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Generation of insect-resistant and glyphosate-tolerant rice by introduction of a T-DNA containing two Bt insecticidal genes and an *EPSPS* gene*

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Abstract: Insect resistance and glyphosate tolerance have been two of the most important traits in the genetic improvement of various crops. In this study, two *Bacillus thuringiensis* (Bt) insecticidal genes, *Cry1Ac* and *Cry1Ig*, and a modified glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene (*G10*) were combined into a single transferred DNA (T-DNA) fragment and introduced into rice by *Agrobacterium*-mediated transformation. A transgenic line with single-copy T-DNA insertion named GAI-14 was found to be highly resistant to striped stem borer and rice leaf roller, and tolerant to glyphosate. Analysis of T-DNA border sequence suggested that the transgenes were inserted at the chromosome 3 and appeared to have not interrupted any known or putative genes. A field trial observed no significant difference in the basic agronomic traits between GAI-14 and the recipient rice.

Key words: Bt gene stacking, Bt resistance management, *EPSPS*, Transgenic rice
doi:10.1631/jzus.B1500056 **Document code:** A **CLC number:** Q182

In questo studio, due geni Bt, Cry1Ac e Cry1Ig, e un gene modificato per la resistenza al glifosato (5-enolpiruvilshikimate-3-phosphate synthase, EPSPS, G10) sono stati clonati in un unico vettore T-DNA e introdotti nel riso con trasformazione mediata da Agrobacterium.

l Cry1Ac e Cry1Ig sono stati accoppiati per rendere meno probabile lo sviluppo della resistenza alla tossina Bt e per migliorare l'attività insetticida del riso transgenico, mentre il G10 è stato utilizzato per conferire la resistenza al glifosato e anche come marcatore di selezione per la trasformazione.

Materiali e Metodi

Riso (*Oryza sativa* spp. *japonica*) cultivar Xiushui 134 è stato usato come ricevente nella trasformazione con *Agrobacterium*.

I geni Cry1Ac, Cry1Ig e G10 hanno queste sequenze

Cry1Ac (GenBank: ADO64599.1)
Cry1Ig (GenBank: AGU13866.1)
G10 EPSPS (Swiss-Prot:Q9RVD3.2).

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Cry1Ig

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ORIGIN

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 121 YPDSLGKRPQ GDLLEALERL GAWVSSNDGR LPISVSGPVR GGTVEVSAER SSQYASALMF
 181 LGPLLPDGLE LRLTGDIKSH APLRQTLDTL SDFGVRATAS DDLRRISIPG GQKYRPGRVL
 241 VPGDYPGSAA ILTAAALLPG EVRLSNLREH DLQGEKEAVN VLREMGADIV REGDTLTVRG
 301 GRPLHAVTRD GDSFTDAVQA LTAAAAFAEG DTTWENVATL RLKECDRISD TRAELERLGL
 361 RARETADSLS VTGSAHLAGG ITADGHGDHR MIMLLTLLGL RADAPLRITG AHHIRKSYPQ
 421 FFAHLEALGA RFEYAEATA
```

Step 1-2:

Sintetizzare i tre geni da clonare nel T-DNA del pCAMBIA1300 ✓

Costruzione cassetta di espressione

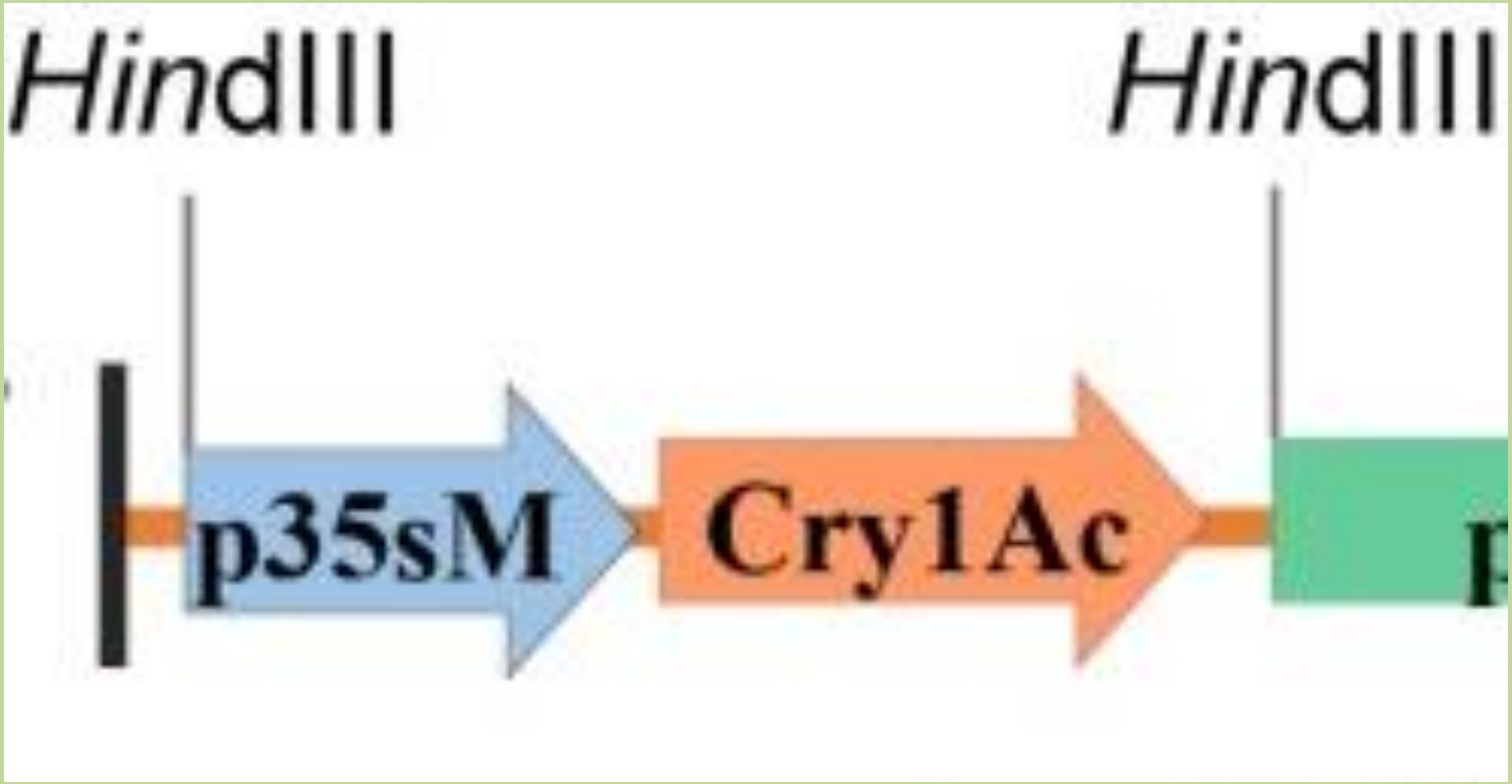
Cassetta di espressione HindIII/HindIII

Promotore (riso), ORF, Term

Promotore: CaMV 35S modificato (promotore 35S originale legato al primo introne del gene di riso Actin1)

ORF: Cry1Ac

Term: non descritto nel paper (!)

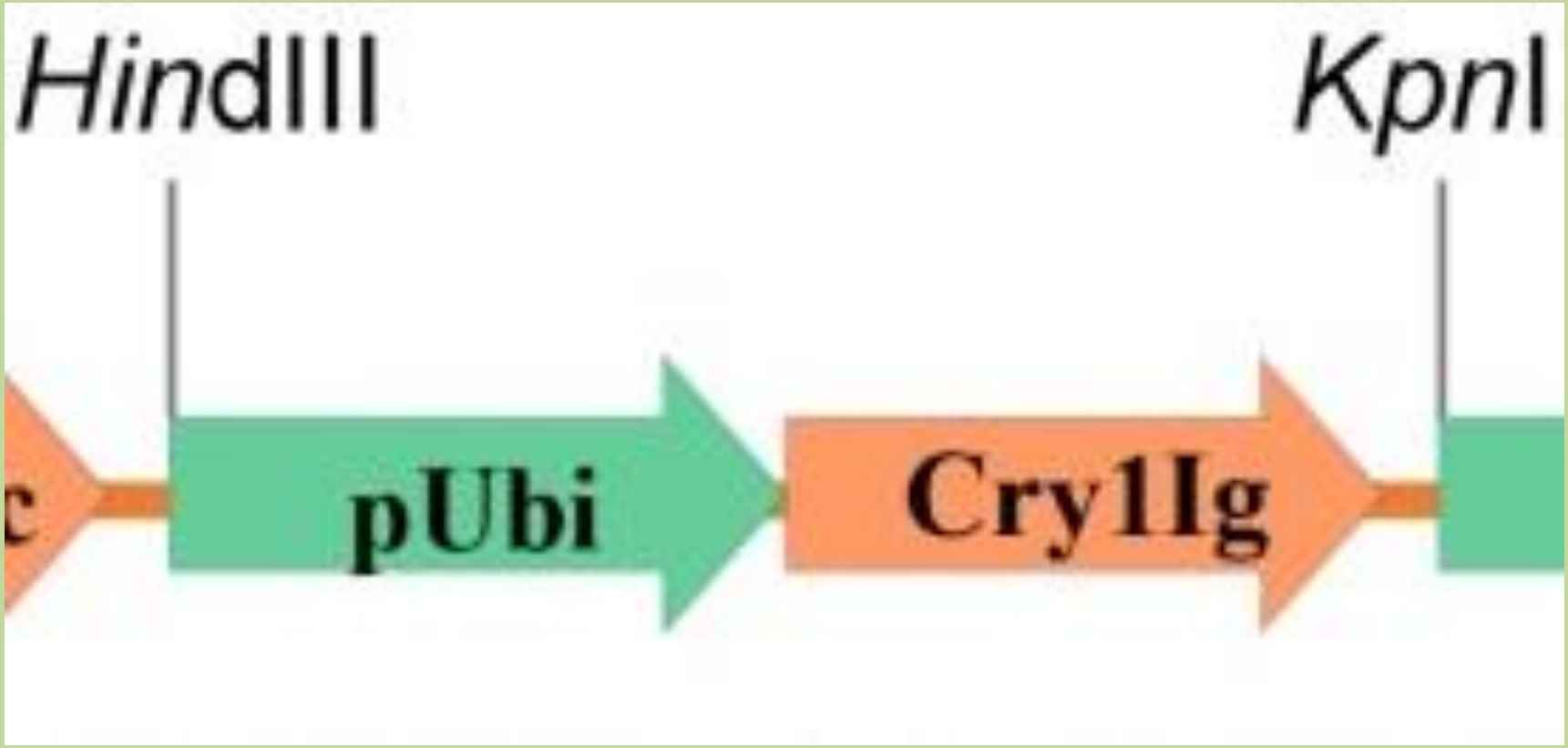


Cassetta di espressione HindIII/KpnI

Promotore: derivato dal gene per ubiquitina di mais (pUBi)

ORF: Cry1Ig

Term: non descritto nel paper (!)

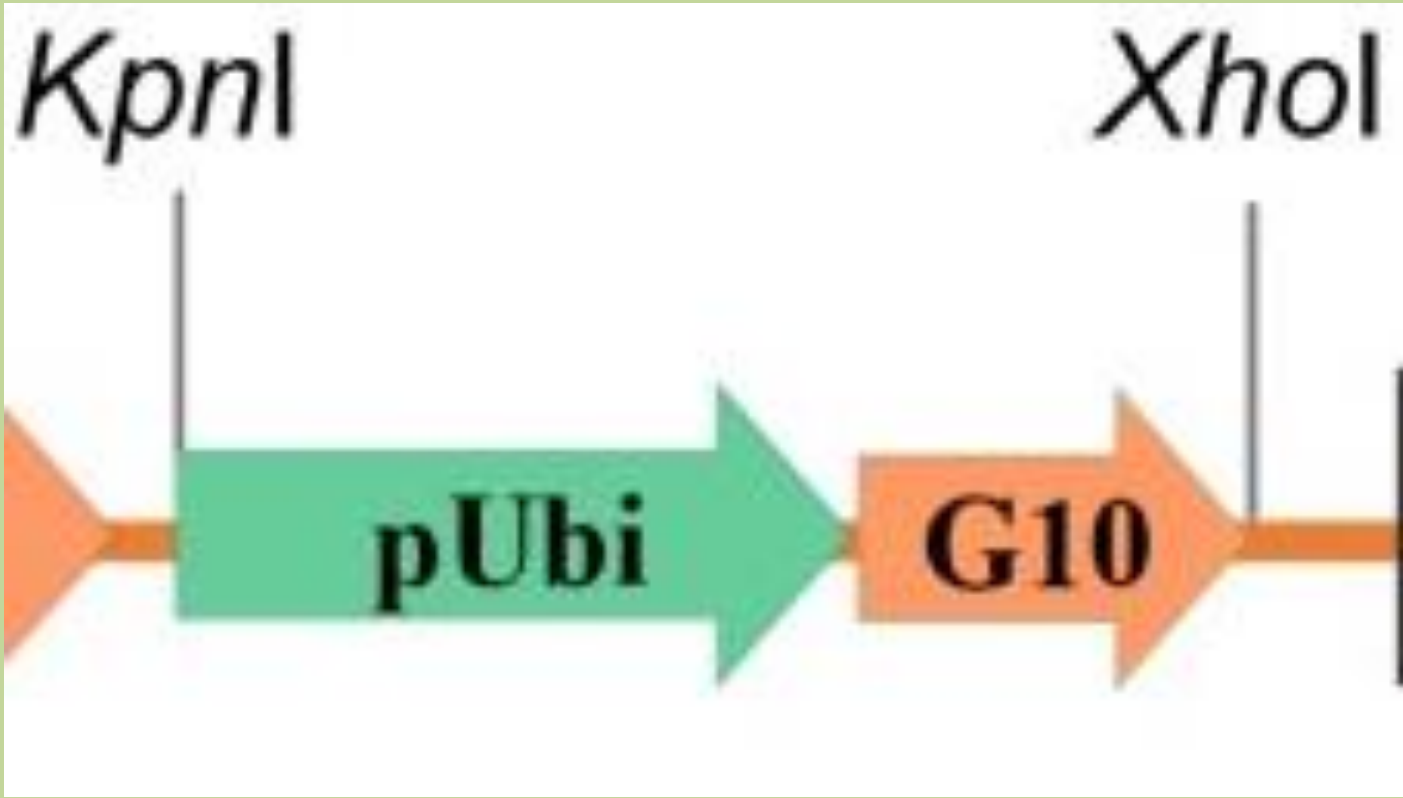


Cassetta di espressione KpnI/XhoI

Promotore: pUBi

ORF: EPSPS G10, fusione traduzionale tra la regione codificante per il peptide di transito plastidico del gene di mais acetohydroxy acid synthase (AHAS, GenBank: NP_001151166.1) ed la regione codificante per la EPSP. La proteina prodotta deve essere localizzata nel cloroplasto.

