
BG643476
TC87127
TG389
TC92082
TC98260
CT190
Chromosome 1
93O2
52M1
dgt
TC102622
TC98523
BI208338
LeCyp1
dgt^{1-1}
$G_{137}R$
dgt^{1-2}
$W_{128}Stop$
dgt^{dp}
$W_{128}C\Delta(129-133)$
(b)
complemented
control
(c)
complemented
mock

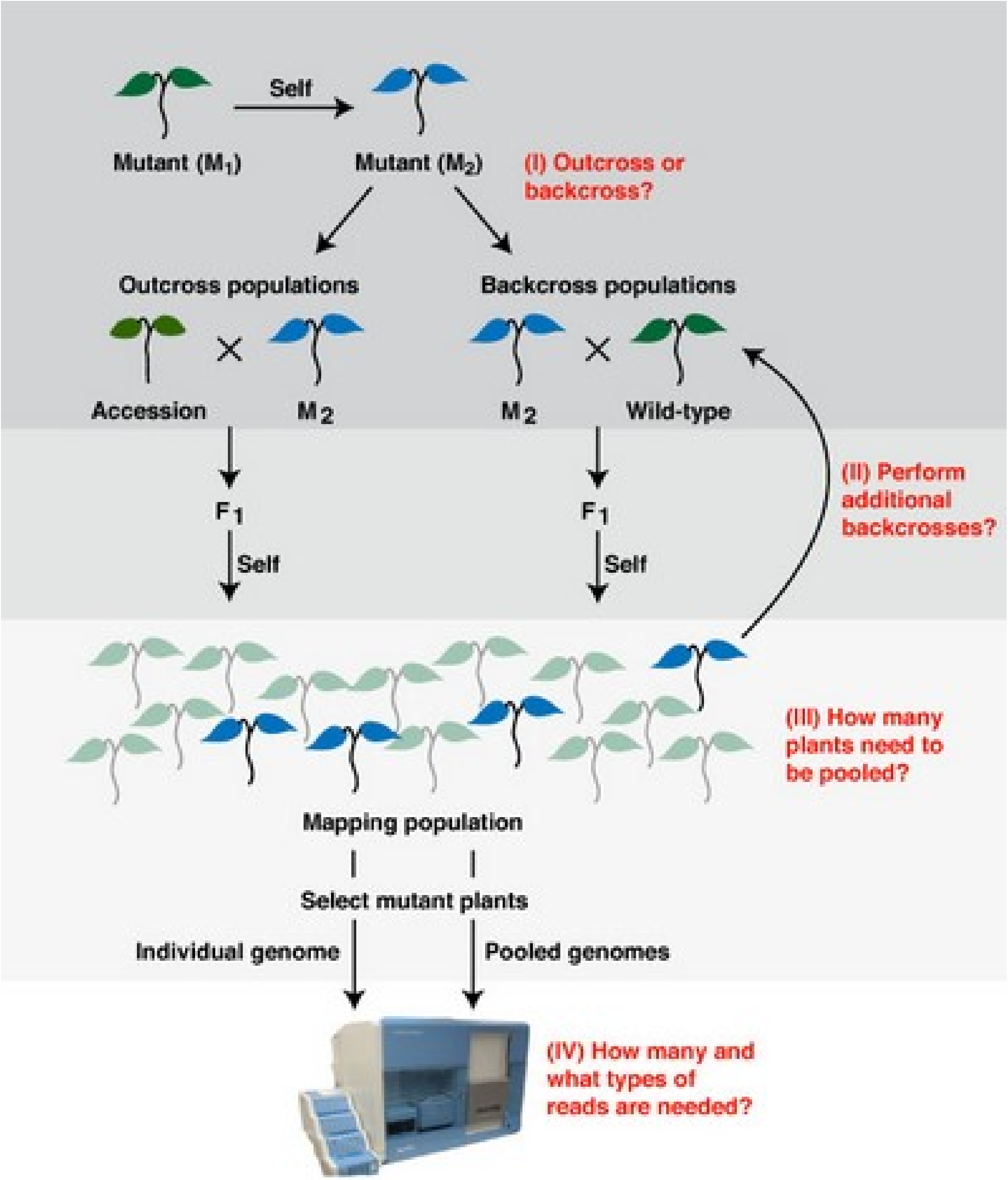
Mutation mapping by deep sequencing

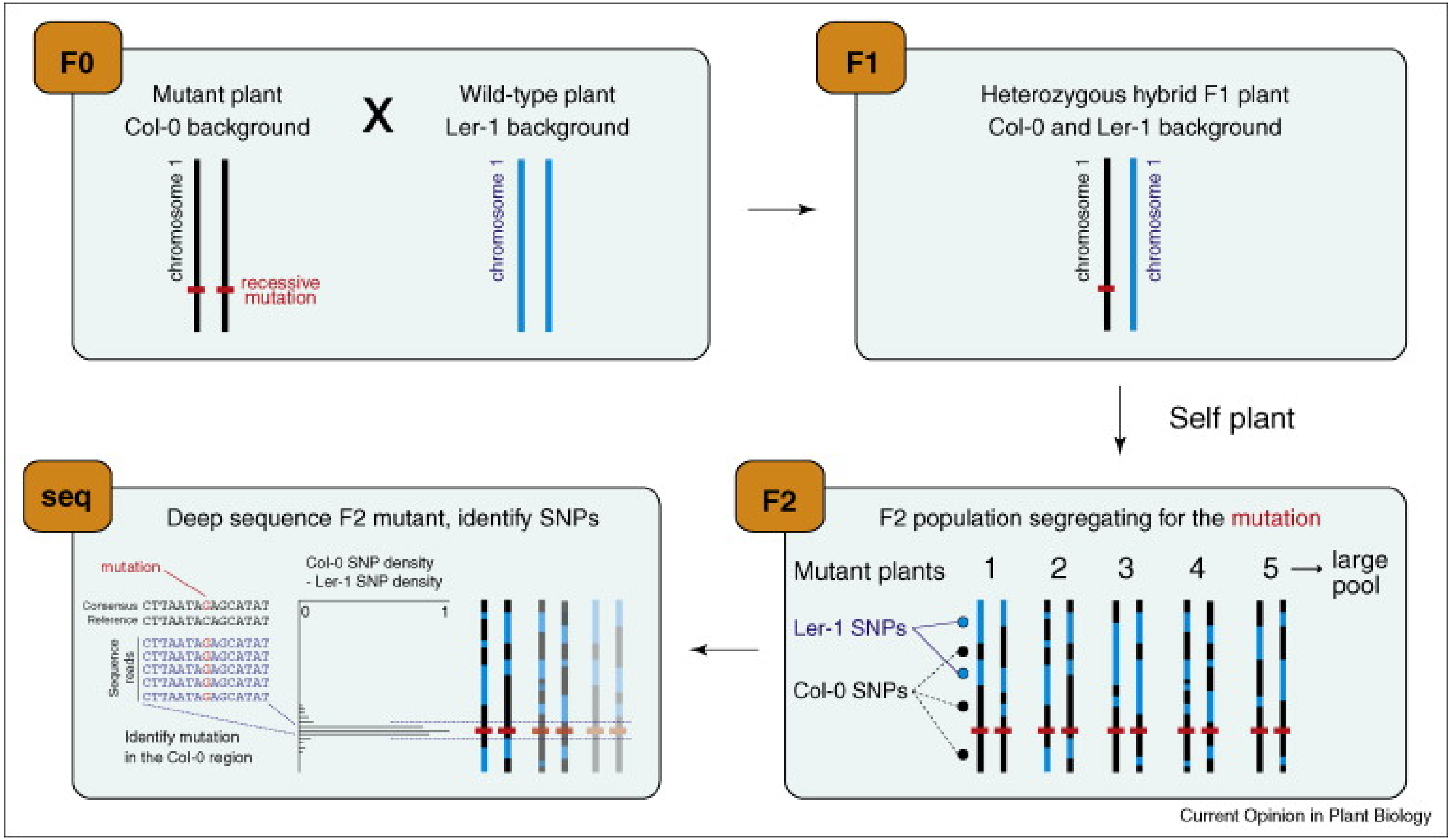
Forward genetic screens have proven extremely powerful in *Arabidopsis thaliana* for assigning genes to specific biological pathways .

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 (M_1)
Mutant (M_2)
(I) Outcross or backcross?
Outcross populations
Backcross populations
Accession
×
M_2
M_2
×
Wild-type
F_1
Self
F_1
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?



Current Opinion in Plant Biology

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

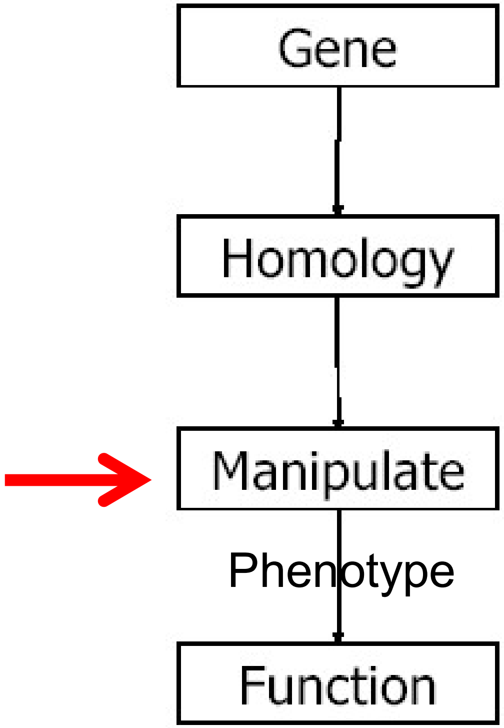
	Outcross populations	**Backcross populations**	**Direct sequencing**	**Deep candidate resequencing (dCARE)**
Generation	F_2	BC_1F_2	$BC_{1-3}F_2$[a]	n/a
Mutants (n)	Approximately 150	Approximately 50	1	As many as possible
Optimal coverage	>25	Approximately 50	>25	n/a
Sequencing type	Paired-end	Paired-end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

GENETICA INVERSA

‘Reverse Genetics’



Gene

Homology

Manipulate

Phenotype

Function

- 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

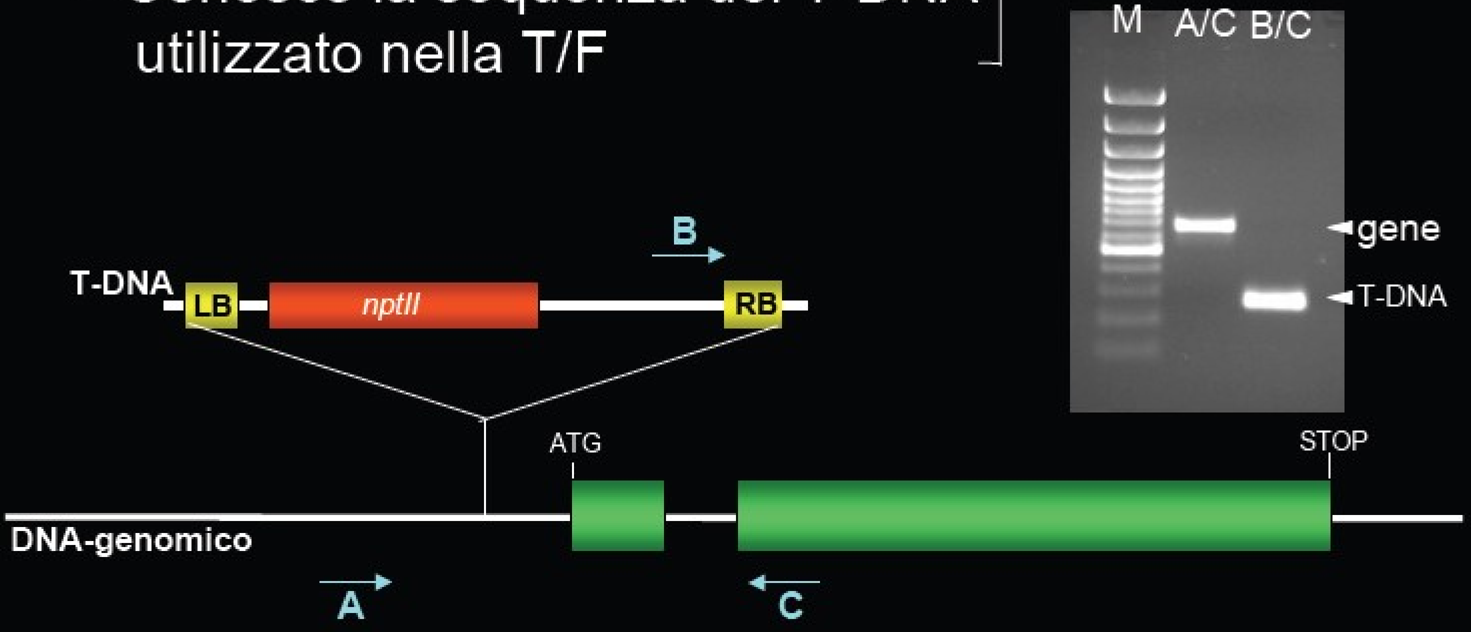
How to choose a gene:

- 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

Approccio di Genetica Inversa:

- Conosco la sequenza del mio gene di interesse
- Conosco la sequenza del T-DNA utilizzato nella T/F

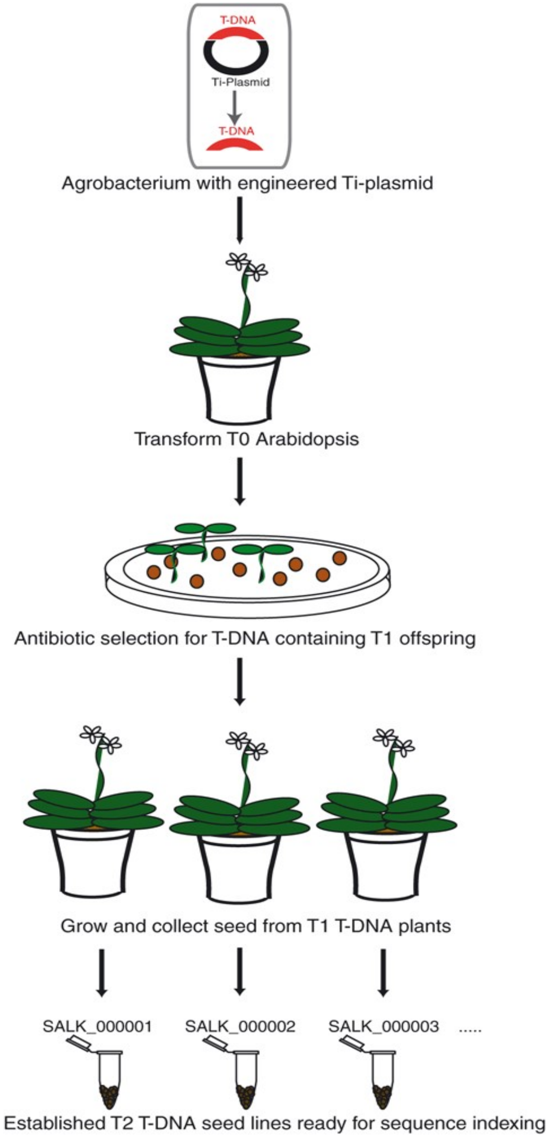
Disegno primers specifici per il gene ed il T-DNA



A+C = **sequenza genomica** B+C = **sequenza T-DNA/genomica**

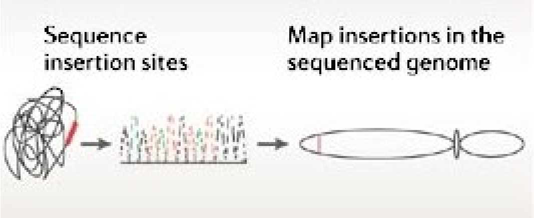
T-DNA insertional mutant collection for reverse genetics (commercially available)

