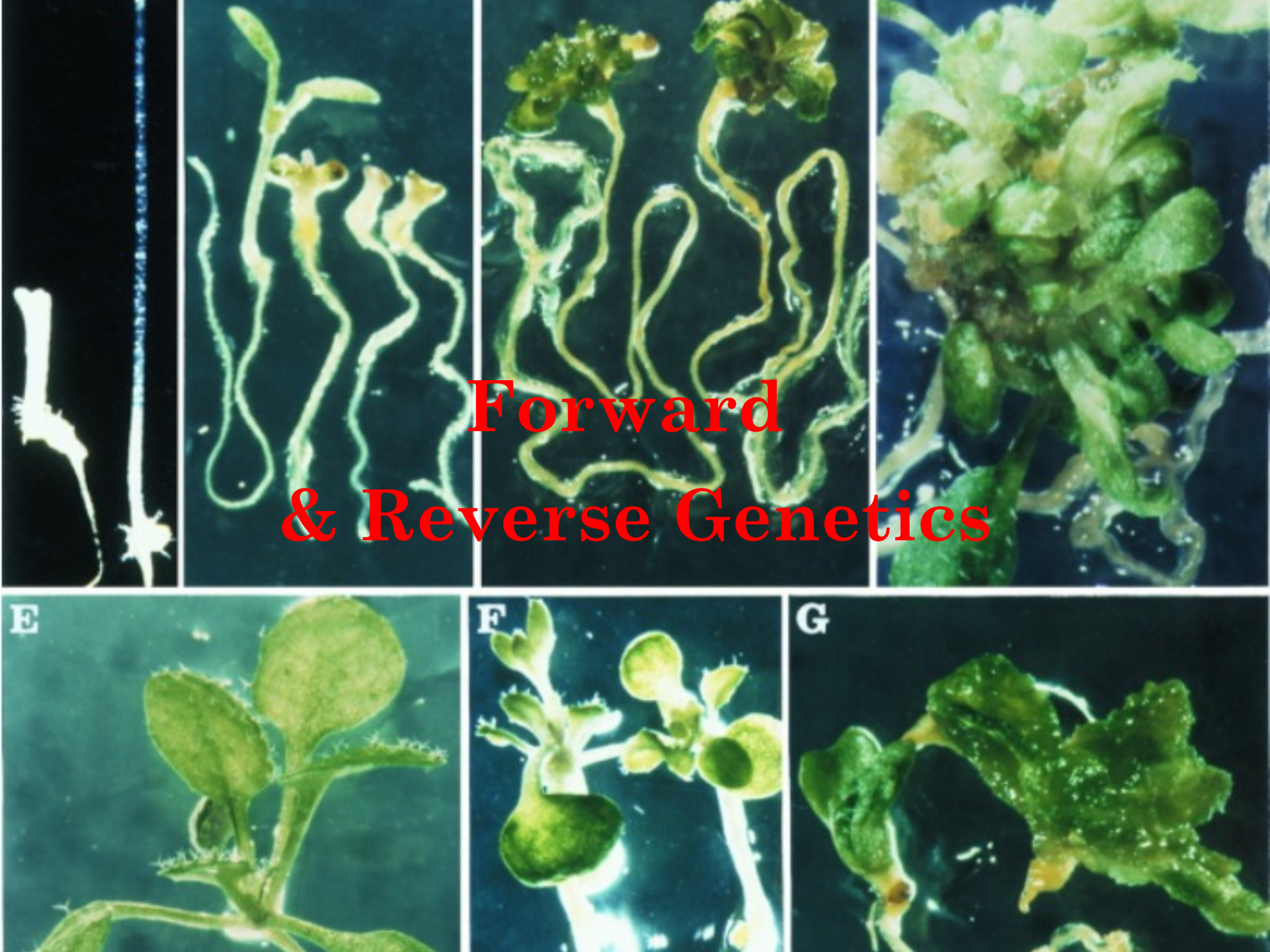

Forward
& Reverse Genetics
E
F
G

Strategie molecolari
per studiare la funzione di un gene

Aumento espressione del gene endogeno:

- **forte costitutiva**
- **ectopica**
- **inducibile**

Riduzione/silenziamento del gene endogeno:

- **AntiSENSO**
- **RNAi**
- **co-soppressione**

✷ **Knock Out del gene.....**

- **Genetica Forward**
- **Genetica Reverse**

Knock Out del gene.....

mutagenesi sito-specifica

v/s

mutagenesi inserzionale “random”

In piante, Drosophila, mammifero

bassa frequenza di ricombinazione omologa

Quindi.....mutagenesi random

O.....Genome editing: Talen, Crisp/Cas9

GENETICA FORWARD E REVERSE

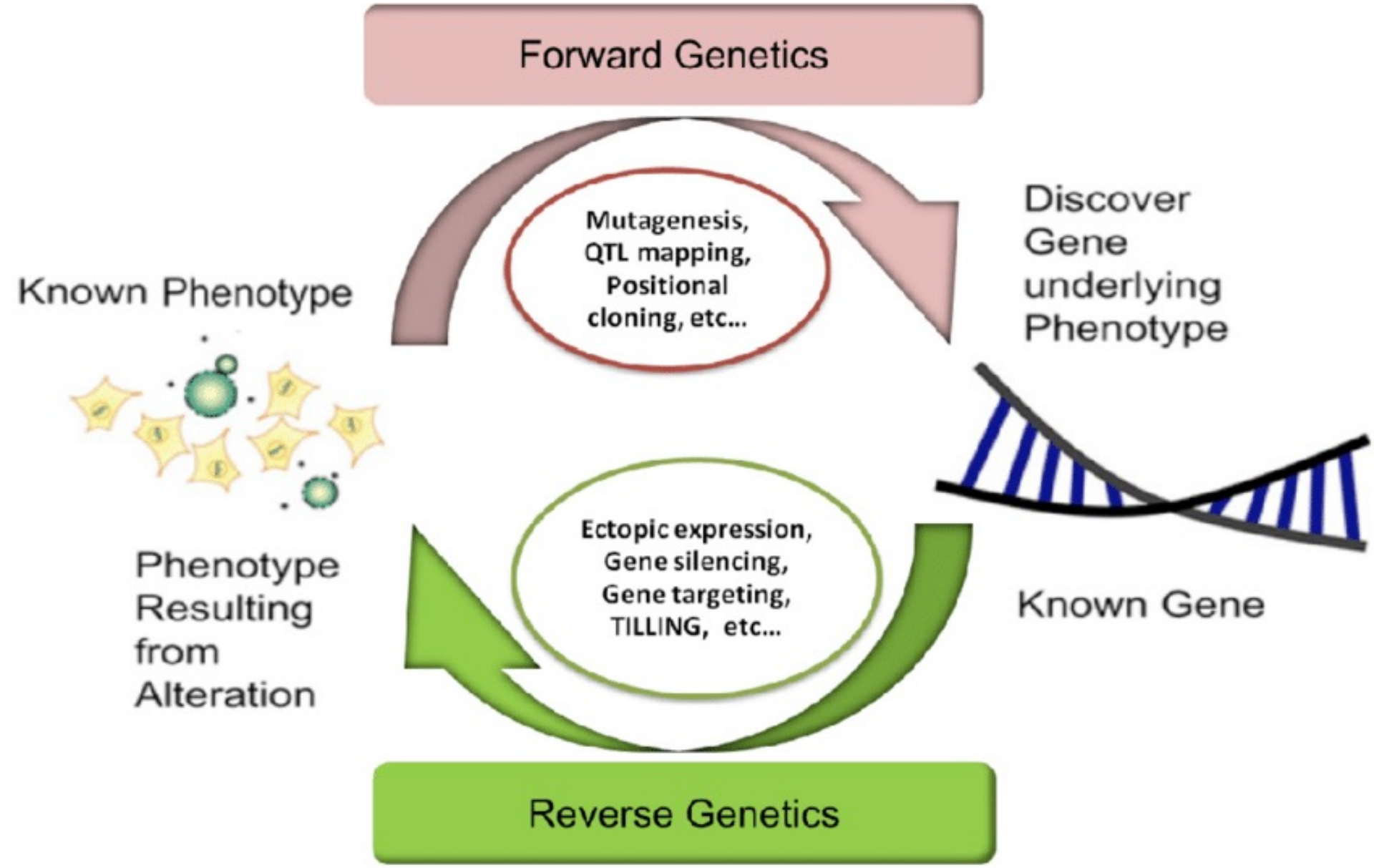
Obiettivo: assegnare una funzione ad ogni gene

Forward Genetics:

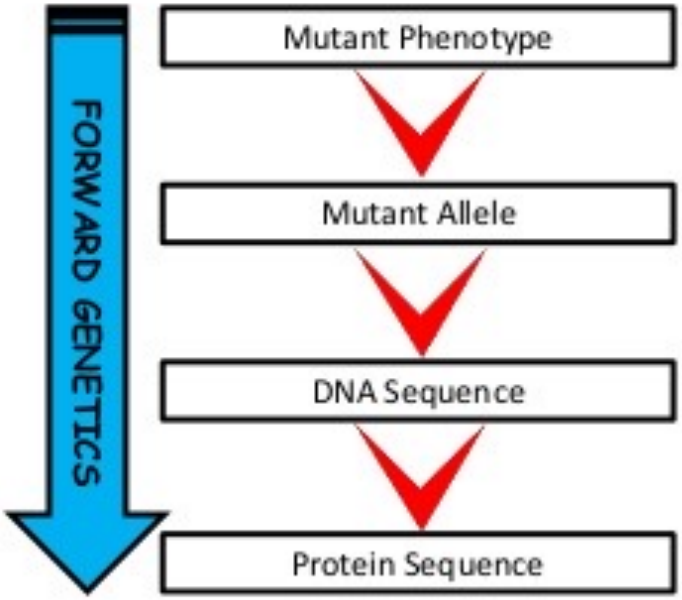
il metodo classico per determinare la funzione di un gene si basa sull'isolamento e analisi di mutazioni in quel gene: mutagenesi sistematica delle sequenze geniche per produrre collezioni di mutazioni (prevalentemente recessive) di tipo "loss of function". Utilizzando varie tecniche di clonaggio si risale alla sequenza genica.

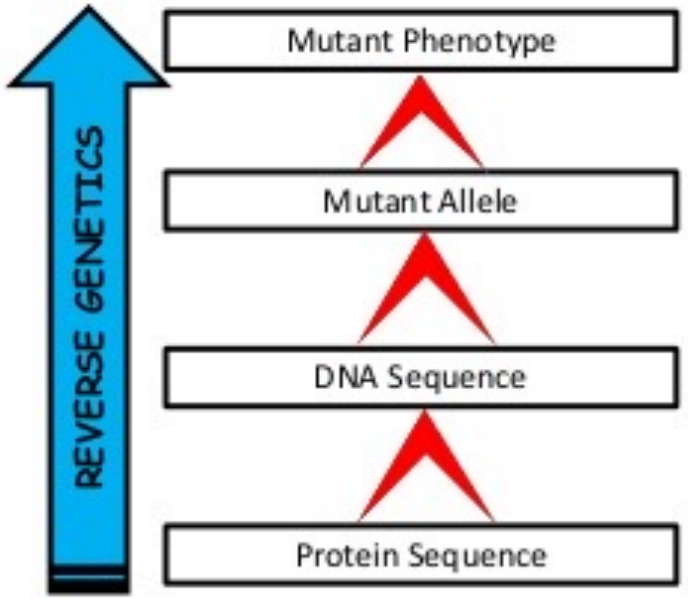
Reverse Genetics:

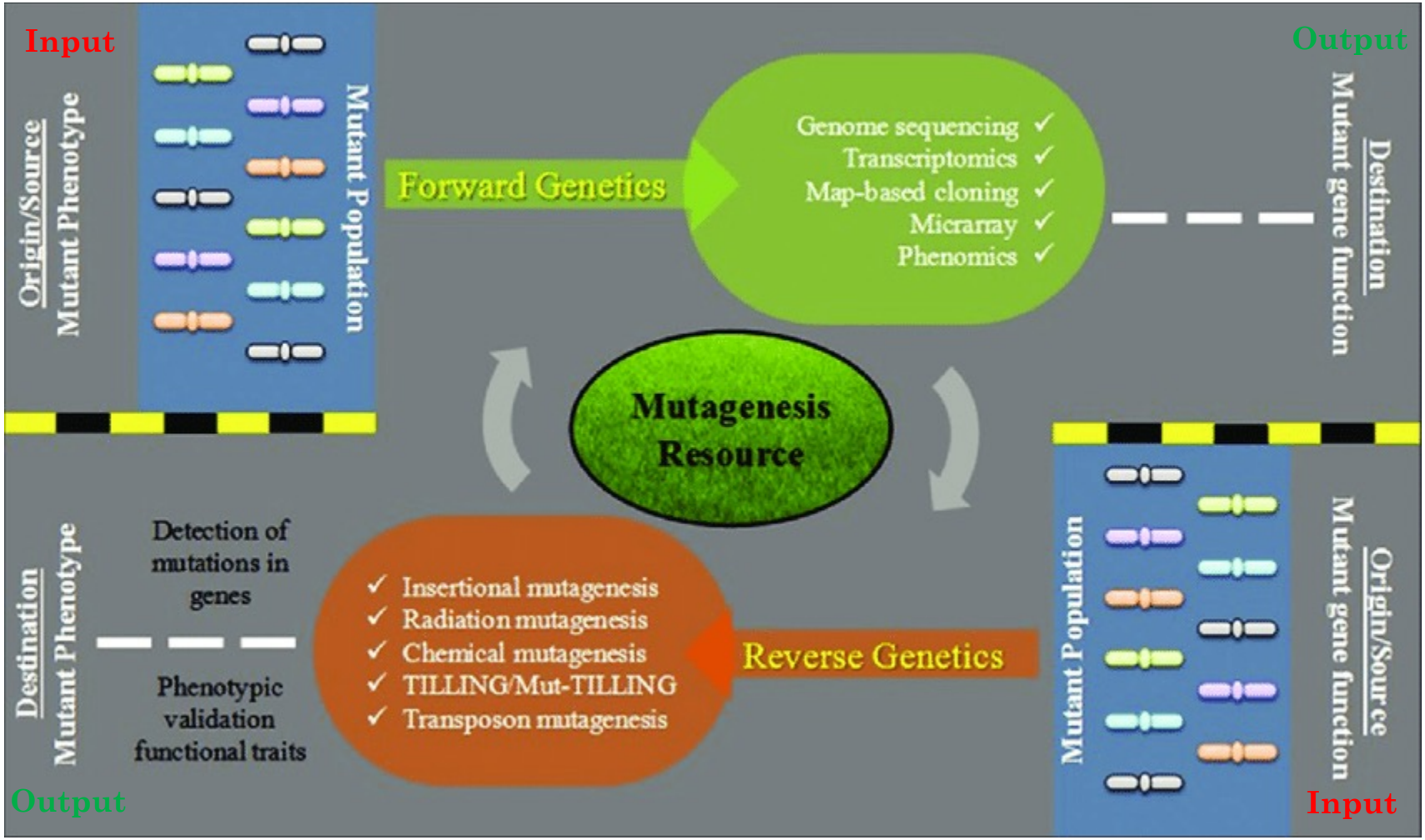
La disponibilità della sequenza del genoma di molte specie ha reso possibile un diverso approccio per la determinazione della funzione di un gene. A partire da una sequenza genica è possibile isolare la linea mutante in cui l'elemento inserzionale è all'interno della sequenza d'interesse. Dallo studio fenotipico del mutante, si può dedurre la funzione del gene.