Term: non descritto



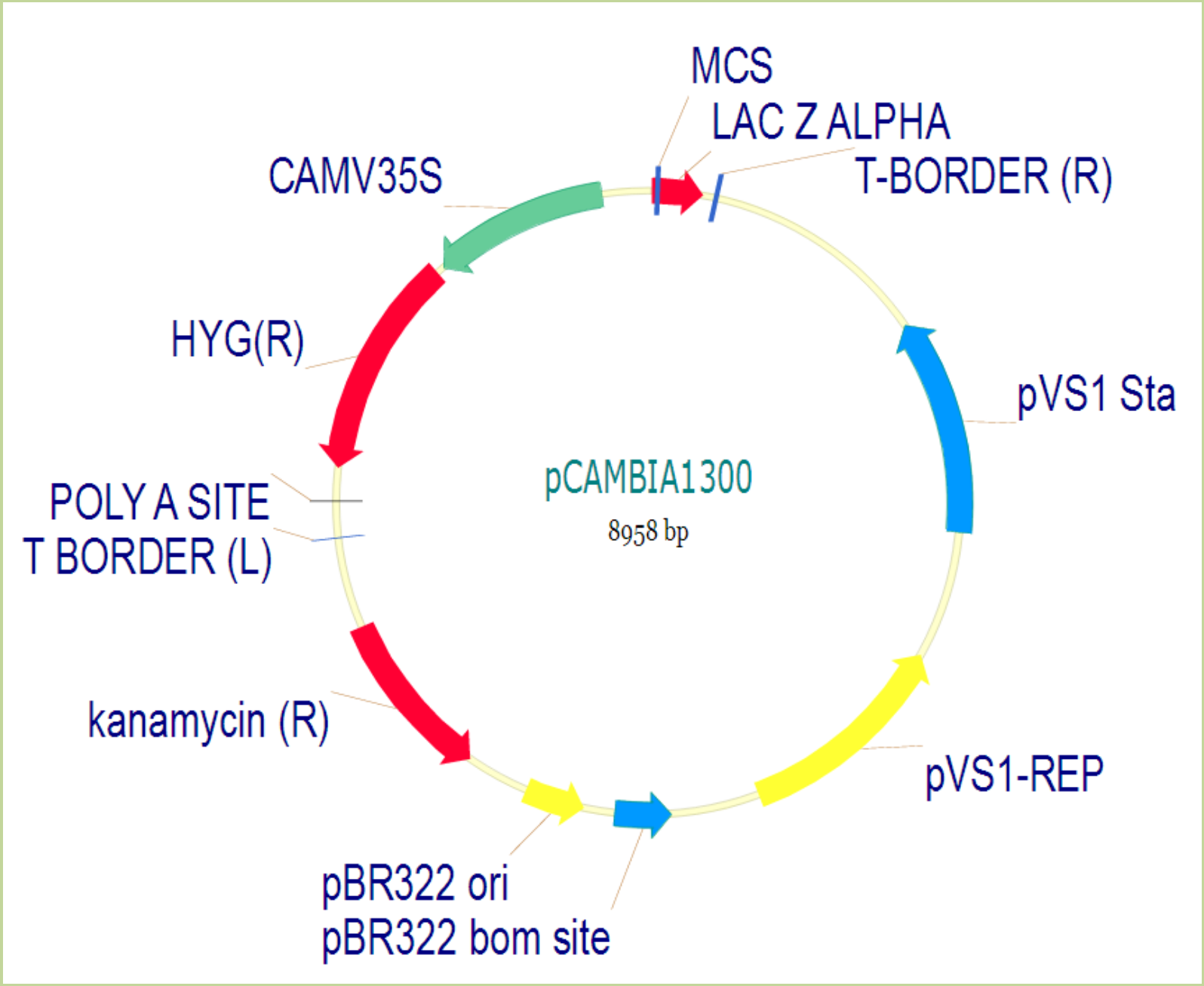
Step 3-4:

Sintetizzare i tre geni da clonare nel T-DNA del pCAMBIA1300 ✓

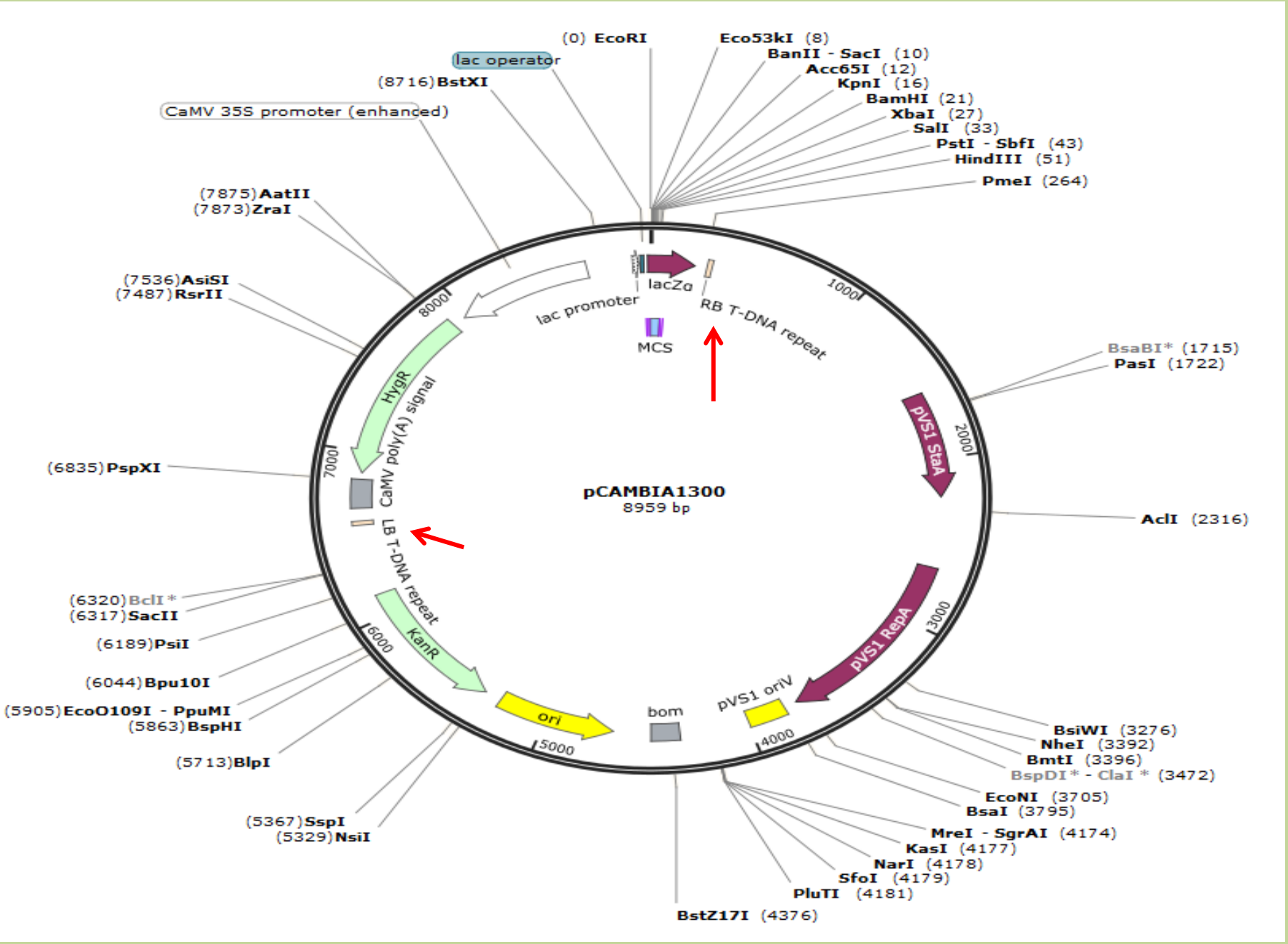
Costruzione cassetta di espressione ✓

Eliminare T-DNA originale del pCAMBIA

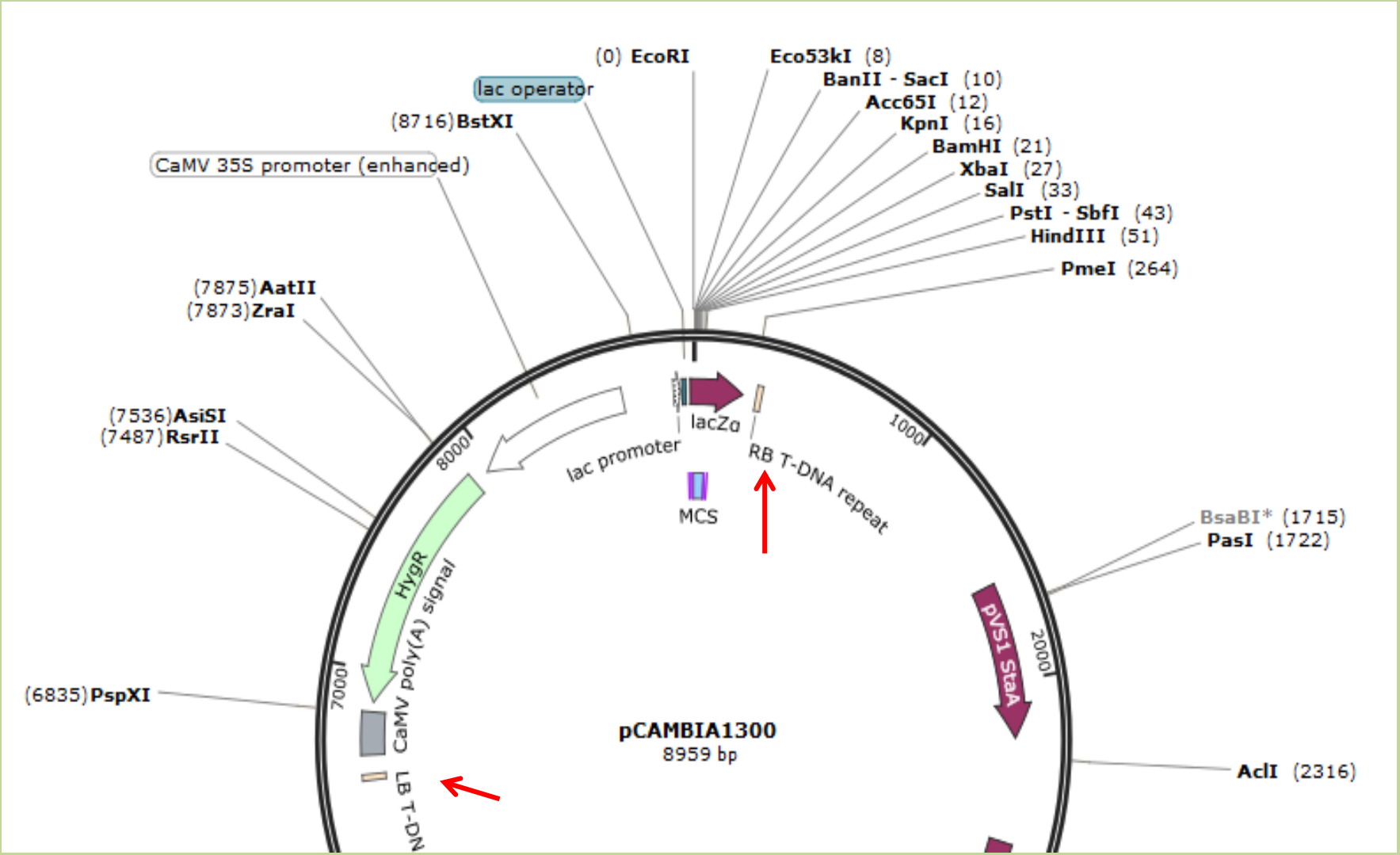
Clonare i tre geni uno dopo l'altro (3 steps di clonaggio)



pVS1 replicon for high stability in *Agrobacterium*



http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA1300/



http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA1300/

XhoI

Did you know this product can be customized
find out more about customization options.

5′. . . C▼T C G A G . . . 3′
3′. . . G A G C T▲C . . . 5′

HindIII

5′. . . A▼A G C T T . . . 3′
3′. . . T T C G A▲A . . . 5′

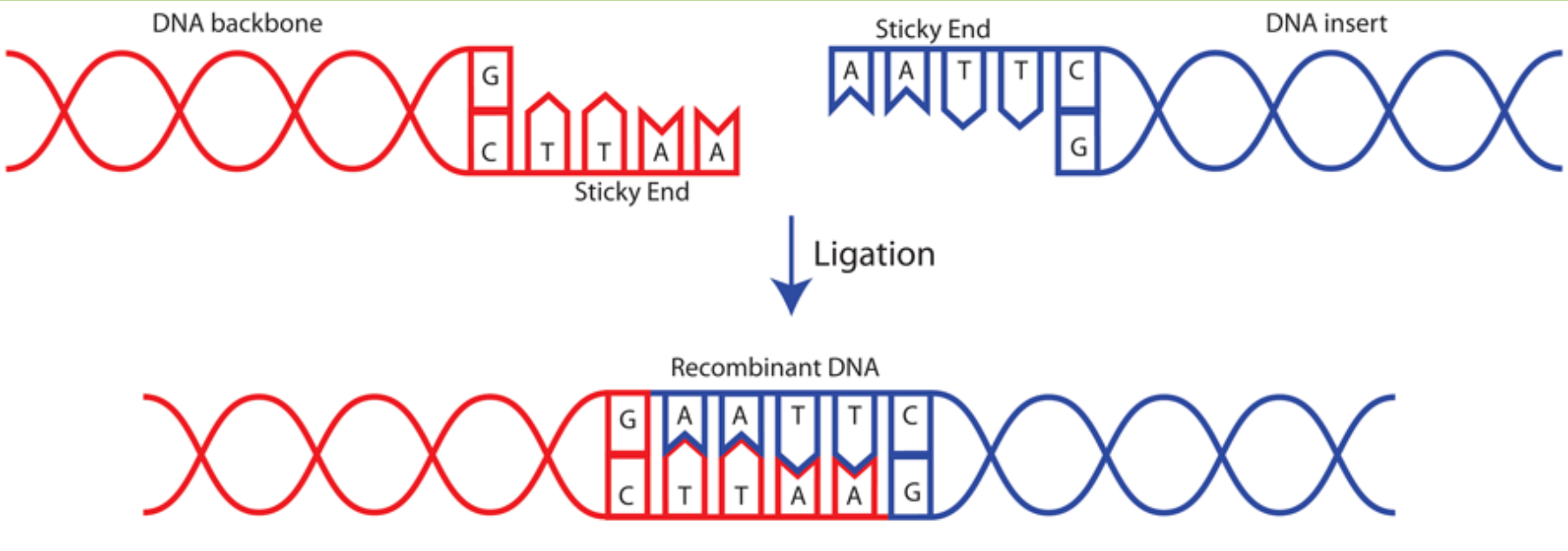
PspXI

KpnI

5′. . . G G T A C▼C . . . 3′
3′. . . C▲C A T G G . . . 5′

5′. . . V C▼T C G A G B . . . 3′
3′. . . B G A G C T▲C V . . . 5′

5'-(A/C/G)C TCGA G(T/G/C)-3' or 5'-VCTCGAGB

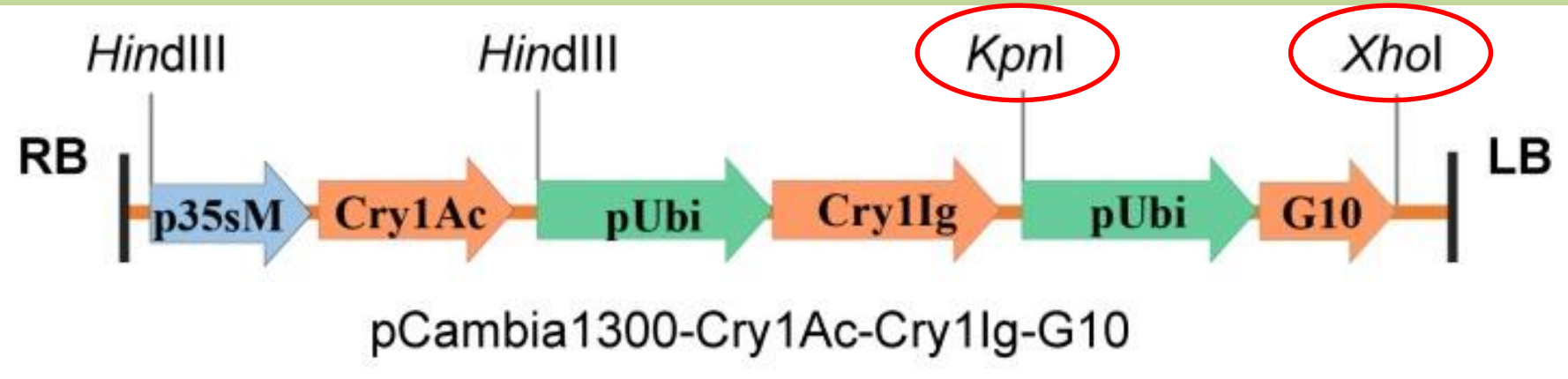


https://www.neb.com/tools-and-resources/selection-charts/compatible-cohesive-ends-and-generation-of-new-restriction-sites#X

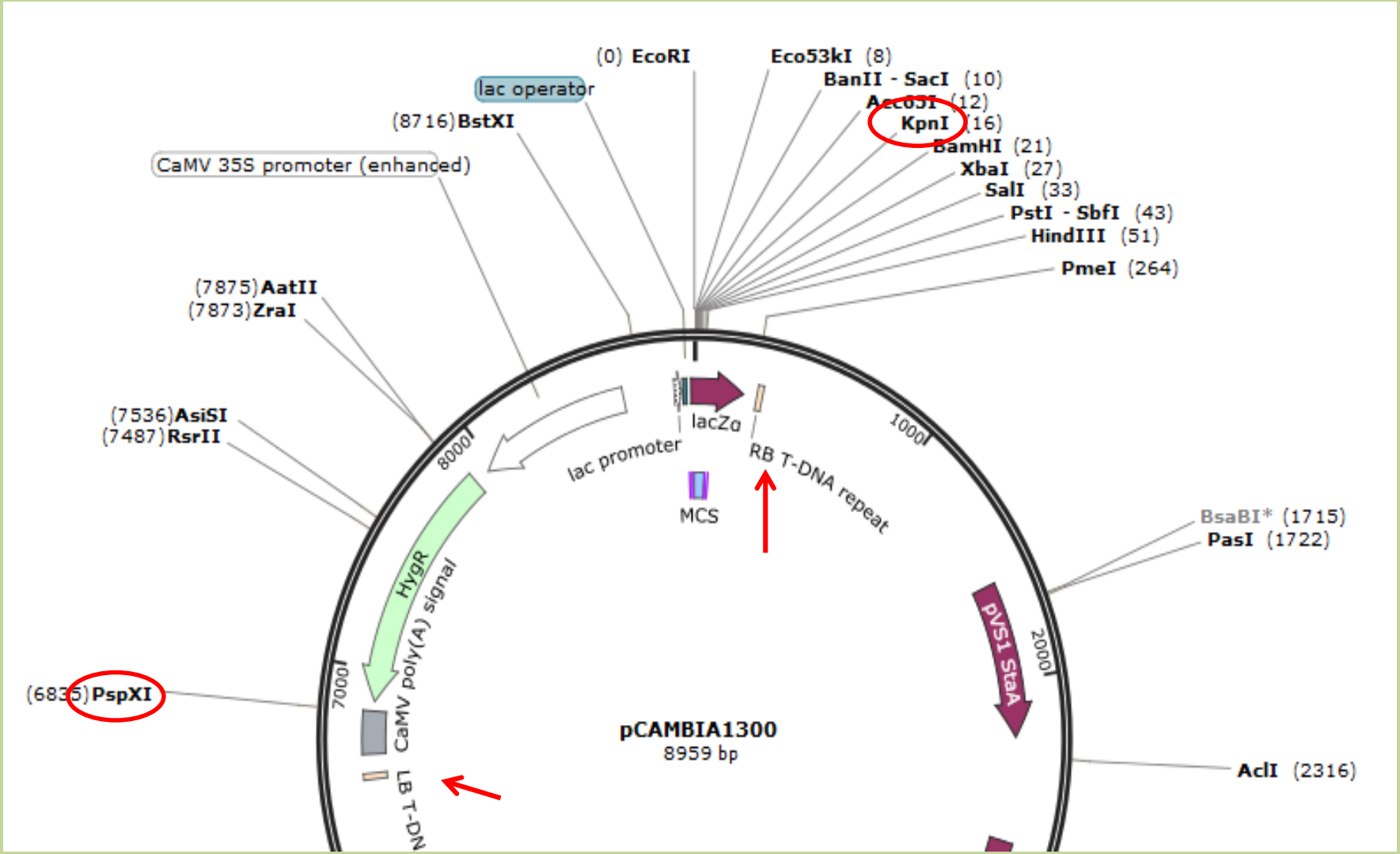
Step 3-4:

Eliminare T-DNA originale del pCAMBIA

Clonare i tre geni uno dopo l'altro (3 steps di clonaggio)



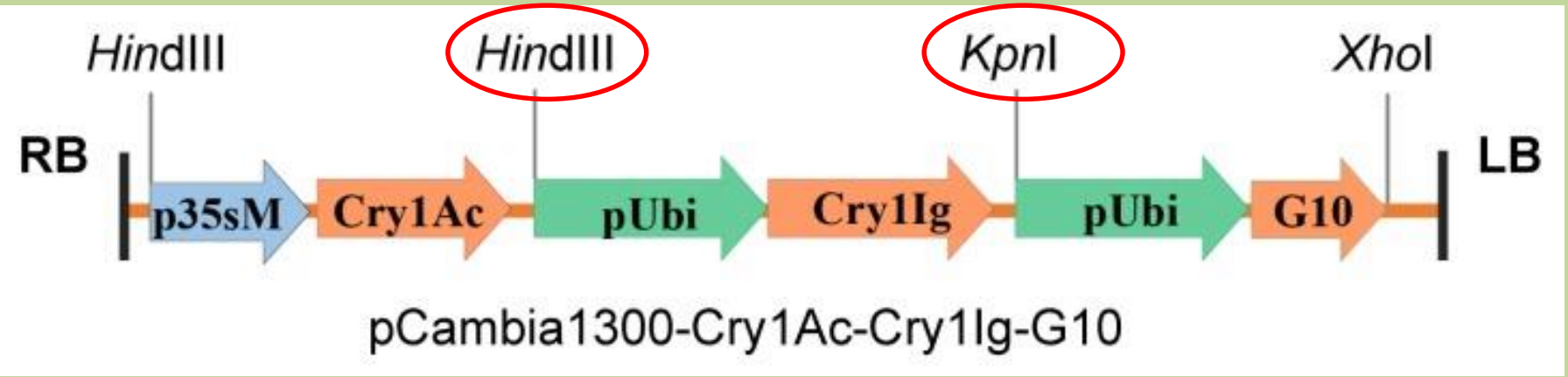
La cassetta di espressione G10 è stata inserita per prima nel T-DNA dopo aver rimosso il gene per la resistenza all'igromicina con taglio KpnI e PspXI→ viene prodotto il vettore 1300-G10.



