Fundamentals of *Agrobacterium* and its applications in plant biotechnology research

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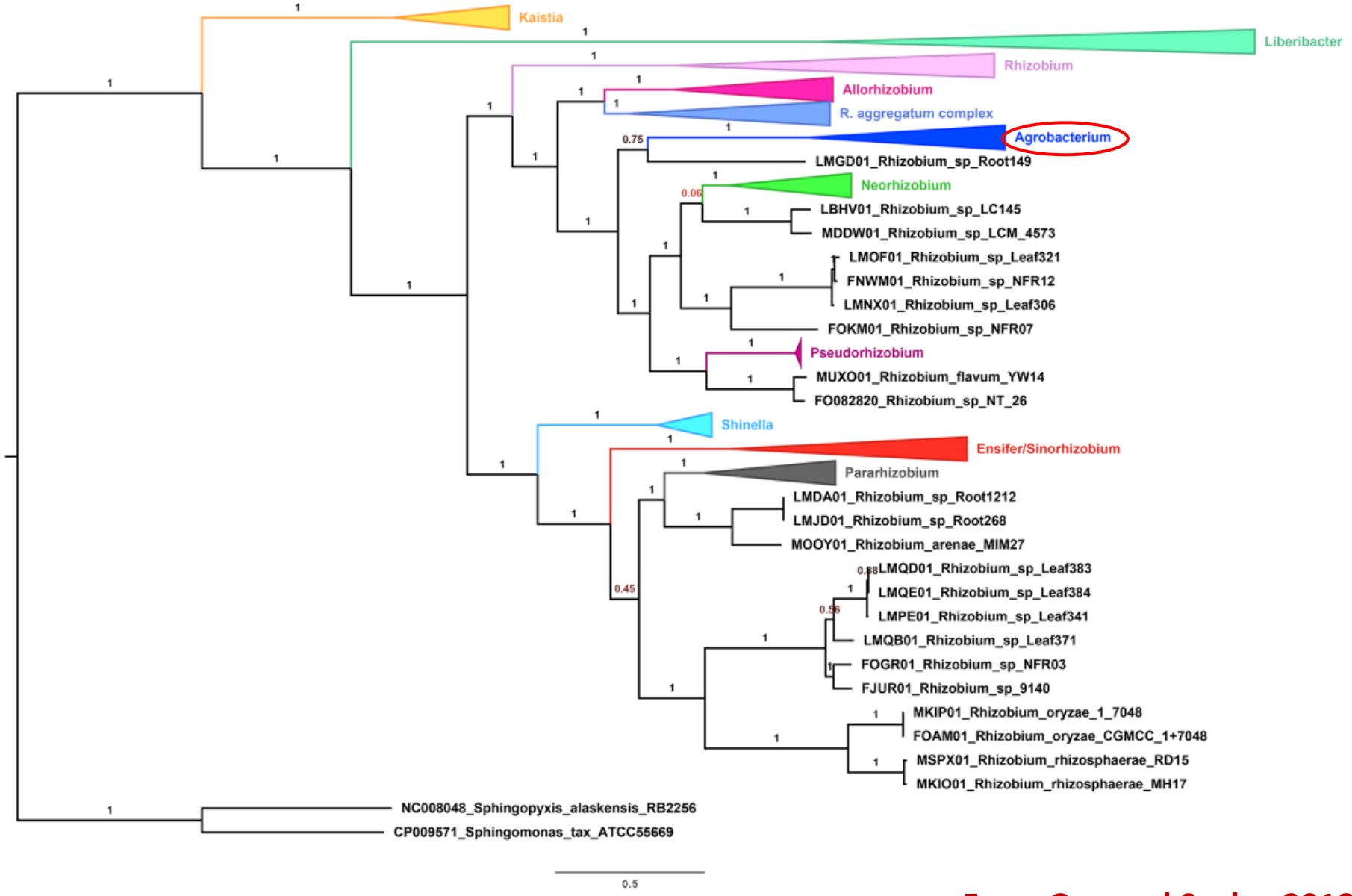
Placement of *Agrobacterium* among the *Rhizobiaceae*

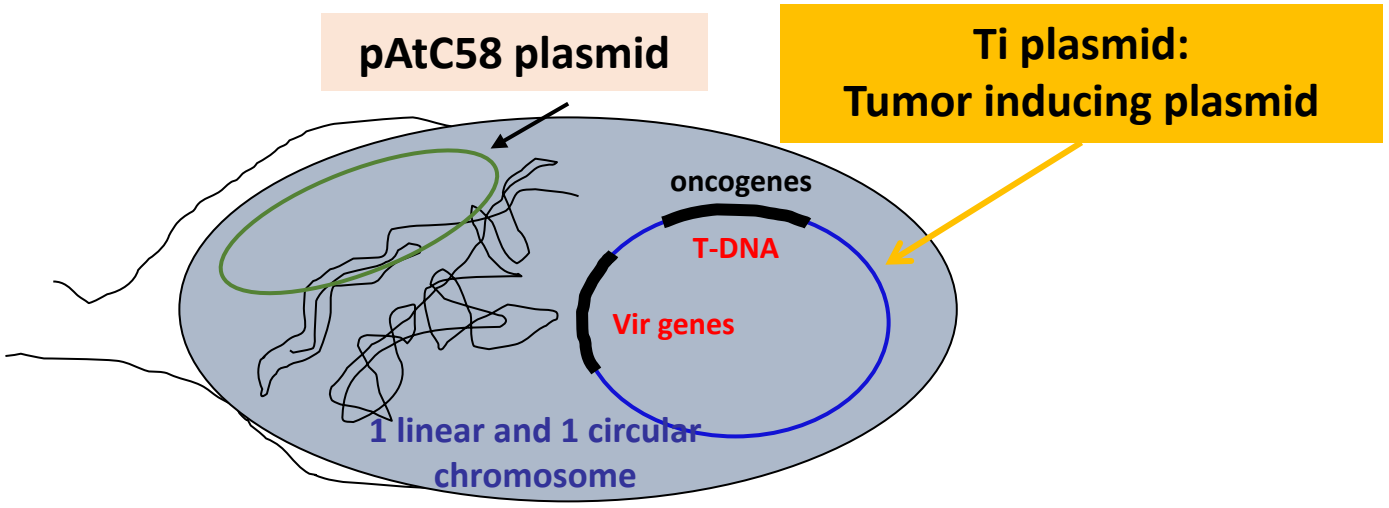
Agrobacterium is a Gram-negative α-proteobacterium