Forward Genetics
Mutagenesis, QTL mapping, Positional cloning, etc...
Known Phenotype
Discover Gene underlying Phenotype
Ectopic expression, Gene silencing, Gene targeting, TILLING, etc...
Phenotype Resulting from Alteration
Known Gene
Reverse Genetics

Forward
Vs
Reverse Genetics



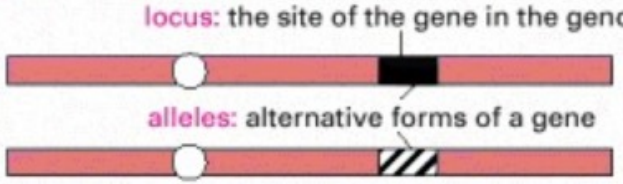


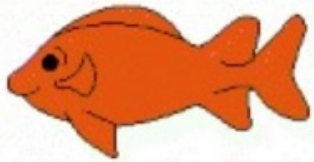

Input
Origin/Source
Mutant Phenotype
Mutant Population
Forward Genetics
Genome sequencing ✓
Transcriptomics ✓
Map-based cloning ✓
Micrarray ✓
Phenomics ✓
Output
Destination
Mutant gene function
Mutagenesis Resource
Destination
Mutant Phenotype
Detection of mutations in genes
Phenotypic validation functional traits
Output
✓ Insertional mutagenesis
✓ Radiation mutagenesis
✓ Chemical mutagenesis
✓ TILLING/Mut-TILLING
✓ Transposon mutagenesis
Reverse Genetics
Mutant Population
Origin/Source
Mutant gene function
Input

GENES AND PHENOTYPES

Gene: a functional unit of inheritance, usually corresponding to the segment of DNA coding for a single protein.

Genome: an organism's set of genes.



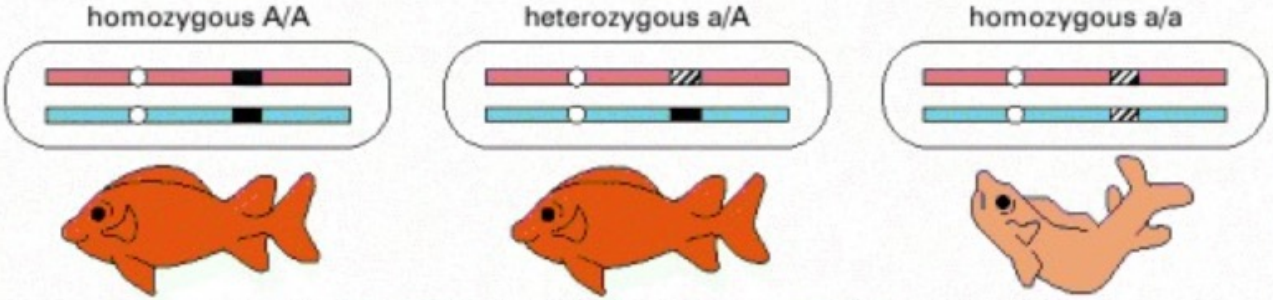


Wild-type: the normal, naturally occurring type

Mutant: differing from the wild-type because of a genetic change (a mutation)

GENOTYPE: the specific set of alleles forming the genome of an individual

PHENOTYPE: the visible character of the individual



allele A is **dominant** (relative to a); allele a is **recessive** (relative to A)

In the example above, the phenotype of the heterozygote is the same as that of one of the homozygotes; in cases where it is different from both, the two alleles are said to be co-dominant.

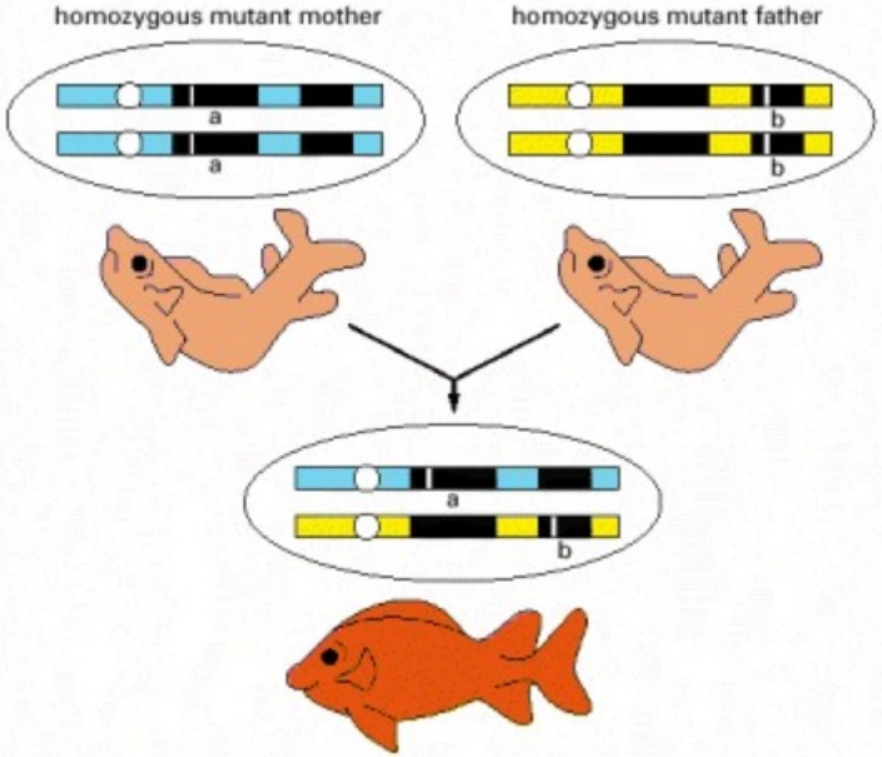
Test di Complementazione tra alleli mutanti

TWO GENES OR ONE?

Given two mutations that produce the same phenotype, how can we tell whether they are mutations in the same gene? If the mutations are recessive (as they most often are), the answer can be found by a complementation test.

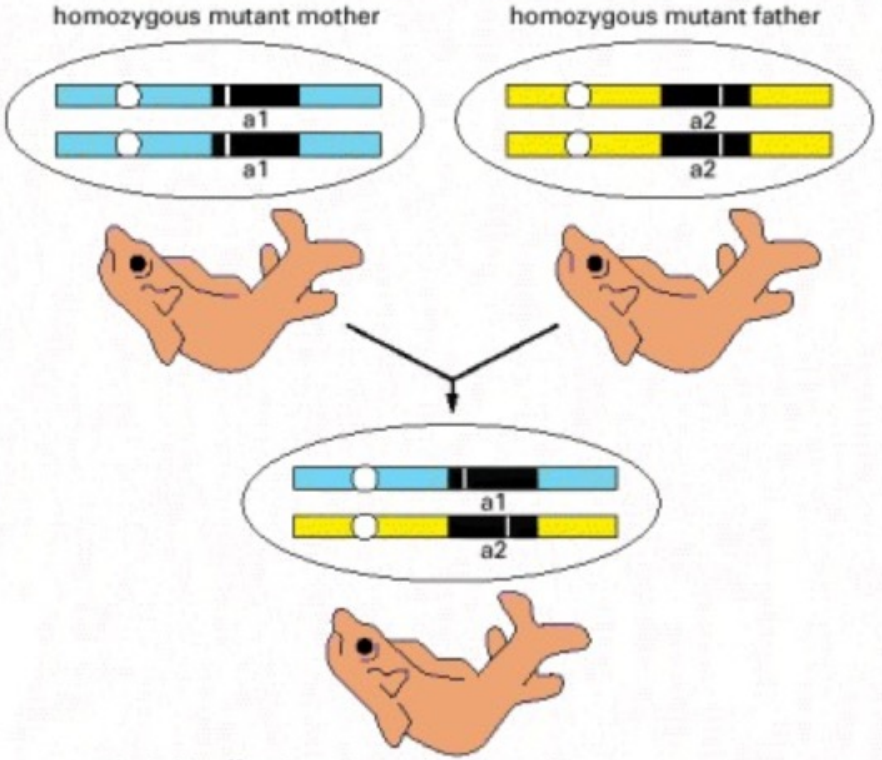
In the simplest type of complementation test, an individual who is homozygous for one mutation is mated with an individual who is homozygous for the other. The phenotype of the offspring gives the answer to the question.

COMPLEMENTATION: MUTATIONS IN TWO DIFFERENT GENES



hybrid offspring shows normal phenotype:
one normal copy of each gene is present

NONCOMPLEMENTATION: TWO INDEPENDENT MUTATIONS IN THE SAME GENE

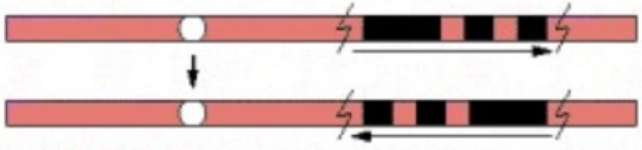


hybrid offspring shows mutant phenotype:
no normal copies of the mutated gene are present

Loss of Function vs Gain of Function mutations

TYPES OF MUTATIONS

POINT MUTATION: maps to a single site in the genome, corresponding to a single nucleotide pair or a very small part of a single gene



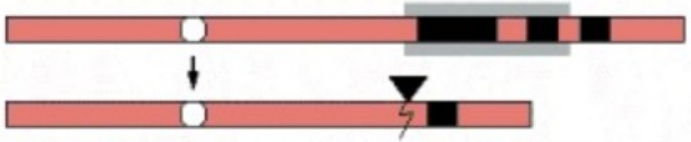
INVERSION: inverts a segment of a chromosome

lethal mutation: causes the developing organism to die prematurely.

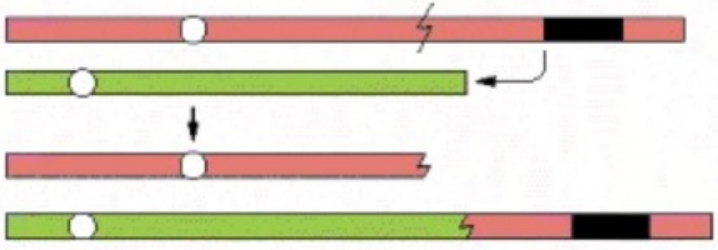
conditional mutation: produces its phenotypic effect only under certain conditions, called the *restrictive* conditions. Under other conditions—the *permissive* conditions—the effect is not seen. For a *temperature-sensitive* mutation, the restrictive condition typically is high temperature, while the permissive condition is low temperature.

loss-of-function mutation: either reduces or abolishes the activity of the gene. These are the commonest class of mutations. Loss-of-function mutations are usually *recessive*—the organism can usually function normally as long as it retains at least one normal copy of the affected gene.

null mutation: a loss-of-function mutation that completely abolishes the activity of the gene.



DELETION: deletes a segment of a chromosome



TRANSLOCATION: breaks off a segment from one chromosome and attaches it to another

gain-of-function mutation: increases the activity of the gene or makes it active in inappropriate circumstances; these mutations are usually *dominant*.

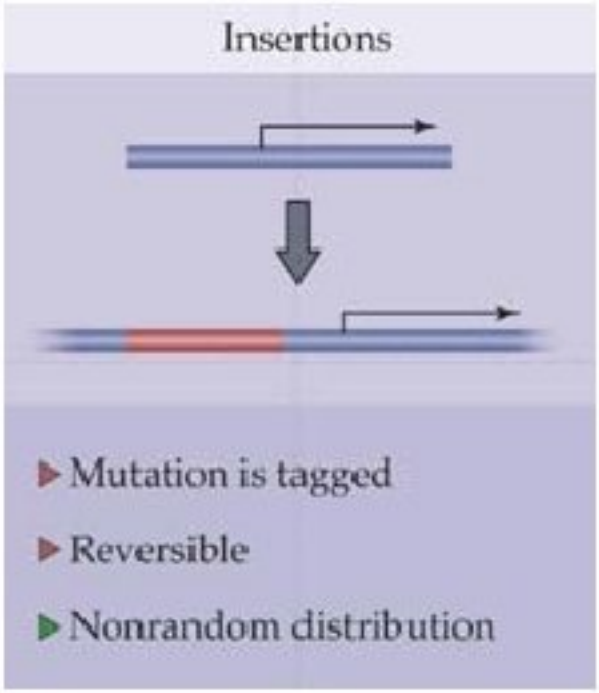
dominant negative mutation: dominant-acting mutation that blocks gene activity, causing a loss-of-function phenotype even in the presence of a normal copy of the gene. This phenomenon occurs when the mutant gene product interferes with the function of the normal gene product.

suppressor mutation: suppresses the phenotypic effect of another mutation, so that the double mutant seems normal. An *intragenic* suppressor mutation lies within the gene affected by the first mutation; an *extragenic* suppressor mutation lies in a second gene—often one whose product interacts directly with the product of the first.

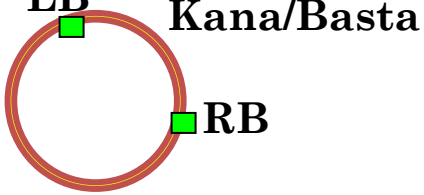
Mutagenesi inserzionale mediata da elementi trasponibili

Facile mappatura dell'inserzione

Rapido clonaggio del gene mutato



Strategia di Isolamento Mutanti Inserzionali



In Planta
Vacuum Infiltration

T0

Raccolta semi T1

Selezione in serra
dei trasformanti T1
resistenti

Autoimpollinazione

Raccolta semi T2

Analisi in vitro
delle plantule Kr

T2

Ks Kr

- Analisi dei mutanti Kana resistenti
- Propagazione di piante omo ed eterozigoti

Ma il T-DNA è correlato (responsabile del) con il fenotipo mutato???

- **Analisi di segregazione**

del marcatore e eventualmente del fenotipo mutante

(inserzione T-DNA in 1 o più loci??)

- **Caratterizzazione molecolare del sito di inserzione**

(T-DNA singolo, a tandem oppure inserzione più complessa??)