Collection of mutants



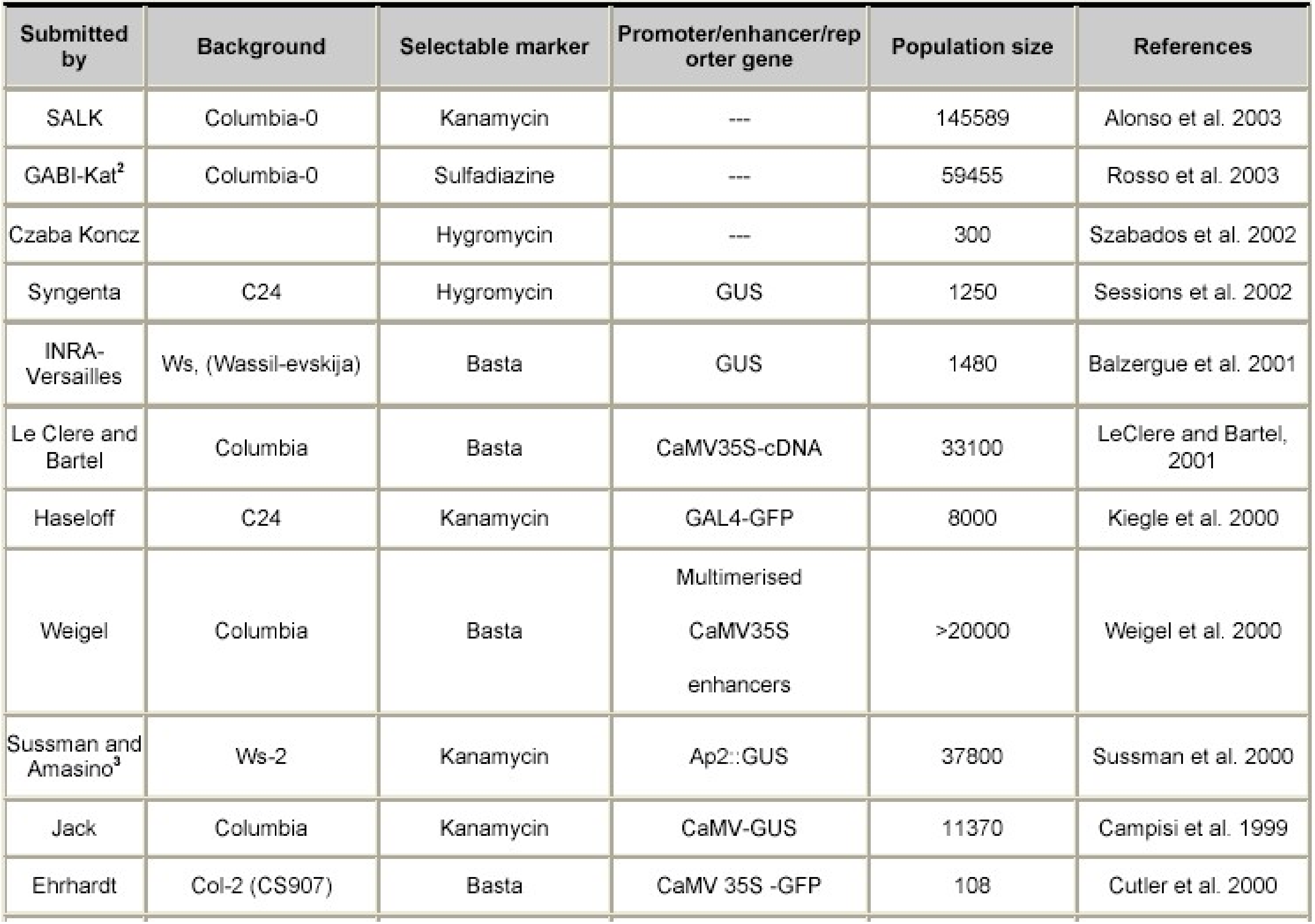
Identification of insertion sites
(TAIL PCR, Inverse PCR, plasmid
rescue)

Catalogued mutants



Commercially available

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

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

Tail-PCR e sequenziamento del DNA vicino al sito di inserzione

Ricerca omologia sul DNA genomico

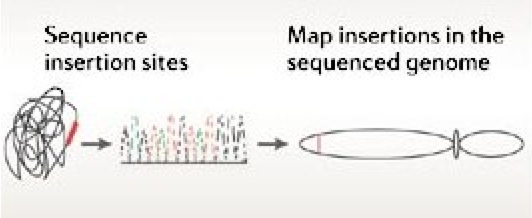
Organizzazione in banche dati

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

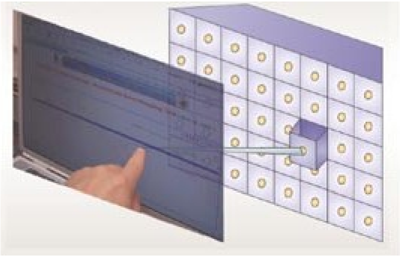
http://signal.salk.edu/tabout.html

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

Catalogued mutants

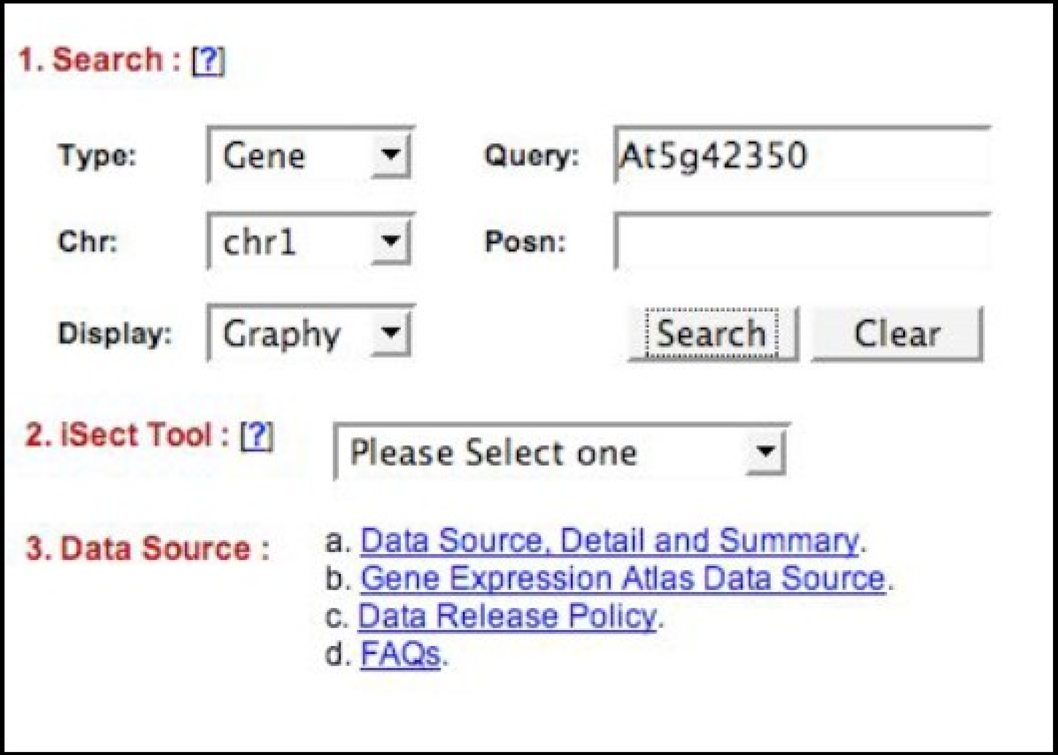


Select on the Internet and buy mutants in the gene or genes of interest



Molecular genotyping

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



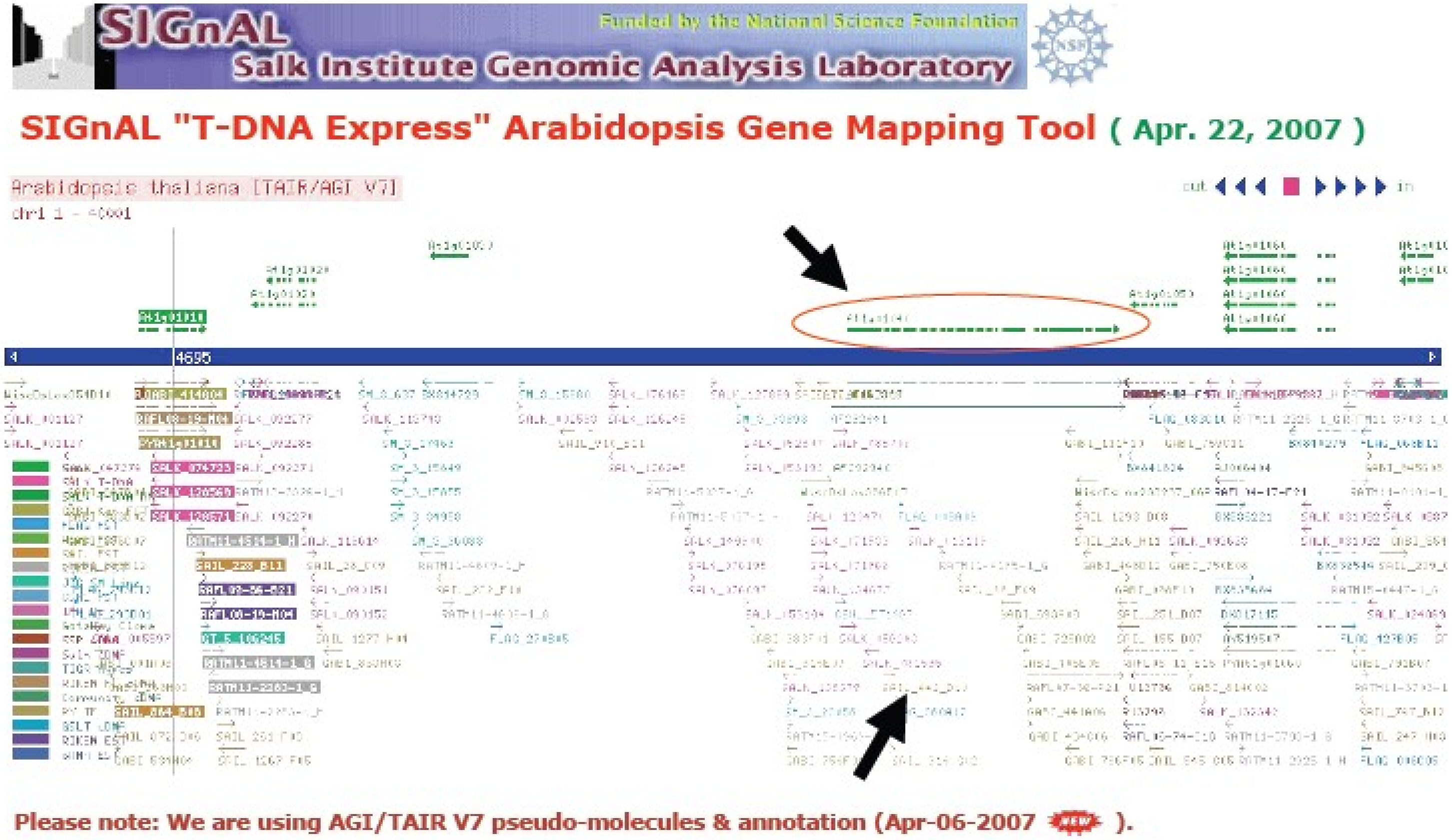
1. Search : [?]

Type: Gene
Query: At5g42350
Chr: chr1
Posn:
Display: Graphy

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.

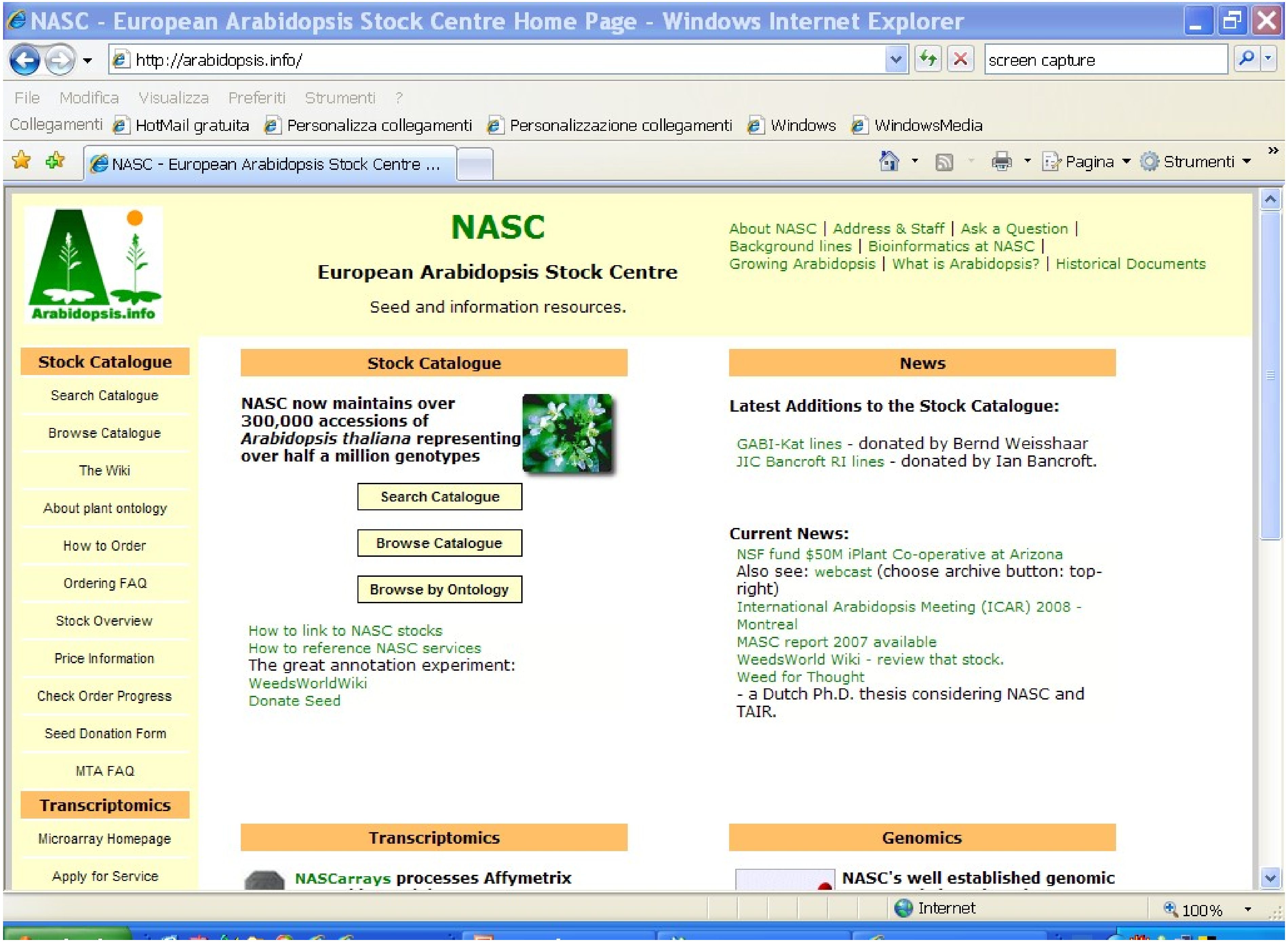


SIGnAL "T-DNA Express" Arabidopsis Gene Mapping Tool (Apr. 22, 2007)

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

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

Ordino il mutante !!



NASC

European Arabidopsis Stock Centre

Seed and information resources.

About NASC | Address & Staff | Ask a Question | Background lines | Bioinformatics at NASC | Growing Arabidopsis | What is Arabidopsis? | Historical Documents

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.