From Gan and Savka, 2018

Genomes of *Agrobacterium tumefaciens* C58

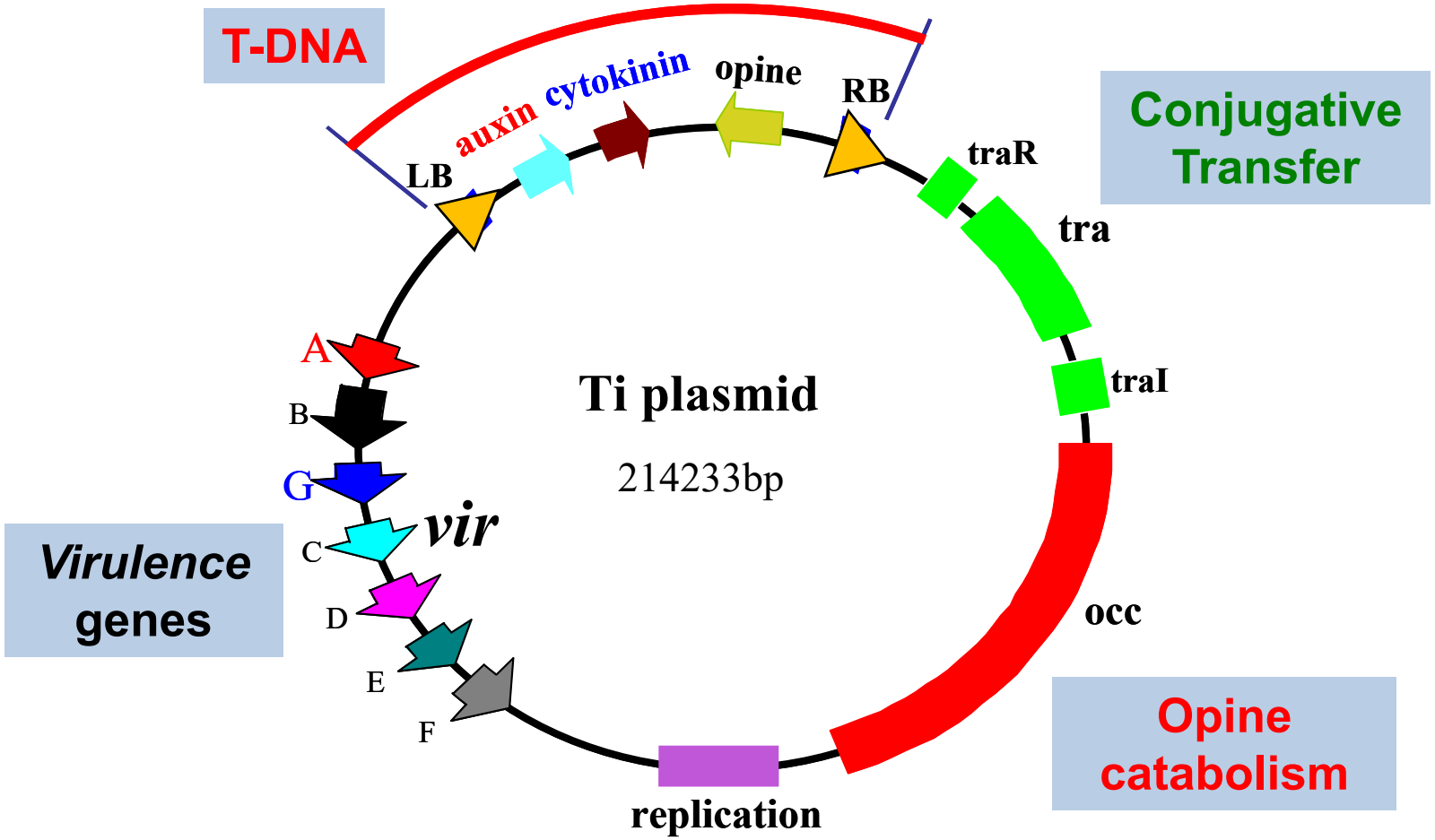
The C58 chromosomal background is in many commonly used *Agrobacterium* strains, including GV3101, EHA101, EHA105, AGL0, and AGL1.
LBA4404 contains the Ach5 chromosomal background



Agrobacterium

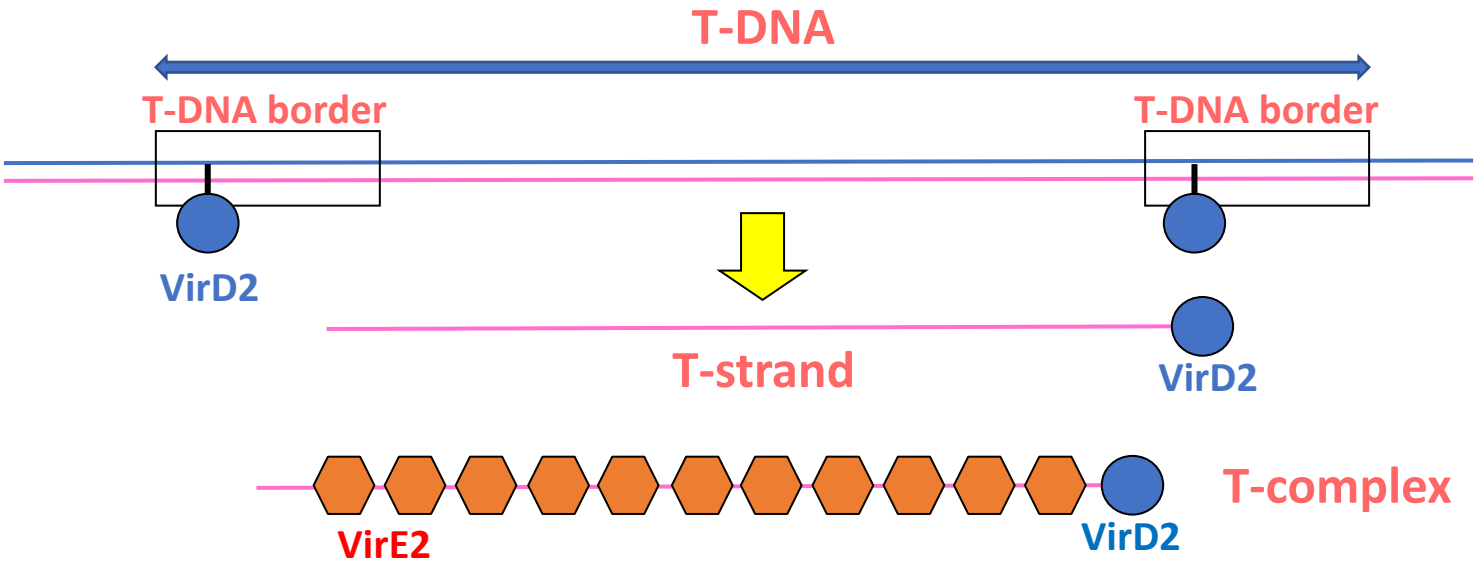
Agrobacterium **transfers oncogenes carried by the T-DNA (transferred DNA) to the plant, resulting in the uncontrolled proliferation of plant cells (Crown Gall tumors).**

The Ti plasmid carries genes required for T-DNA transfer, Ti plasmid conjugation, and opine metabolism



Processing of the T-DNA out of the Ti-plasmid and its subsequent export from the bacterium into the plant cell requires Virulence proteins encoded by the Ti-plasmid.