- **Clonaggio delle "Flanking Sequences"**

Analisi di segregazione

Fenotipo «selvatico»

T2 Plants	aa(Kr/Kr)	Aa(Kr/Ks)	AA(Ks/Ks)	Total
	96	193	94	383
	1	2	1	

autoimpollinazione

T3 Plants	aa(Kr)	Aa(Kr/Ks)	Ks	Total
a	24	47	26	97
b	25	46	24	95
c	27	55	28	110
e	121	0	0	121

aa gene inattivo in omozigosi
Aa una copia gene inattiva, una selvatica

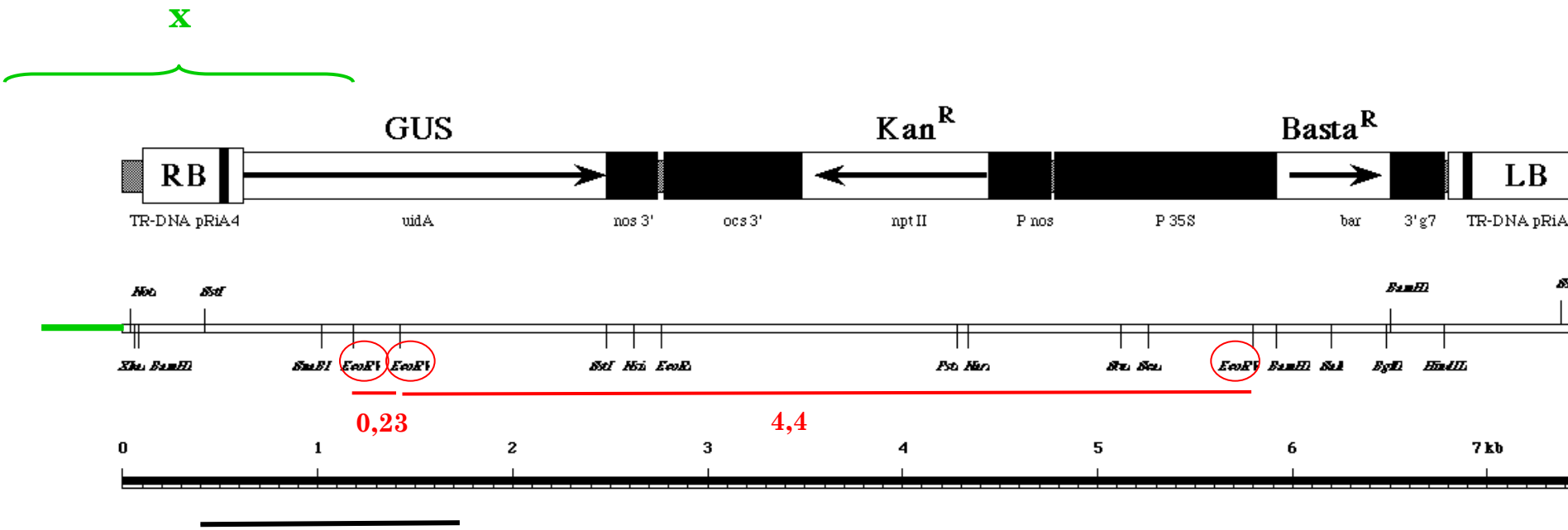
Ma il T-DNA è correlato (responsabile del) con il fenotipo mutato???

- **Analisi di segregazione del marcatore e del fenotipo mutante (inserzione T-DNA in 1 o più loci??)**

- **Caratterizzazione molecolare del sito di inserzione (T-DNA singolo, a tandem oppure inserzione più complessa??)**

- **Clonaggio delle "Flanking Sequences"**

pGKB5 T-DNA



probe

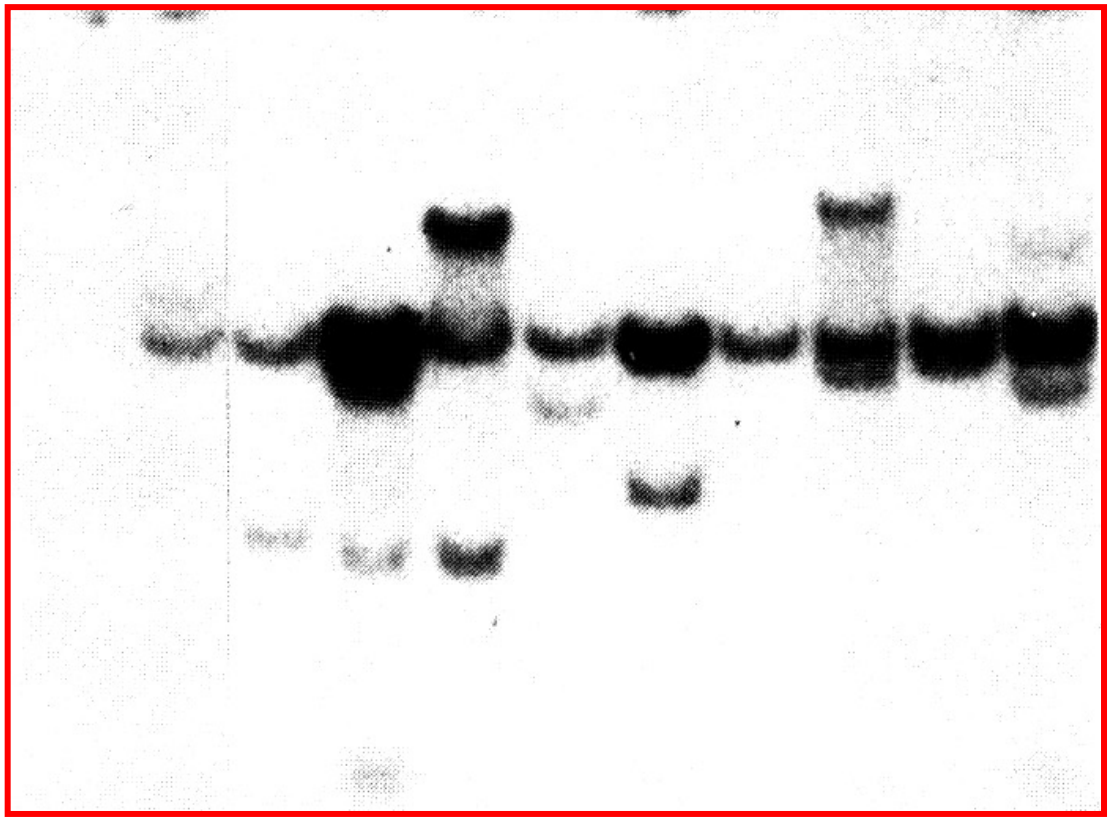
Digestione EcoRV:

0.23 Kb e 4,4 Kb interne al T-DNA

X Kb fino al sito EcoRV sulla flanking sequence

Caratterizzazione molecolare del sito di inserzione

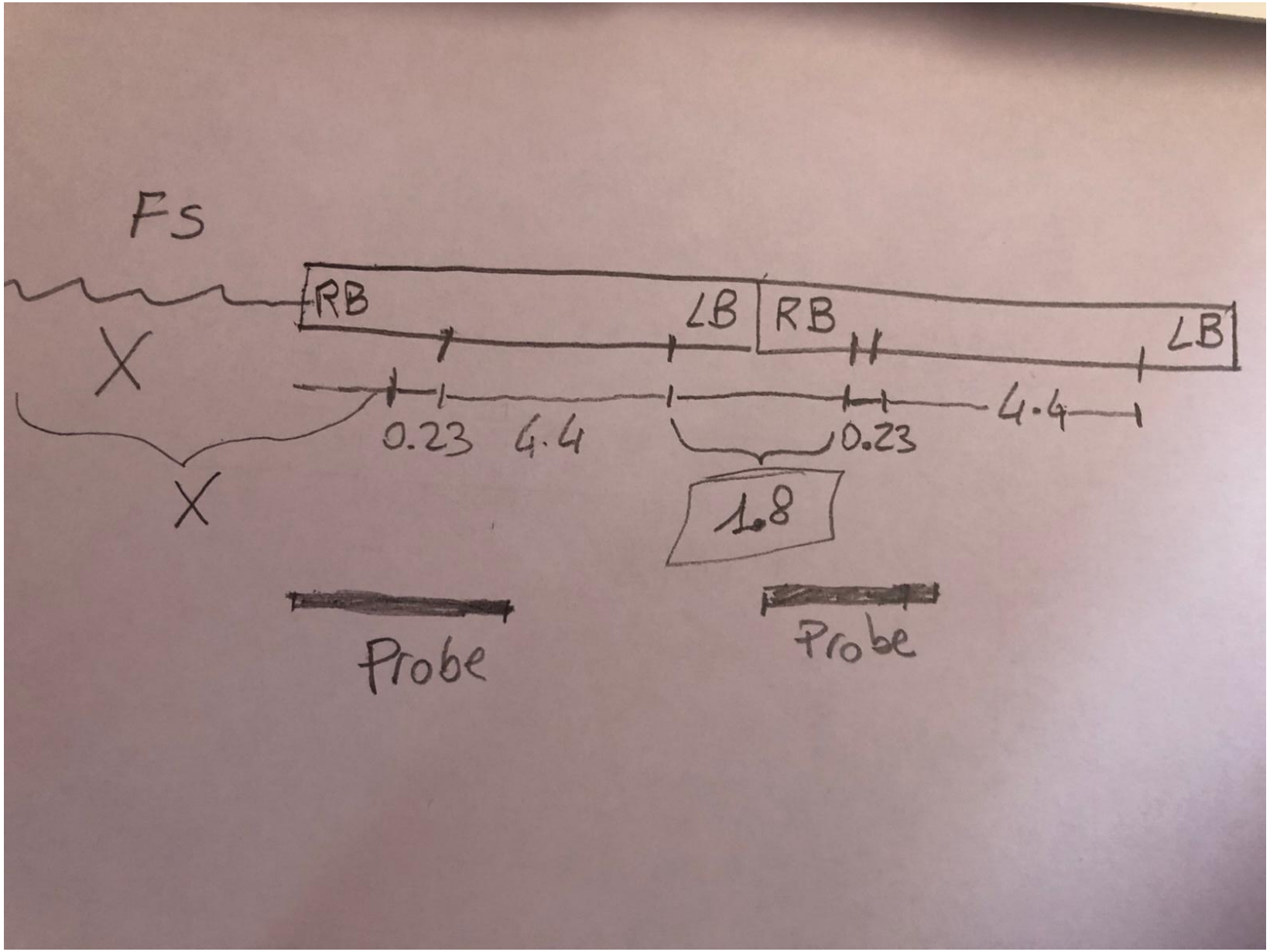
NT



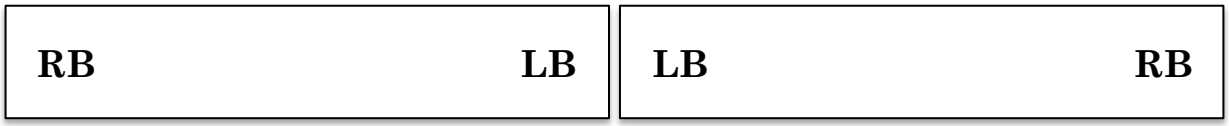
4,4 Kb

1,8 Kb
RB-LB IR

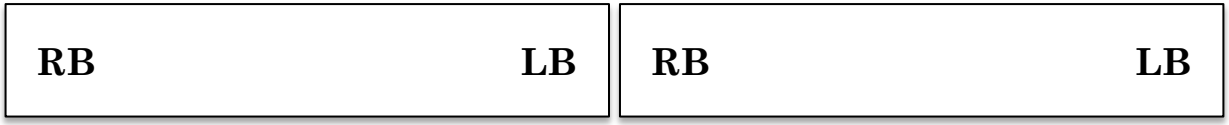
0,23 Kb

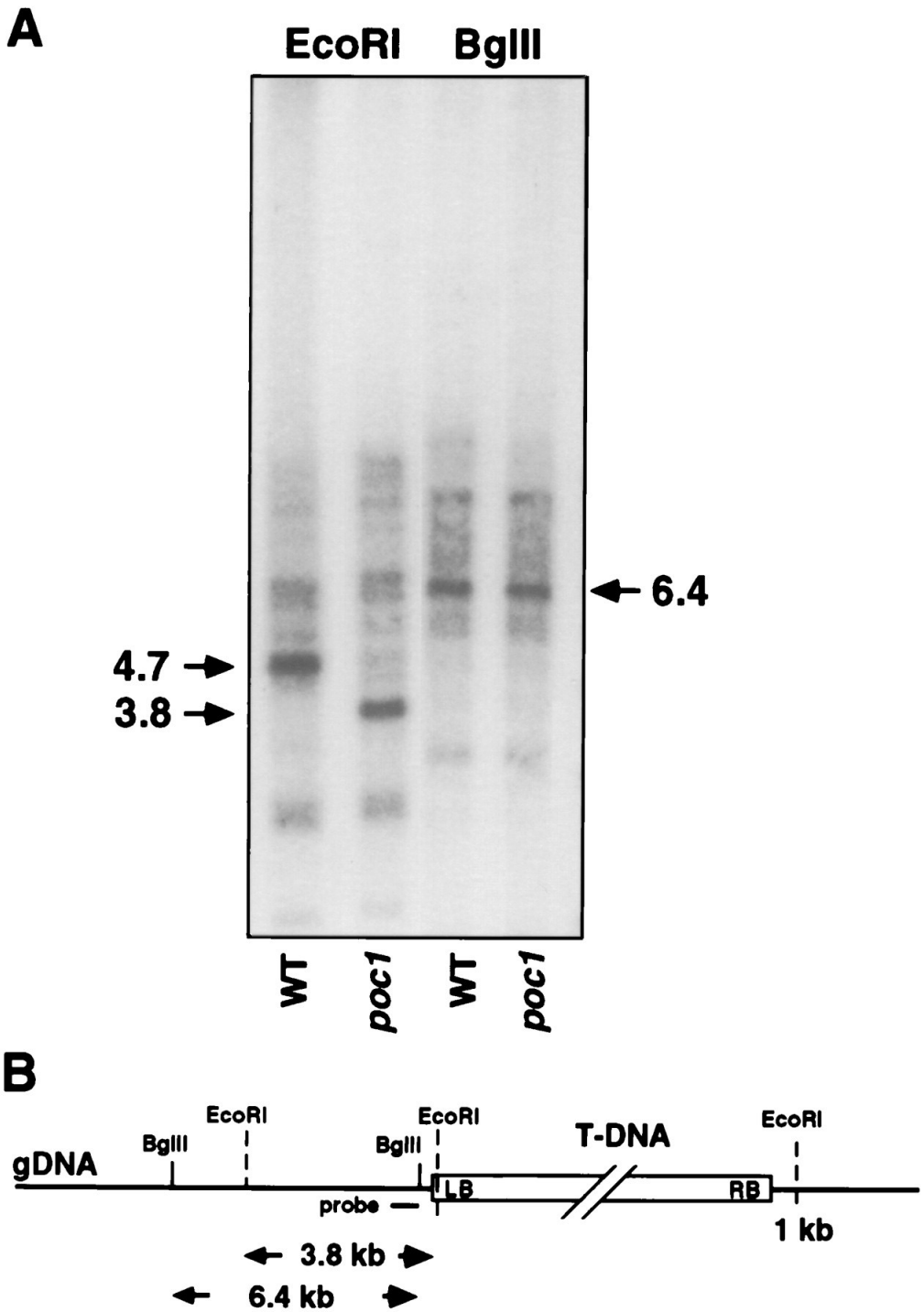

Fs
RB
LB
RB
LB
X
X
0.23
4.4
0.23
4.4
1.8
Probe
Probe

Coda-Coda



Testa-Coda





Identification of the genomic region flanking the T-DNA inserted in *poc1*.