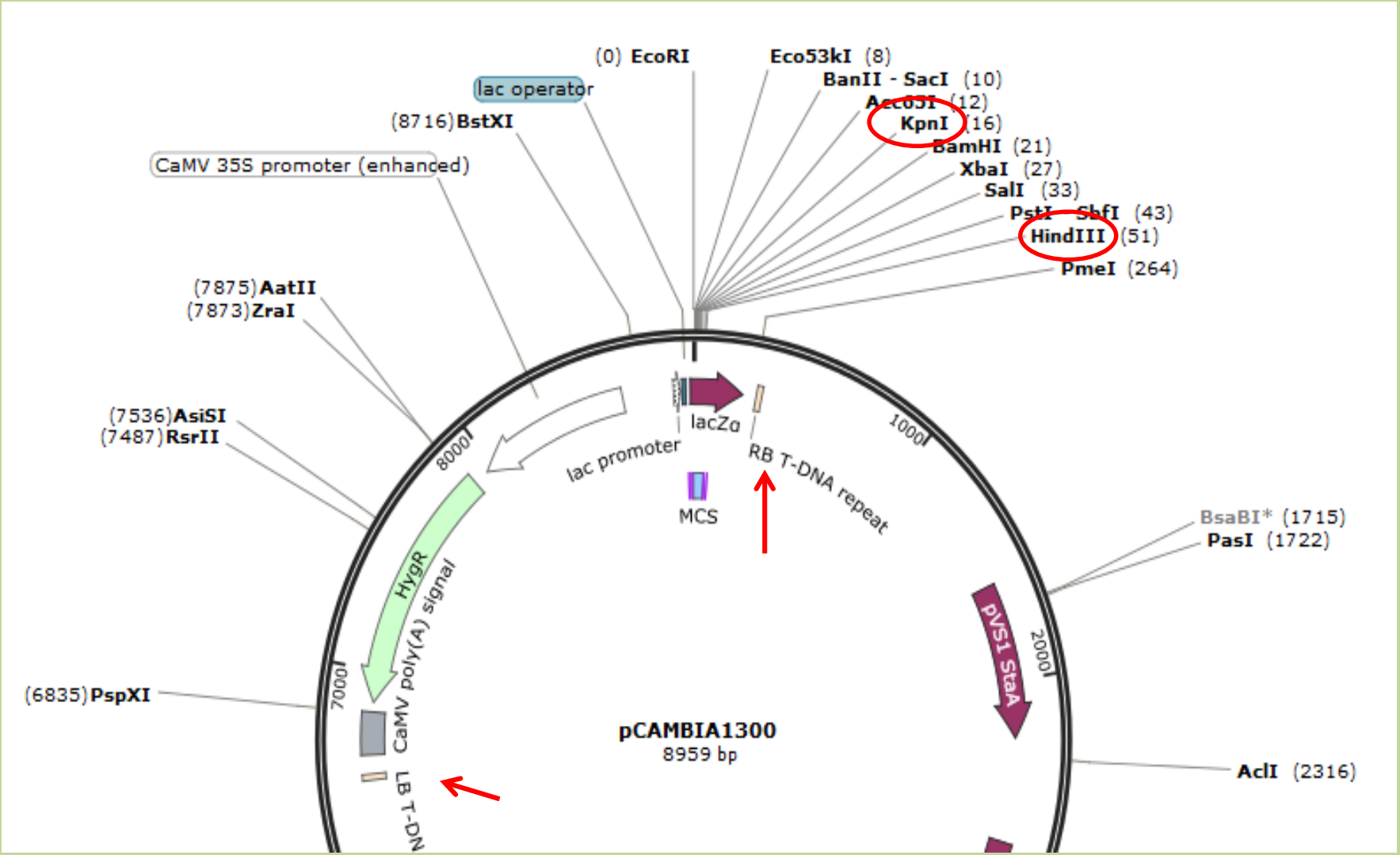
http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA1300/

Eliminare T-DNA originale del pCAMBIA

Clonare i tre geni uno dopo l'altro (3 steps di clonaggio)



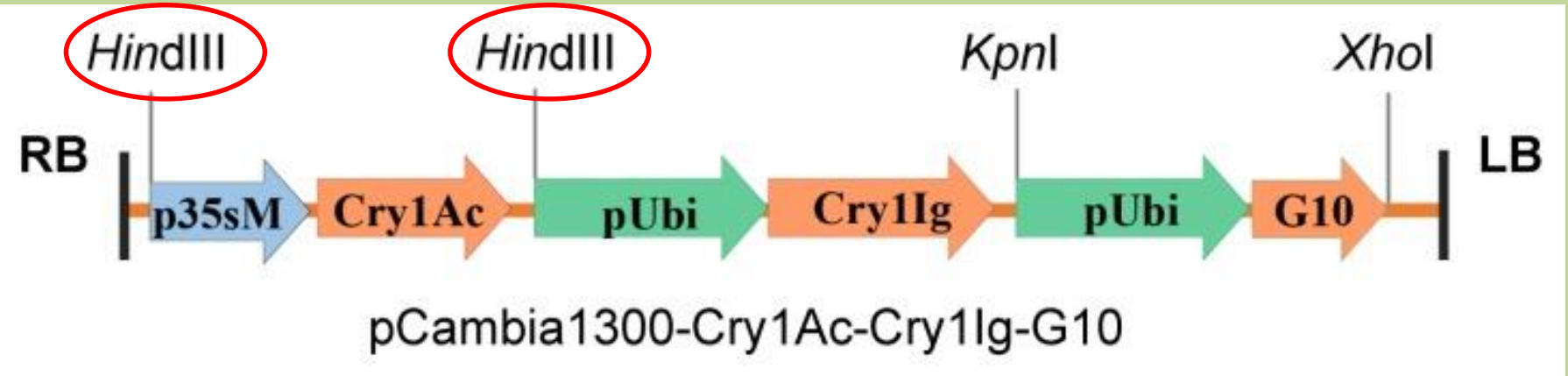
Successivamente la cassetta Cry1Ig è stata inserita ne plasmide 1300-G10dopo digestion con HindIII and KpnI generando il vettore 1300-Cry1Ig-G10.



http://www.snapgene.com/resources/plasmid_files/plant_vectors/pCAMBIA1300/

Eliminare T-DNA originale del pCAMBIA

Clonare i tre geni uno dopo l'altro (3 steps di clonaggio)



Infine la cassetta Cry1Ac è stata inserita nel vettore 1300-Cry1Ig-G10 pre-digerito con HindIII per produrre il vettore finale pCambia1300-Cry1Ac-Cry1Ig-G10

Come controllare orientamento?

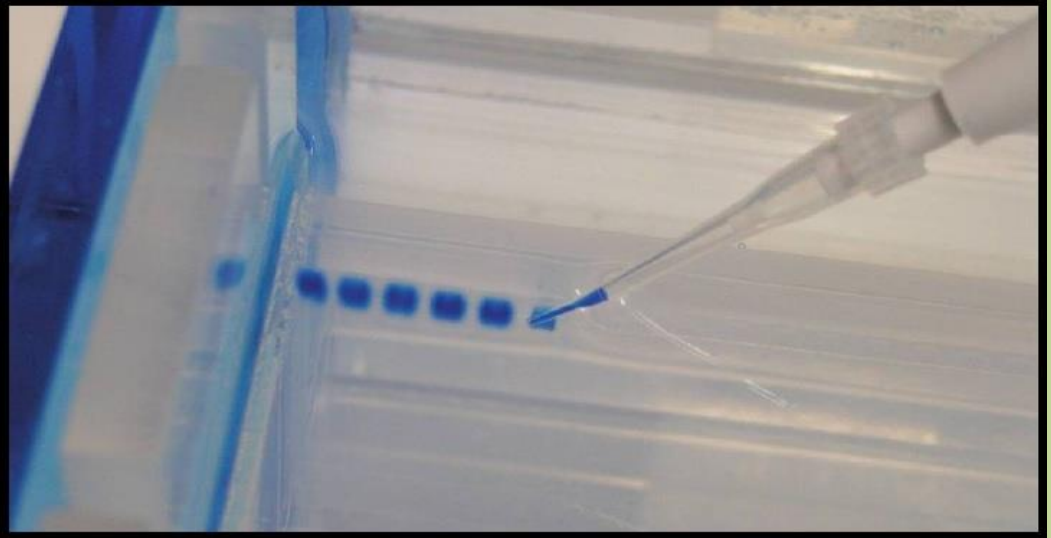
Reagents	HindIII
10X buffer	4 µl
DNA (1µg/µl)	4.0 µl
*Hind*III (10u/µl)	2.0 µl
Water	30.0 µl
Fin vol	40.0 µl

Close the microtubes and incubate at 37°C for 2h.

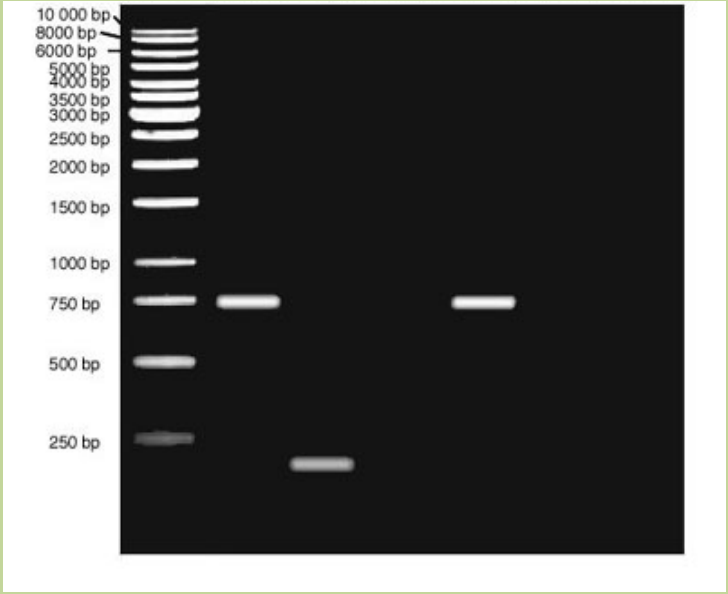
Reagents	HindIII/KpnI
10X buffer	4 µl
DNA (1µg/µl)	4.0 µl
*Hind*III (10u/µl)	2.0 µl
KpnI	2.0 µl
Water	28.0 µl
Fin vol	40 µl

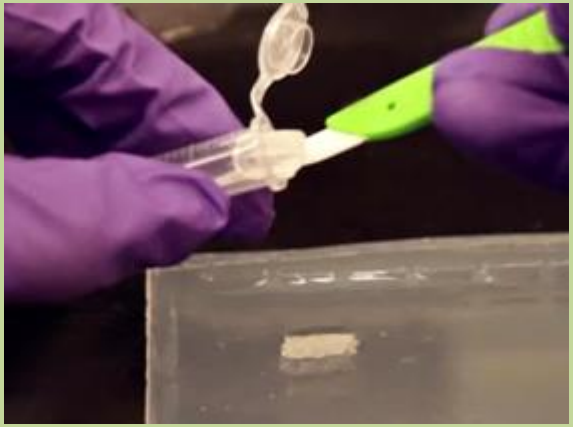
https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder?enzyme1={CB454480-8A3F-4BC8-843C-CB05AAFD0D57}&enzyme2={DA2537A1-428C-4E01-B72E-7E957F7A9654}

Loading the Gel



Carefully place the pipette tip inside a well and gently/slowly expel the sample. The sample should sink into the well.