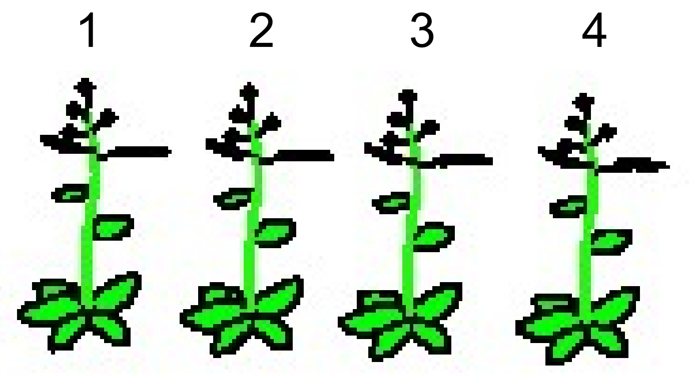
Transcriptomics

NASCarrays processes Affymetrix

Genomics

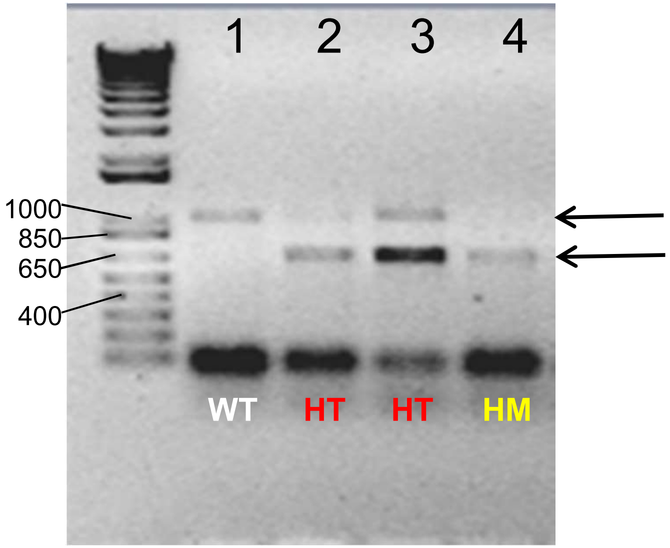
NASC's well established genomic

Mutant genotyping

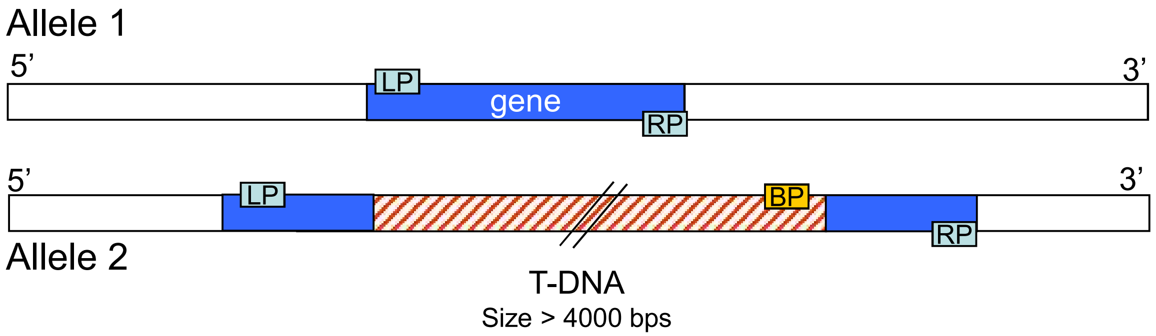


Genomic extraction

PCR

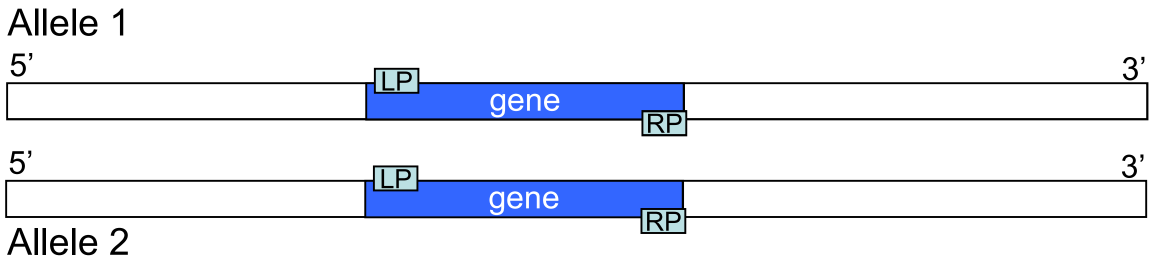


Mutant genotyping

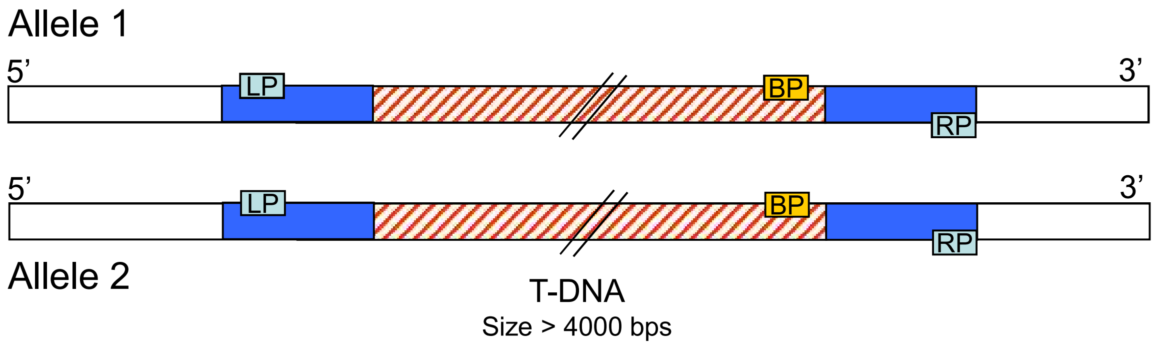


PCR primer pairs

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

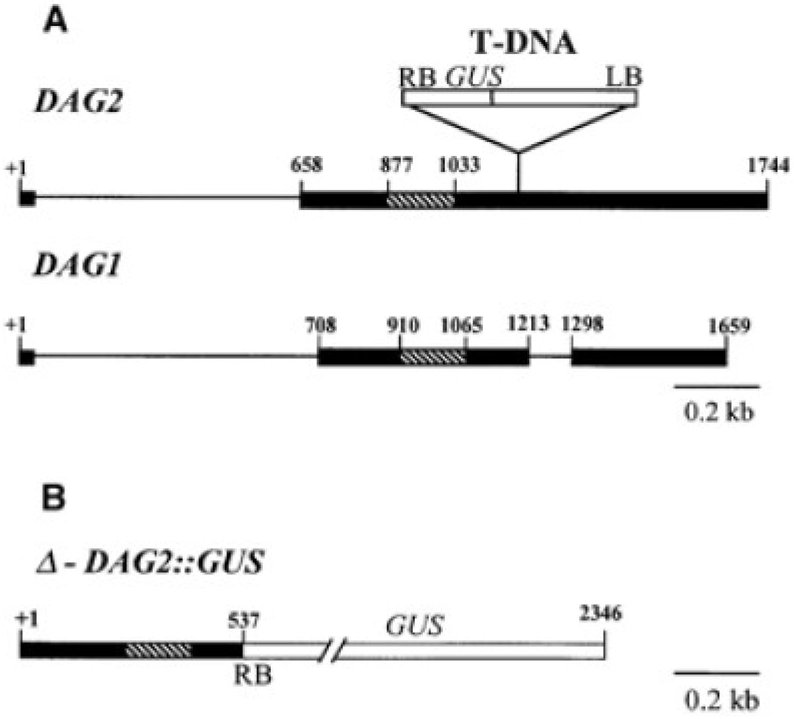


LP + RP Product
LP + BP No product
BP + RP No product

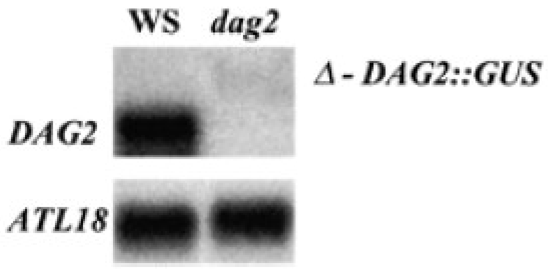


LP + RP No product
LP + BP No product
BP + RP Product

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

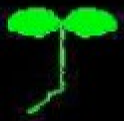


Se il gene è distrutto non si dovrebbe avere la presenza del trascritto

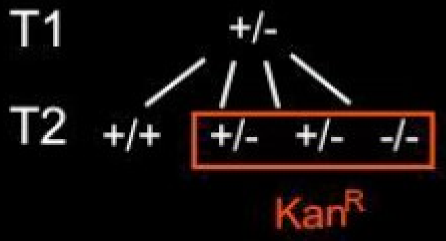


La T/F con *Agrobacterium* ha una efficienza medi di 1,5 inserzioni di T-DNA per linea

Più inserzioni nella mia linea?



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

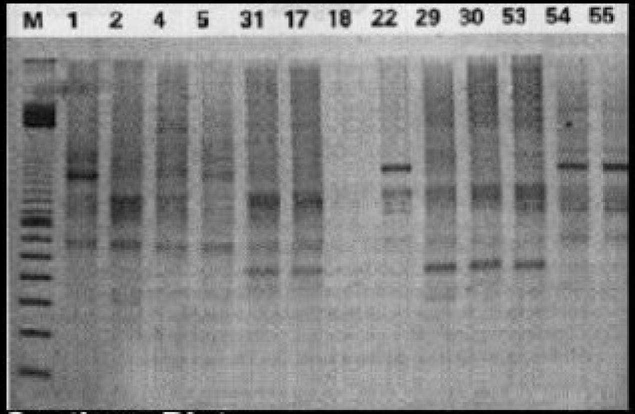


T1 +/-

T2 +/+ +/- +/- -/-

Kan^R

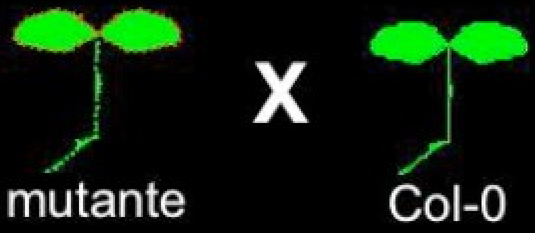
- = Allele selvatico
- = T-DNA



Southern Blot

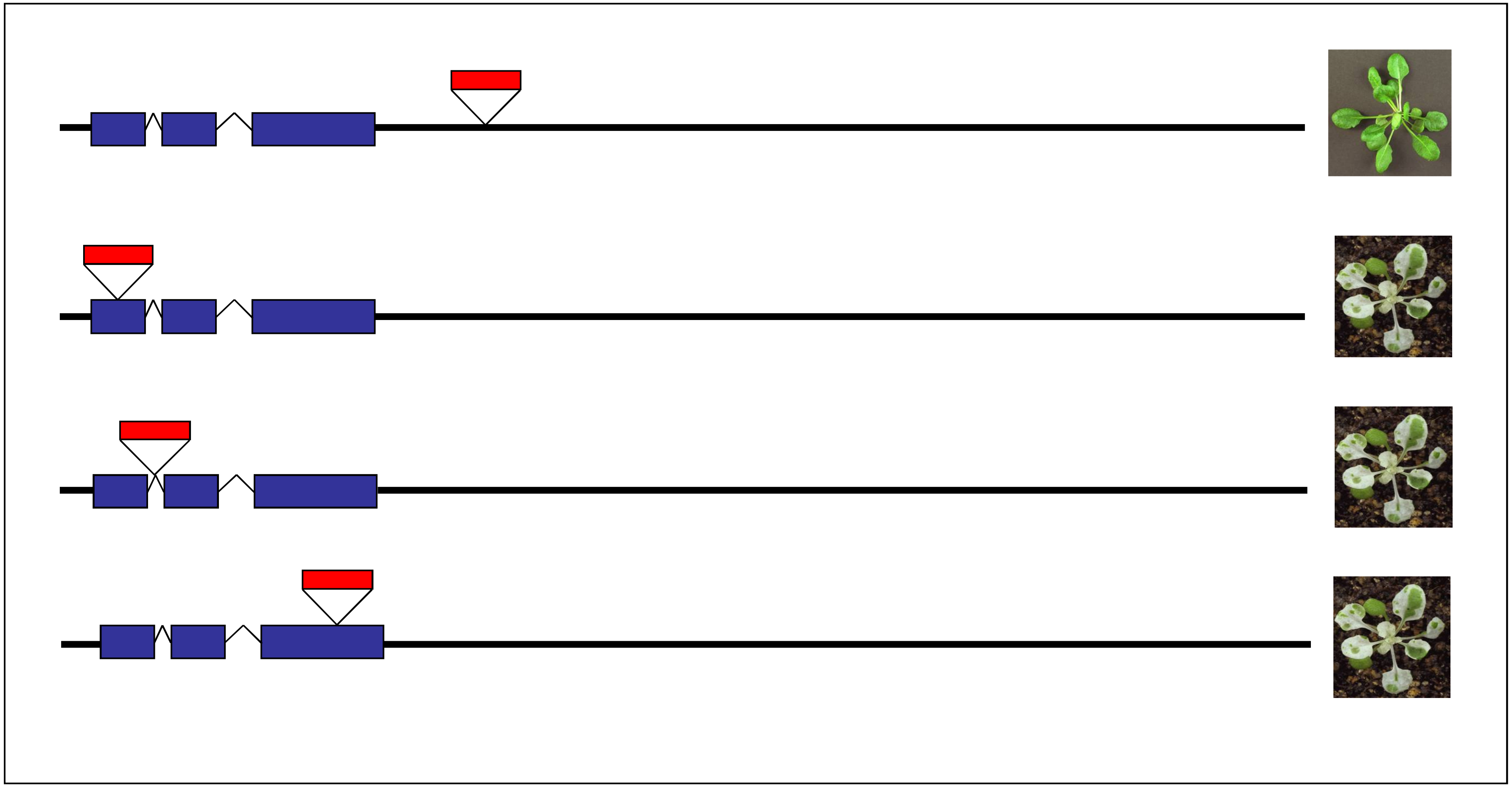
→ Analisi del pattern di bande

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 $(1/2)^n$

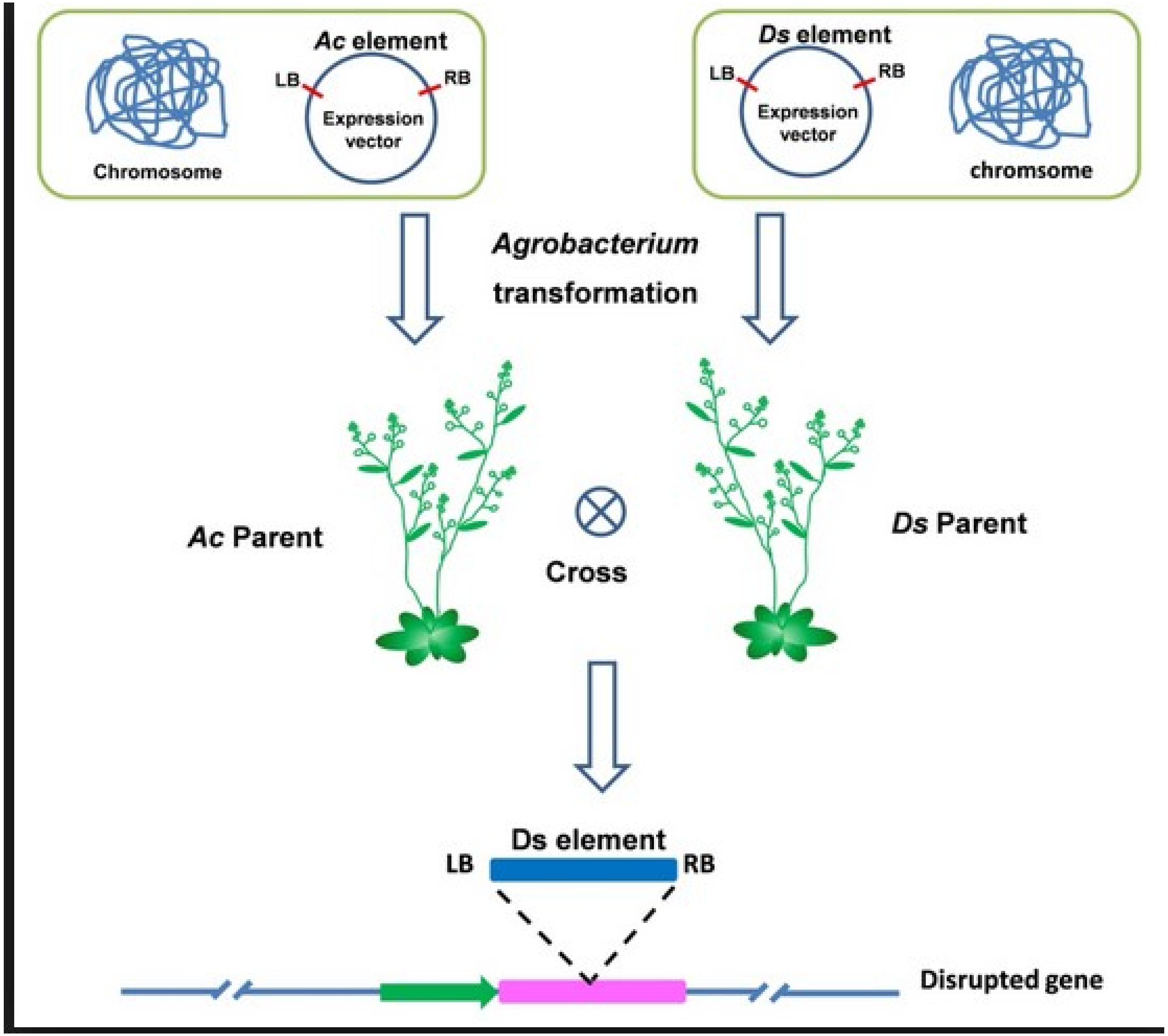
Analyze phenotype

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


Chromosome
Ac element
LB
RB
Expression vector
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)