A protein called **VirD2** nicks the T-DNA region of the Ti-plasmid at 25 bp **border repeat sequences** flanking the T-DNA. VirD2 subsequently covalently attaches to the single-stranded "T-strand" which is released from the plasmid. VirD2 leads the T-strand out of the bacterium through a Type IV protein secretion system.



T-strands are thought to be coated by **VirE2** protein in the plant, forming a "T-complex"

Disarming *Agrobacterium* Ti-plasmids

Disarming a Ti-plasmid means deleting the oncogenes.

Early versions only deleted the oncogenes, leaving the opine synthase genes.

Subsequent versions deleted the entire T-DNA region, including the border repeat sequences.

The resulting disarmed plasmids contain virulence (*vir*) genes, an origin of replication, and sometimes other plasmid functions.

Frequently used disarmed *Agrobacterium* strains

Strain name	Chromosomal background	Ti/Ri-plasmid derivation	Antibiotic resistance[a]	Reference
AGL-0	C58	pTiBo542	rif	Lazo et al., 1991
AGL-1	C58	pTiBo542	rif, carb	Lazo et al., 1991
C58-Z707	C58	pTiC58	kan	Hepburn et al., 1985
EHA101	C58	pTiBo542	rif, kan	Hood et al., 1986
EHA105	C58	pTiBo542	rif	Hood et al., 1993
GV3101::pMP90	C58	pTiC58	rif, gent	Koncz & Schell, 1986
LBA4404	Ach5	pTiAch5	rif	Ooms et al., 1982
NT1(pKPSF2)	C58	pTiChry5	ery	Palanichelvam et al., 2000
SHA17	K599	pRi2659	---	Markin et al., 2007

[a]carb, carbenicillin; ery, erythromycin; gent, gentamicin; kan, kanamycin; rif, rifampicin

Modified from Lee and Gelvin, 2008

Growth of *Agrobacterium*

Agrobacterium is a soil microorganism.

Although biovar I strains (formerly *A. tumefaciens*) will grow in temperatures up to 37°C, some strains (e.g. C58) can lose their Ti-plasmid when grown at elevated temperatures, and biovar II strains (formerly *A. rhizogenes*) will not grow well at 37°C.

In the laboratory, *Agrobacterium* strains are routinely grown at 28-30°C. Growth is 2-3 times slower than that of *E. coli* grown in the equivalent medium.

Growth can be monitored using a spectrophotometer. A_{600}=0.83 or Klett=100 is ~10^9 cfu/ml

Growth of *Agrobacterium*

Agrobacterium can be grown in rich or minimal medium.

Rich medium: Luria-Bertani (LB) can be used, but some strains prefer the lower salt of YEP medium (5 g/l rather than 10 g/l of NaCl).

Minimal medium: Most strains can grow on minimal defined medium using either glucose or sucrose as a carbon source (most commonly used *E. coli* K-12 laboratory strains cannot use sucrose as a carbon source).

AB minimal medium is commonly used. (Many *E. coli* laboratory strains are auxotrophic and cannot grow on AB minimal medium, especially with sucrose).

Storage of *Agrobacterium*

Agrobacterium **cells are highly desiccation-sensitive. It is very difficult to revive bacteria from dry plates.**

Agrobacterium **cells survive on plates best when kept at room temperature (*E. coli* plates are best kept at 4°C). Do not use if the colonies look dry.**

For long-term storage in glass vials, we grow *Agrobacterium* cultures in rich medium containing the appropriate antibiotics overnight. Add 1 ml culture to 1 ml sterile glycerol (final 50% glycerol) or to 0.5 ml DMSO (final 33% DMSO). Mix the cultures thoroughly, then quick-freeze in either liquid N_2 or in a dry ice-ethanol bath. Store at -80°C.

When streaking out cultures from storage, DO NOT thaw the tubes; rather, "scratch" the frozen surface with a sterile toothpick.

For frequently used strains, make an additional frozen "working stock" from which to retrieve cultures.

Antibiotic selection with *Agrobacterium*

Most *Agrobacterium* strains are sensitive to many commonly used antibiotics:

	Useful range (μg/ml)	
	In plates	In liquid
Kanamycin	50-100	25-50
Gentamicin	50-100	50-100
Spectinomycin	100-150	50-100
Rifampicin	10-100	10-100
Carbenicillin[1,2]	100	50-100

[1]Strains based on the Ach5 chromosomal background, such as LBA4404, are inhibited by carbenicillin at concentrations >5 μg/ml even if they contain a β-lactamase gene.

[2]Carbenicillin, rather than ampicillin, is used for *Agrobacterium* because ampicillin degrades more rapidly. *Agrobacterium* has a longer doubling time than does *E. coli*, and takes longer to grow.

Antibiotic selection with *Agrobacterium*

Difficult-to-use antibiotics with *Agrobacterium*:

Tetracycline—Many strains based on the C58 chromosomal background have a natural resistance to low levels of tetracycline (2-3 μg/ml on plates), and even with a tet-resistance gene cannot tolerate more than 5-10 μg/ml tetracycline in plates.
Luo et al. (2001) deleted the *tet* locus from the C58 chromosome, generating an *Agrobacterium* strain (NTL4) that is highly sensitive to tetracycline unless a tet-resistance gene is introduced into the strain.

Streptomycin—Many strains can easily develop spontaneous resistance to streptomycin, so this antibiotic should not be used.

Opine utilization as a selection for the presence of a Ti/Ri-plasmid in *Agrobacterium*

Most Ti/Ri plasmids contain opine utilization genes (octopine, nopaline, mannopine, agropine, agrocinopine, succinamopine, etc.). Ti plasmids used to be characterized by the class of opine utilization conferred upon the bacterium.

Octopine, nopaline, and mannopine can be used as the sole carbon and nitrogen source for strains harboring the appropriate Ti plasmid. One must use defined minimal medium, omit the sugar source, and delete the nitrogen source from the salts. It takes almost a week for single colonies to grow.