***(A)* Southern blot analysis of total genomic DNA from wild type (WT) and *poc1* digested with *Eco*RI or *Bgl*II and hybridized with a 0.5-kb probe from the cloned region flanking the T-DNA left border (LB).**

(*B*) Diagram of the T-DNA insert within the *poc1* genomic DNA (gDNA) illustrating the locations of the restriction sites, the size of the restriction fragments generated, and the fragment used as the probe.

Ma il T-DNA è correlato (responsabile del) con il fenotipo mutato???

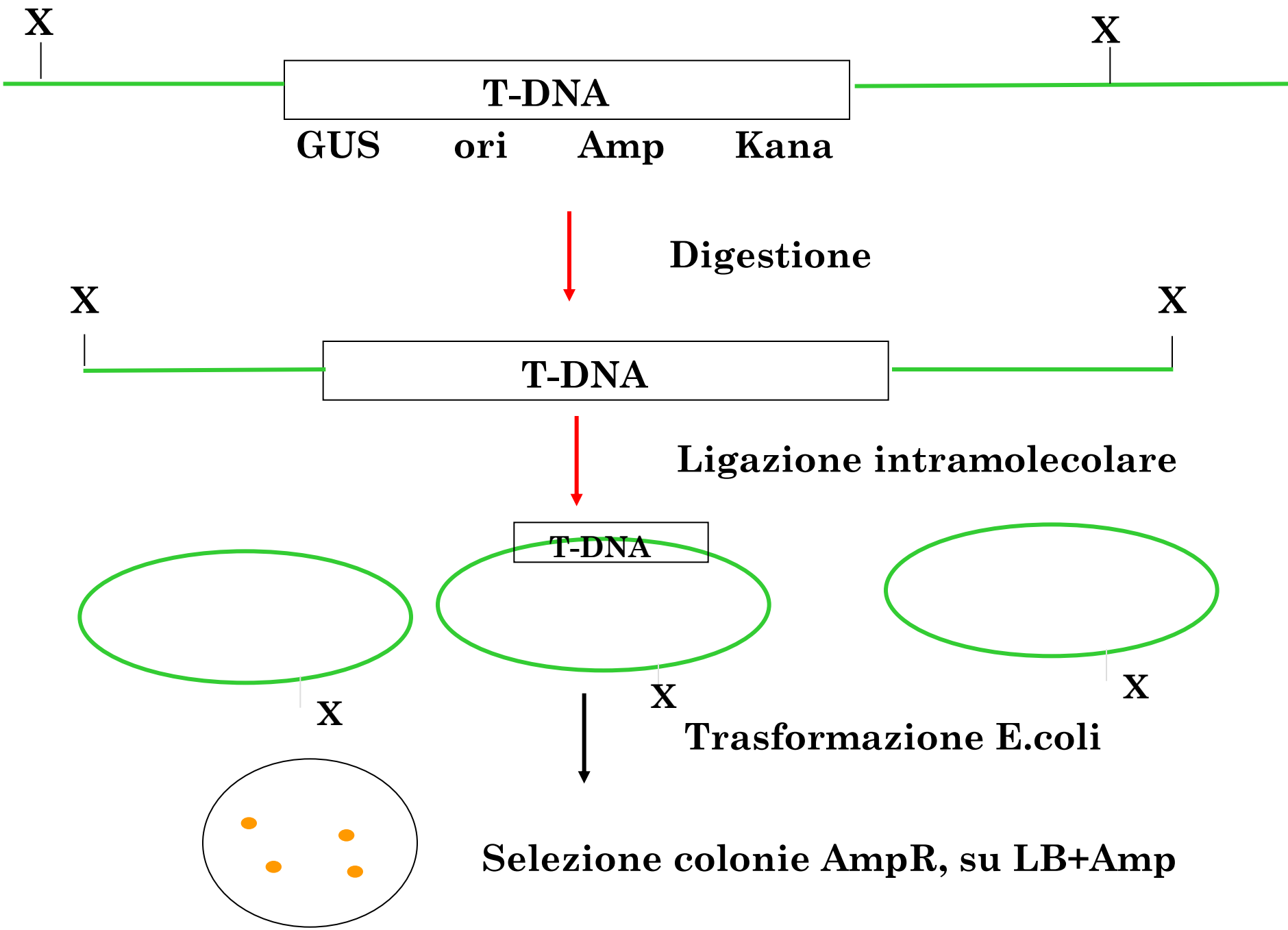
- **Analisi di segregazione del marcatore e del fenotipo mutante (inserzione T-DNA in 1 o più loci??)**

- **Caratterizzazione molecolare del sito di inserzione (T-DNA singolo, a tandem oppure inserzione più complessa??)**

- **Clonaggio delle "Flanking Sequences"**

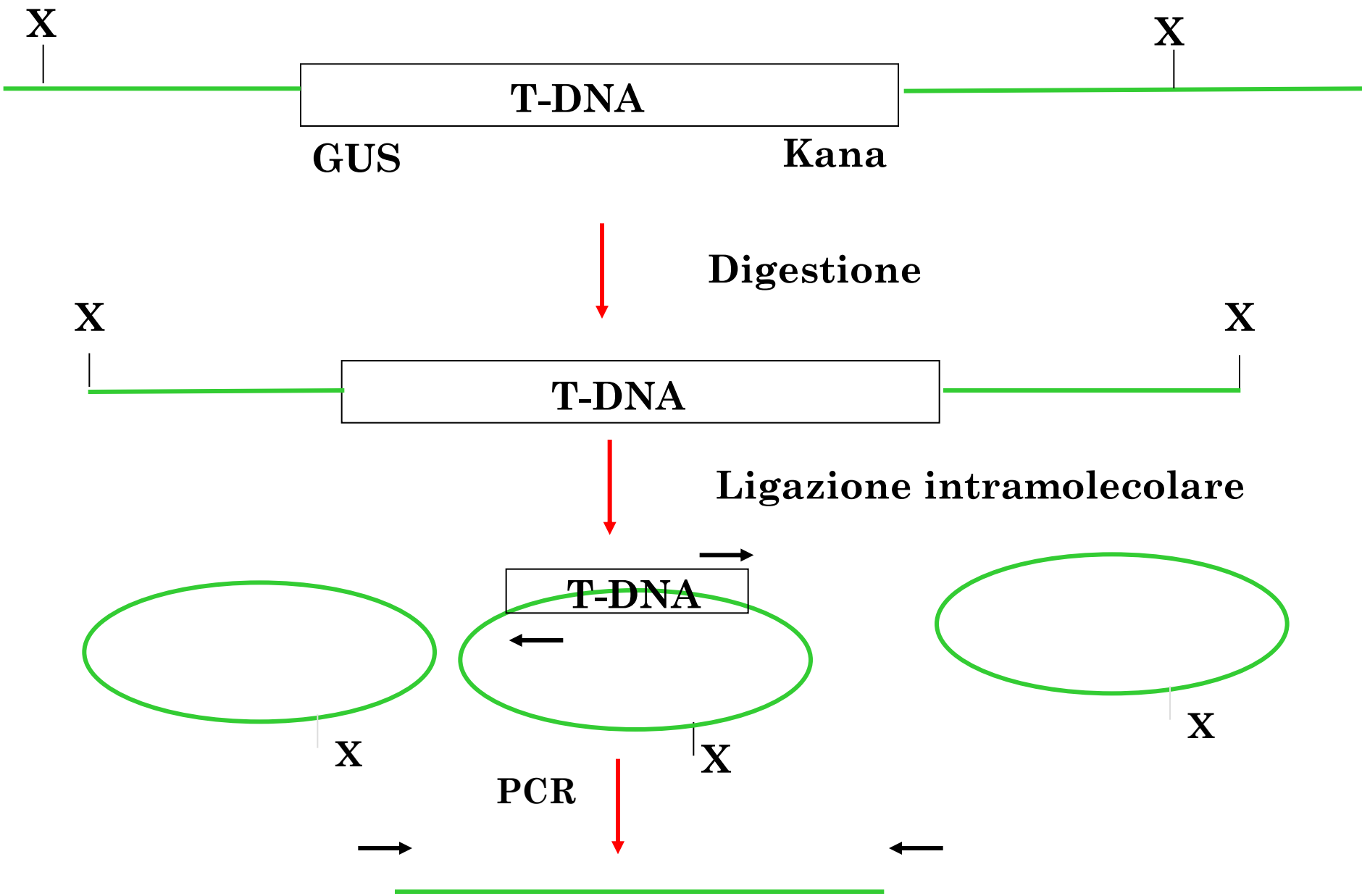
Strategie di clonaggio delle “Flanking Sequences”

- **Plasmid Rescue**
- **Inverse PCR (IPCR)**
- **Libreria genomica del mutante (probe T-DNA)**


X
X
T-DNA
GUS
ori
Amp
Kana
Digestione
X
X
T-DNA
Ligazione intramolecolare
T-DNA
X
X
X
Trasformazione E.coli
Selezione colonie AmpR, su LB+Amp

Strategie di clonaggio delle “Flanking Sequences”

- **Plasmid Rescue**
- **Inverse PCR (IPCR)**
- **Libreria genomica del mutante (probe T-DNA)**


X
X
T-DNA
GUS
Kana
Digestione
X
X
T-DNA
Ligazione intramolecolare
T-DNA
X
X
X
PCR

Gene Targeting in Mice/hES cells

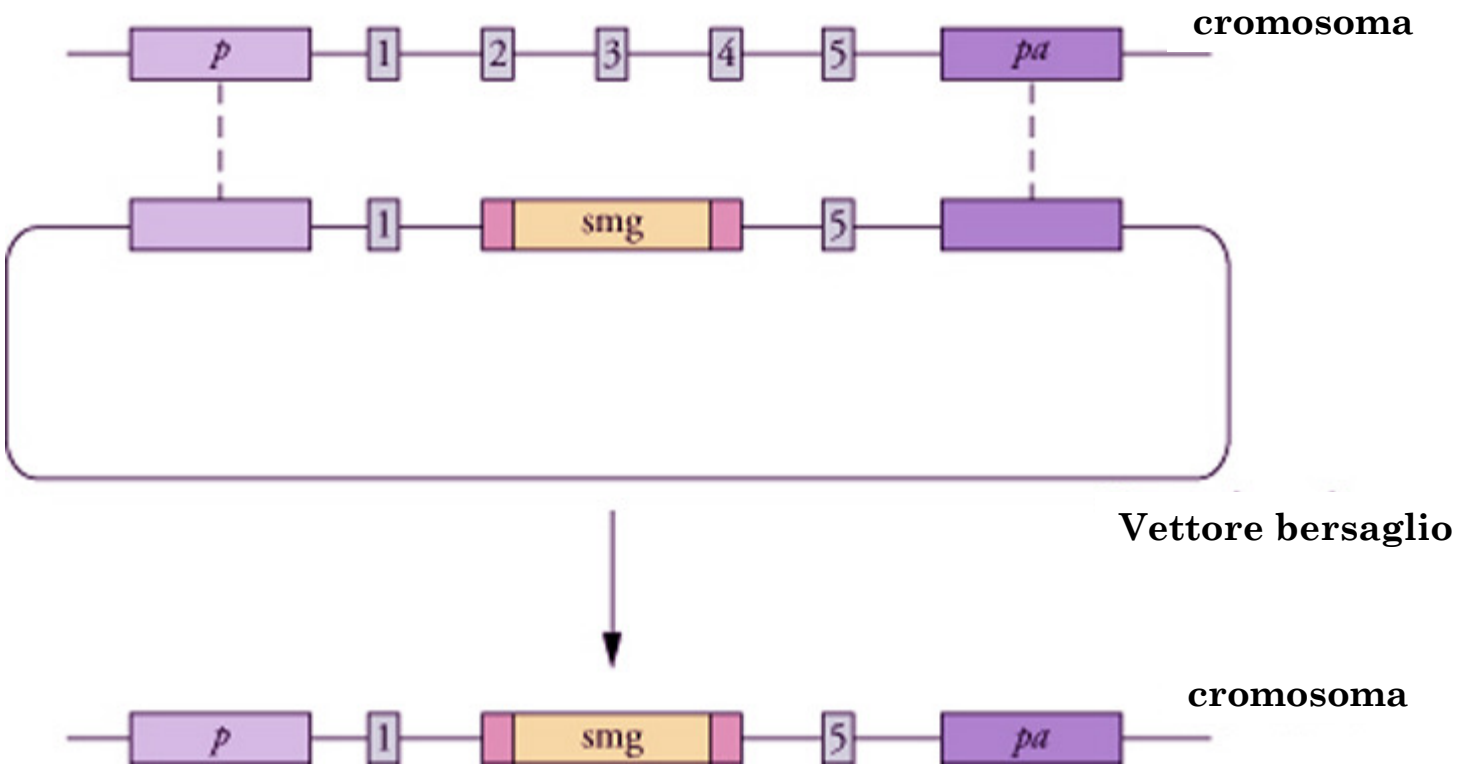
Il Gene Targeting è mediato da Ricombinazione omologa

Nelle cellule di mammifero la ricombinazione omologa è un evento
molto raro (a differenza del lievito)
La frequenza di questo evento aumenta se il grado di omologia di sequenza tra il DNA introdotto (esogeno) e il gene bersaglio (endogeno) è molto elevata

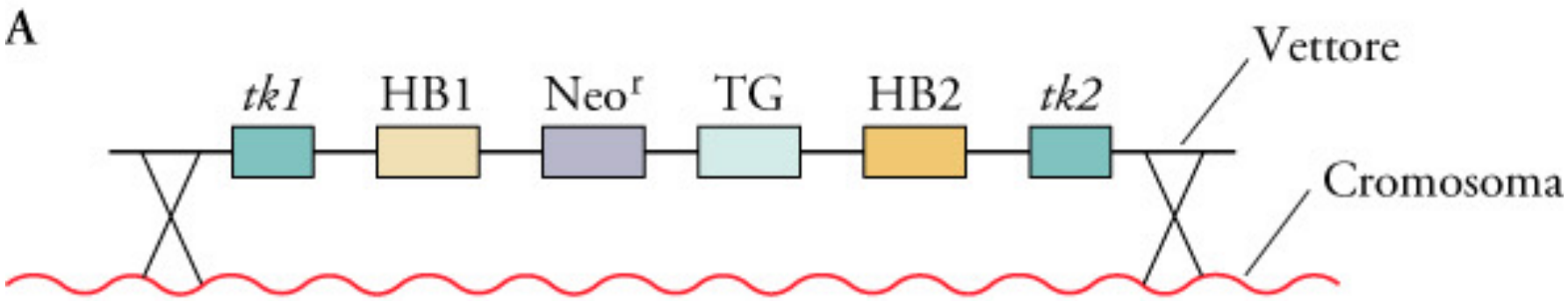
Per questo il clone di DNA esogeno è una sequenza isogenica
(derivante dallo stesso ceppo murino)