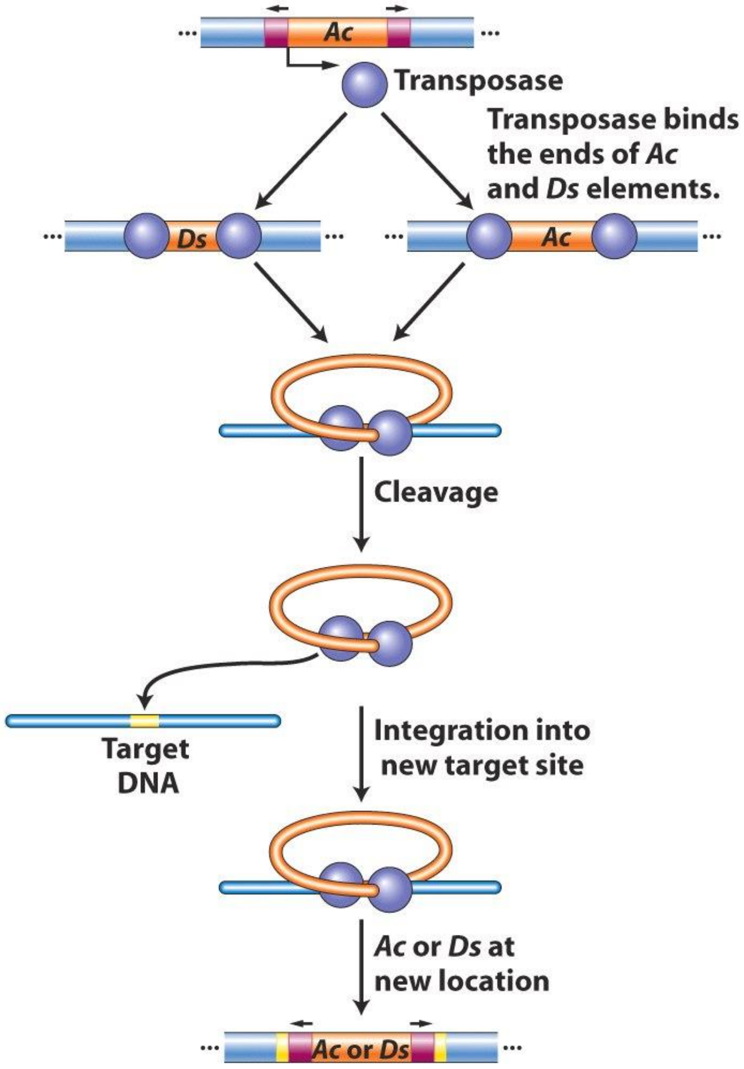
- Ds is the transposon (does not encode a transposase)
- *Ac* is an immobile element encoding the transposase

- 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

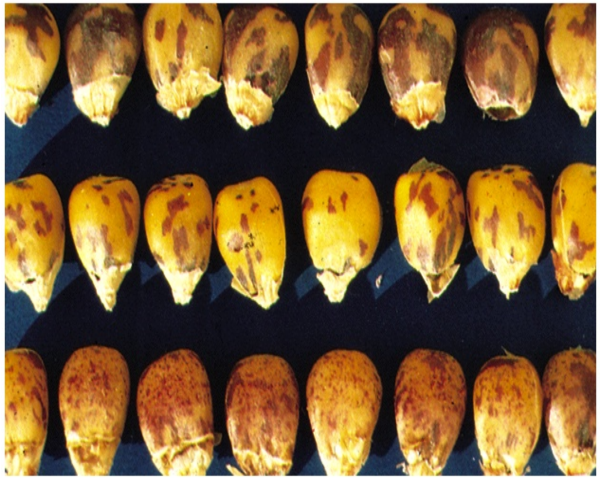
MUTAGENESI TRAMITE TRASPOSONI



Esempio: il sistema Ac/Ds (Activator/Dissociator)

Due linee transgeniche, che recano una l’elemento Ac e l’altra l’elemento Ds vengono incrociate.

Nella progenie la TRASPOSASI Ac mobilizza l’elemento Ds, generando piante F1 che sono MOSAICI GENETICI (Barbara McClintock)



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.

MUTAGENESI TRAMITE TRASPOSONI

Gli elementi Ds traspongono preferibilmente in siti vicini al sito donatore, per cui le linee sono disegnate in modo da permettere:

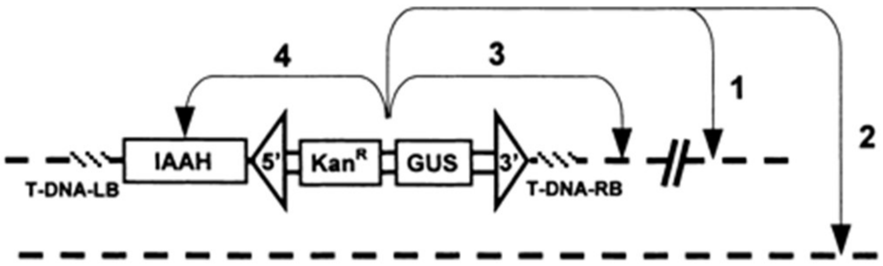
a) la selezione positiva per la presenza di Ds originati da nuove trasposizioni

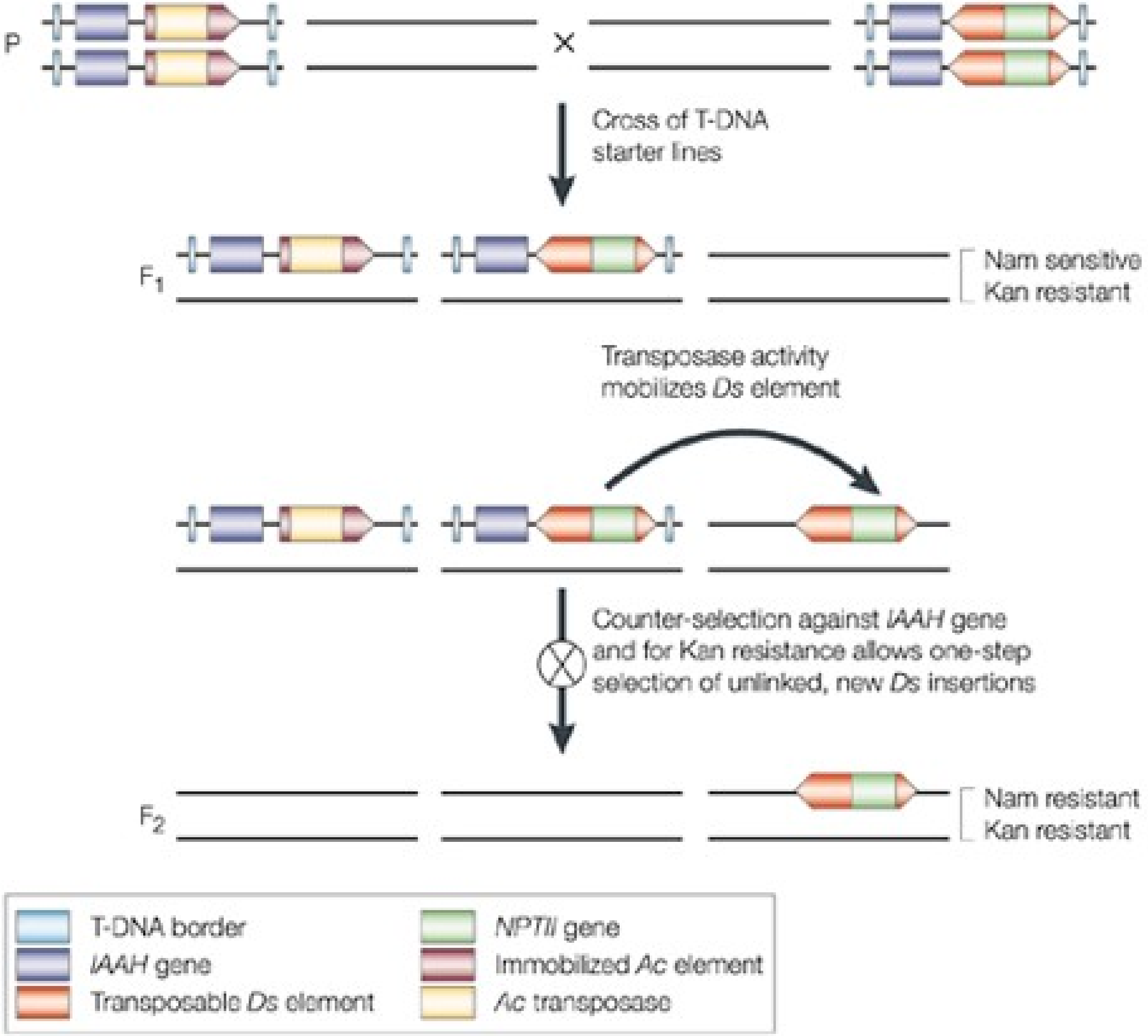
b) la selezione negativa contro Ac e contro il locus Ds donatore.

Il T-DNA che contiene Ac reca anche il gene per l'indolacetammide idrolasi (IAAH), che conferisce sensibilità al naftalene acetammide (NAM).

L'elemento Ds porta il gene NPTII (resistenza alla kanamicina)

Il T-DNA per l'elemento Ds donatore porta anche il gene IAAH, in modo da selezionare contro la presenza del Ds trasposto vicino al Ds donatore.





Nature Reviews | Genetics

VANTAGGI DELLA MUTAGENESI TRAMITE TRASPOSONI

1) La mutazione è reversibile: in presenza della trasposasi, il trasposone può escindersi nuovamente dal locus mutagenizzato, ripristinando il locus WT e confermando che il fenotipo è legato all'inserzione
2) Poiché gli eventi di trasposizione avvengono preferibilmente vicino la sito donatore, si può utilizzare questa strategia per effettuare mutagenesi mirate su determinate regioni cromosomiche

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.

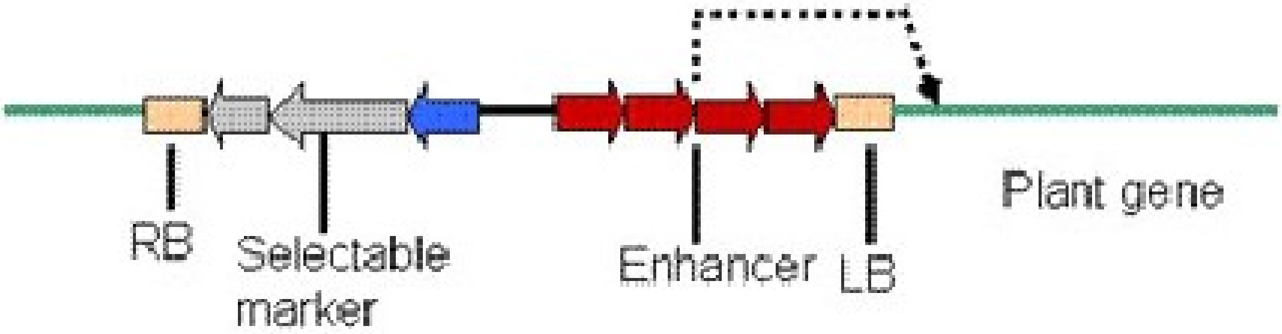
1) Generation of gain of function mutants by activation tagging

-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.

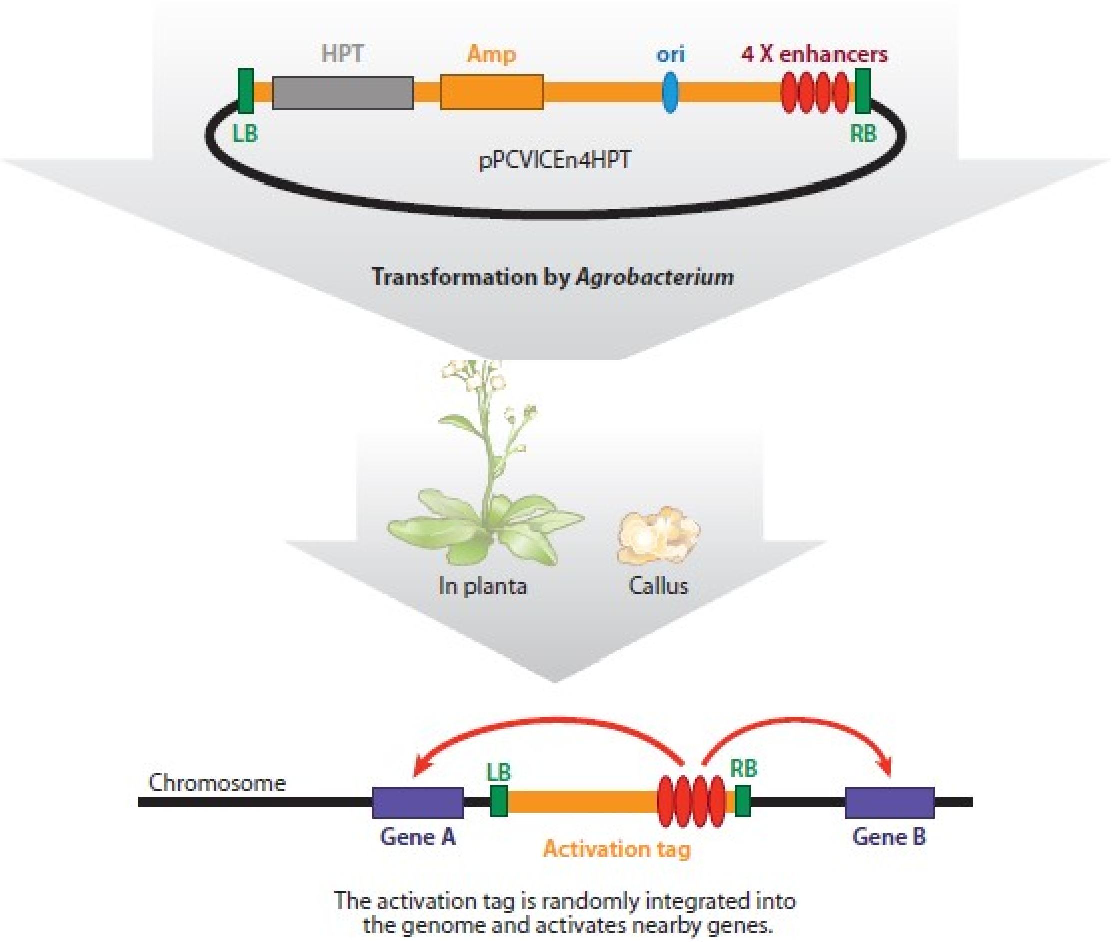


Figure 1

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.

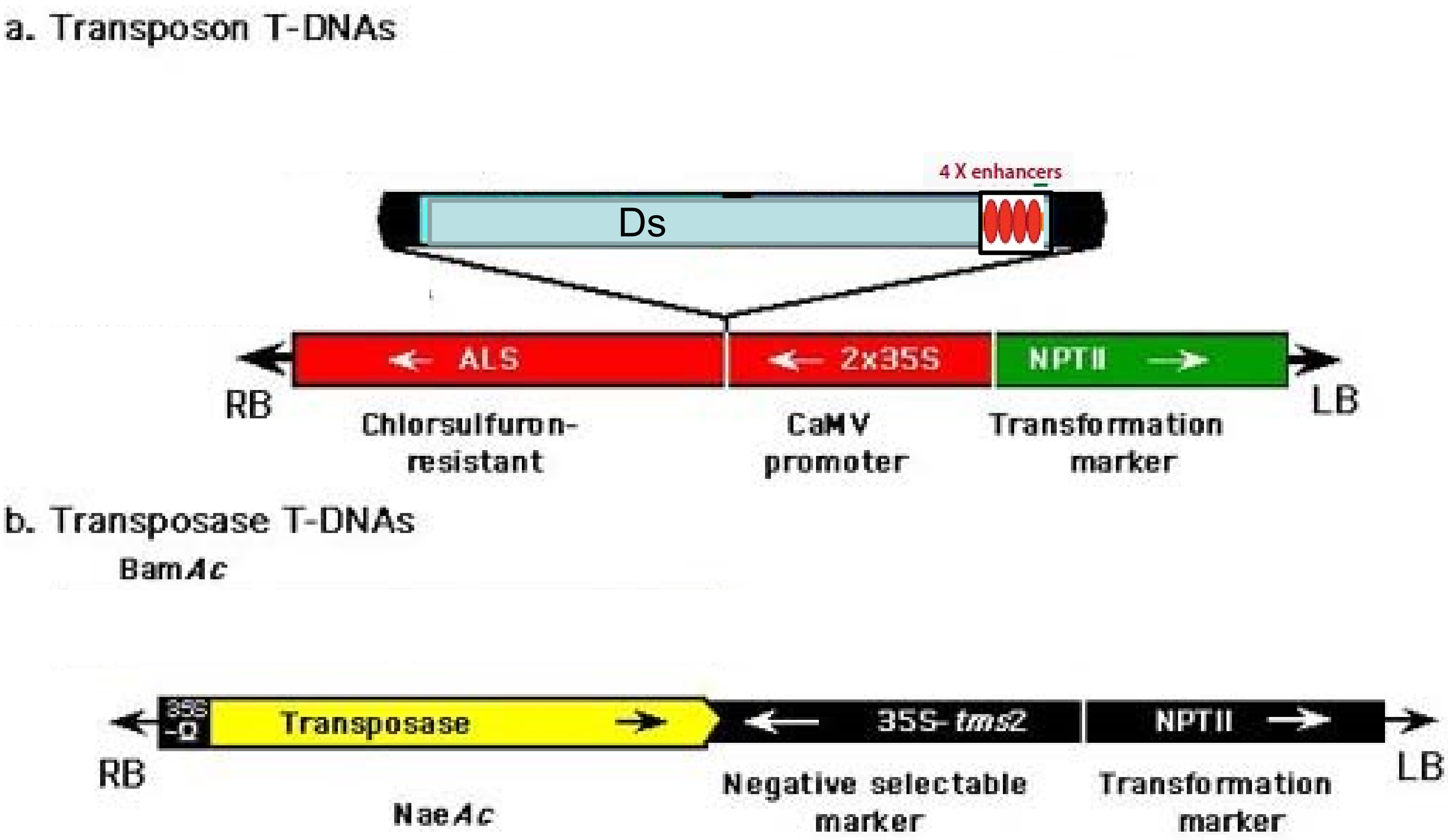
Transposon-based Activation Tagging

- Two transposons used:
    - Ac/Ds Transposon

    - En1 Transposon

Construct for transposon-based Activation Tagging

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



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

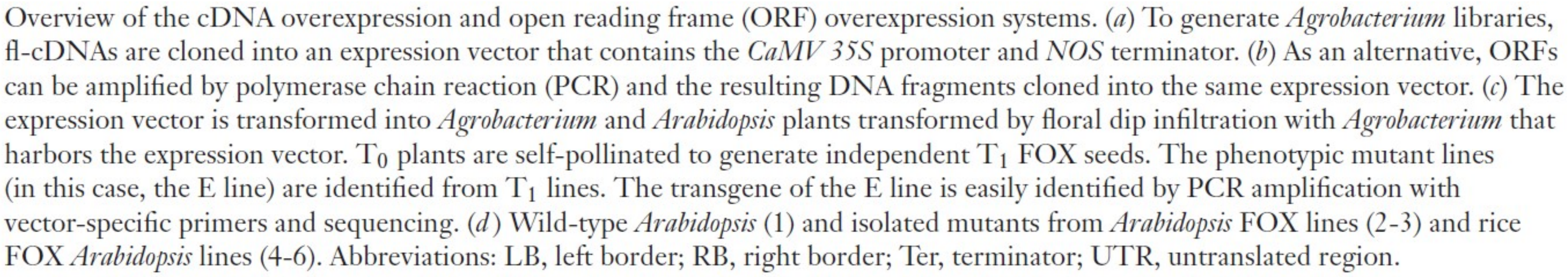
Arabidopsis lines that overexpresses random cDNAs driven by the *CaMV 35S* promoter.
More than 30,000 *Arabidopsis* transgenic plants have been generated