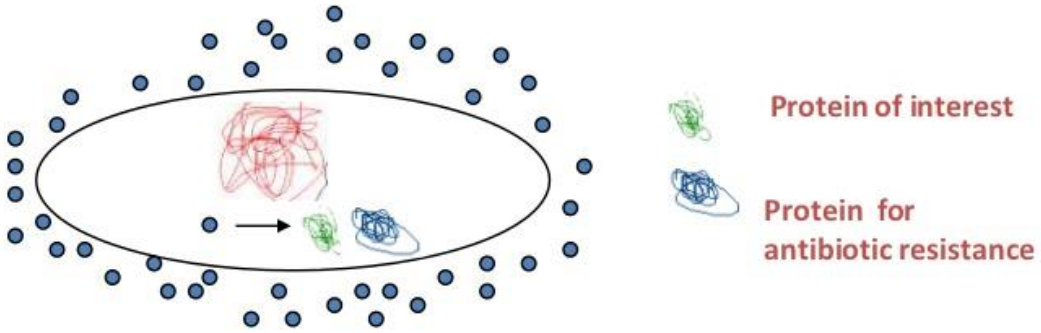




COMPONENT	20 µl REACTION
T4 DNA Ligase Buffer (10X)	2 µl
Vector DNA (4 kb)	50 ng (0.020 pmol)
Insert DNA (1 kb)	37.5 ng (0.060 pmol)
Nuclease-free water	to 20 µl
T4 DNA Ligase	1 µl

- Gently mix the reaction by pipetting up and down and microfuge briefly.
- For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
- For blunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours

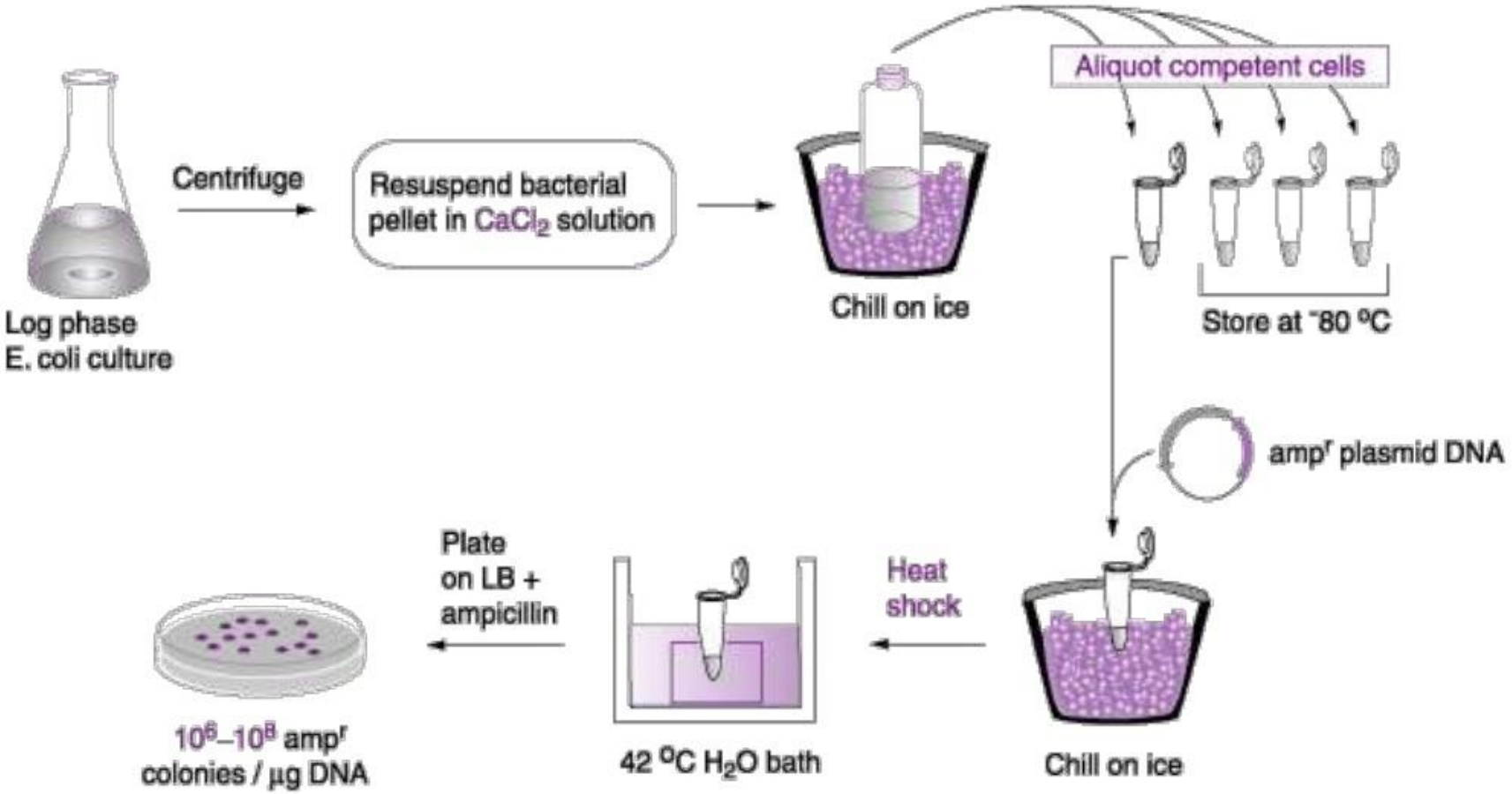
Key Steps for Transformation



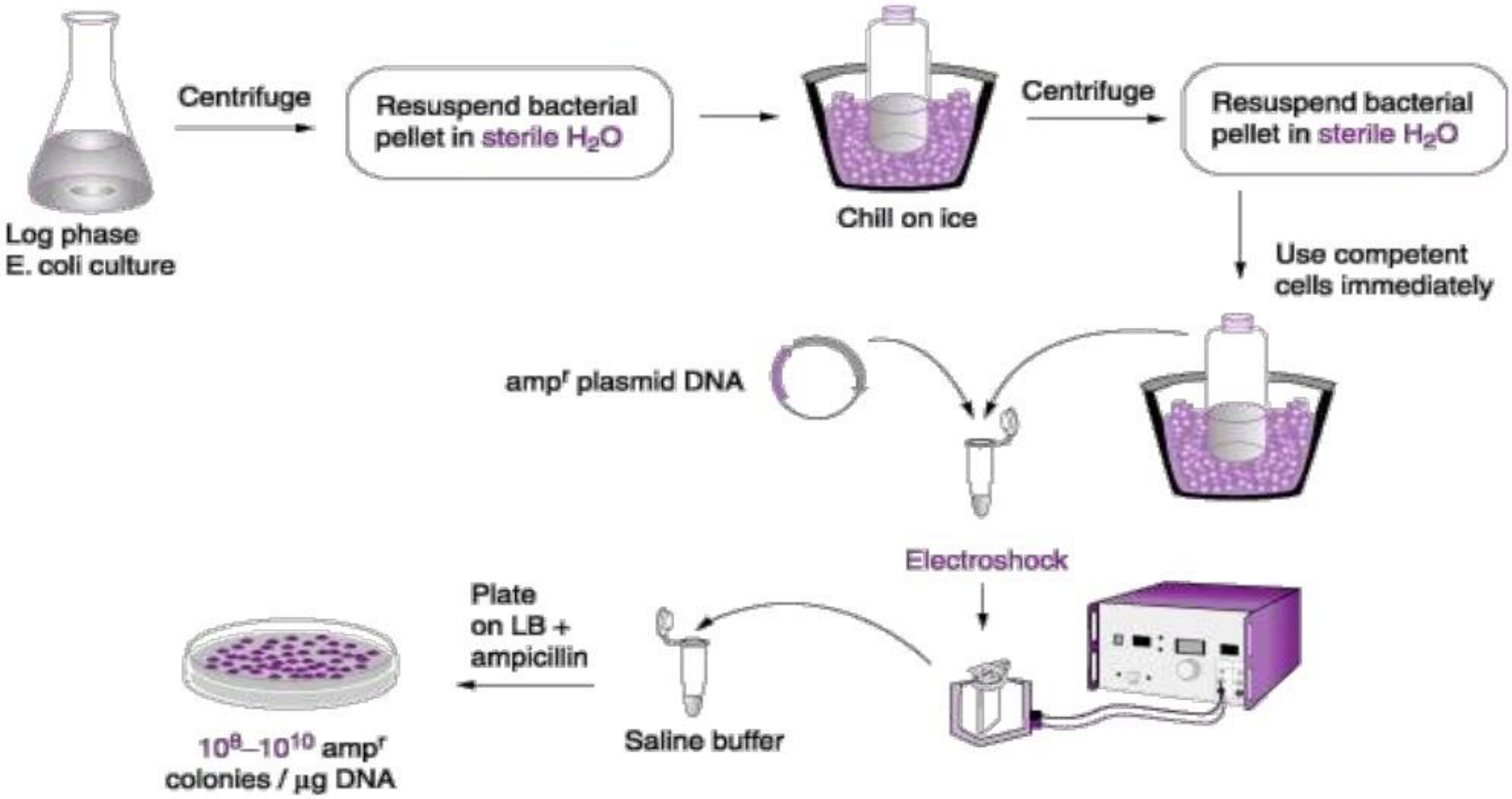
Plasmid DNA enters the bacterial cell and the genes are expressed.

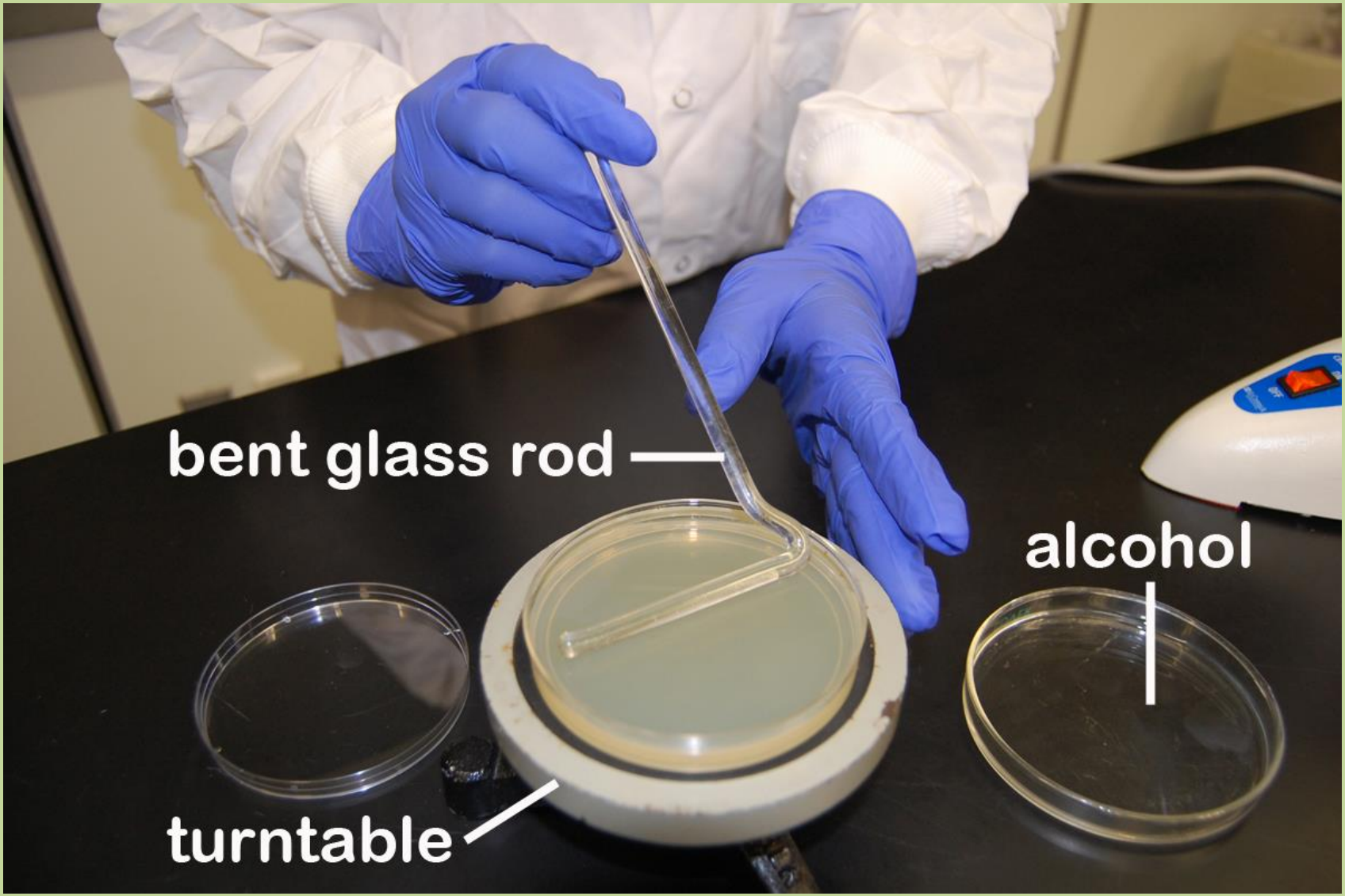
- Bacterial cell suspension is placed in $CaCl_2$ solution
- Cells must be in *log phase* of growth.
- Cells are kept on ice until heat shock treatment
- Heat shock at 42 ˚C for one minute
- Recover period in LB broth
- Cells are spread on appropriate selection plates

CHEMICAL TRANSFORMATION WITH CALCIUM CHLORIDE



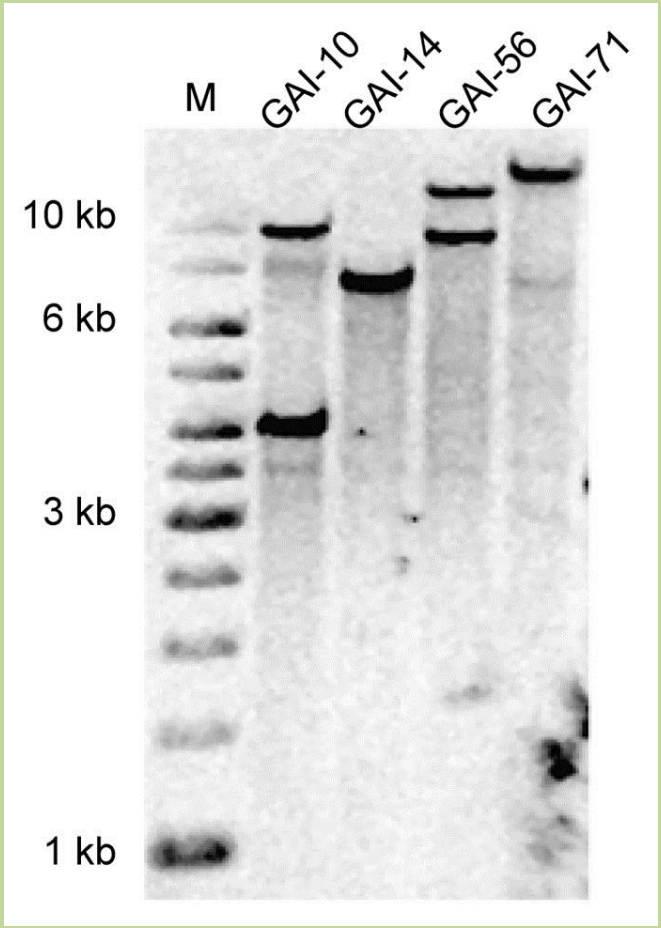
TRANSFORMATION BY ELECTROPORATION




bent glass rod
alcohol
turntable

Il plasmide binario è stato inserito nel ceppo di Agrobacterium tumefaciens LBA4404 con elettroporazione.

La trasformazione di riso con Agrobacteriumè stata svolta come da protocollo (Hiei et al. 1994), con l'eccezione che i calli di riso sono stati selezionati con glifosato 2 mmol/L



Southern blot analysis of the selected transgenic events
Genomic DNAs prepared from GAI-10, 14, 56, and 71 plants were digested with *Bam*HI and then hybridized with a digoxigenin (DIG)-labelled probe specific to gene *G10*.