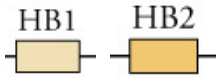
Gene targeting mediante ricombinazione omologa

Comporta l'introduzione di una mutazione in un gene specifico e puo essere considerata come una forma di "site-directed in vivo mutagenesis" e quindi è utilizzata per lo studio della funzione genica

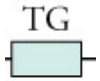


SELEZIONE POSITIVA-NEGATIVA cellule ES

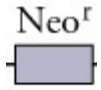




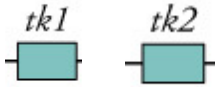
Seq di DNA per RICOMBINAZIONE OMOLOGA nel genoma bersaglio



TRANSGENE di interesse



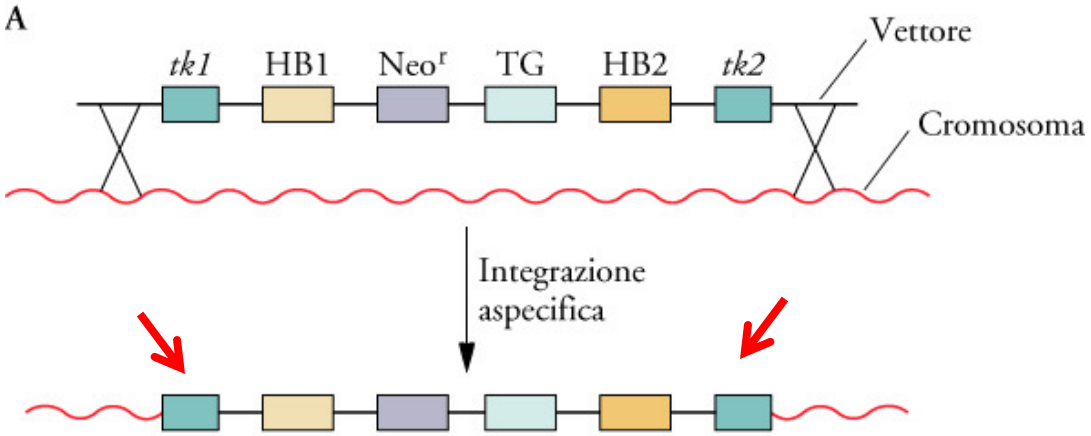
Gene che conferisce resistenza alla G418



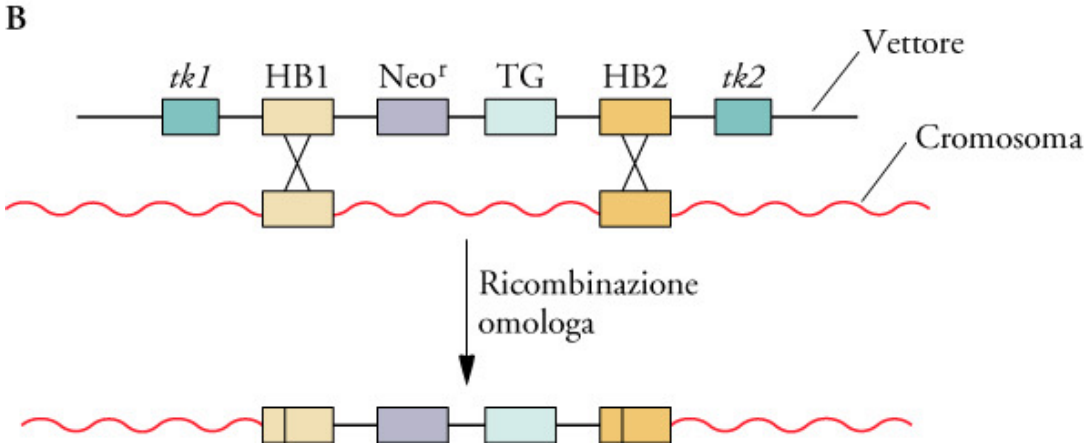
Geni della Timidina Kinasi (virus herpes simplex). In presenza di GANCICLOVIR: MORTE

SELEZIONE POSITIVA e NEGATIVA

ASPECIFICA



SPECIFICA



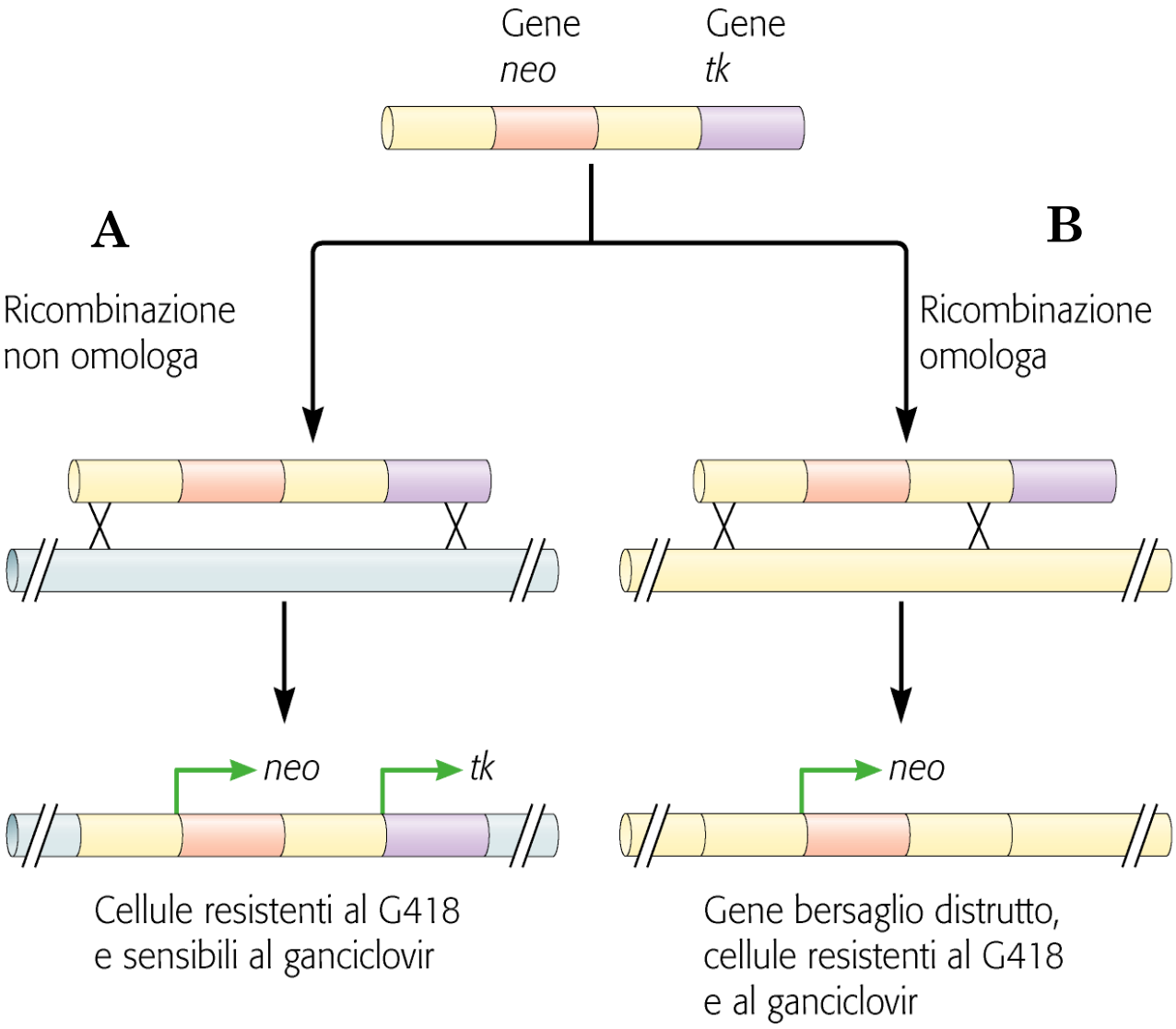
1) **Aggiunta di G418: SELEZIONE POSITIVA di cellule che hanno INTEGRATO il DNA**

2) **Aggiunta di GANCICLOVIR: SELEZIONE NEGATIVA di cellule con INTEGRAZIONE ASPECIFICA**

La selezione positiva/negativa

A: risultato di integrazione aspecifica. Si selezionano le cellule positivamente per la resistenza a G-418, conferita dal gene Neo, e negativamente in presenza del gancyclovir, che viene trasformato dalla timidina chinasi in composti tossici. Le cellule contenenti il vettore inserito random (A) conterranno il gene TK, e saranno uccise in presenza di gancyclovir

B: risultato di ricombinazione omologa. Il prodotto del doppio crossover tra i blocchi omologhi HB1 e HB2 non contiene i due geni della timidina kinasi (tk1 e tk2), quindi sono resistenti al gancyclovir, e anche al G-418 grazie alla presenza del gene Neo.



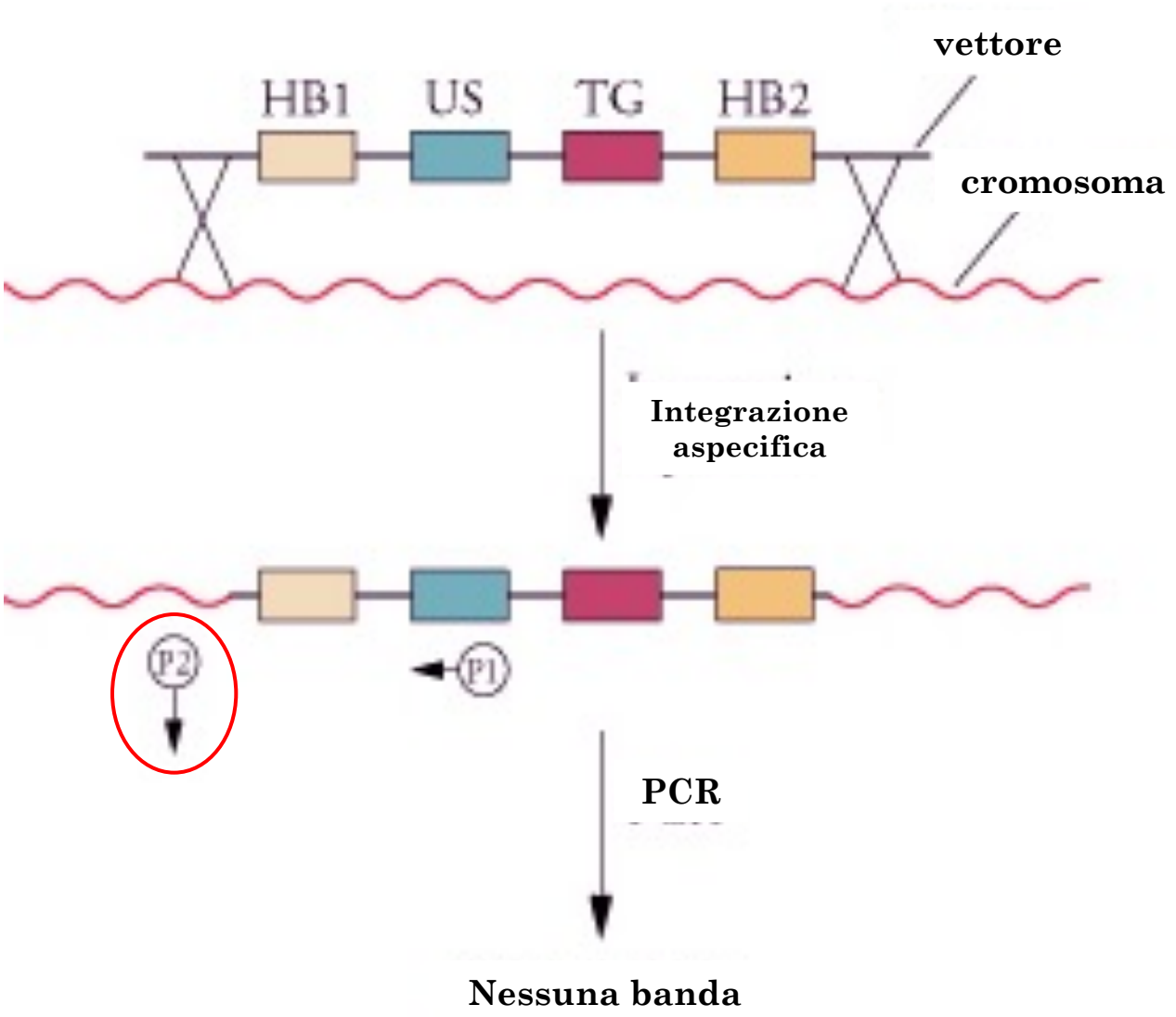
Analisi dell'integrazione mediante PCR

A: integrazione aspecifica

Uno dei primers (P2) non è in grado di appaiarsi al sito cromosomico P2. P1 invece si ibrida ad una sequenza US che si trova sul DNA esogeno.