Problem cDNA may not be full length

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

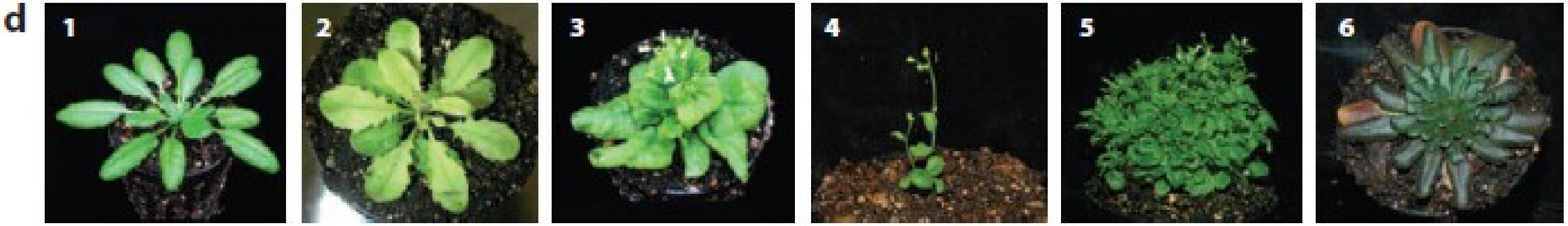
3) Gain-of-function mutants generated by the FOX Hunting System

The full-length cDNA over-expressing gene (FOX) hunting system is an alternative gain-of-function approach that uses fl-cDNAs



Overview of the cDNA overexpression and open reading frame (ORF) overexpression systems. (*a*) To generate *Agrobacterium* libraries, fl-cDNAs are cloned into an expression vector that contains the *CaMV 35S* promoter and *NOS* terminator. (*b*) As an alternative, ORFs can be amplified by polymerase chain reaction (PCR) and the resulting DNA fragments cloned into the same expression vector. (*c*) The expression vector is transformed into *Agrobacterium* and *Arabidopsis* plants transformed by floral dip infiltration with *Agrobacterium* that harbors the expression vector. T_0 plants are self-pollinated to generate independent T_1 FOX seeds. The phenotypic mutant lines (in this case, the E line) are identified from T_1 lines. The transgene of the E line is easily identified by PCR amplification with vector-specific primers and sequencing. (*d*) Wild-type *Arabidopsis* (1) and isolated mutants from *Arabidopsis* FOX lines (2-3) and rice FOX *Arabidopsis* lines (4-6). Abbreviations: LB, left border; RB, right border; Ter, terminator; UTR, untranslated region.

See figure in the next slide


d
1
2
3
4
5
6

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

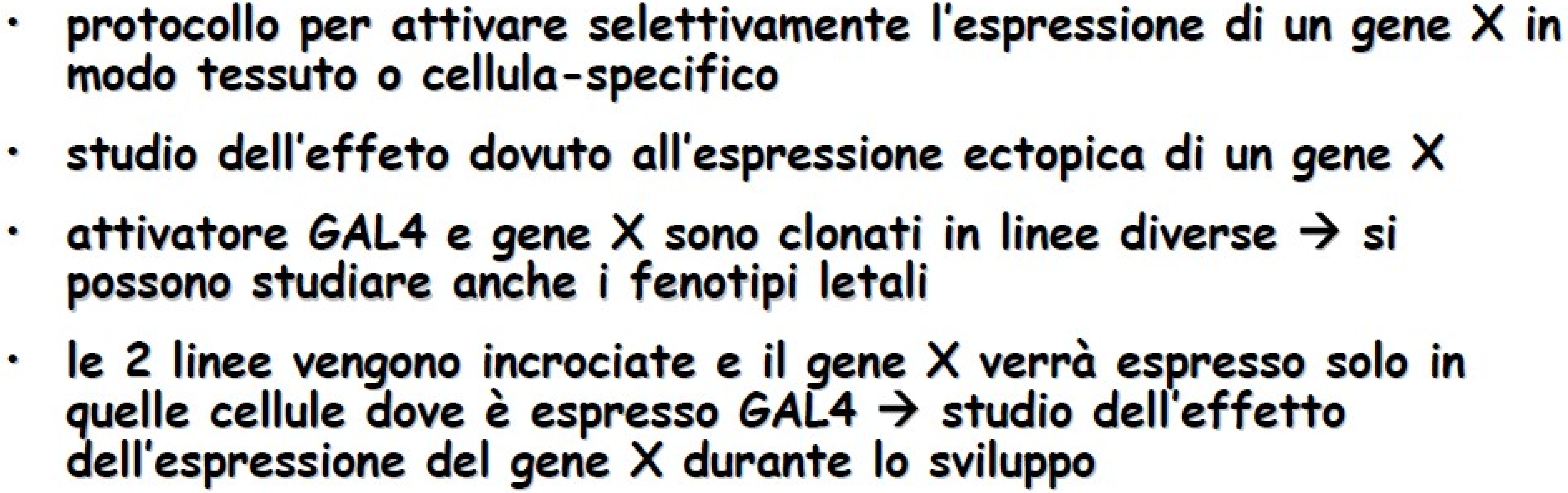
Usually the gene is placed under the CaMV 35S promoter

Inducible promoter

Dominant phenotype

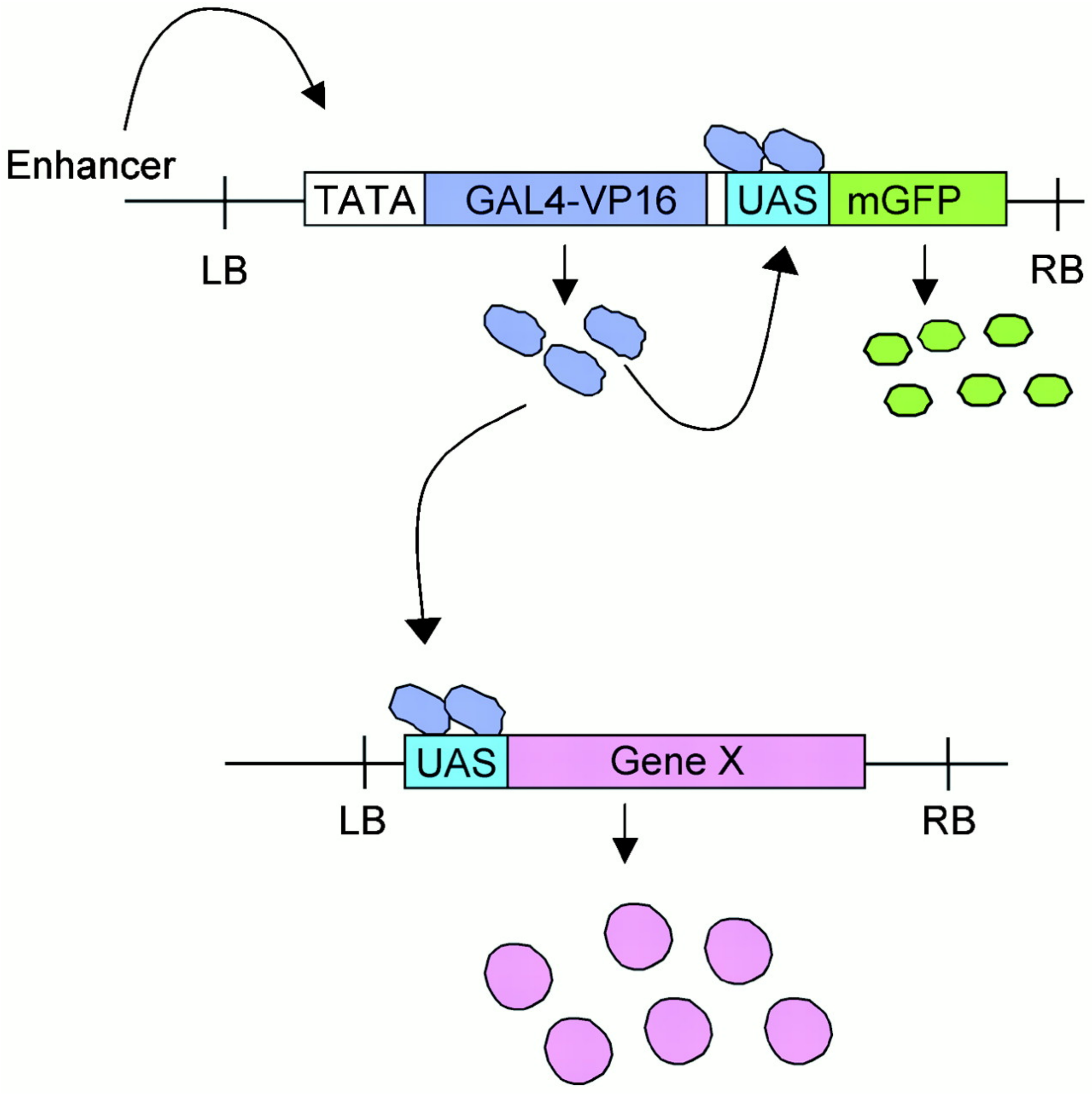
Espressione ectopica in specifici tessuti per generare fenotipi dominanti

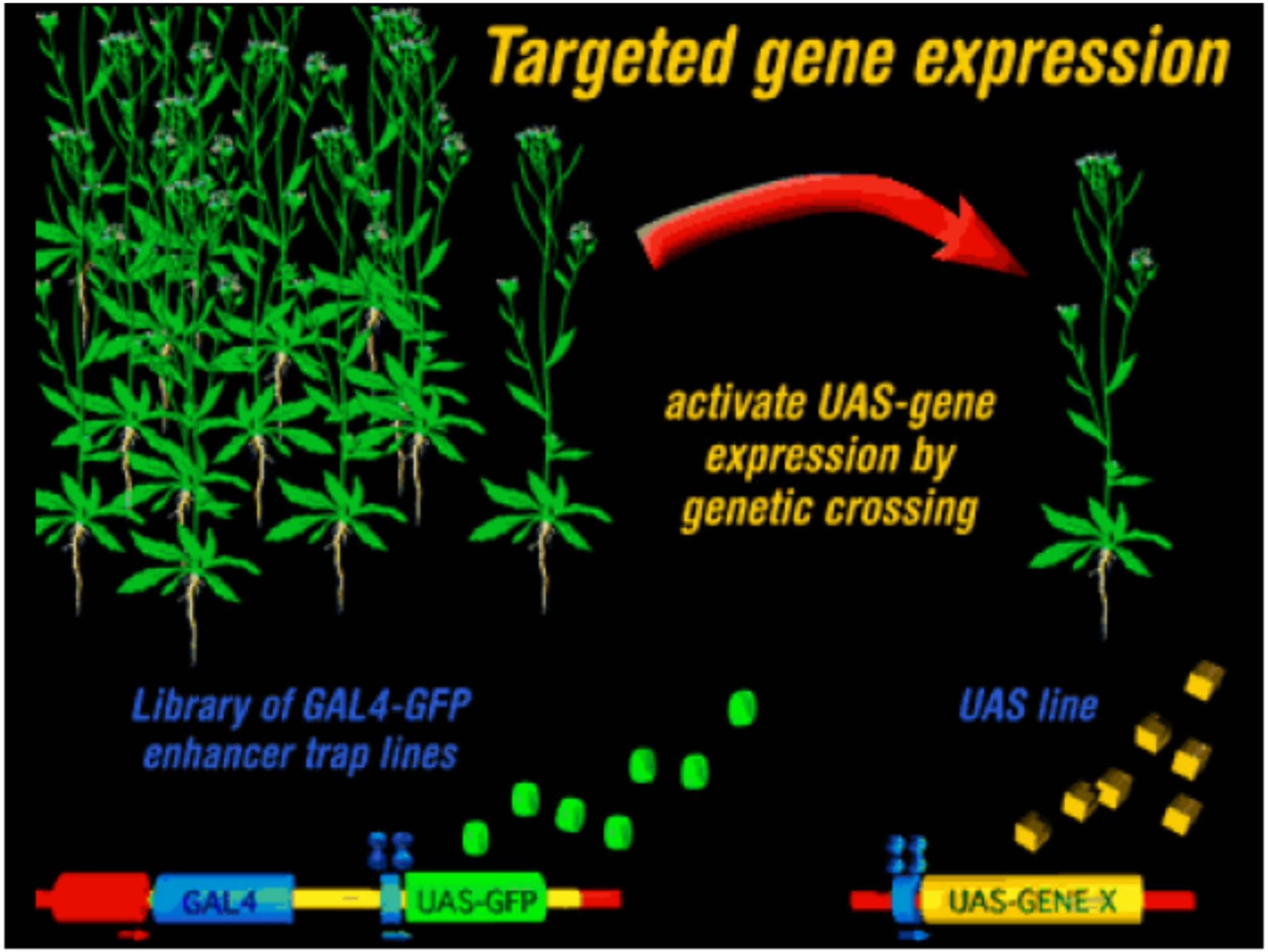
Sistema GAL4-UAS

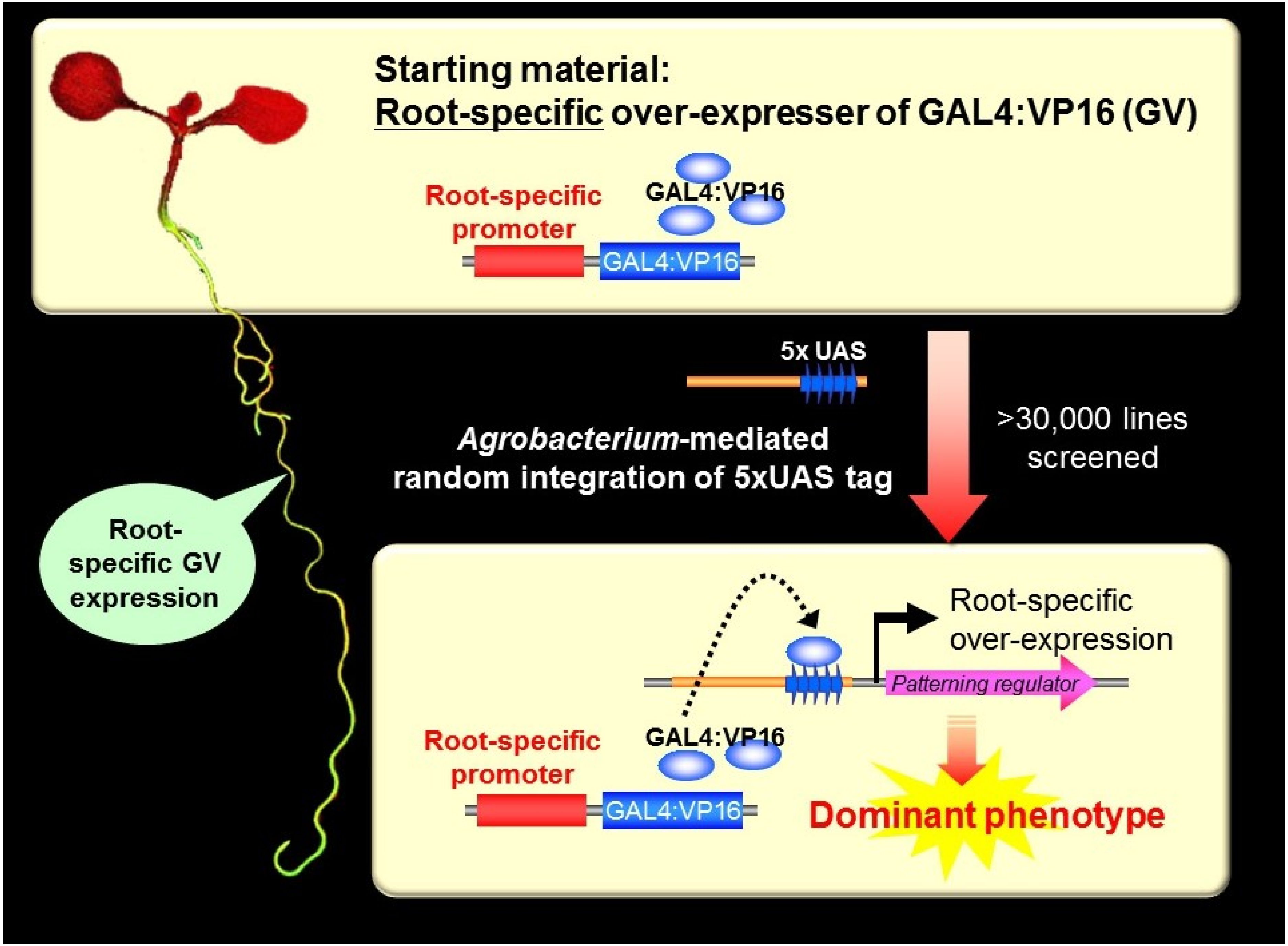


- protocollo per attivare selettivamente l’espressione di un *gene* X in modo tessuto o cellula-specifico
- studio dell’effeto dovuto all’espressione ectopica di un *gene* X
- attivatore *GAL4* e *gene* X sono clonati in linee diverse → si possono studiare anche i fenotipi letali
- le 2 linee vengono 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