M: the DNA ladder

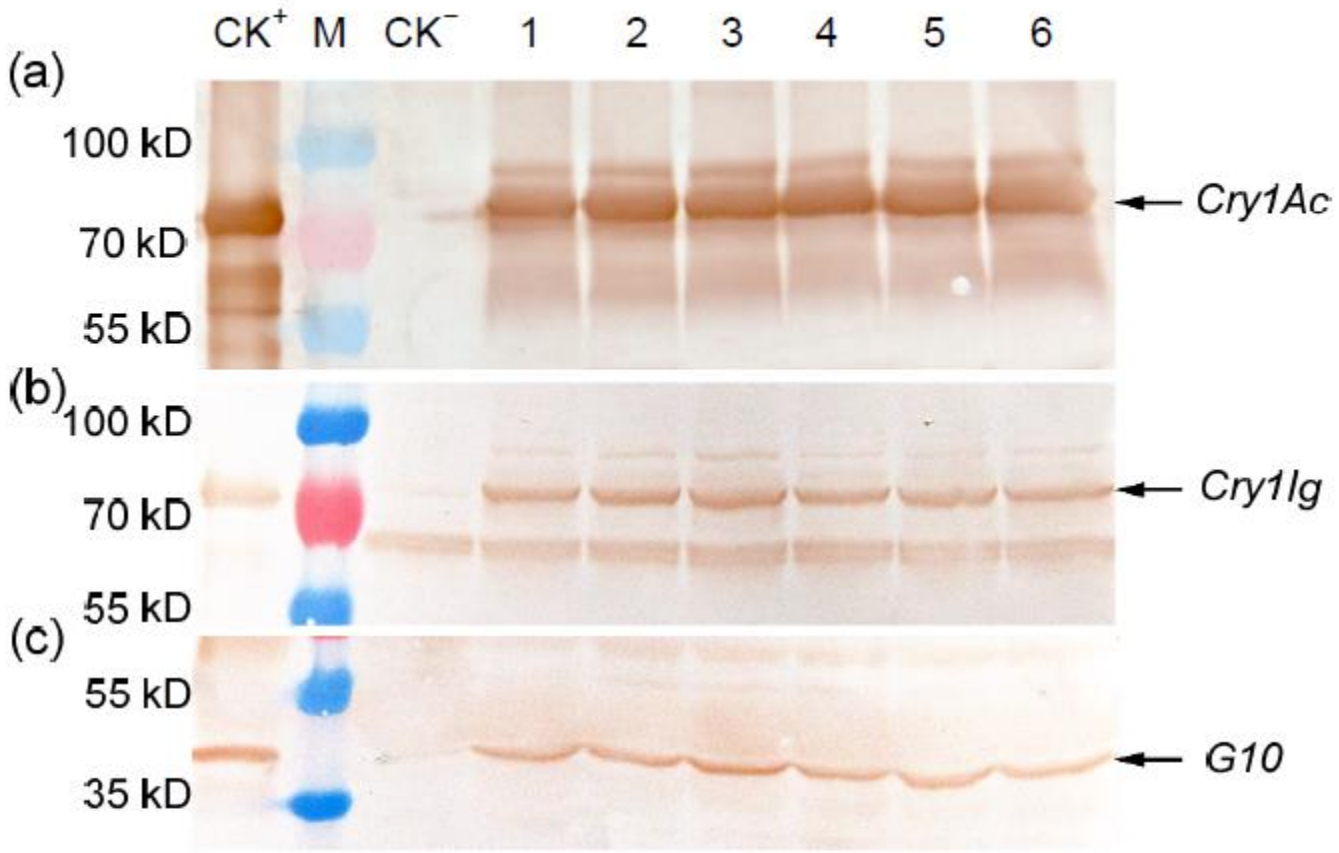


Fig. 3 Western blot analysis of transgene expression
Six plants were selected. Each sample was blotted with a primary antibody against Cry1Ac (a), Cry1Ig (b), and G10 (c), respectively. Prokaryotic expressed Cry1Ac, Cry1Ig, and G10 were used as a positive control (CK^+), respectively. The sample prepared from non-transgenic rice was used as a negative control (CK^-)

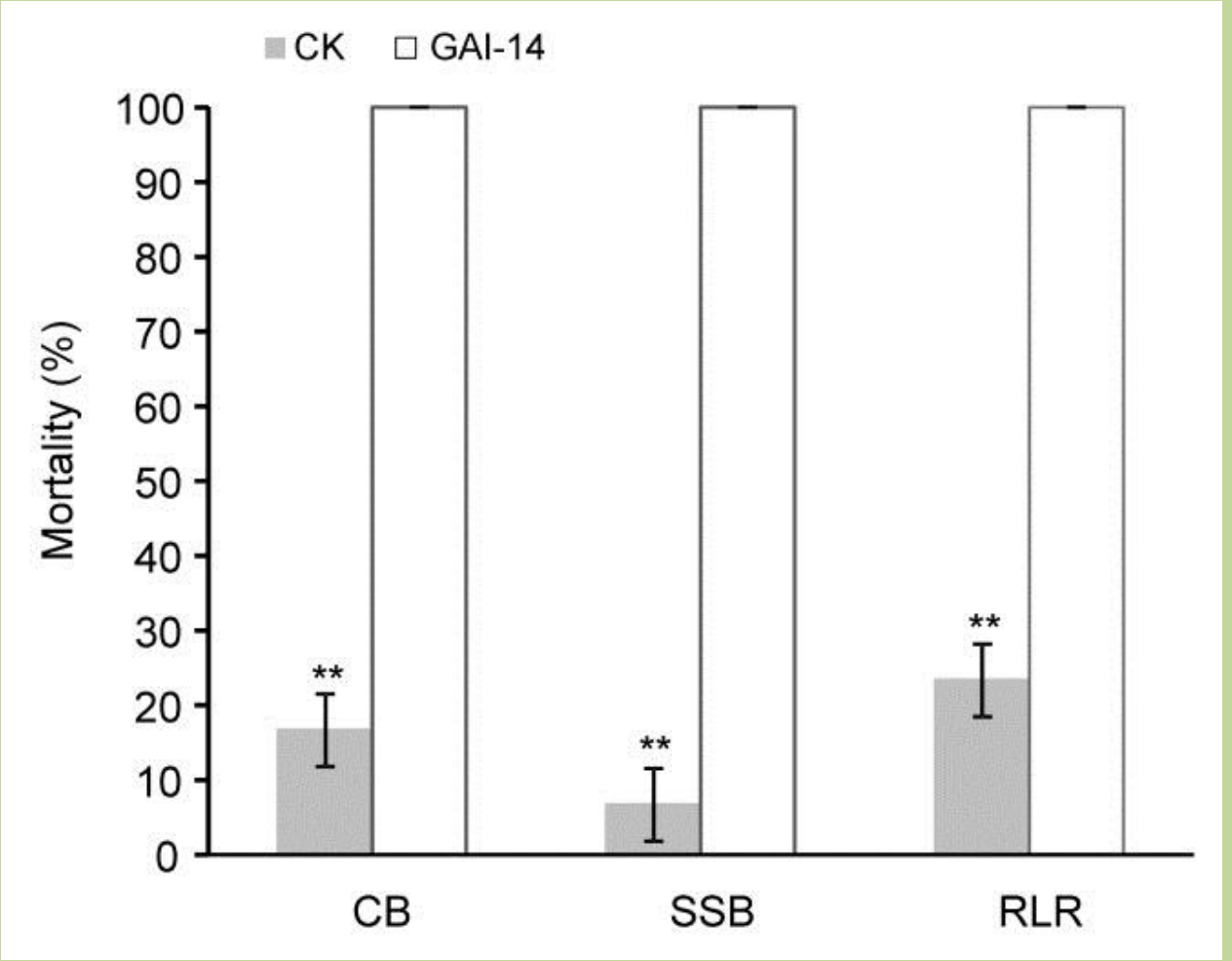
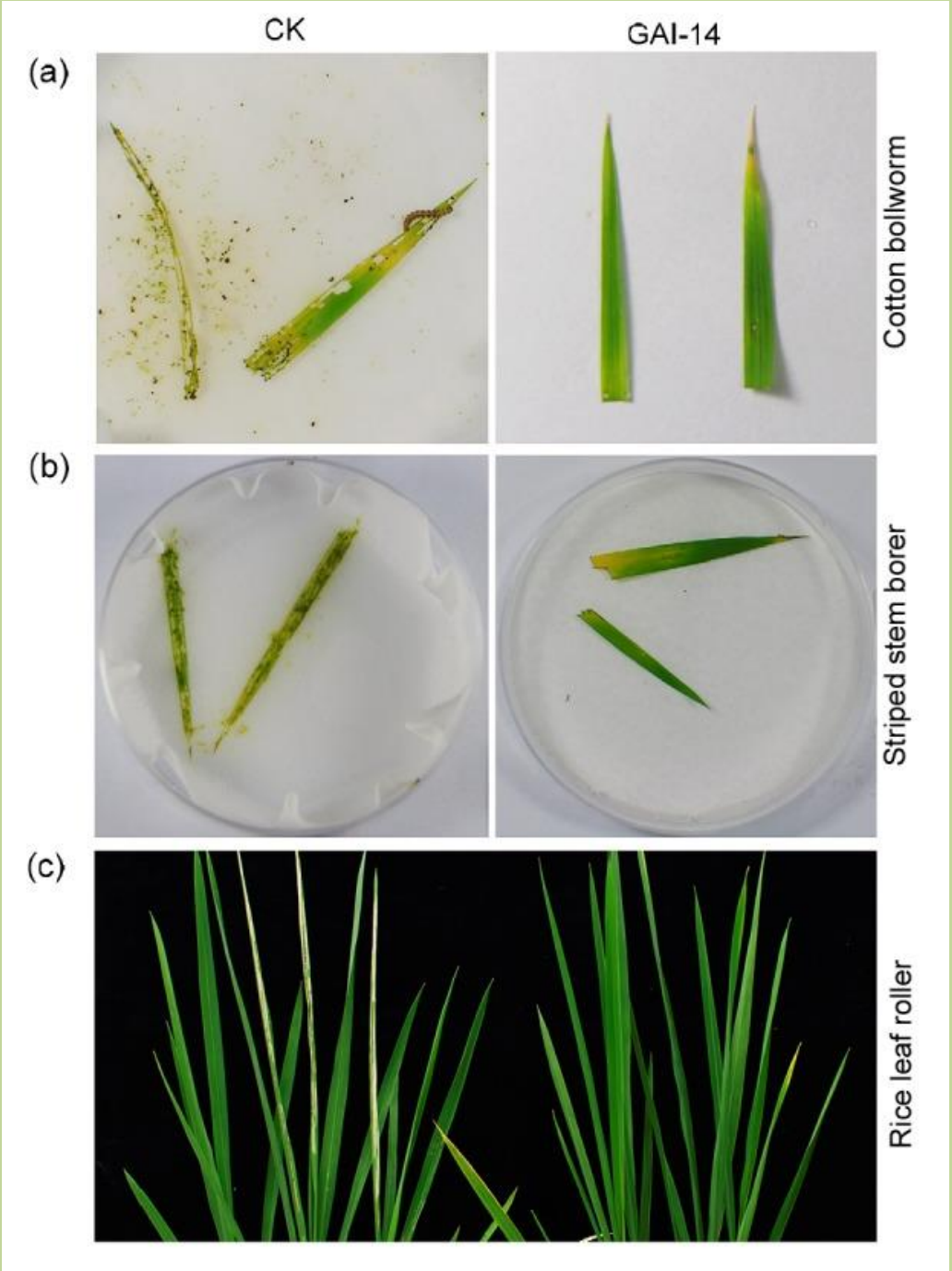
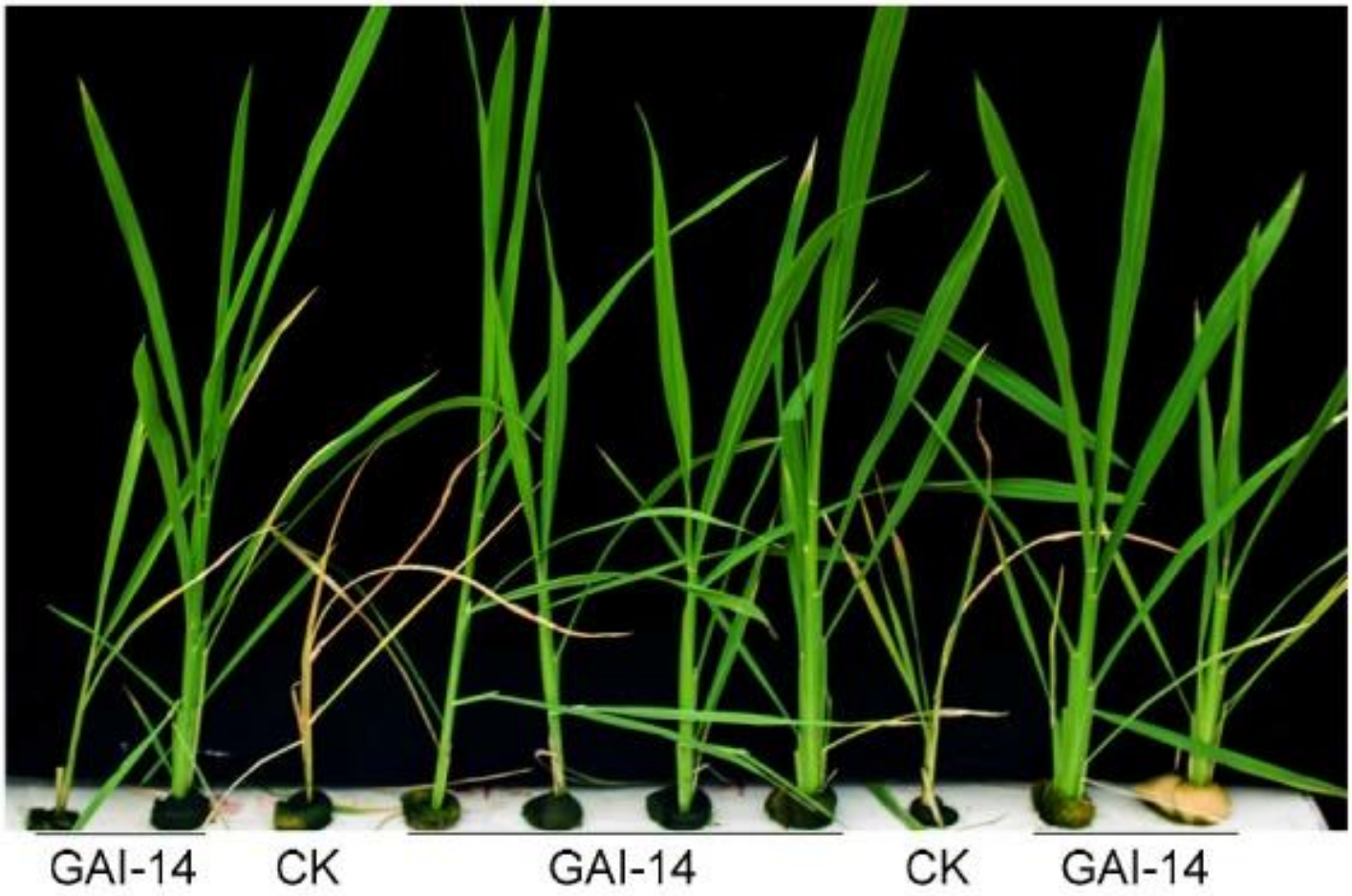


Fig. 4 Mortalities of cotton bollworm (CB), striped stem borer (SSB), and rice leaf roller (RLR) feeding on GAI-14

Non-transgenic rice at the same growing stage was used as the control (CK). Data are expressed as mean±standard deviation (*n*=3). Mortalities of all insects feeding on GAI-14 plants were 100%. ** on the bars indicated extremely significant difference between CK and GAI-14 (*P*<0.01, Kruskal Wallis test)

The bioassay was conducted with cotton bollworm (a), striped stem borer (b), and rice leaf roller (c), respectively. Non-transgenic rice at the same growing stage was used as the control (CK)





The GAI-14 plants were sprayed with Roundup diluted at 1:140 (v/v). Non-transgenic rice at the same growing stage was used as a control (CK)

Table 2 Evaluation of basic agronomic performance between GAI-14 and Xiushui 134

Plant	Plant height (cm)	Panicles per plant	Grains per panicle	1000-grain weight (g)
GAI-14	69.05±2.24	11.43±2.49	112.53±6.16	24.55±0.67
Xiushui 134	68.31±1.97	12.10±2.53	110.15±8.32	24.22±0.49
P-value	0.19	0.34	0.40	0.50

The value was shown as mean±standard deviation (n=30). Data were analyzed by Student's t-test in SPSS

La linea transgenica GAI-14 ha dimostrato un'ottima resistenza agli insetti nei confronti dei principali parassiti del riso e un'alta tolleranza al glifosato. Benché non sia stata osservata alcuna differenza nelle prestazioni agronomiche tra GAI-14 e il il controllo non transgenico, ulteriori prove in campo devono essere intraprese per esaminare la stabilità ereditaria dei geni introdotti e determinare l'impatto dell'inserzione T-DNA sui tratti agronomici in condizioni di campo.