Because commonly used BactoAgar preparations frequently have trace contaminants of usable carbon and nitrogen, use "agar purified" or "agarose". "Agar purified" is very expensive, so use small plates.

Note: octopine, nopaline, and mannopine are no longer commercially available.

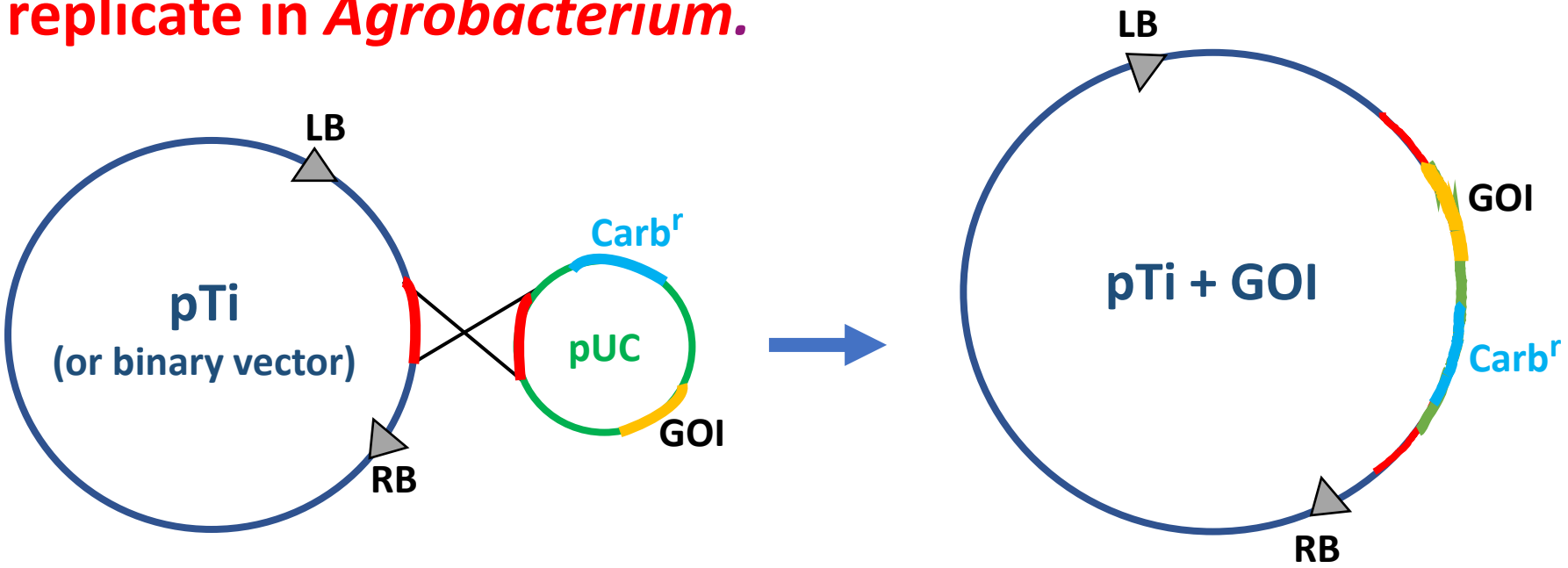
Types of T-DNA vectors

Cointegrate systems
Binary vector systems
Ternary vector systems
Multiple T-DNAs

Types of T-DNA vectors: Cointegrate systems

Cointegrate systems use homology between a plasmid that cannot replicate in *Agrobacterium* and a plasmid (pTi or other) that can replicate. The two plasmids are joined by homologous recombination.

For example, plasmids containing a ColE1 origin of replication (*oriColE1*; pBR, pUC, pBS, etc.) cannot replicate in *Agrobacterium.*



Types of T-DNA vectors: Binary vector systems

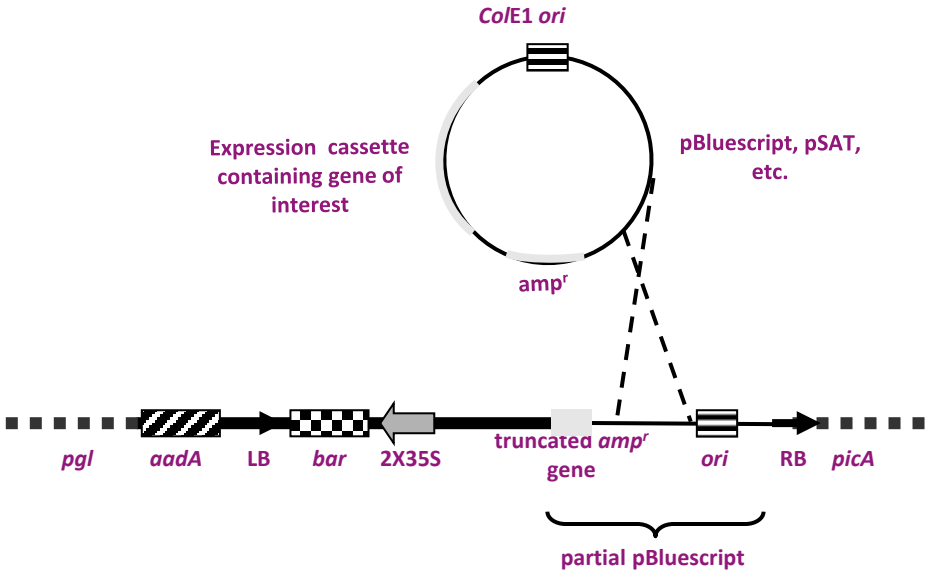
Hoekema et al. (1983) first showed that T-DNA and the *vir* regulon could be separated onto two different replicons that, when in the same bacterial cell, could function to deliver T-DNA to plants.

In practice, the replicons are usually two different plasmids, one containing T-DNA (the T-DNA binary vector) and the other the *vir* regulon (*vir* helper plasmid).