Root-specific GV expression

5x UAS

Agrobacterium-mediated random integration of 5xUAS tag

>30,000 lines screened

Root-specific over-expression

Patterning regulator

Root-specific promoter

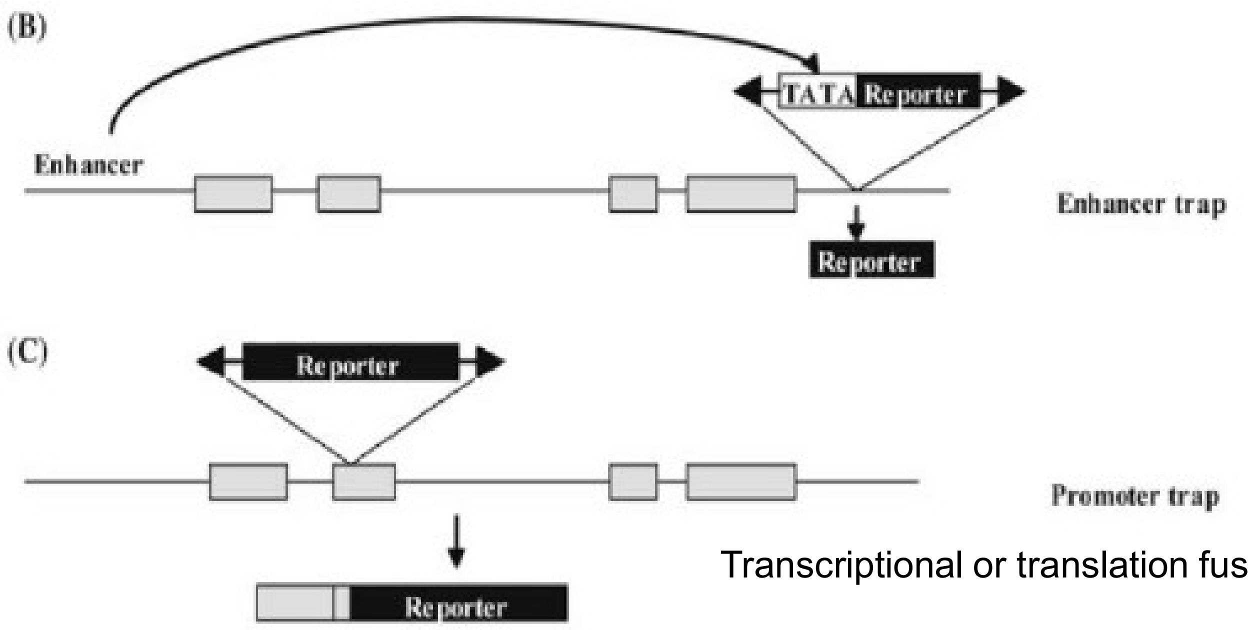
GAL4:VP16

GAL4:VP16

Dominant phenotype

Promoter/ enhancer trapping

To find genes and corresponding regulatory sequences with specific expression patterns



Transcriptional or translation fusions are possible

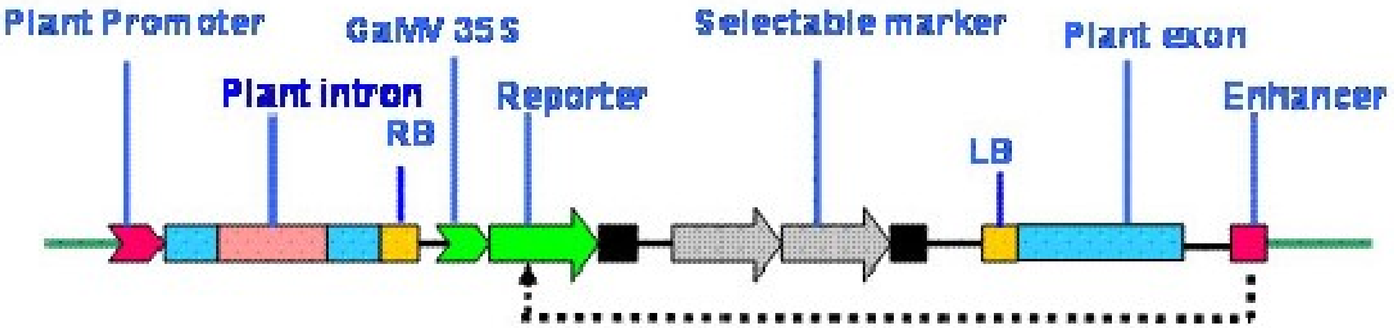
An et al., 2005 PMB 59, 111

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).

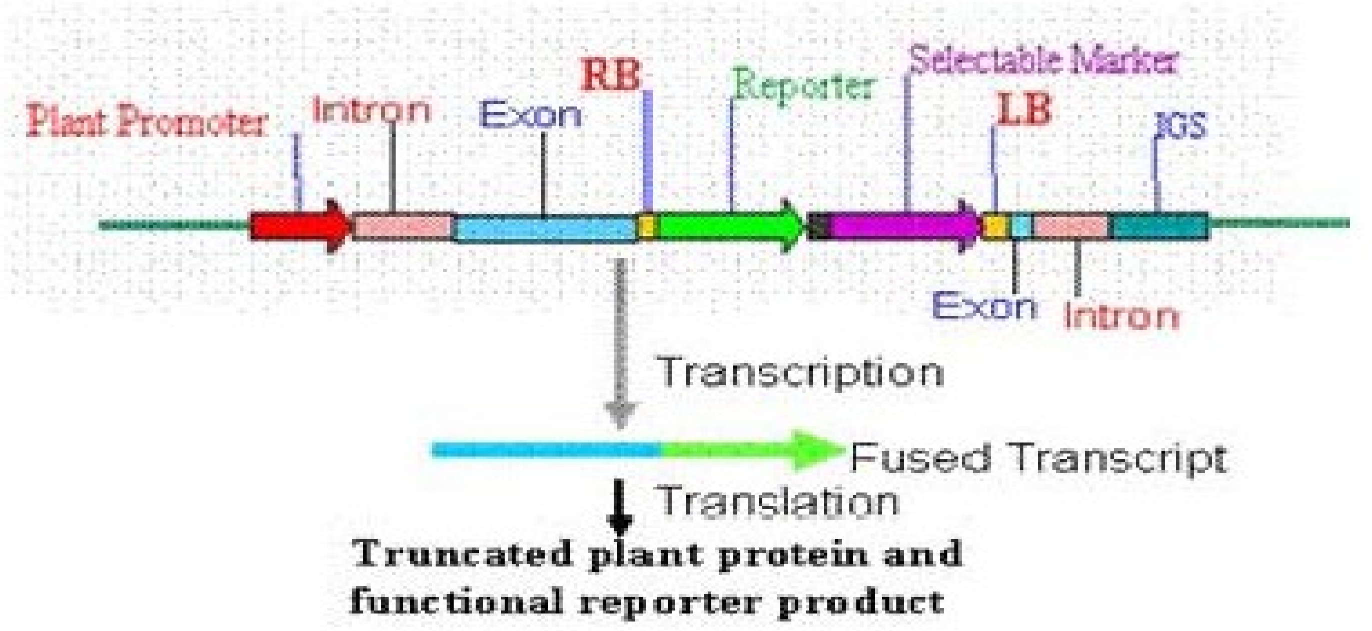
Note- The marker gene will be expressed only when the element insert in proximity of a transcriptional unit (minimal promoter construct) or within the transcription unit of a gene generating either a transcriptional or an in-frame translational fusion (promoterless construct).

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.

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.

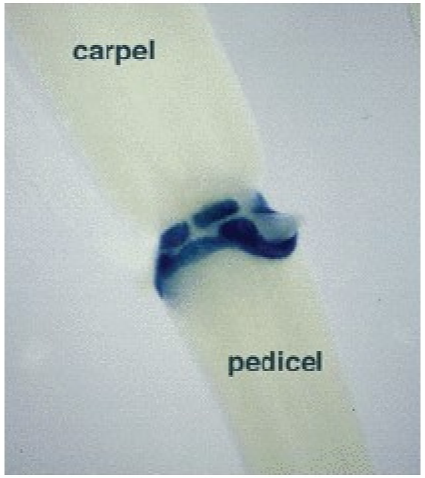
Un vantaggio di questa tecnica rispetto all’analisi di mutanti consiste nel fatto che anche se l’inserzione del T-DNA (enhancer trap) avviene in un gene essenziale la pianta sarà in quasi tutti i casi vitale essendoci nella generazione T_1 una condizione di emizigosi.

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.

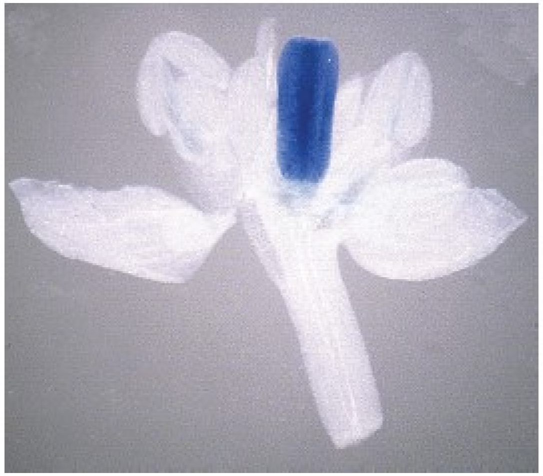
Enhancer trap (GUS) in *Arabidopsis*

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

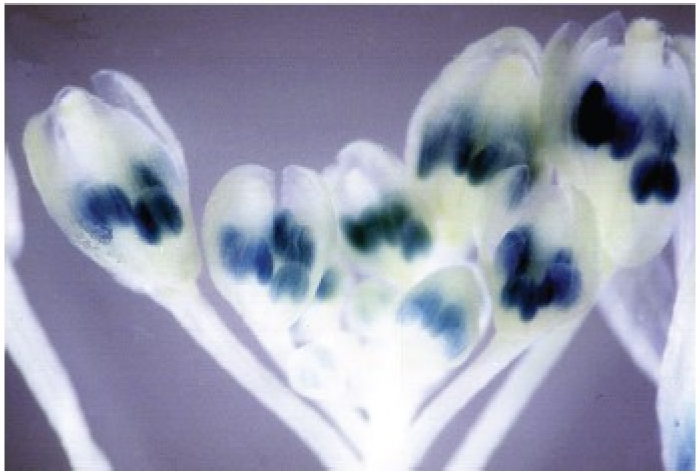
Espressione diffusa



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

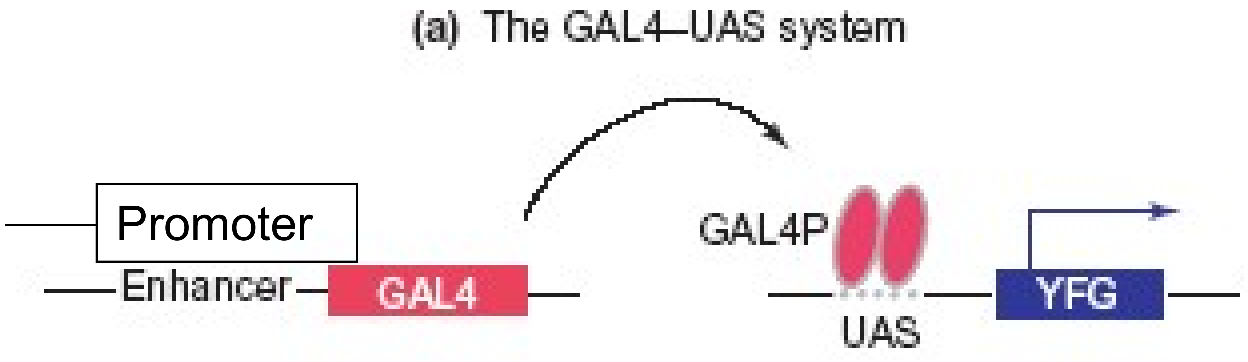
- gene trap strategy selects for insertions into genes -> is very useful in plants with large amount of non-genic DNA
- Dependency on ‘in frame’ insertions -> two-third of all “hits” on genes were not recognized
- expression of marker relies on the transcriptional activity of the surrounding gene, and 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.

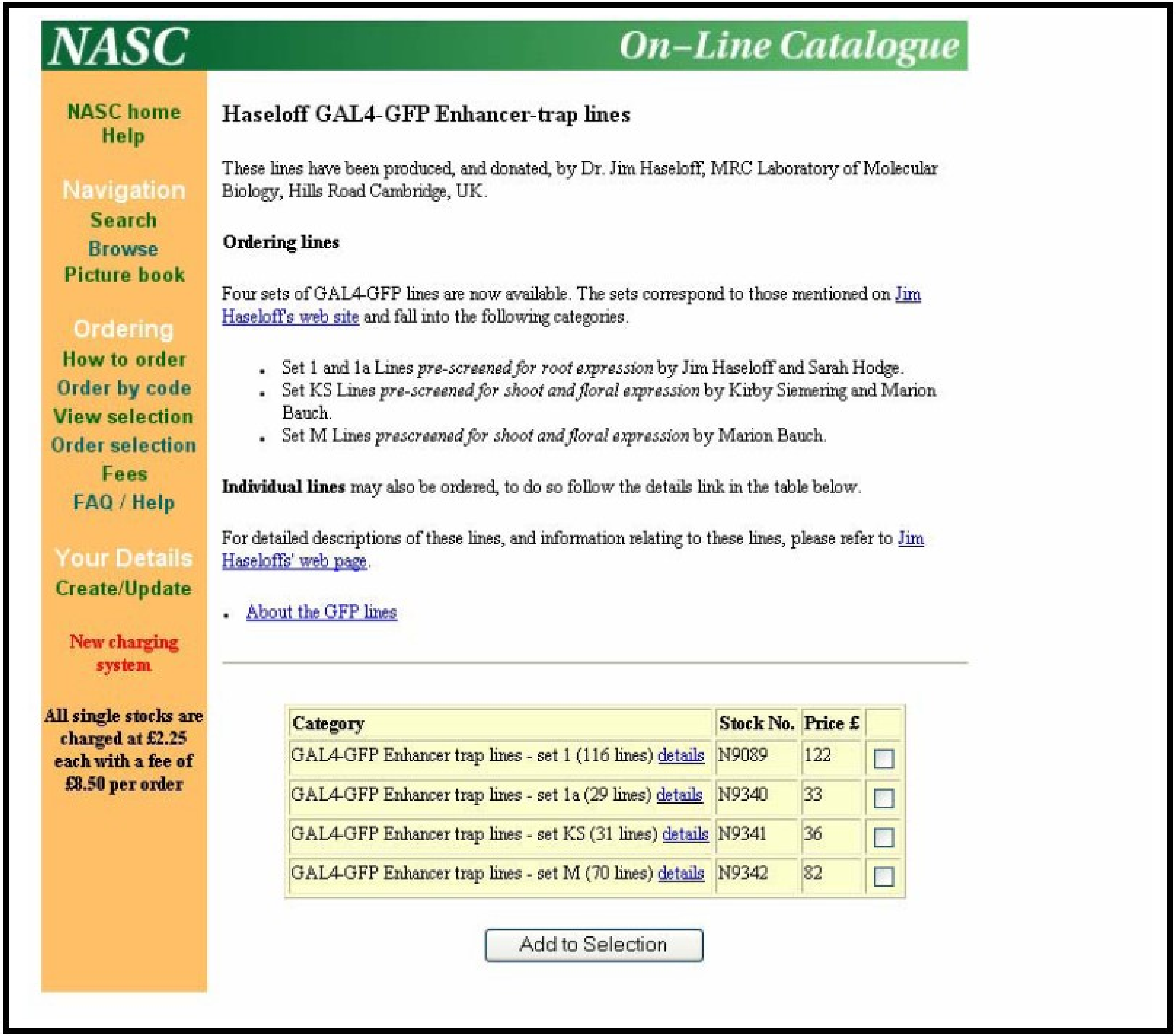
Formed by fusing a DNA-binding fragment of the yeast activator GAL4 to a highly acidic portion of the herpes simplex virus protein VP16 (ref. 11; also called Vmw65).