Quindi non si ha amplificazione

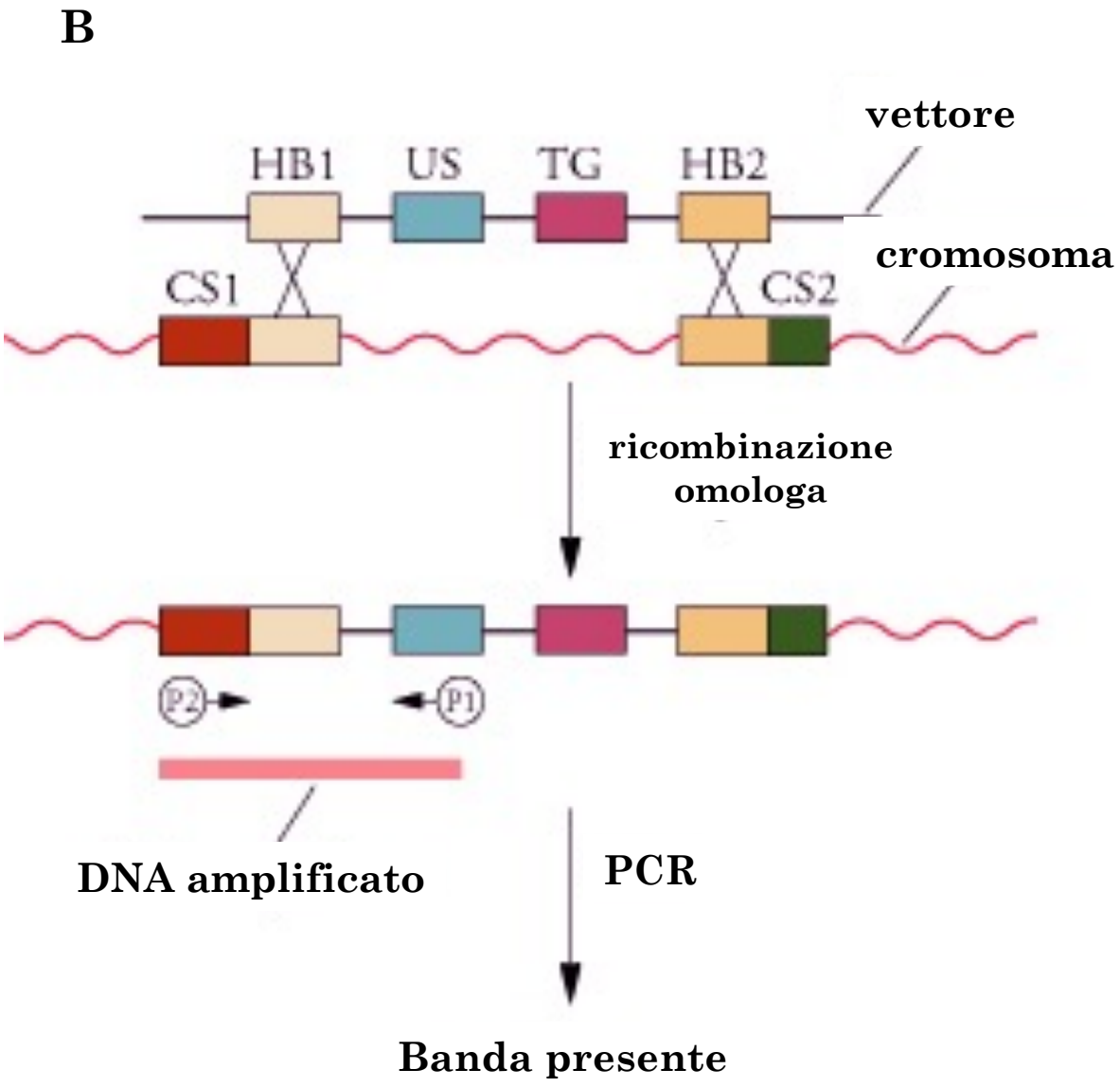
A



Analisi dell' integrazione mediante PCR

B: ricombinazione omologa

La ricombinazione omologa tra le sequenze di DNA HB1 e HB2 del DNA esogeno che sono complementari ai siti cromosomici CS1 e CS2, crea regioni di ibridazione per P1 e P2 che si trovano ad una distanza predeterminata. L ' amplificazione così genera il prodotto di dimensioni attese.



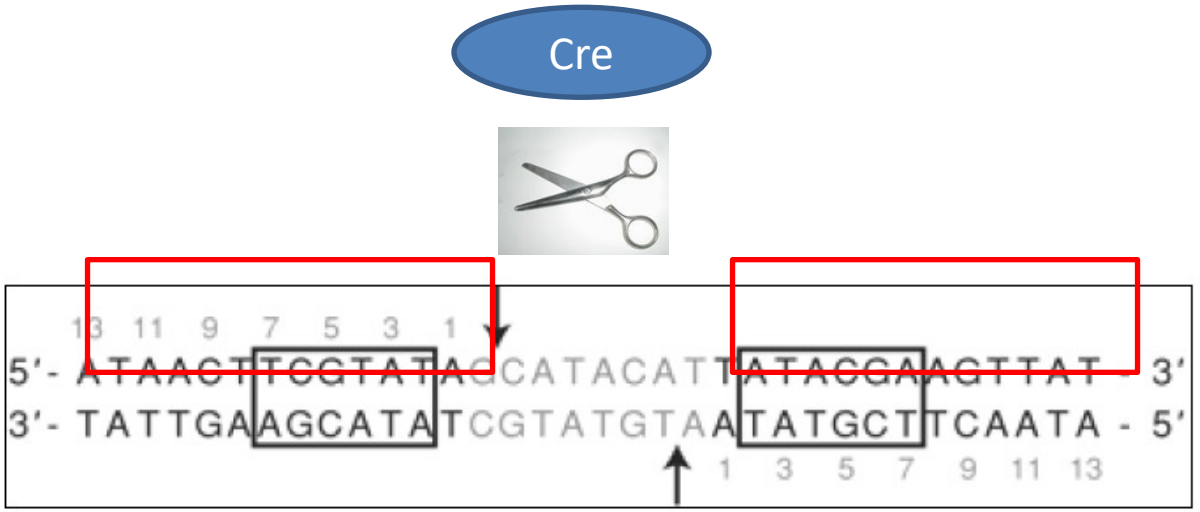
Cre-Lox System

Il sistema Cre/Lox deriva dal fago P1

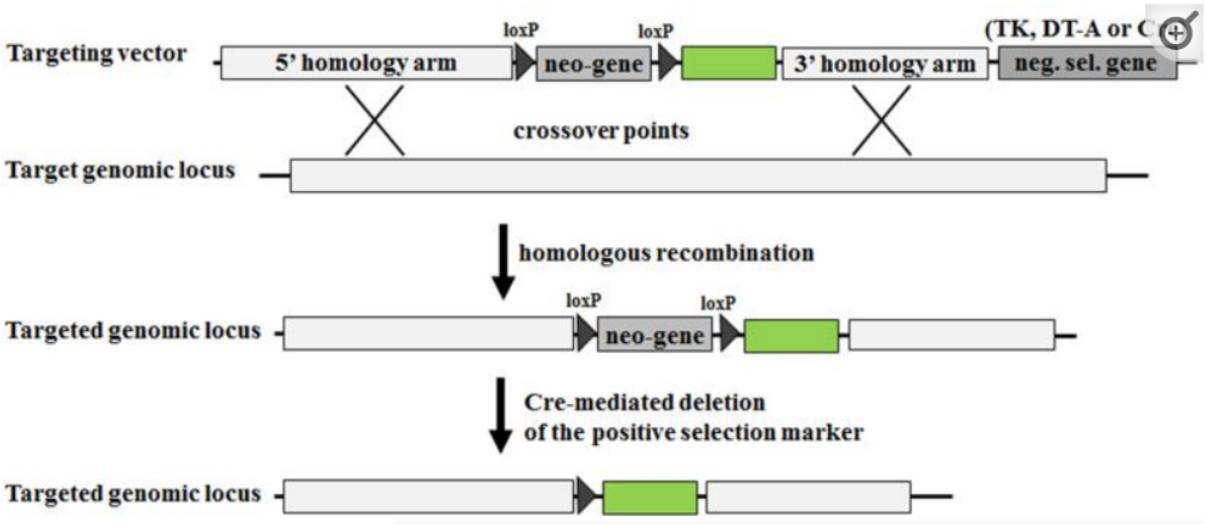
Il sistema è detto "floxing" in quanto consiste di sequenze fiancheggianti il gene e la ricombinazione mediata da Cre

Cre: Ricombinasi sequenza-specifica (catalizza la ricombinazione tra siti Lox)

LoxP: Locus di crossover (2 palindromi 13bp + regione centrale di 8nt)

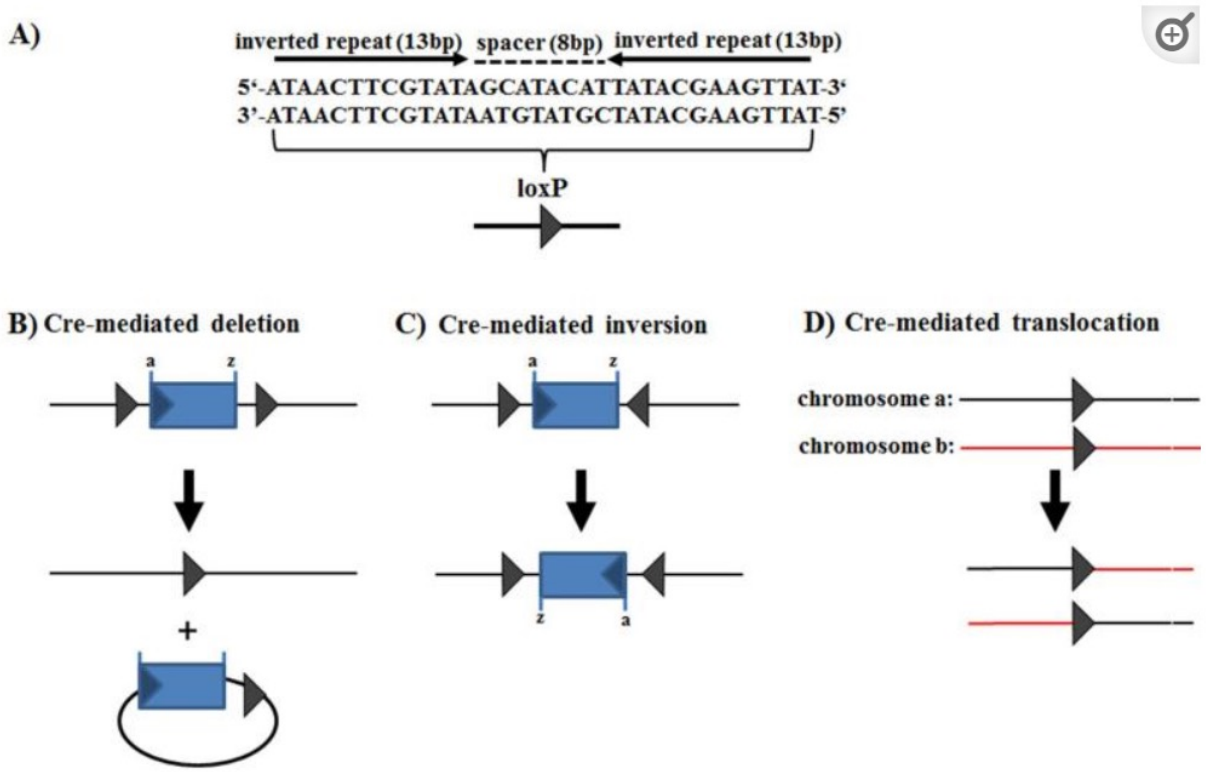


la ricombinasi CRE riconosce i siti LoxP e ricombina le sequenze di DNA adiacenti