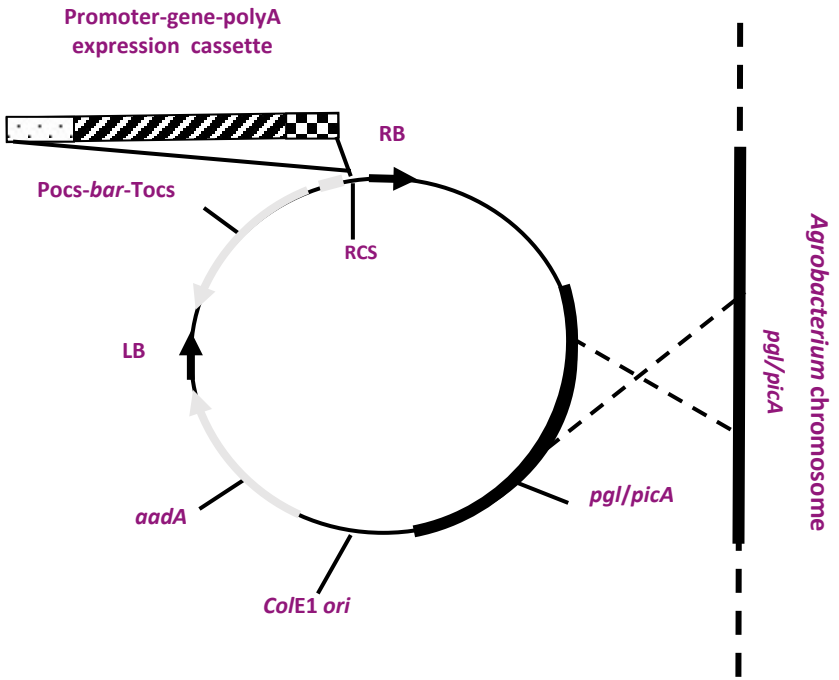
However, one or both of these two components can also be integrated into the *Agrobacterium* chromosome. Oltmanns et al. (2010) showed that launching T-DNA from the *Agrobacterium* chromosome resulted in plants with lower copy number of integrated T-DNA. However, transformation frequency was also decreased.

Engineering a "chromosomal launch" system at the *Agrobacterium pgl/picA* chromosome locus

This locus is "neutral": Disruption does not inhibit transformation



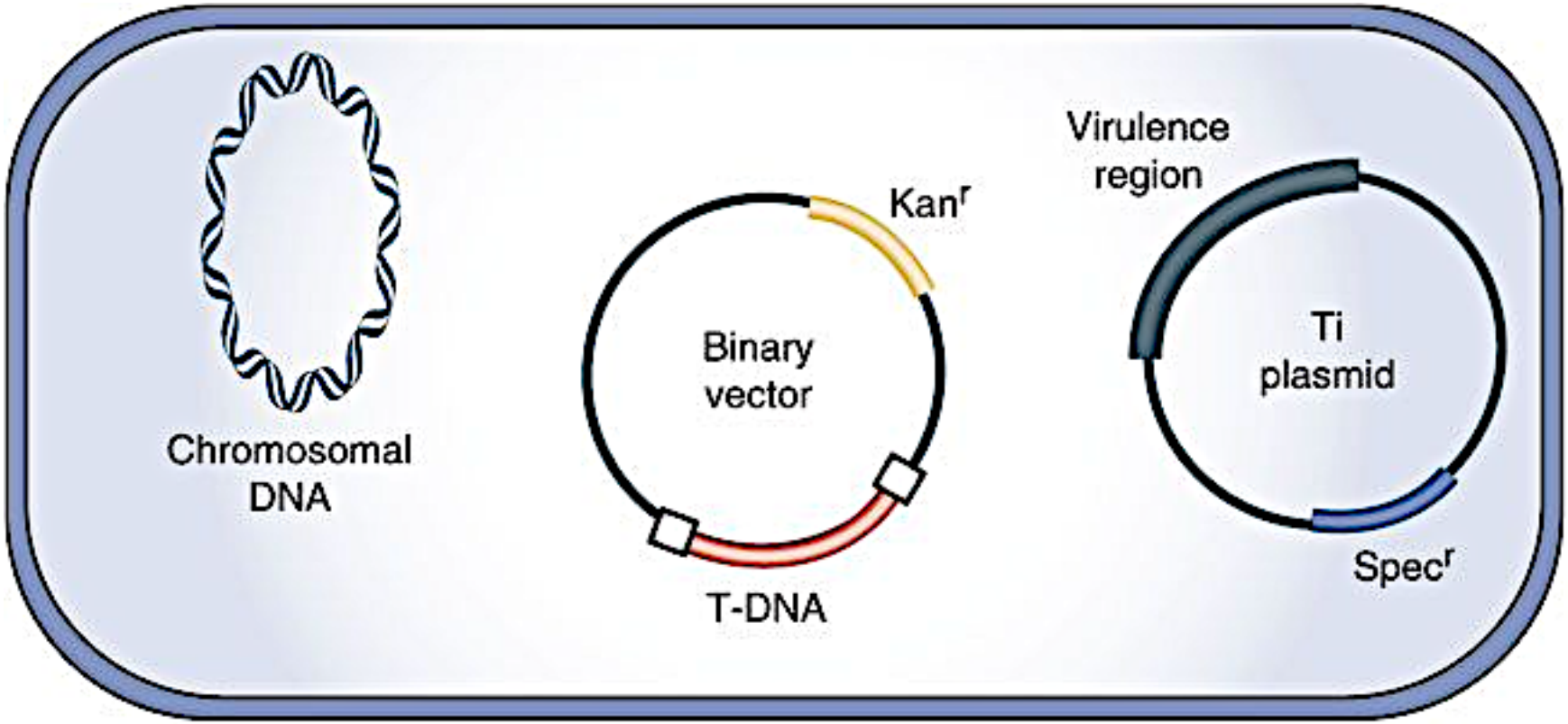
One-step recombination into an engineered *Agrobacterium* chromosome