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)]



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.

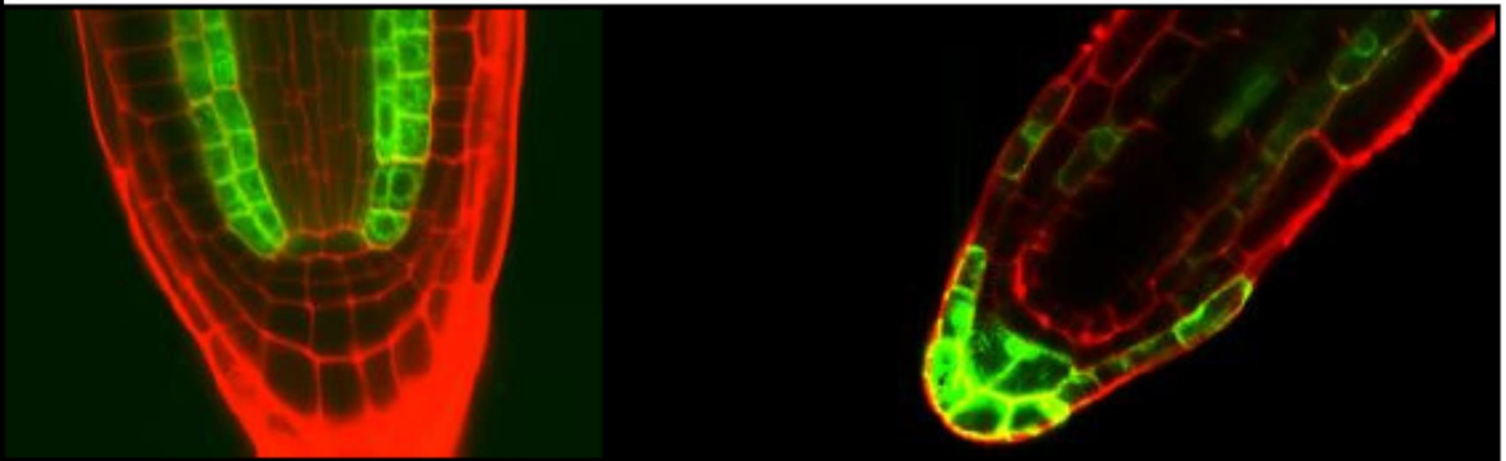
- Set 1 and 1a Lines *pre-screened for root expression* by Jim Haseloff and Sarah Hodge.
- Set KS Lines *pre-screened for shoot and floral expression* by Kirby Siemering and Marion Bauch.
- Set M Lines *prescreened for shoot and floral expression* by Marion Bauch.

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

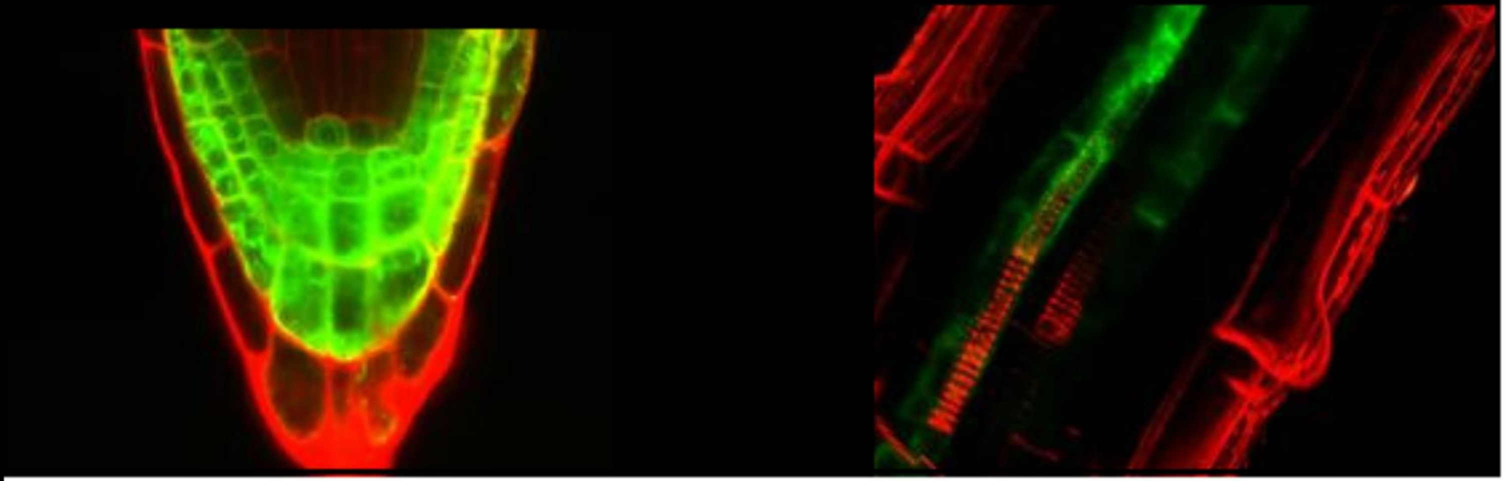
For detailed descriptions of these lines, and information relating to these lines, please refer to Jim Haseloffs' web page.

- 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	☐



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



REVERSE GENETICS
ALTRI APPROCCI

1. Come faccio a studiare i fenotipi letali?
2. Come posso studiare i fenotipi associati al mio gene di interesse se per questo non è ancora disponibile un mutante inserzionale?

- TILLING
- ANTISENSO
- RNAi

Il T-DNA ed i trasposoni NON sono facilmente applicabili a tutte le specie vegetali

TILLING (Targeting Induced Local Lesions IN Genomes)

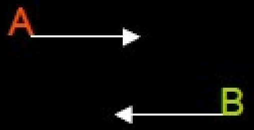
Mutagenesi chimica
Ethyl methanesulfonate)
→
Screening dei mutanti tramite pooling
→
Identificazione della linea con un determinato gene con una mutazione puntiforme

<u>Vantaggi:</u>

- E' una procedura semplice e più ampiamente utilizzabile
- Non necessita di colture cellulari e non genera linee transgeniche
- Produce serie alleliche con linee K.O. o solamente "attenuate"

L’endonucleasi Cel-I identifica i mismatch dell’appaiamento tra wt e mutante

Primer gene specifici

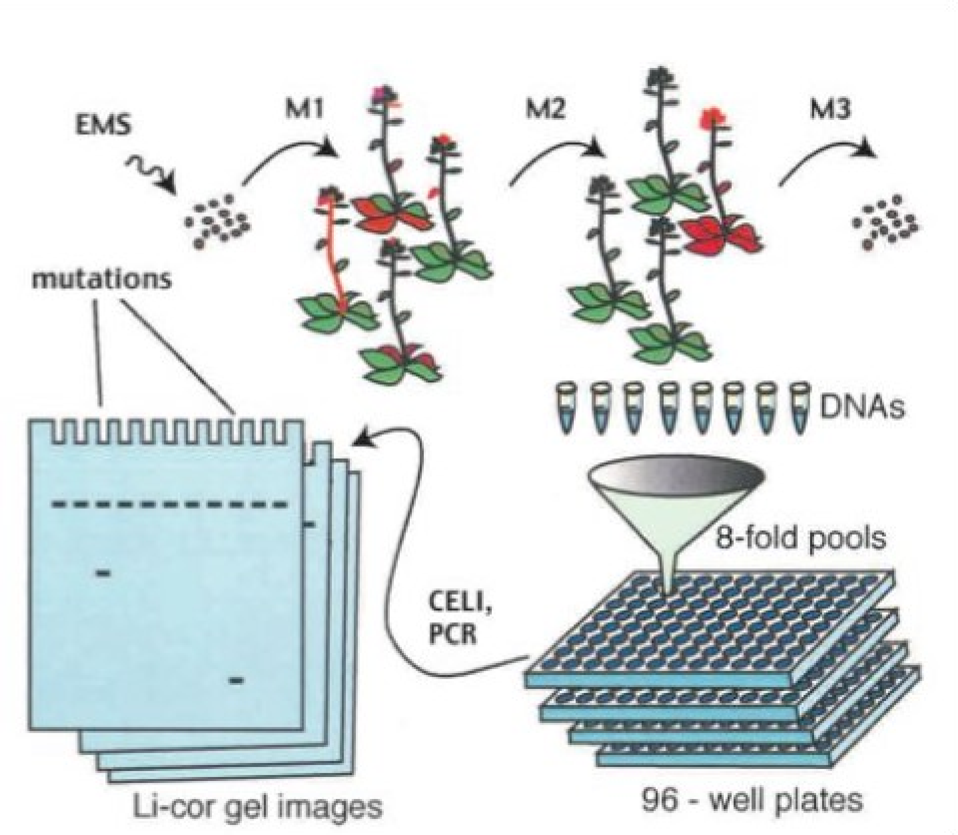


A e B due marcatori differenti

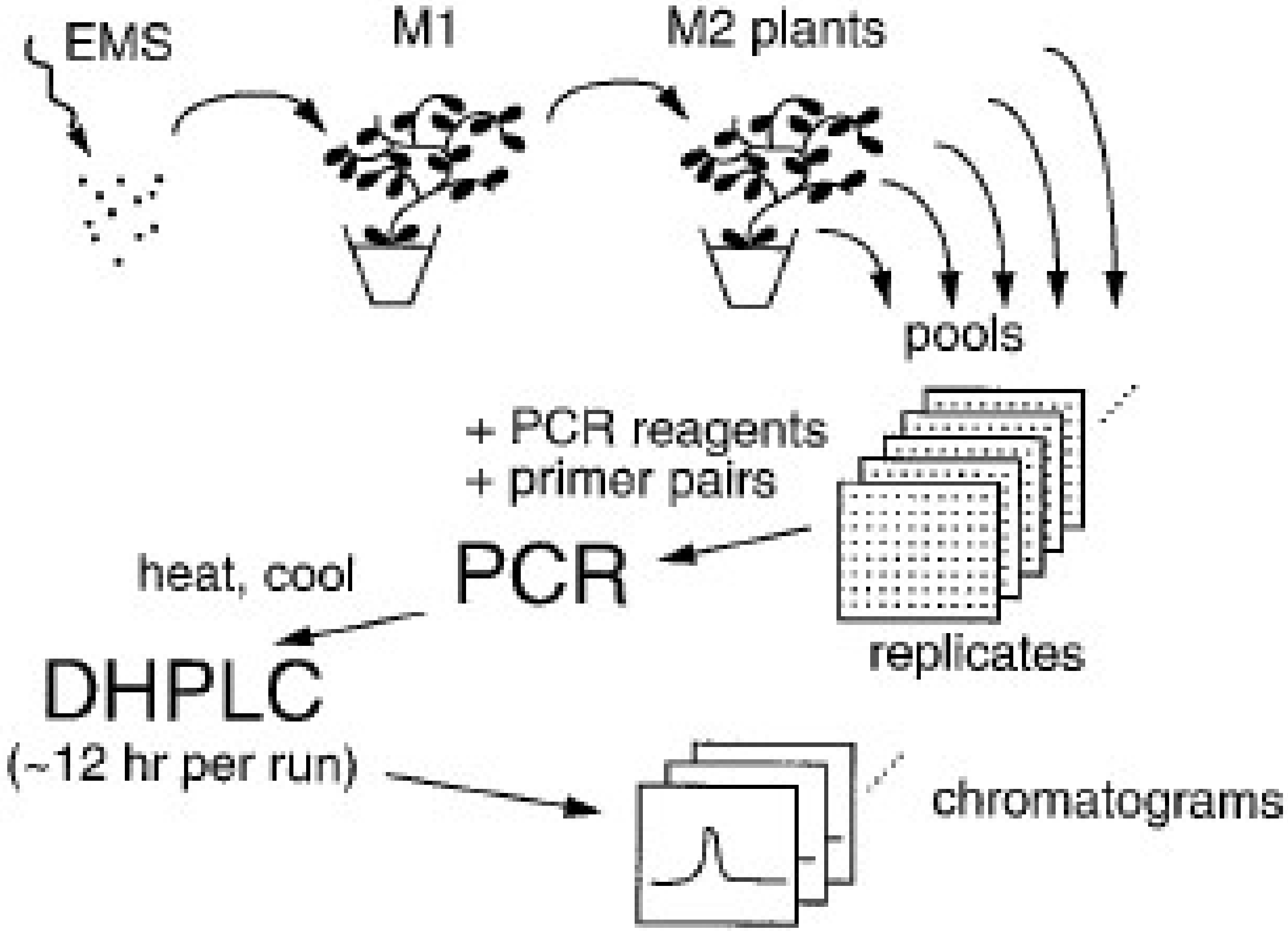
PCR sul wt e nel mutante → cicli di appaiamento dei frammenti amplificati

Heteroduplex

high-throughput TILLING - how it works



Targeting Induced Local Lesions IN Genomes (TILLING)