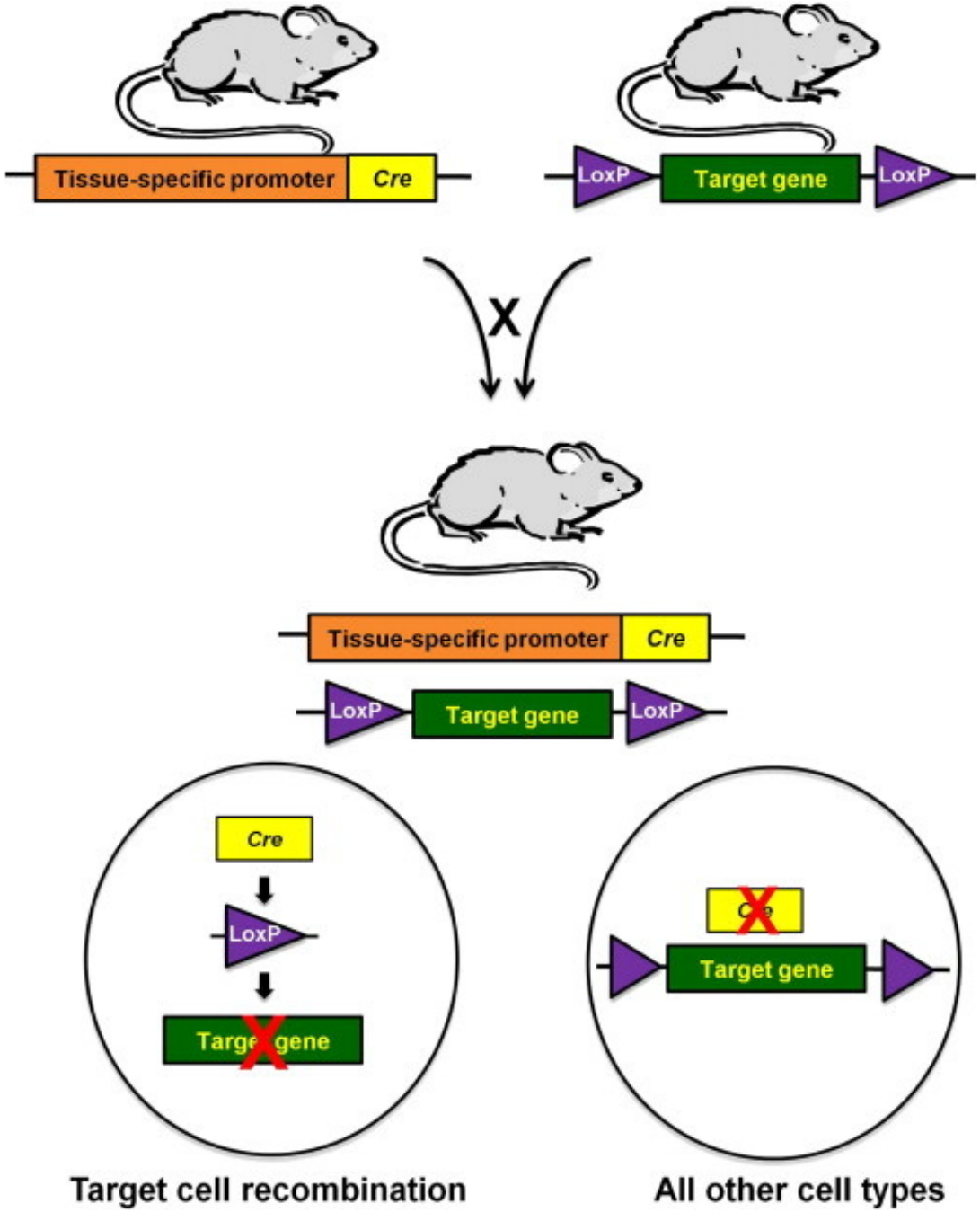
Typical gene targeting strategy

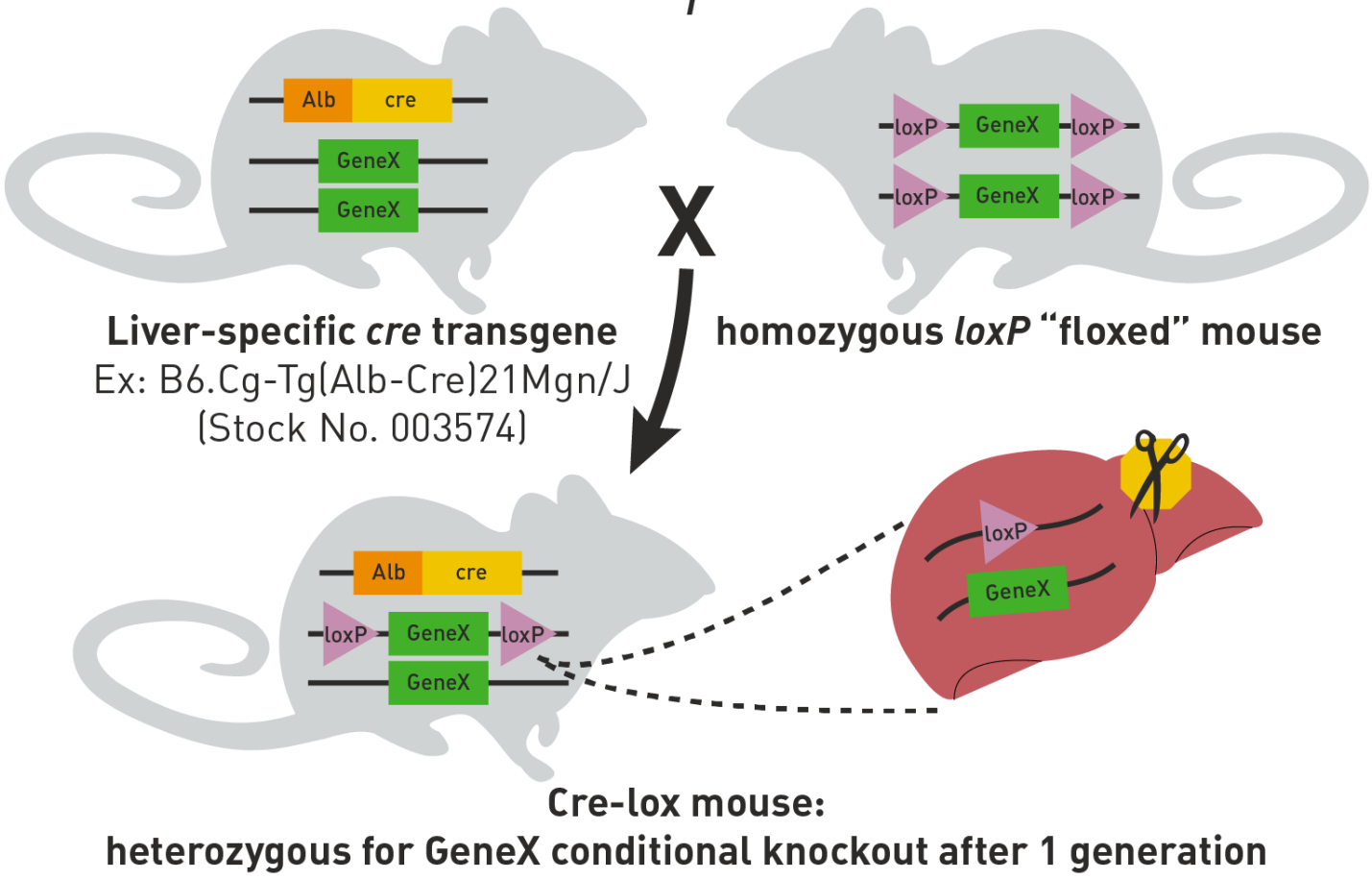
A targeting vector is typically composed of three basic units: (i) a 5' homology arm; (ii) a gene marker for positive selection (e.g. neomycin resistance gene (neo)); (iii) a 3' homology arm; and (iv) a negative selection marker (neg. sel. Marker), such as thymidine kinase, diphtheria toxin fragment A (DT-A), or, if the positive selection marker is flanked by loxP sites, Cre recombinase gene (Cre). Furthermore, any desired DNA sequence of interest (here green box) can be inserted between the homology arms of the targeting vector, in order to introduce it into the target genome by homologous recombination. Homologous recombination between the targeting vector and the target cognate chromosomal region results in the disruption of one genomic copy of the targeted genomic locus and loss of the vector's negative selection marker gene. Crossover points are depicted by "X". The floxed (loxP sites flanked) positive selection marker gene can be removed by expressing Cre recombinase in the recombinant ESCs or by crossing the chimeric mice with Cre-expressing transgenic mice (see also Fig. 4A).



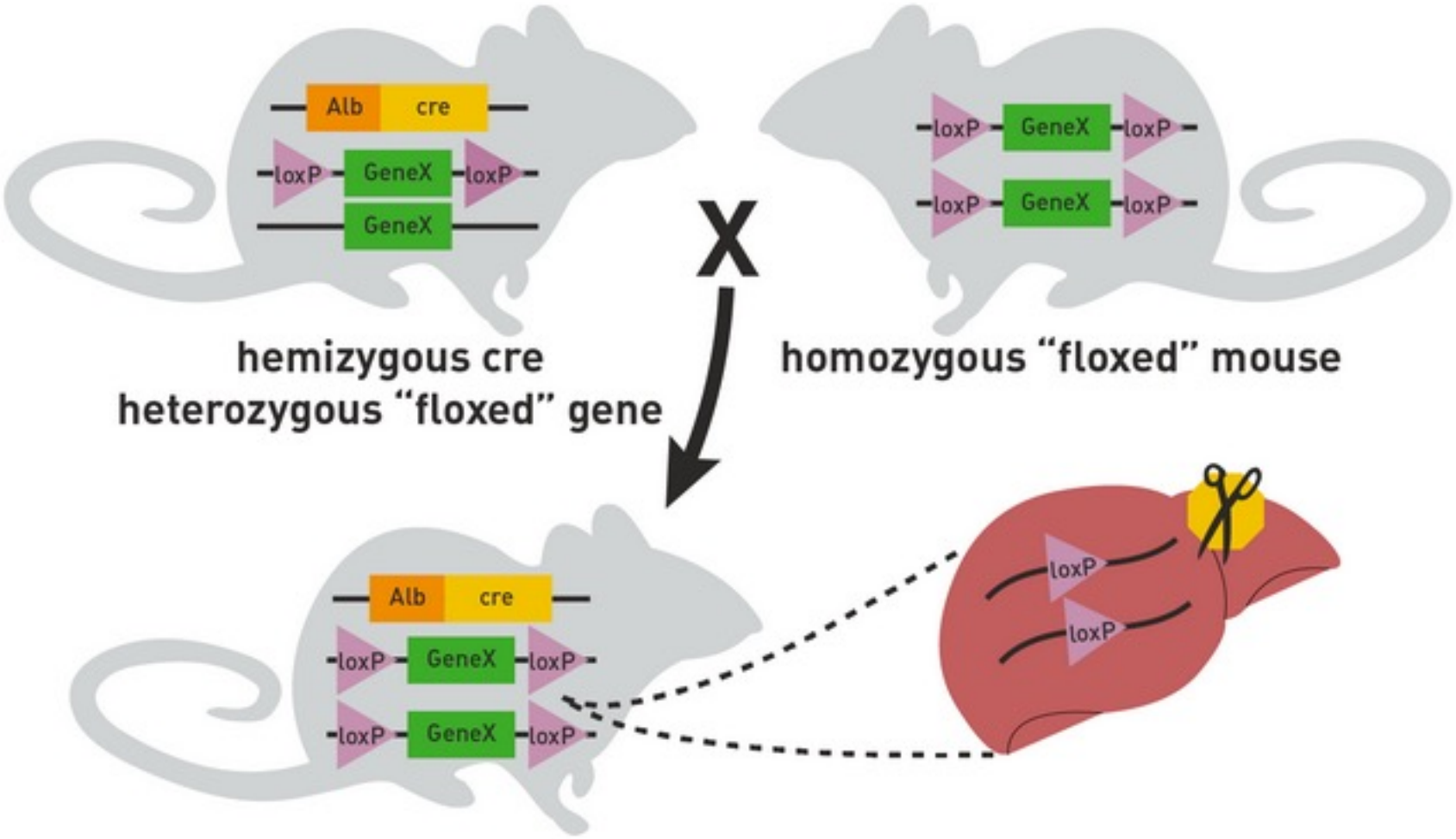
LoxP structure and Cre recombinase-mediated recombinations

(A) Single loxP site that contains two inverted 13 bp repeats, separated by an asymmetric 8 bp long sequence. The type of Cre-mediated recombination is dependent on the orientation and location of the loxP sites: (B) Cre excises a circular molecule from between two loxP sites placed in the same orientation; (C) Cre inverts the DNA sequence between two loxP sites positioned in opposite orientation; (D) Cre-mediated recombination between two different linear DNA molecules (e.g. chromosomes), each containing a loxP site, resulting in the exchange of the DNA regions flanking the loxP sites. Figure was modified from Torres and Kuehn (111).


Tissue-specific promoter
Cre
LoxP
Target gene
LoxP
X
Tissue-specific promoter
Cre
LoxP
Target gene
LoxP
Cre
LoxP
Target gene
Target cell recombination
Target gene
All other cell types

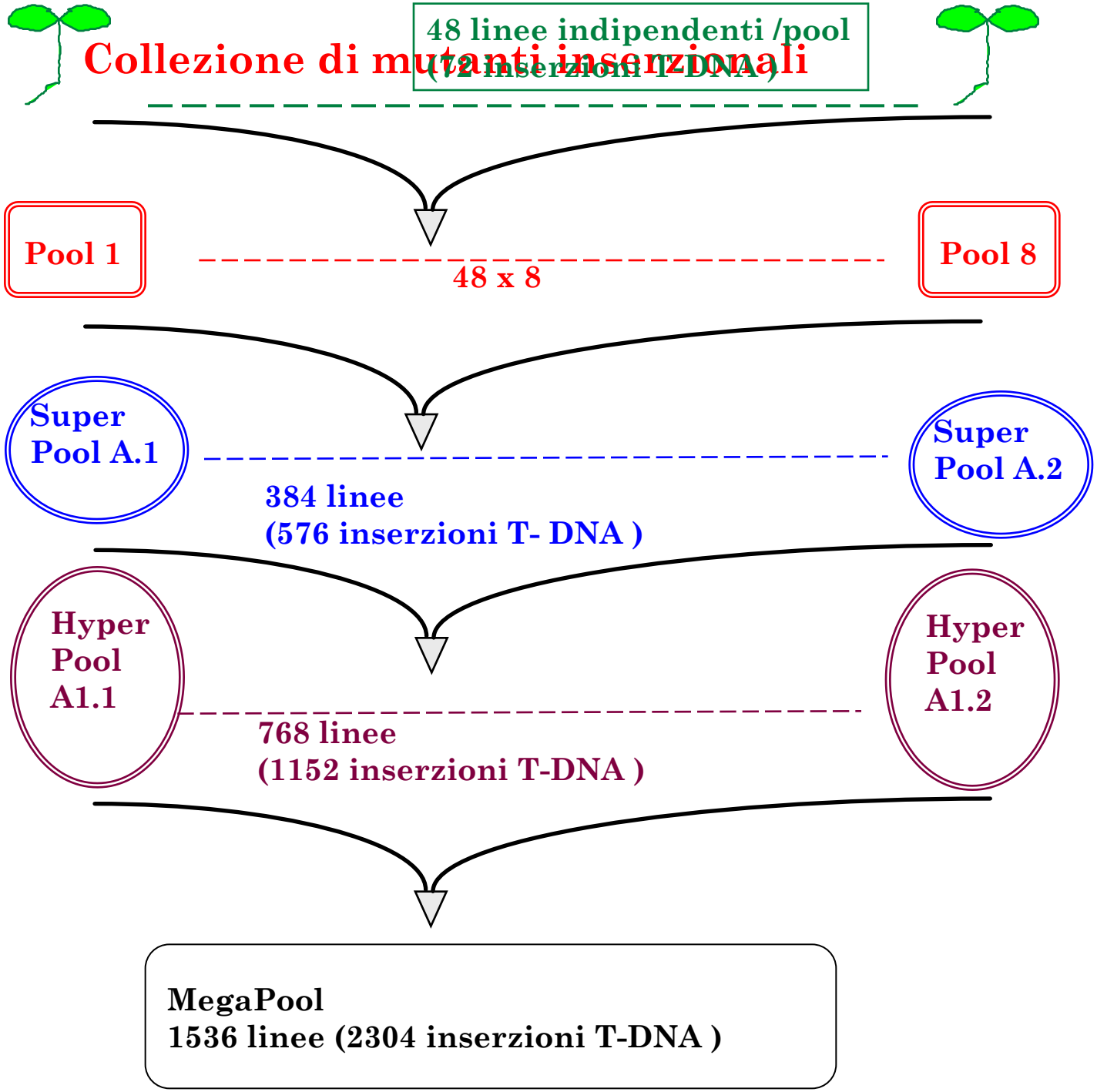


Approximately 50% of the offspring will be heterozygous for the loxP allele and heterozygous for the cre transgene. Mate these mice back to the homozygous *loxP*-flanked mice. Approximately 25% of the progeny from this mating will be homozygous for the *loxP*-flanked allele and heterozygous for the *cre* transgene. These will be your experimental mice.