ENZYMES USED IN MOLECULAR BIOLOGY

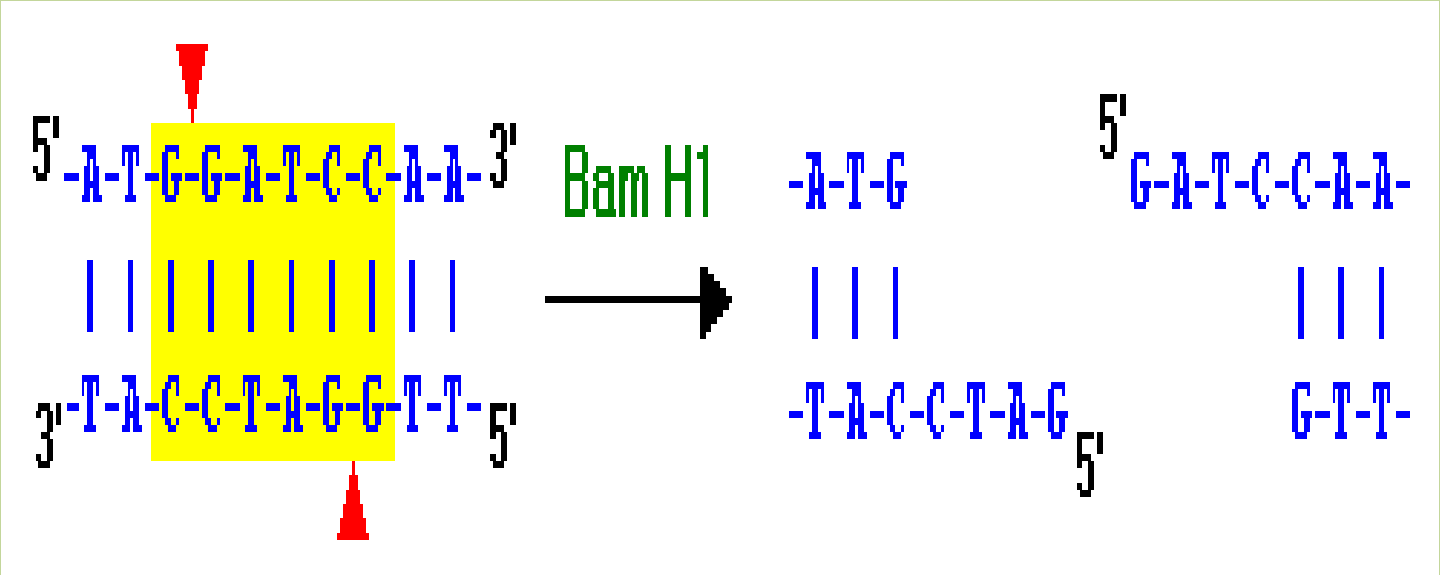
Alkaline phosphatase	Removes phosphate groups from 5' ends of DNA (prevents unwanted re-ligation of cut DNA)
DNA ligase	Joins compatible ends of DNA fragments (blunt/blunt or complementary cohesive ends). Uses ATP
DNA polymerase I	Synthesises DNA complementary to a DNA template in the 5'-to-3'direction. Starts from an oligonucleotide primer with a 3' OH end
Exonuclease III	Digests nucleotides progressiviely from a DNA strand in the 3' -to-5' direction
Polynucleotide kinase	Adds a phosphate group to the 5' end of double- or single-stranded DNA or RNA. Uses ATP
RNase A	Nuclease which digests RNA, not DNA
Taq DNA polymerase	Heat-stable DNA polymerase isolated from a thermostable microbe (*Thermus aquaticus*)

RESTRICTION ENZYMES

- **The restriction enzymes most used in molecular biology labs cut within their recognition sites and generate one of three different types of ends.**

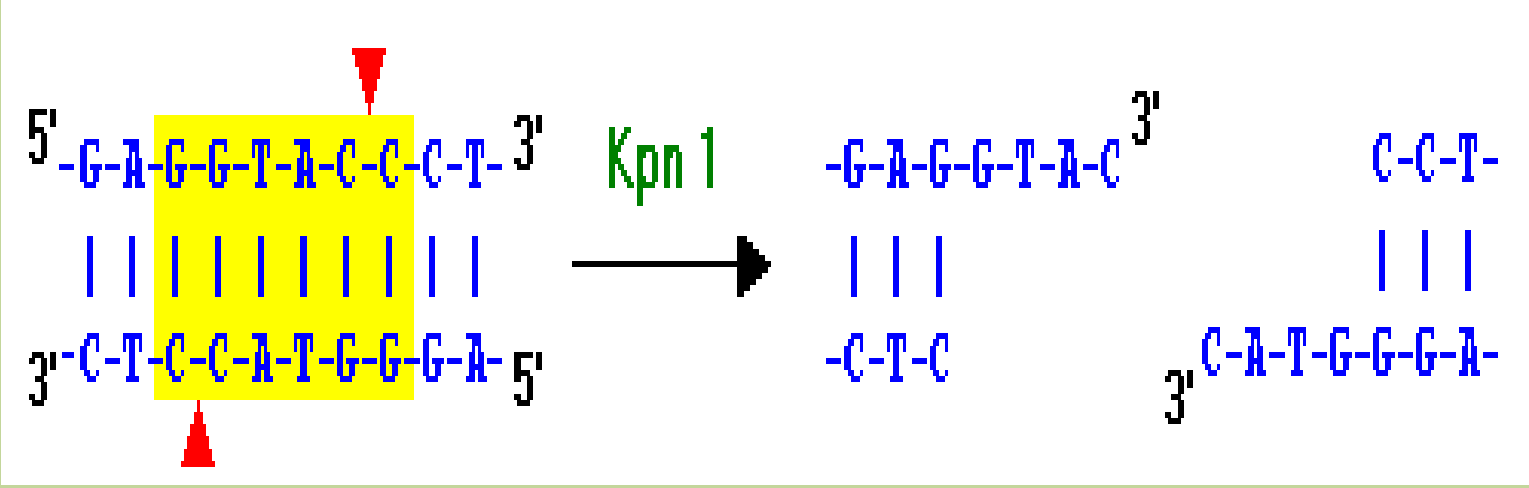
5’ OVERHANGS

- **5' overhangs:** The enzyme cuts asymmetrically within the recognition site such that a short single-stranded segment extends from the 5' ends. *Bam* HI cuts in this manner.



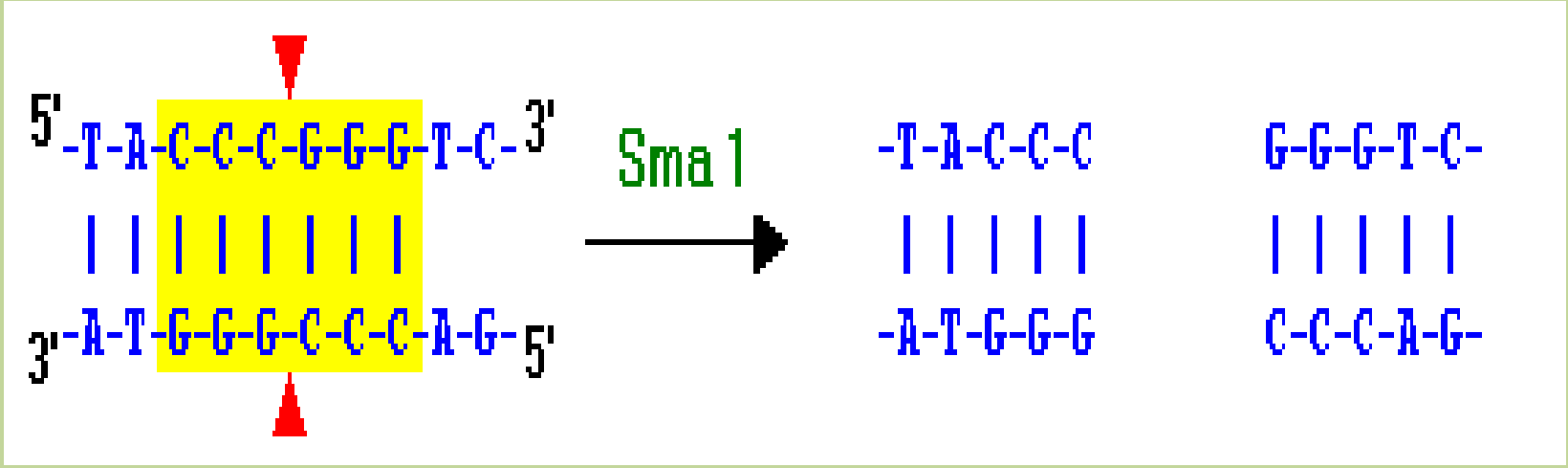
3’ OVERHANGS

- **3' overhangs:** Again, we see asymmetrical cutting within the recognition site, but the result is a single-stranded overhang from the two 3' ends. *Kpn*I cuts in this manner.



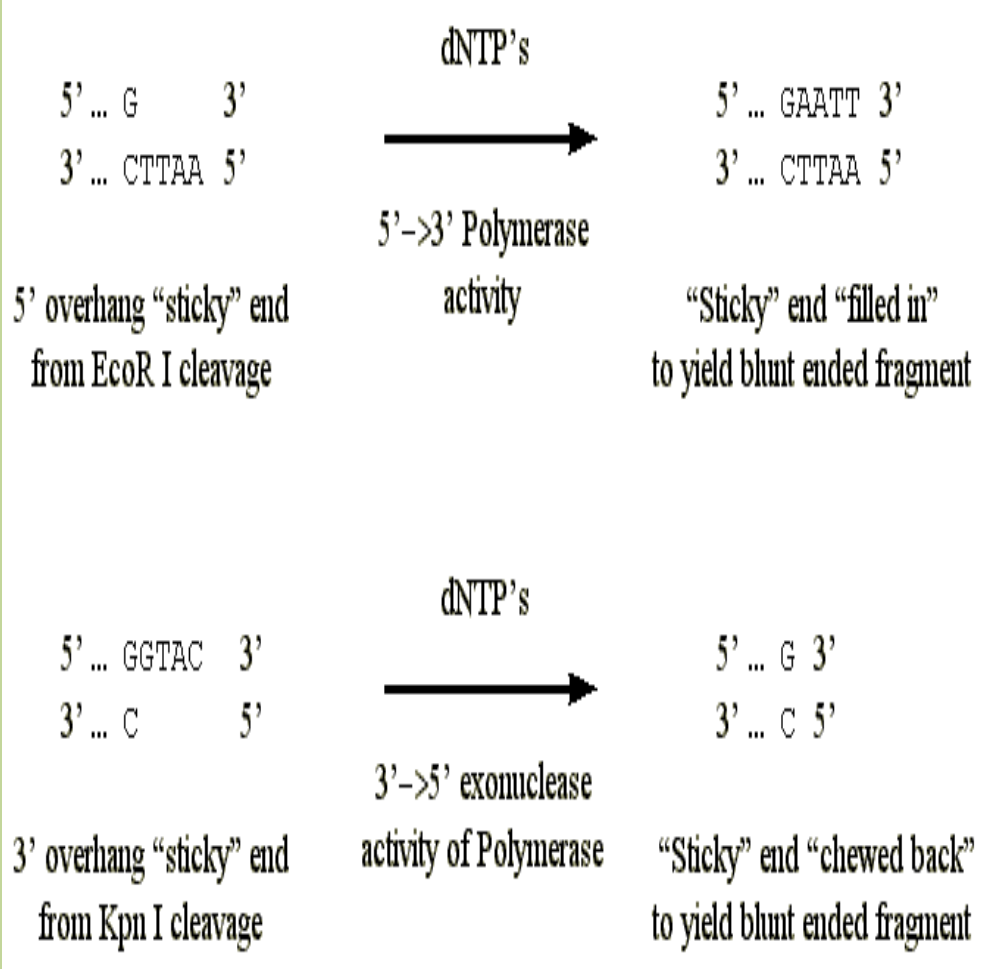
BLUNT ENDS

- **Blunts:** Enzymes that cut at precisely opposite sites in the two strands of DNA generate blunt ends without overhangs. *Sma*I is an example of an enzyme that generates blunt ends.



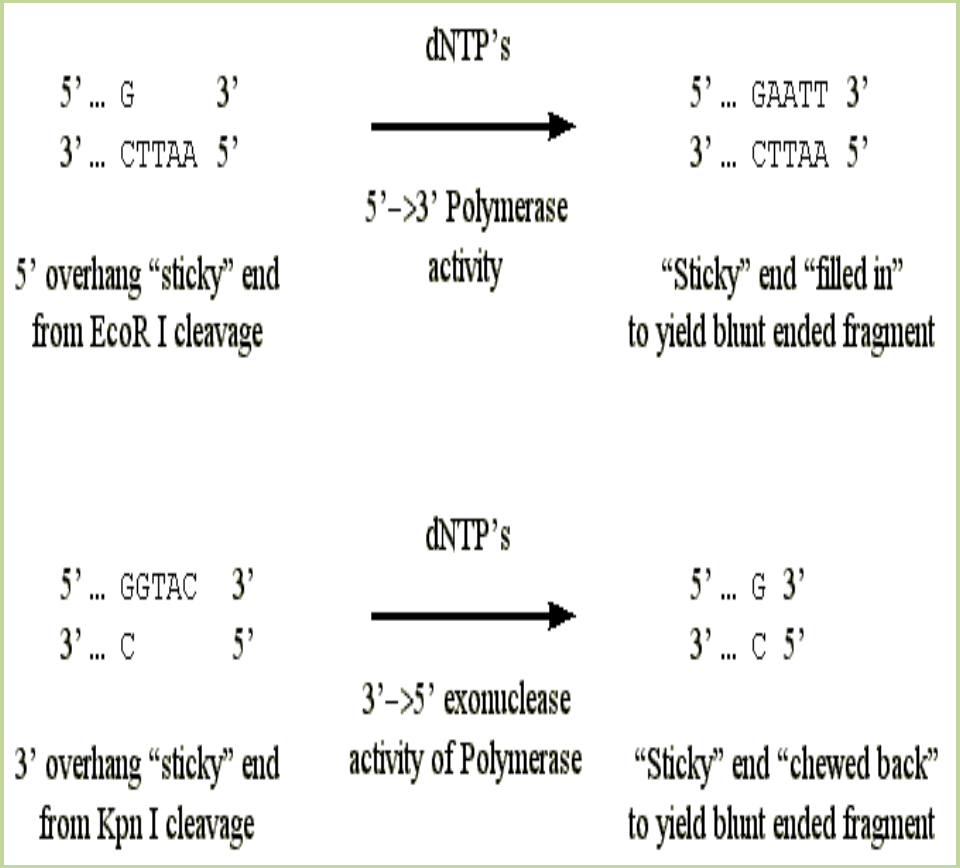
Converting a 5’ overhang to blunt end

- Both Klenow and T4 DNA polymerase can be used to fill in 5’ protruding ends with dNTPs
- Used in joining DNA fragments with incompatible ends
- Once the ends have been blunted, ligation can proceed



Converting a 3’ overhang to a blunt end

- T4 DNA polymerase has a 3’-5’ exonuclease activity
- In the presence of excess dNTPs will convert a 3’ protruding end to a blunt end
- Ligation can know proceed

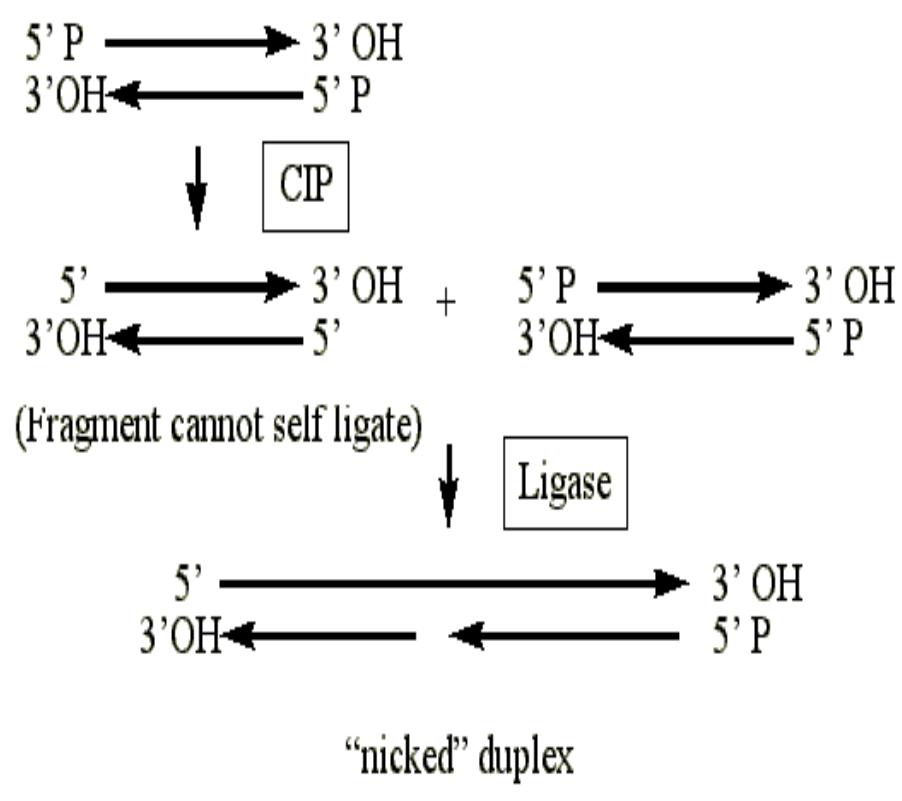


DIRECTIONAL CLONING

- Often one desires to insert foreign DNA in a particular orientation
- This can be done by making two cleavages with two different **restriction** enzymes
- Construct foreign DNA with same two **restriction** enzymes
- Foreign DNA can only be inserted in one direction

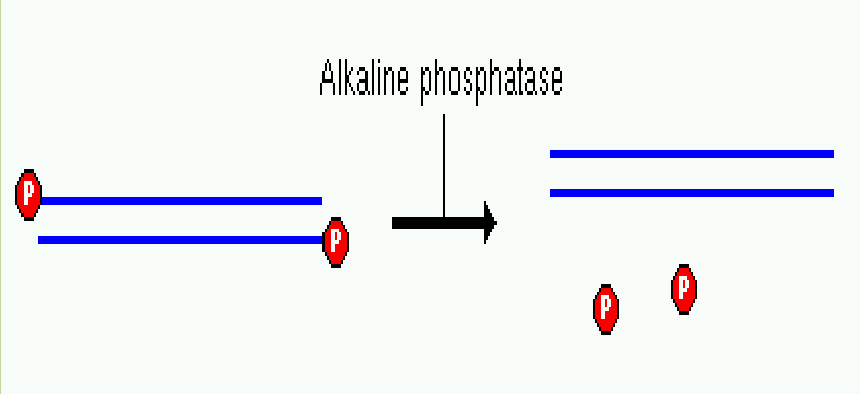
- Good efficiency of ligation of foreign DNA into a vector can be achieved if both the vector and the insert DNA are cut with 2 different restriction enzymes which leave single stranded ends (cohesive ends).