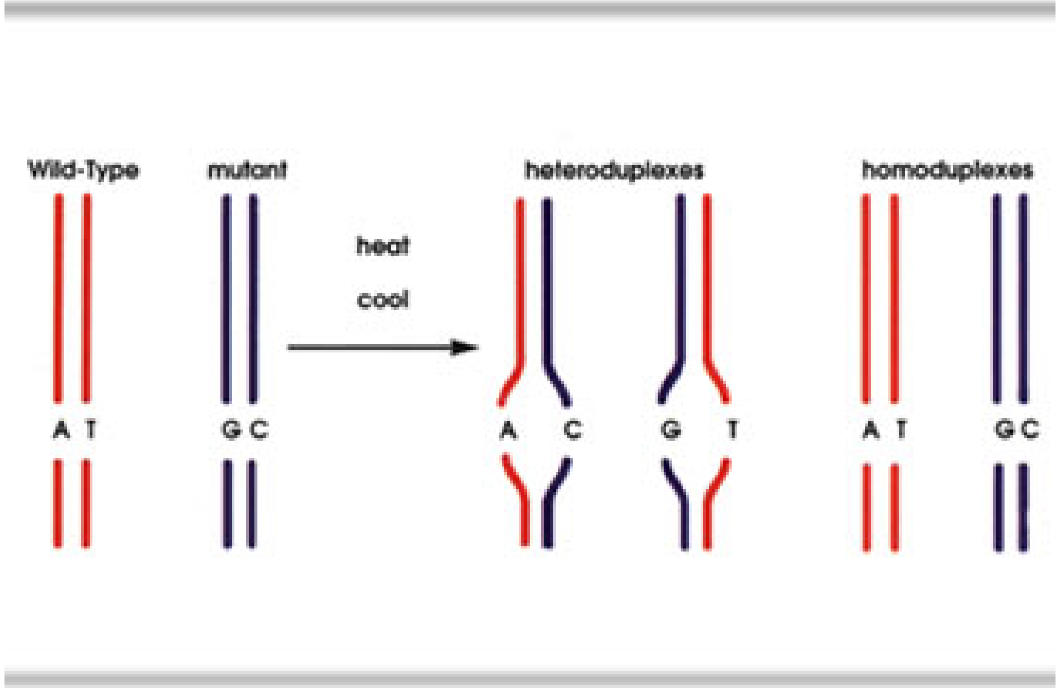


DHPLC= HPLC denaturante

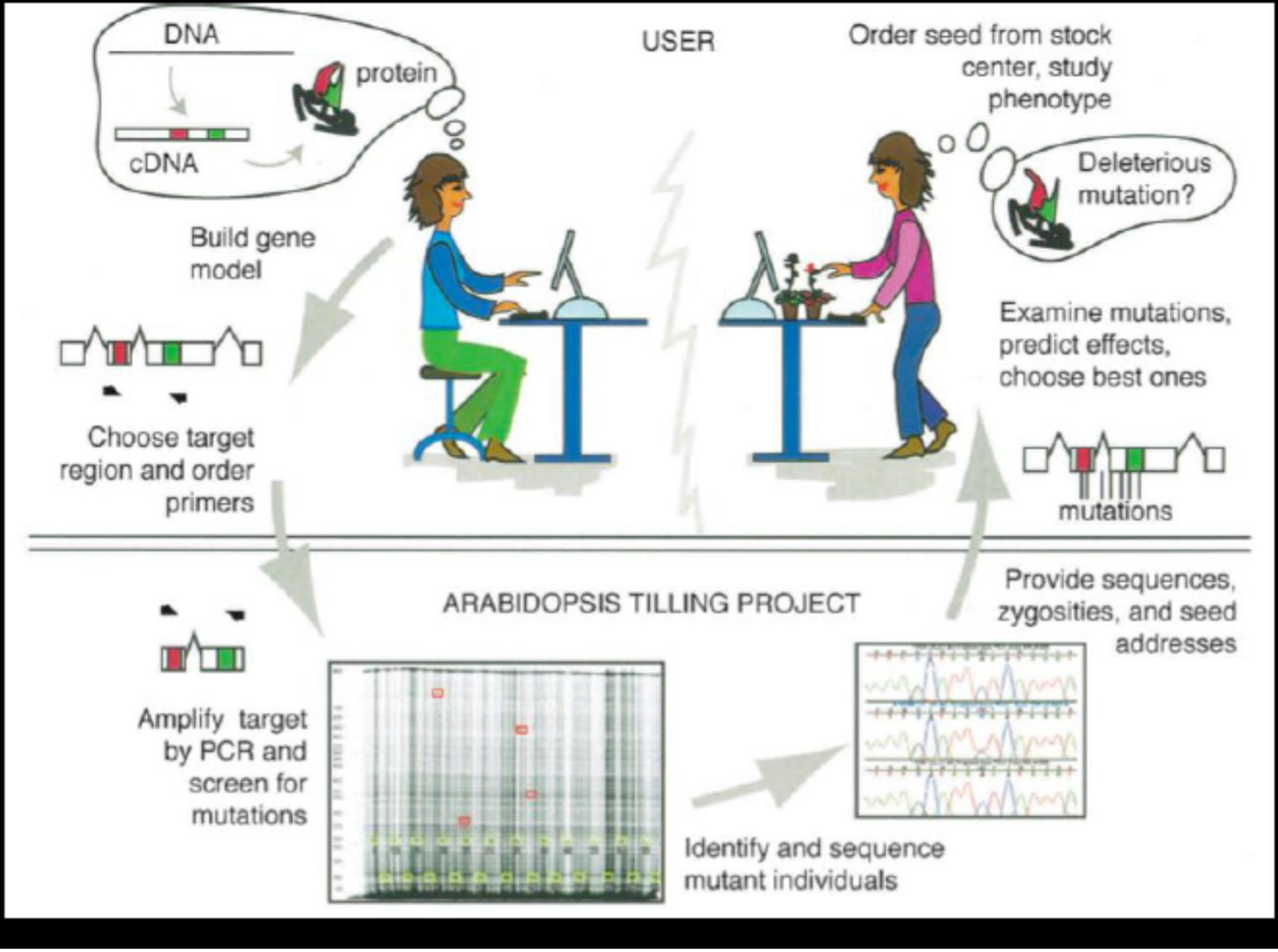
- Duplex si formano quando un frammento amplificato di DNA mutato ed uno non mutato vengono denaturati termicamente e lasciati ricombinare.

- Su una colonna cromatografica, l'eteroduplex è solitamente più veloce (meno trattenuto) dell'omoduplex

- Può essere impiegata per rilevare ogni tipo di mutazione (SNPs, inserzioni, delezioni e tandem repeat)



high-throughput TILLING - how it works



USER

DNA

protein

cDNA

Build gene model

Choose target region and order primers

Order seed from stock center, study phenotype

Deleterious mutation?

Examine mutations, predict effects, choose best ones

mutations

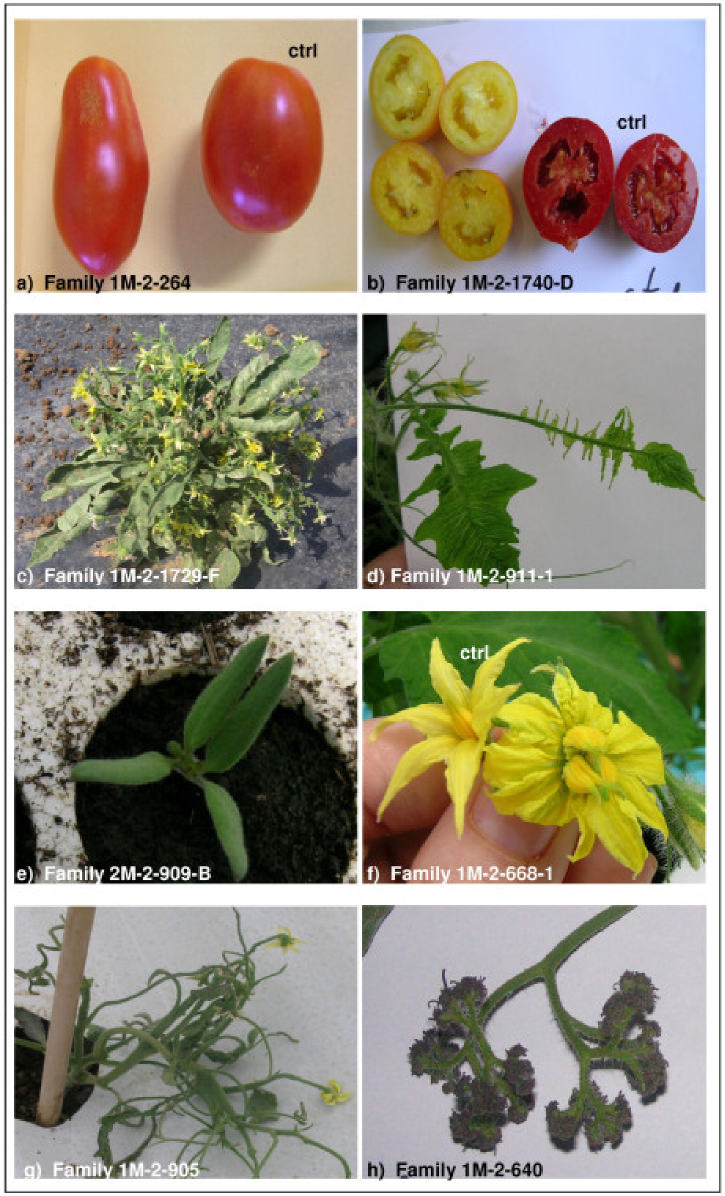
ARABIDOPSIS TILLING PROJECT

Amplify target by PCR and screen for mutations

Identify and sequence mutant individuals

Provide sequences, zygosities, and seed addresses

TILLING in pomodoro



Mutation density in 0.7% EMS and 1% EMS Red Setter populations

Target gene		**No. of screened M3 families**		**No. of identified mutations**		**Overall mutation density**	
Name	**Amplicon size (kb)**	**0.7% EMS**	**1% EMS**	**0.7% EMS**	**1% EMS**	**0.7% EMS**	**1% EMS**
Rab11a	0.407	1,373	713	1	3	1/559 kb	1/97 kb
PG	2.587	2,791	963	7	2	1/1031 kb	1/1246 kb
Exp1	1.025	3,885	1,284	14	6	1/284 kb	1/219 kb
RIN	1.331	3,885	1,284	4	8	1/1293 kb	1/214 kb
Gr	1.409	3,885	1,284	5	3	1/1095 kb	1/603 kb
Lcy-b	1.274	3,801	1,252	4	3	1/1211 kb	1/532 kb
Lcy-e	1.414	3,630	1,185	6	0	1/855 kb	-
Total/mean	**9.447**			**41**	**25**	**1/574 kb**	**1/322 kb**

The accession numbers of the analyzed seven target genes are the following: *Rab11a* [GenBank:AJ245570], *PG* [GenBank:M37304], *Exp1* [GenBank:AF548376], *RIN* [GenBank:AF448522], *Gr* [GenBank:DQ372897], *Lcy-b* [GenBank:CQ788383], *Lcy-e* [GenBank:Y14387]. The number of screened M3 families, the number of identified mutations and the overall mutation density, estimated as described in Methods, are reported both for 0.7% and 1% EMS Red Setter populations.

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iTILLING: A Personalized Approach to the Identification of Induced Mutations in Arabidopsis[1][C][OA]

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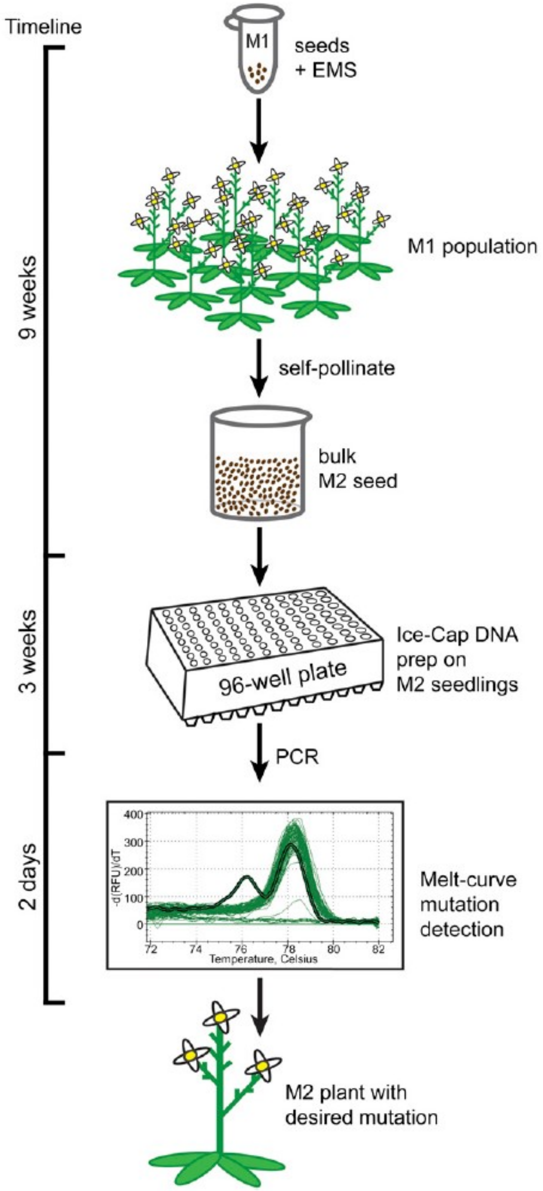

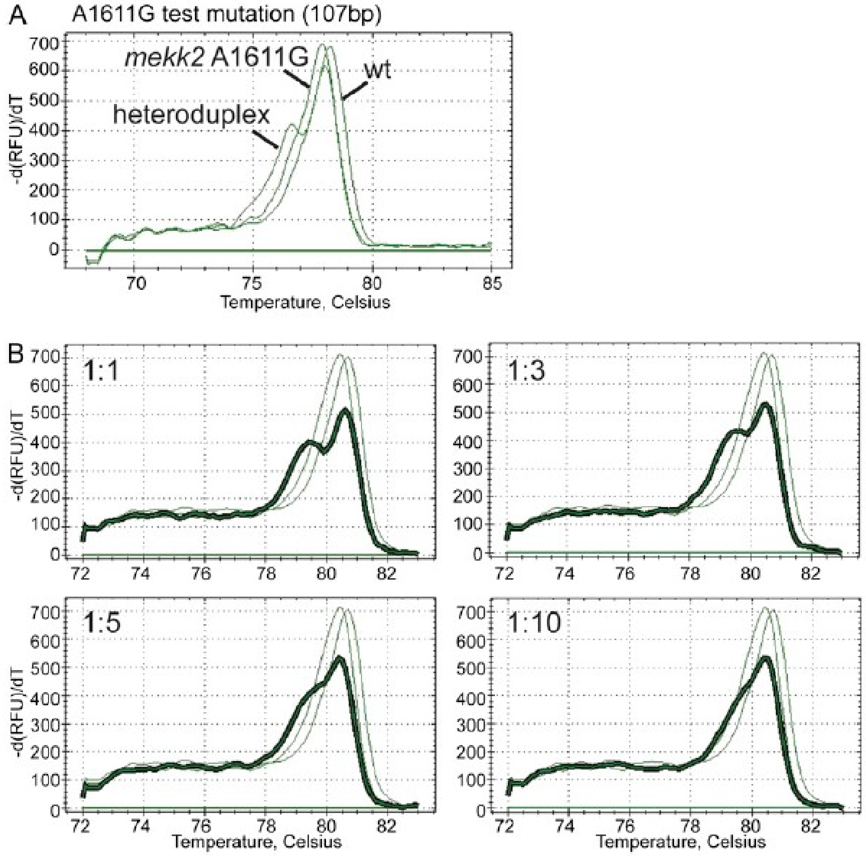

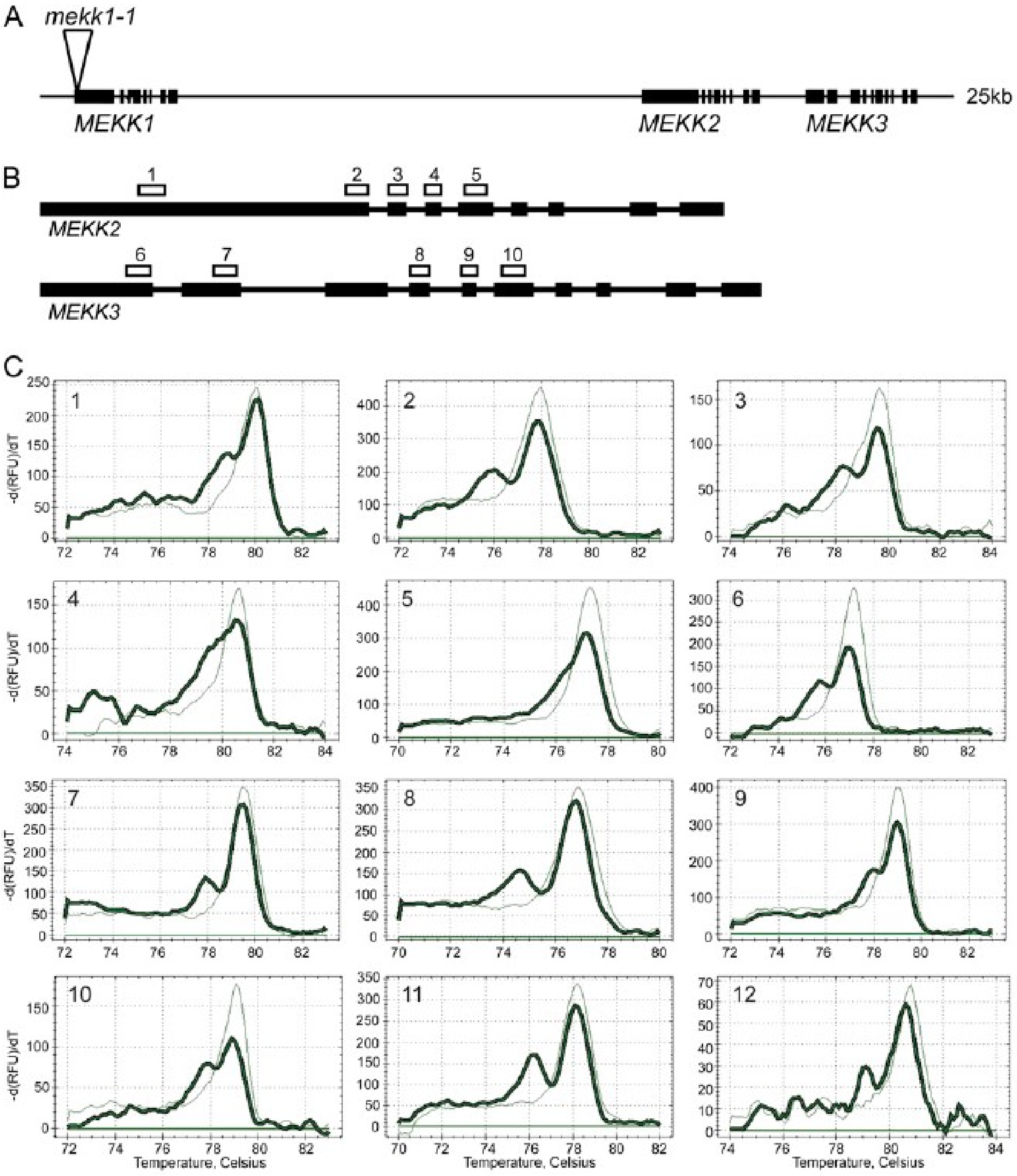
A
mekk1-1
MEKK1
MEKK2
MEKK3
25kb
B
1
2
3
4
5
MEKK2
6
7
8
9
10
MEKK3
C
1
2
3
4
5
6
7
8
9
10
11
12
-d(RFU)/dT
Temperature, Celsius