Alb
cre
loxP
GeneX
loxP
GeneX
X
loxP
GeneX
loxP
loxP
GeneX
loxP
hemizygous cre
heterozygous "floxed" gene
homozygous "floxed" mouse
Alb
cre
loxP
GeneX
loxP
loxP
GeneX
loxP
loxP
loxP

Reverse Genetics

Collezione di mutanti inserzionali



Protocollo di Screening mediante PCR

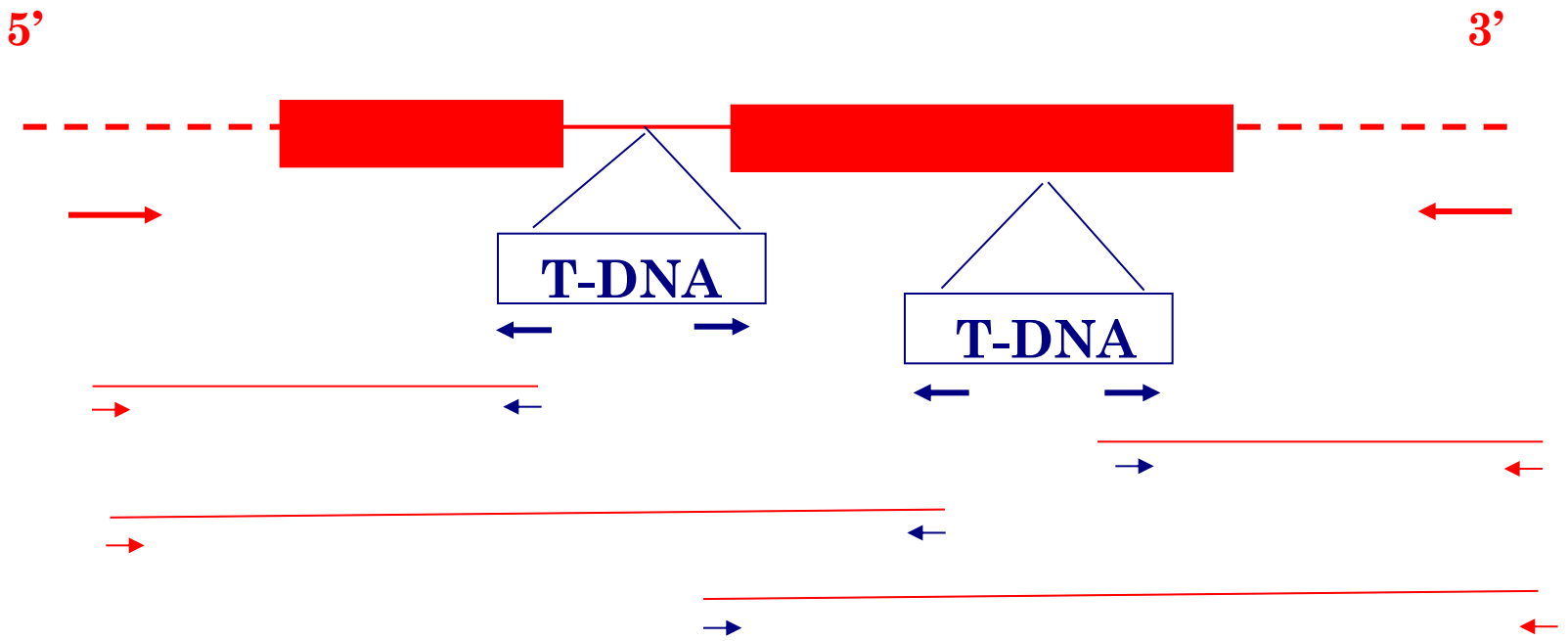
- **Screening per PCR di n HP:**
  combinazione di un primer gene-specifico (F/R)
  ed un primer T-DNA (RB/LB)

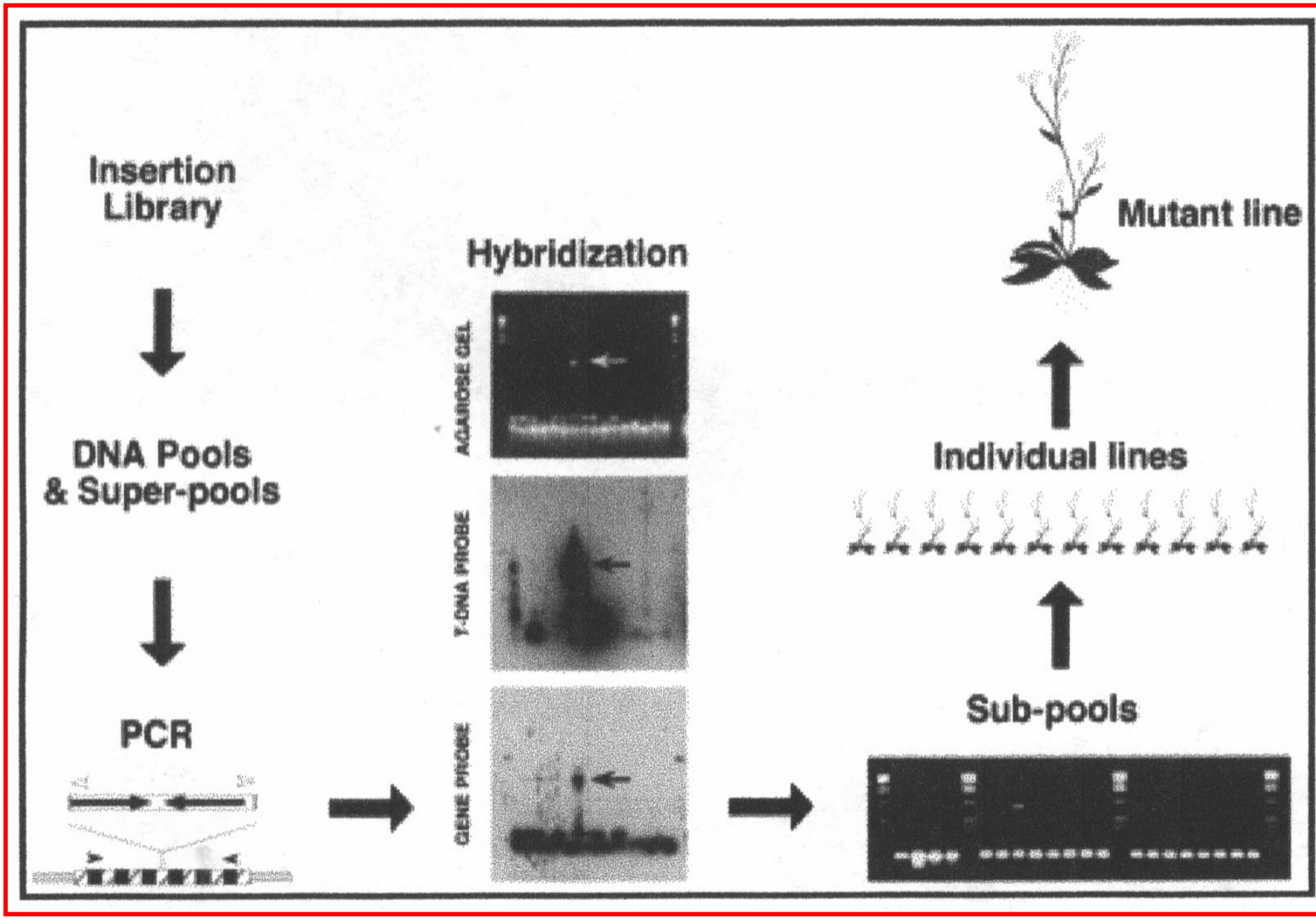
- **Qualità dei primers:** **specificità-sensibilità**
  background con i primers T-DNA

- **Analisi per ibridazione (transfer "sandwich"):**
  sonda T-DNA (RB+LB)
  sonda gene-specifica

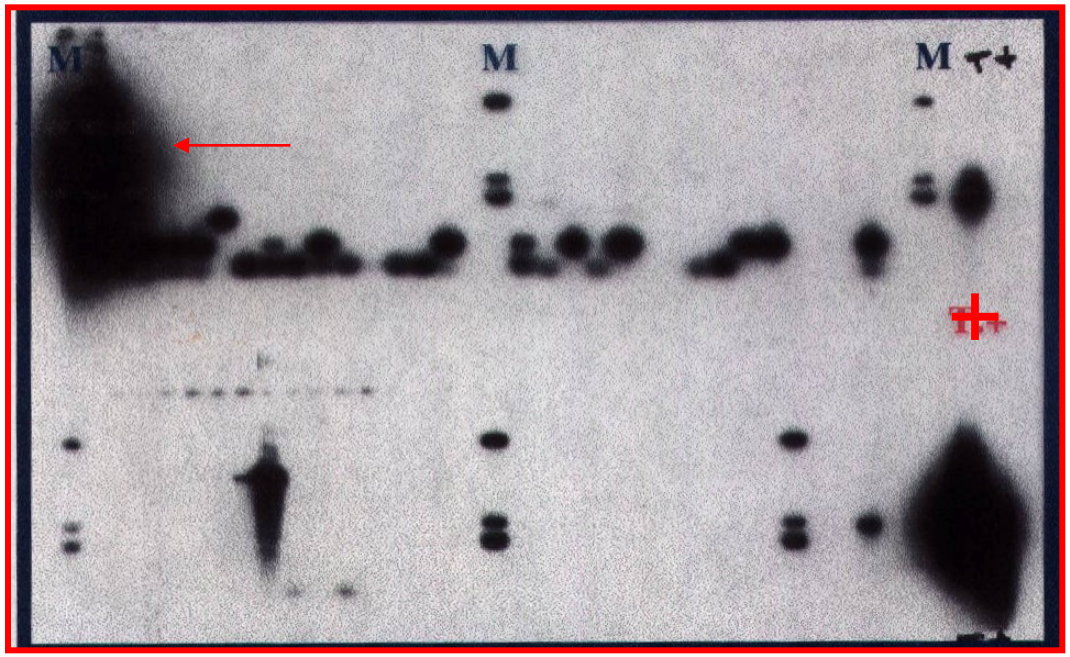
- **Segnali coincidenti con le 2 sonde:**
  PCR di conferma con primers T-DNA nested
  o con primers gene-specifici
  sequenza del frammento amplificato

Scelta dei Primers gene-specifici

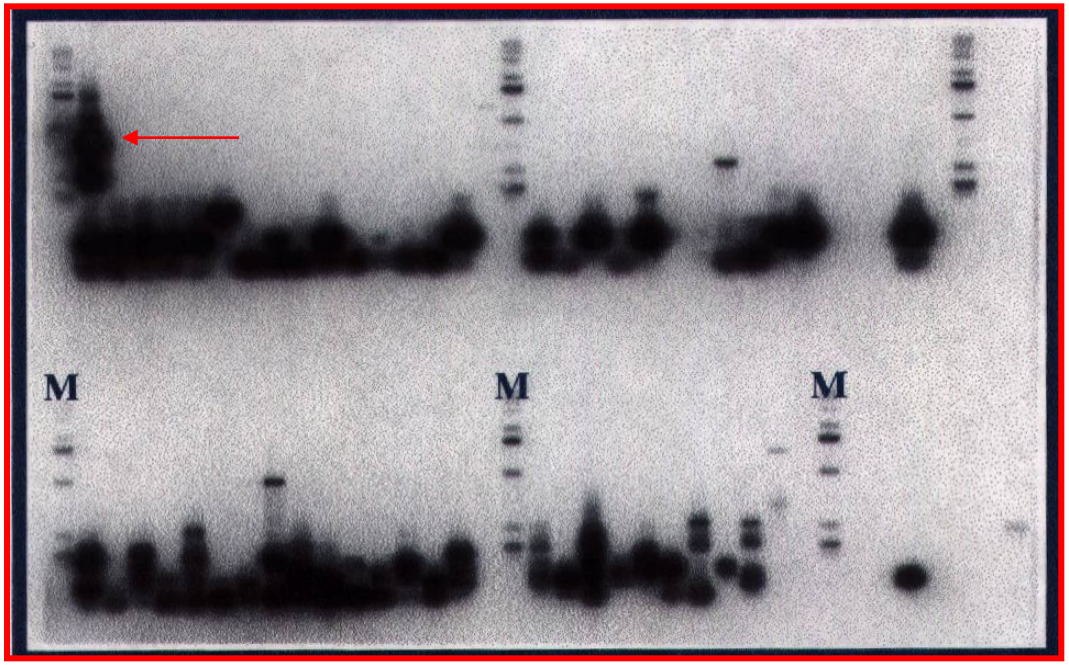


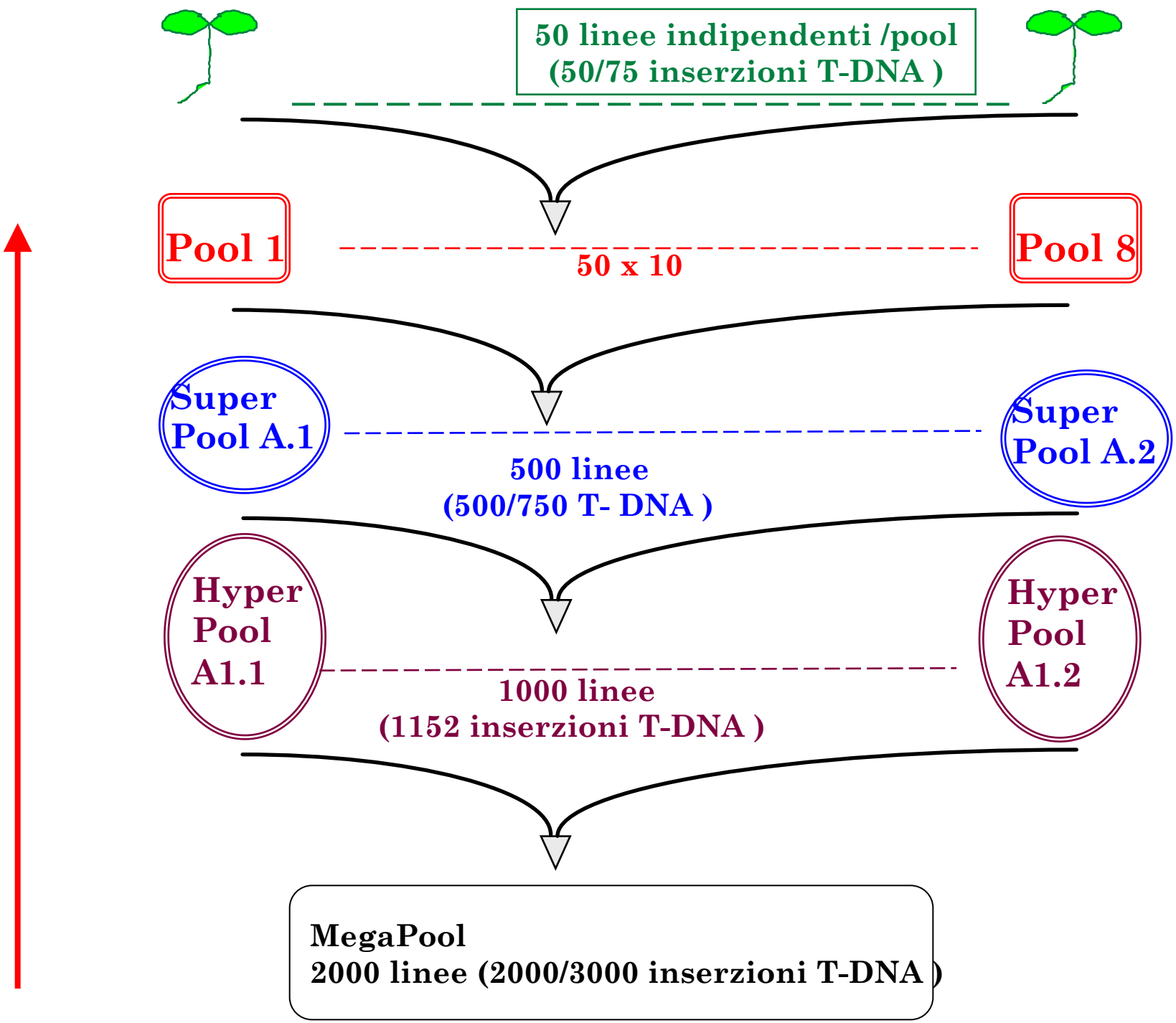

Insertion Library
DNA Pools & Super-pools
PCR
Hybridization
AGAROSE GEL
T-DNA PROBE
GENE PROBE
Sub-pools
Individual lines
Mutant line

Sonda DAG1



Sonda T-DNA




50 linee indipendenti /pool
(50/75 inserzioni T-DNA)
Pool 1
50 x 10
Pool 8
Super
Pool A.1
500 linee
(500/750 T- DNA)
Super
Pool A.2
Hyper
Pool
A1.1
1000 linee
(1152 inserzioni T-DNA)
Hyper
Pool
A1.2
MegaPool
2000 linee (2000/3000 inserzioni T-DNA)