Two-step recombination into a native *Agrobacterium* chromosome

Lee et al., 2001; Oltmanns et al., 2010

Commonly used T-DNA binary vector system



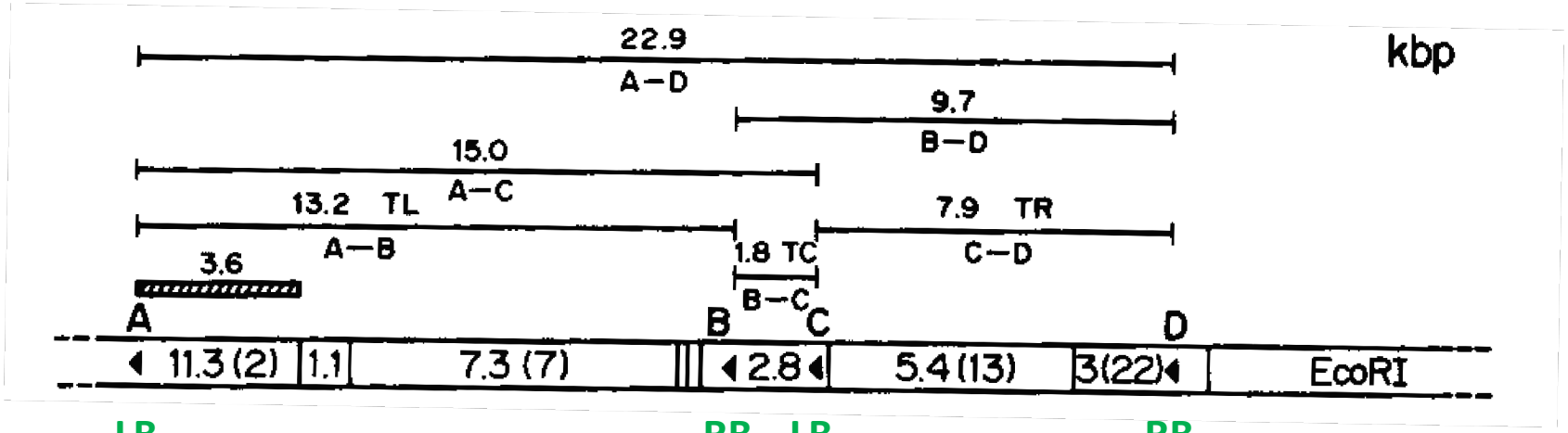
Multiple T-DNAs

Multiple T-DNAs on the same plasmid are common on native Ti/Ri-plasmids. T-DNAs can be transferred and integrated into the plant genome independently or linked.

Similarly, multiple T-DNAs can be engineered onto the same binary vector, or multiple compatible binary vectors can be introduced into the same *Agrobacterium* cell.

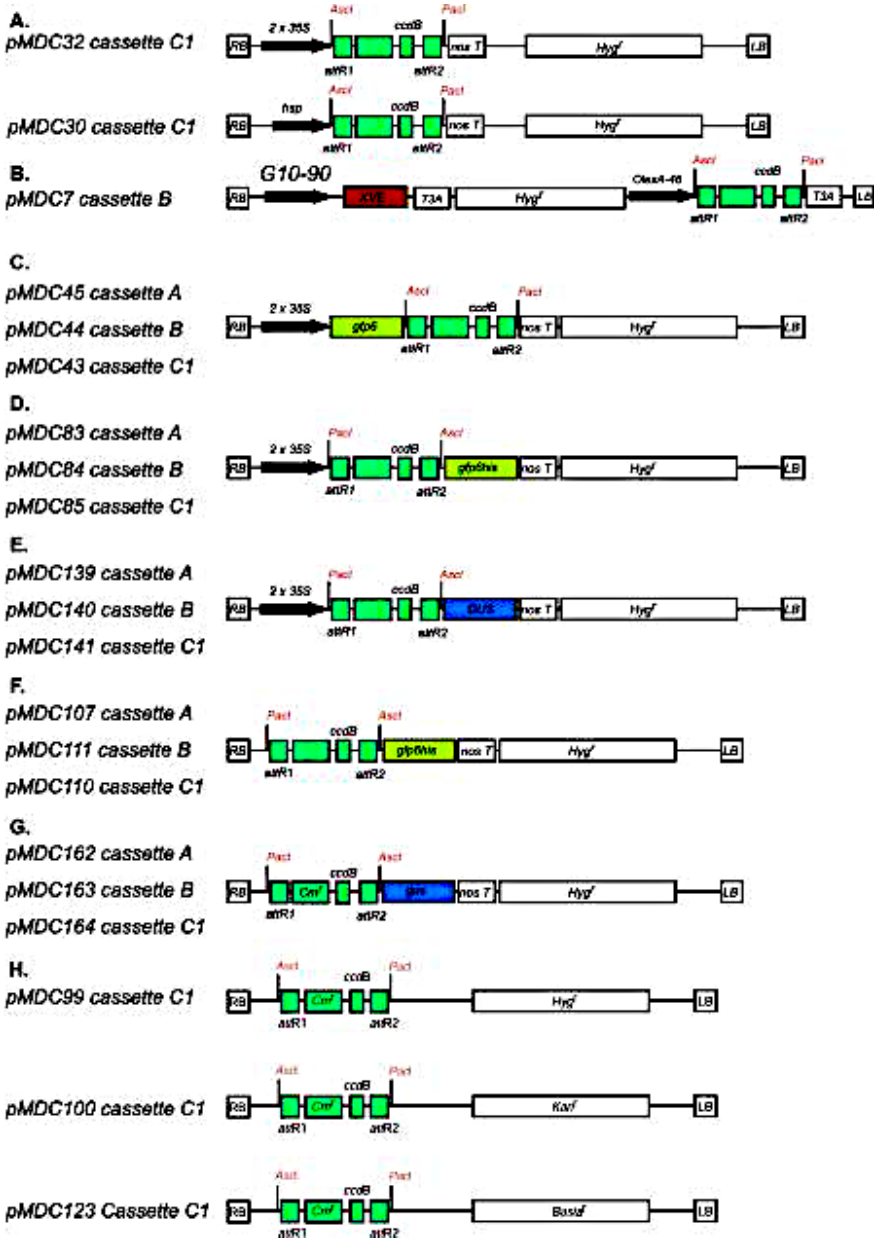
Independently transferred T-DNAs (from the same or different *Agrobacterium* cells) often link/insert into the same locus in the plant genome (De Neve et al., 1997).