- The DNA is ligated in only one direction, and there is only a low background of non-recombinant plasmids.
- If only one restriction enzyme is used to cut the vector and insert, then efficiency of ligation is lower, DNA can be inserted in two directions and tandem copies of inserts may be found.
- To avoid high background of non-recombinants, **alkaline phosphatase** is used to remove 5' phosphate groups from the cut vector to prevent self-ligation.

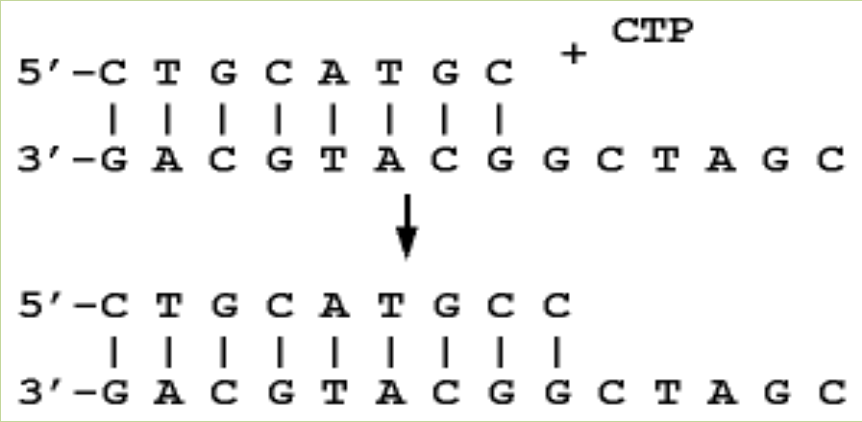
Alkaline phosphatase

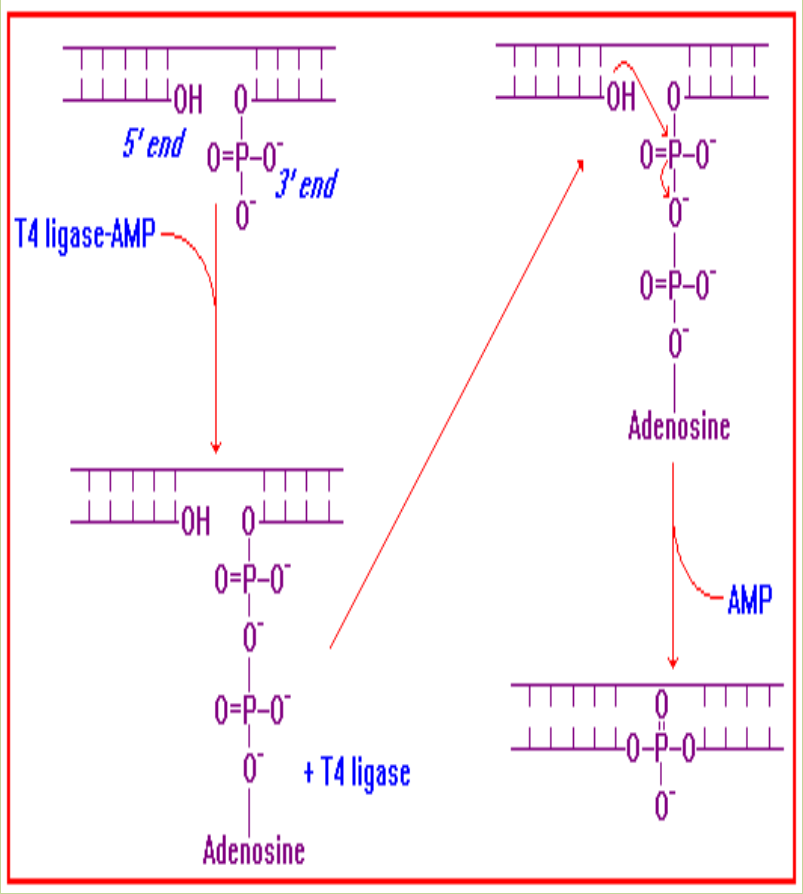


- **Alkaline phosphatase removes 5' phosphate groups from DNA and RNA. It will also remove phosphates from nucleotides and proteins. These enzymes are most active at alkaline pH**

ENZYMES USED IN MOLECULAR BIOLOGY







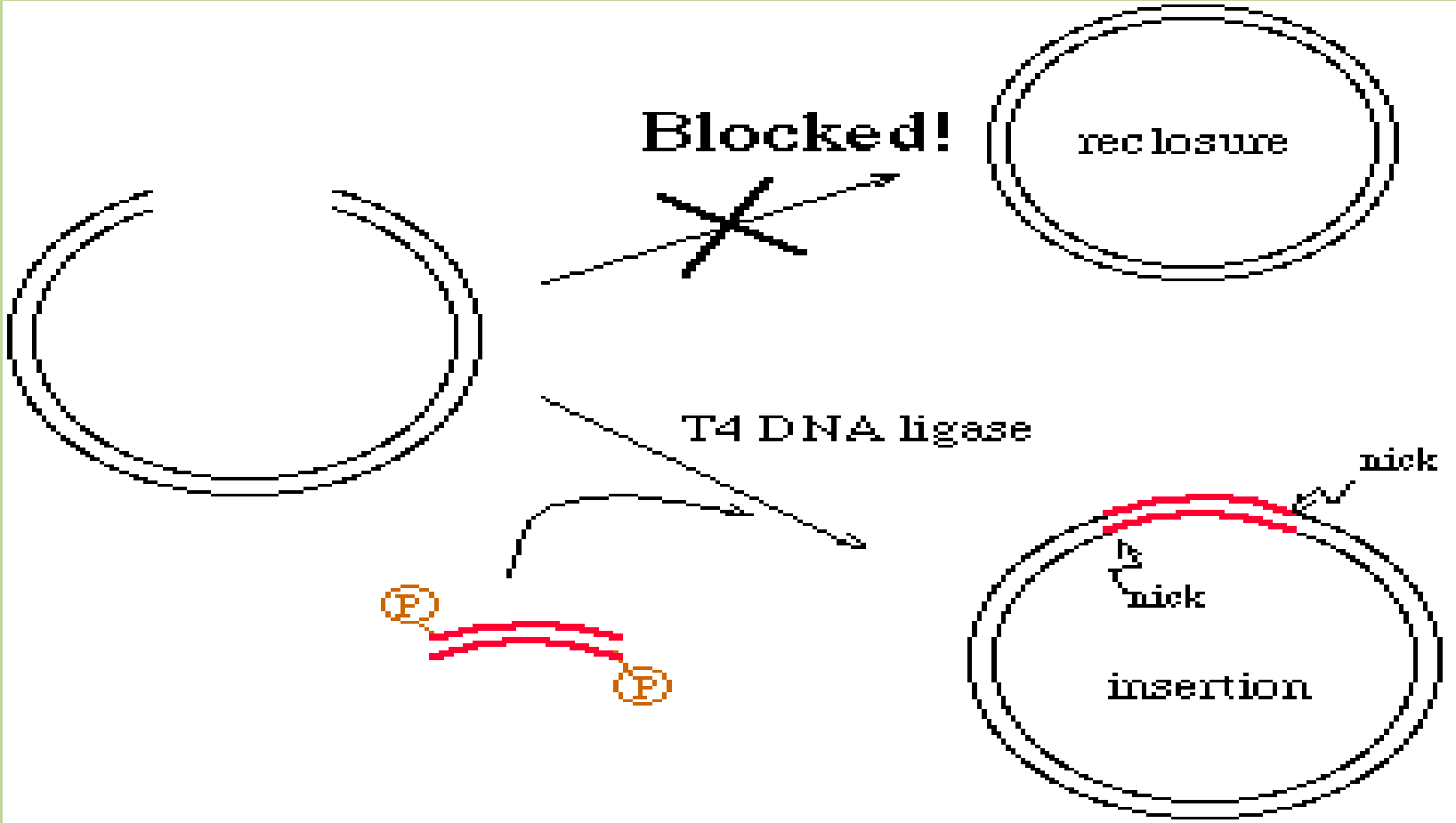
Alkaline phosphatase

- There are two primary uses for alkaline phosphatase in DNA manipulations:

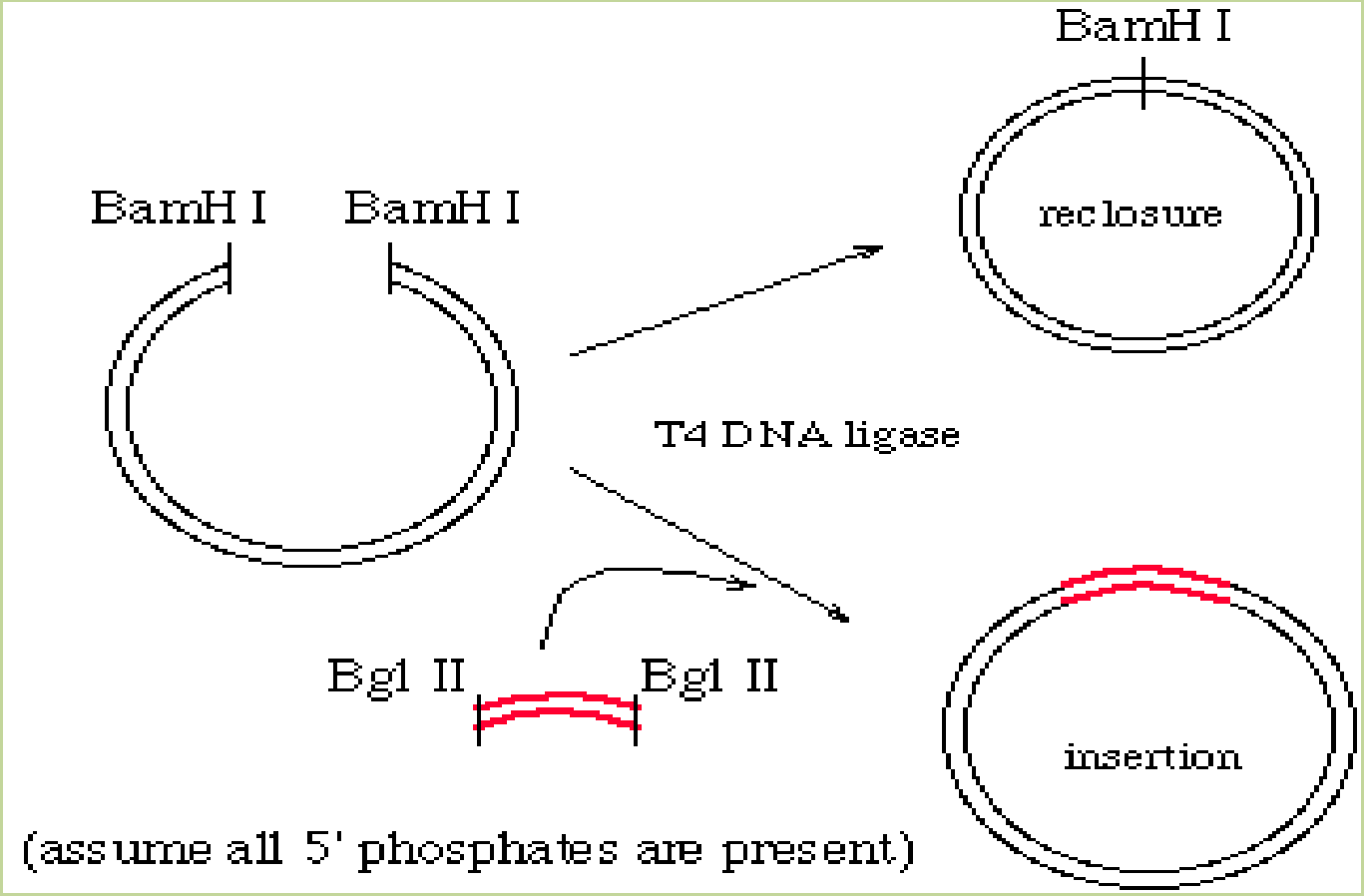
- **Removing 5' phosphates from plasmid and bacteriophage vectors** that have been cut with a restriction enzyme. In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation of other DNA fragments into the vector (e.g. subcloning).