Multiple T-DNAs of the octopine-type Ti plasmid pTiA6



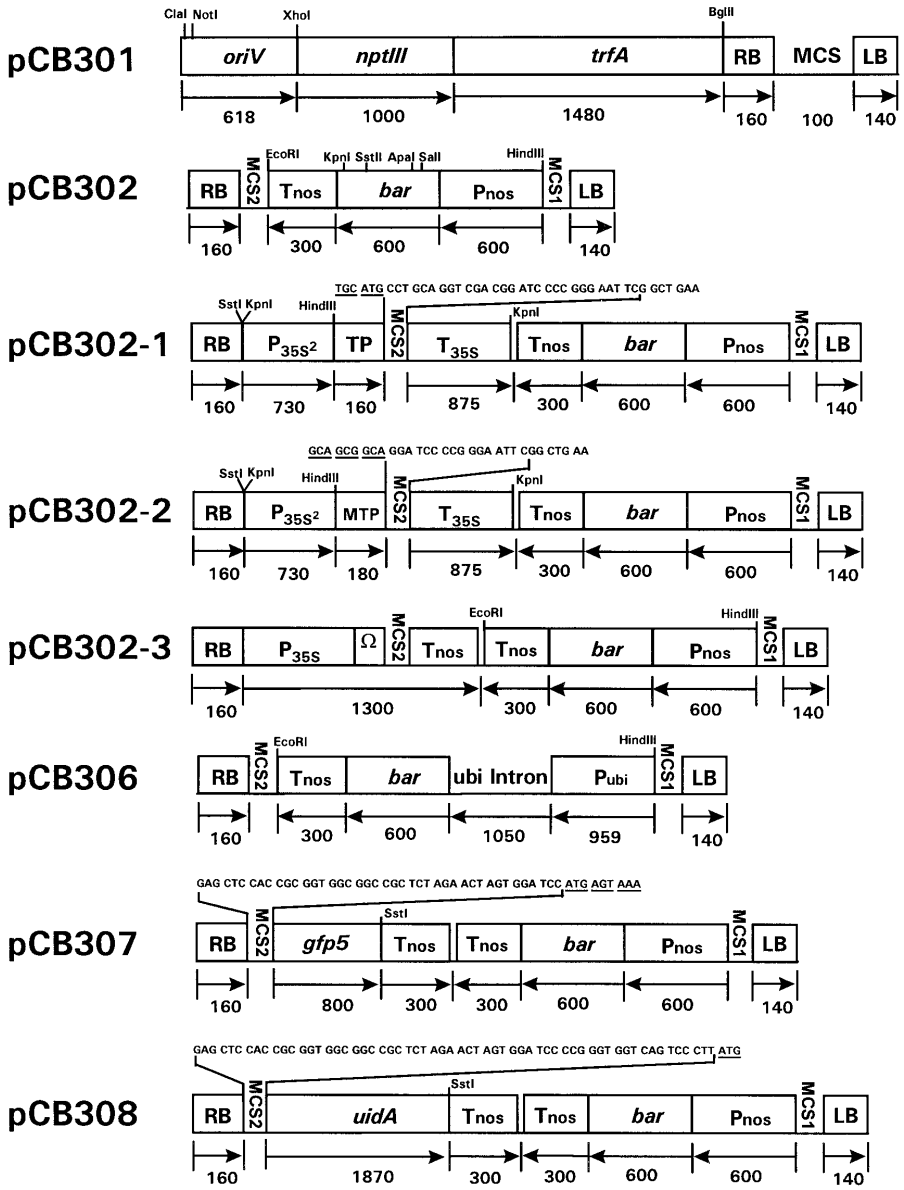
Gateway-compatible pMDC T-DNA binary vectors

(Curtis and Grossniklaus, 2003)



pCB T-DNA binary vectors

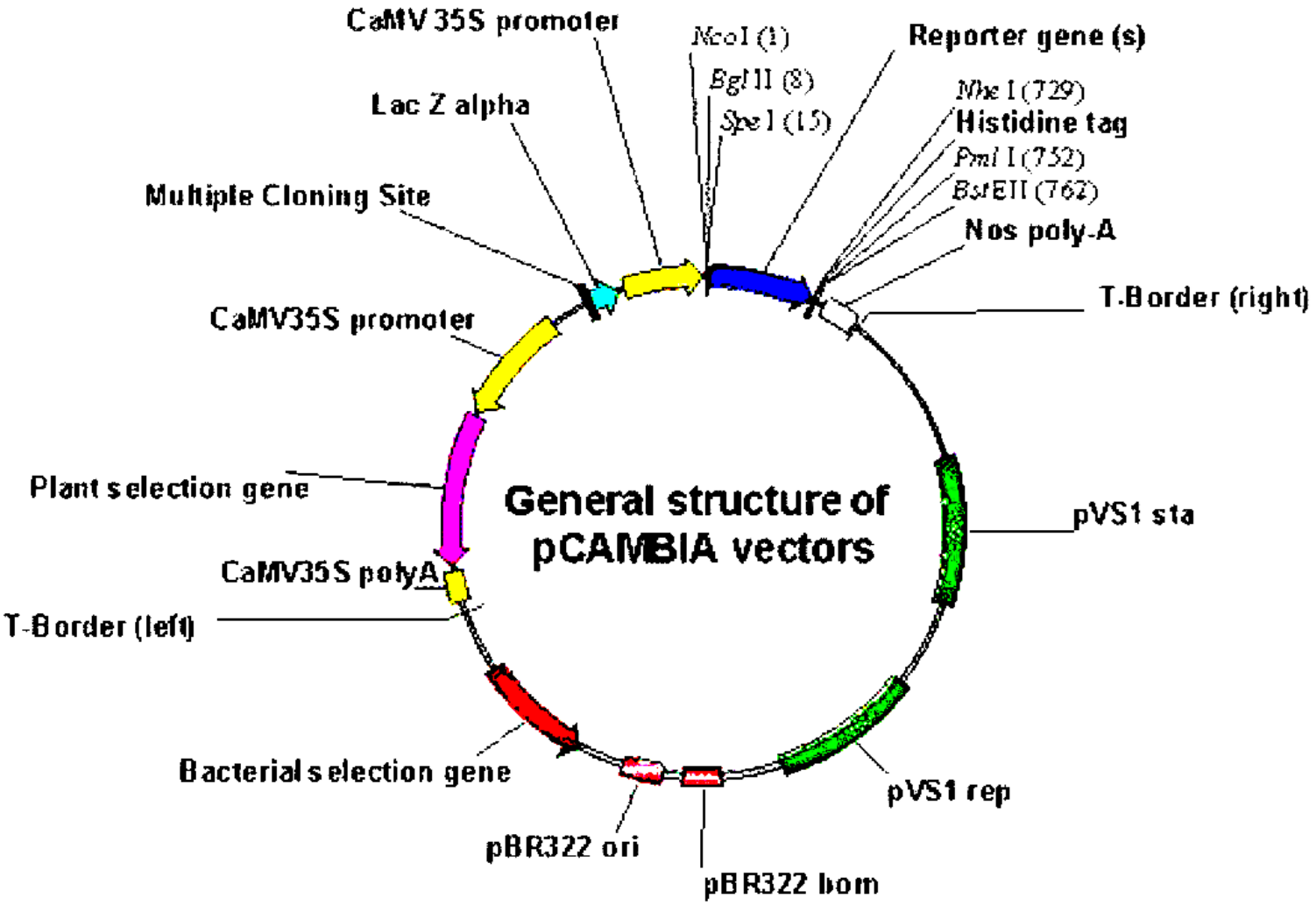
(Small size; Xiang et al., 1999)



pCAMBIA T-DNA binary vectors

(High copy number in bacteria, small size, stable in *Agrobacterium*)