- **Removing 5' phosphates from fragments of DNA prior to labeling with radioactive phosphate**. Polynucleotide kinase is much more effective in phosphorylating DNA if the 5' phosphate has previously been removed

DEPHOSPORYLATED VECTOR



R.E.S WITH COMPATIBLE ENDS



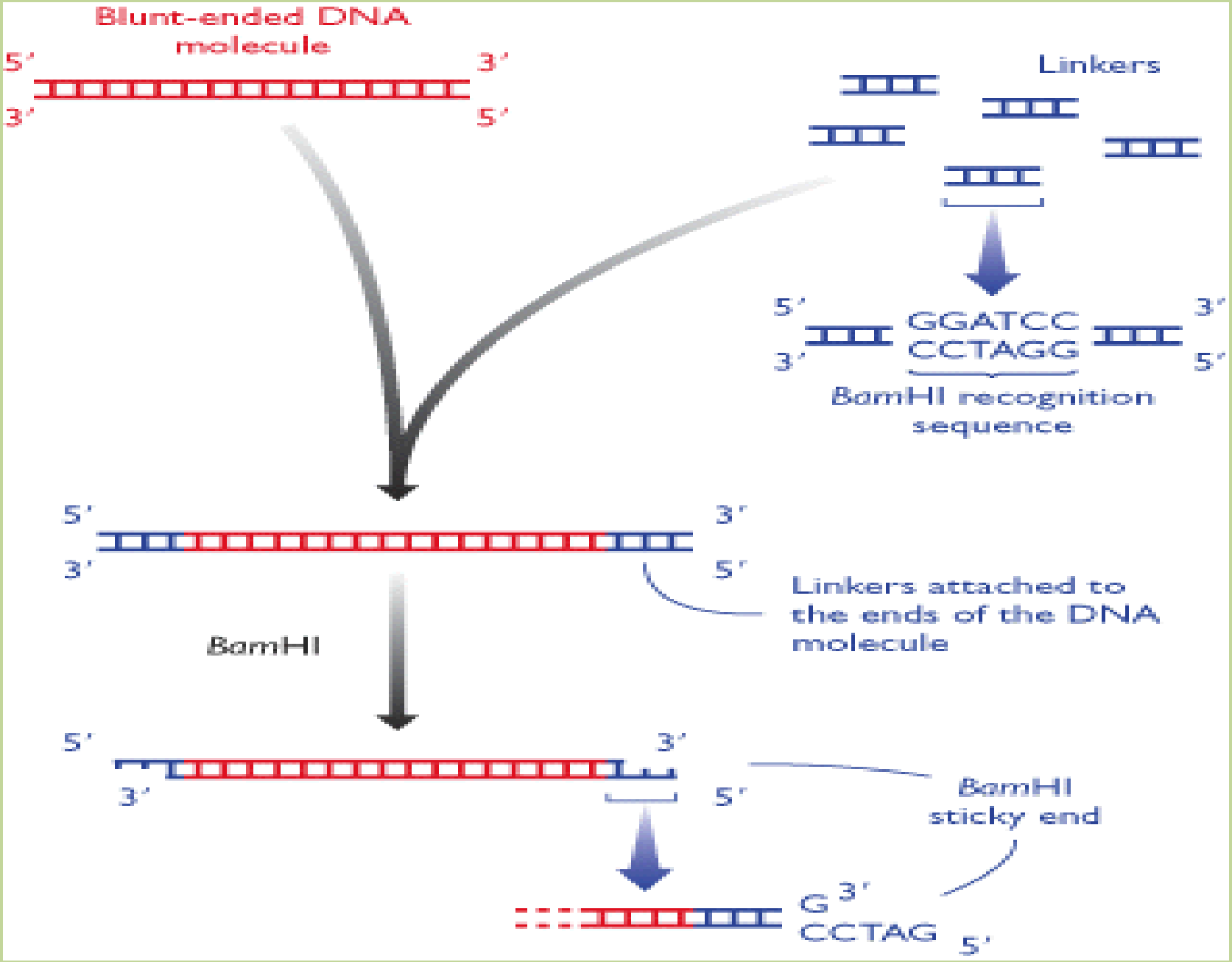
Generating a new R.E. site at a blunt end

- **Use linkers to generate a new R.E.**
- **Linkers are used to place sticky ends on to a blunt-ended molecule**
- **Short blunt ended synthetic ds DNA containing a R.E. site**
- **Experimental design:**

  (i) Blunt ended DNA + linker (T4 ligase)

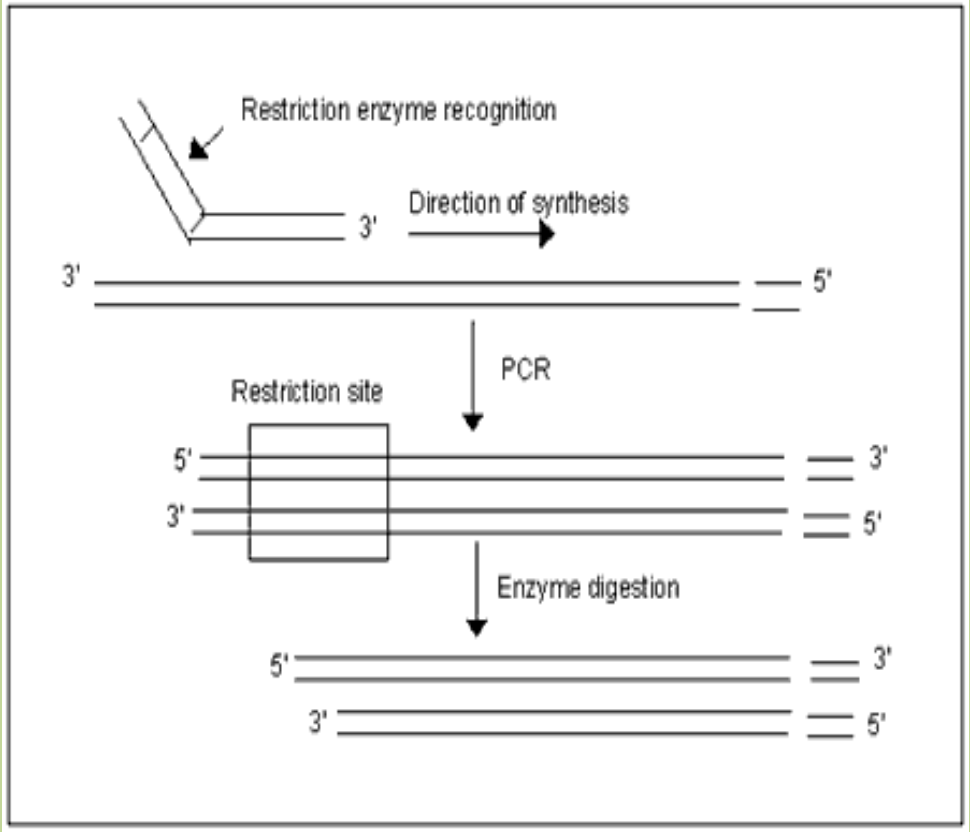
  (ii) Digest with appropriate R.E.

  (iii) Ligate to vector

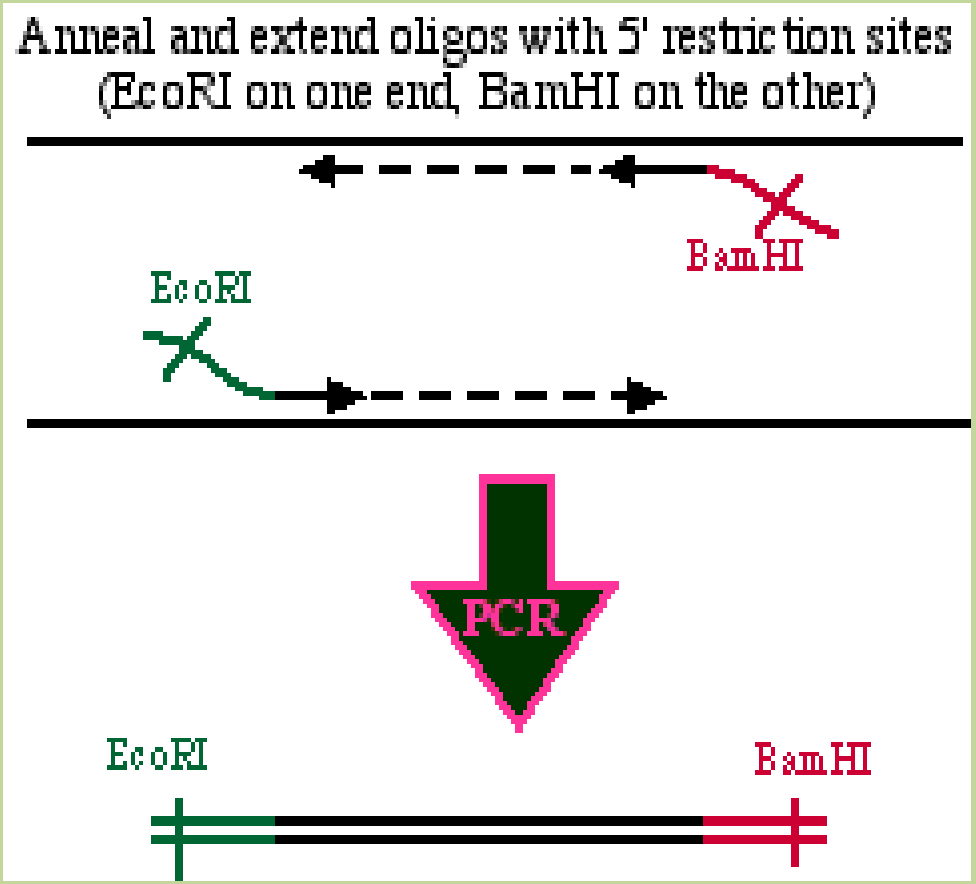

Blunt-ended DNA molecule
5′
3′
3′
5′
Linkers
5′
GGATCC
CCTAGG
3′
3′
5′
BamHI recognition sequence
5′
3′
3′
5′
Linkers attached to the ends of the DNA molecule
BamHI
5′
3′
3′
5′
BamHI sticky end
G
3′
CCTAG
5′

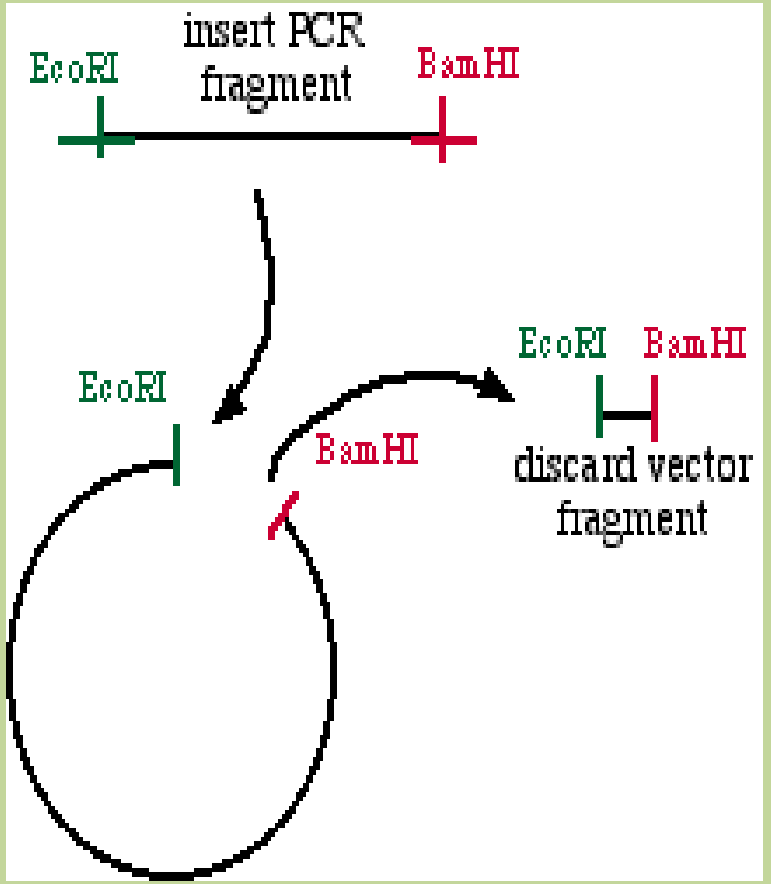
Introducing a R.E. site by PCR

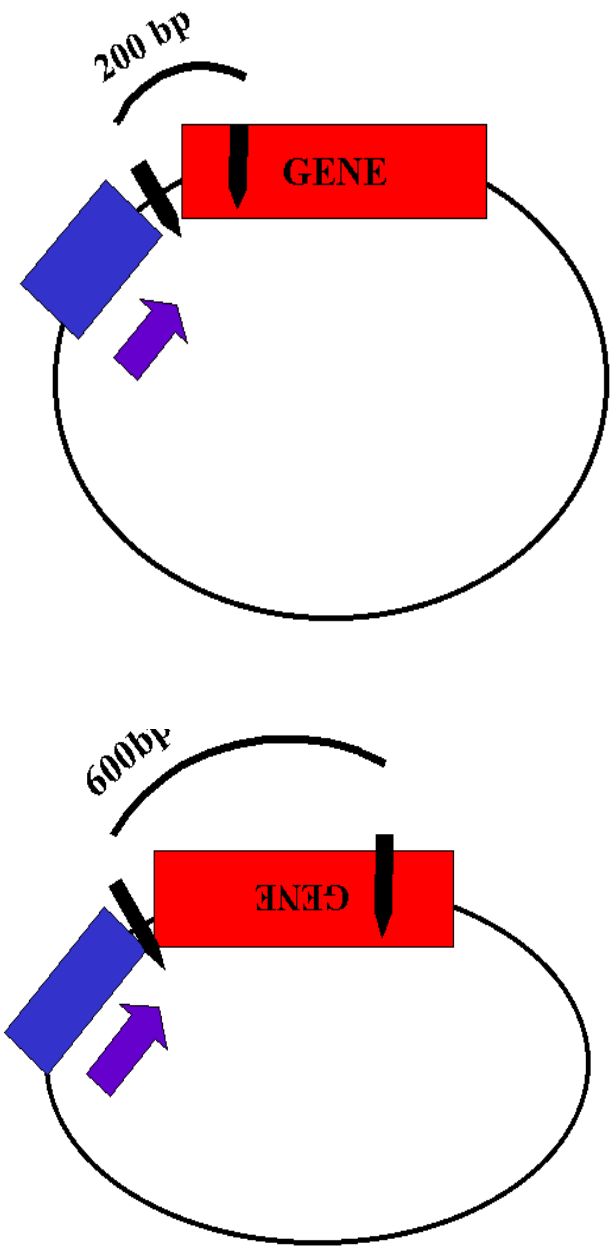
- R.E. site is designed into the 5’ end of the PCR primer
- PCR fragment is digested with appropriate R.E., purified and ligated into plasmid vector

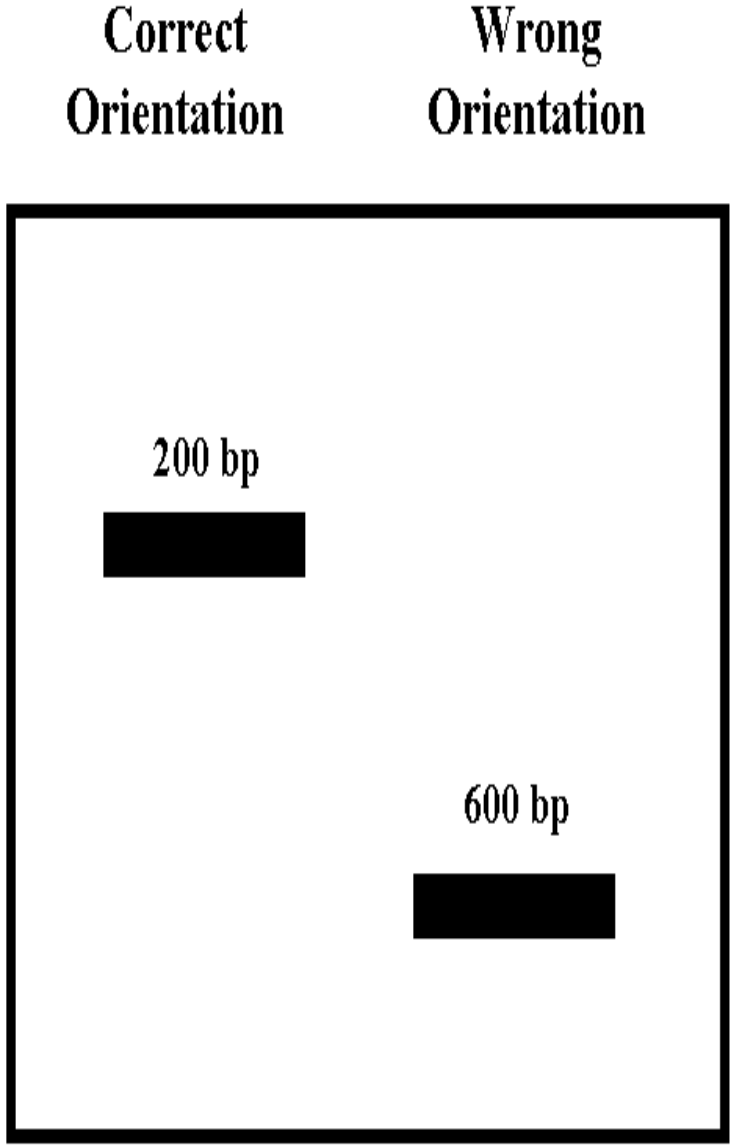


USING DIFFERENT R.E.s






200 bp
GENE
600bp
GENE


Correct Orientation
Wrong Orientation
200 bp
600 bp