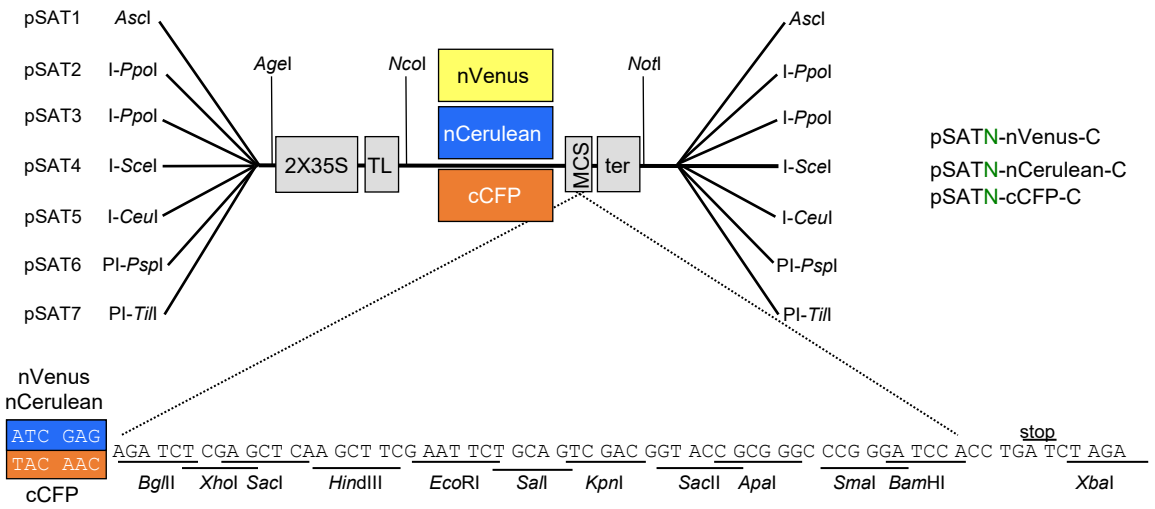
Richard Jefferson, Cambia Labs

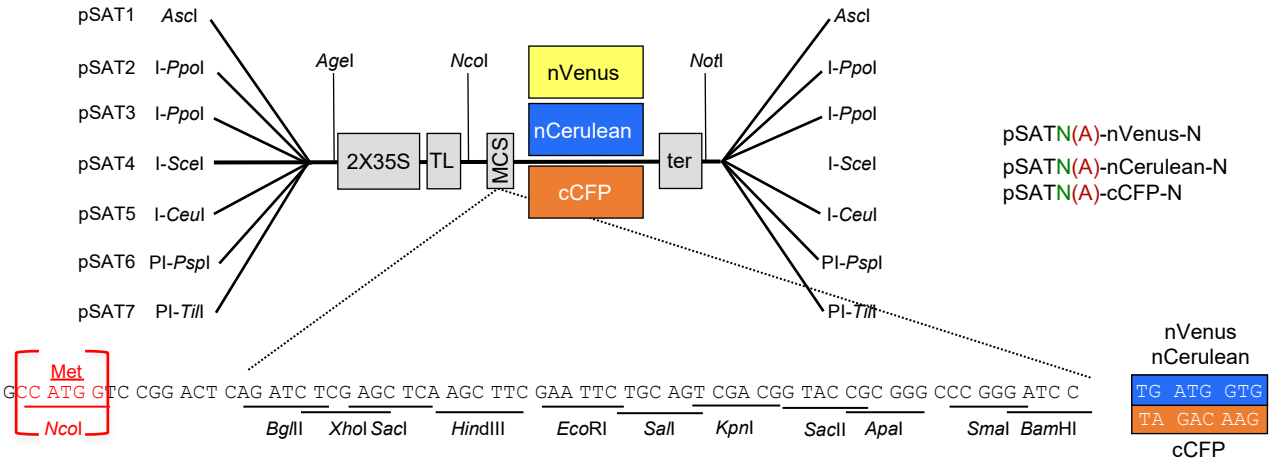


The pSAT fluorescence protein tagging system

Lee and Gelvin, 2014

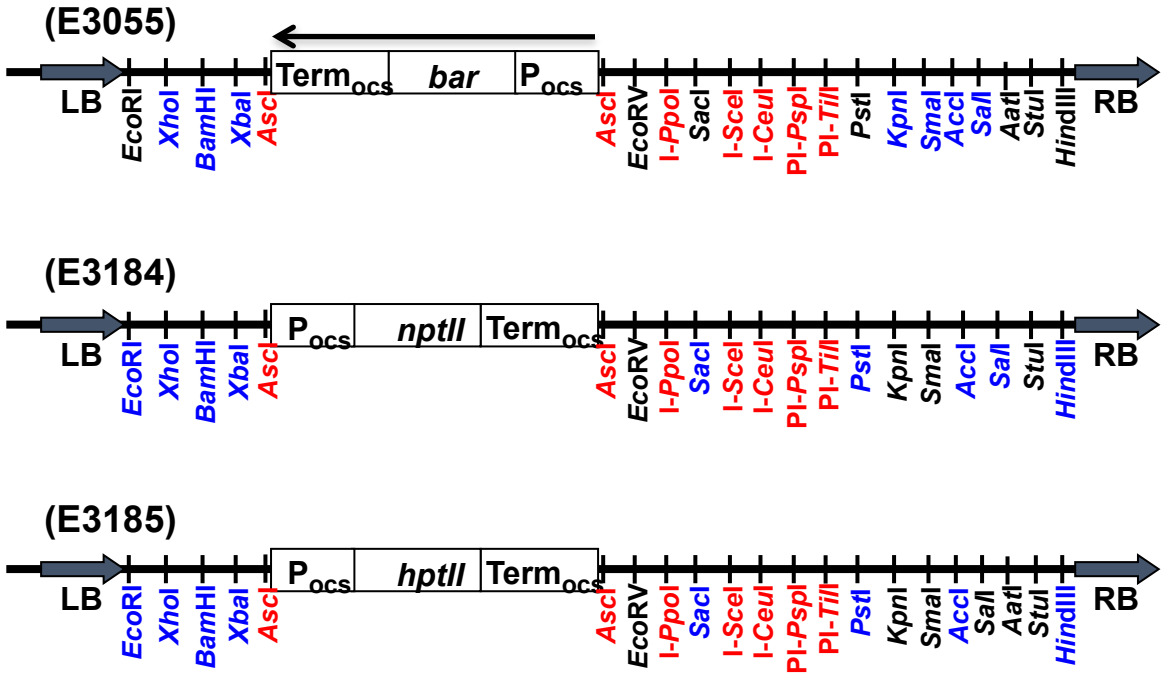
Genes of interest can be cloned into expression cassettes, which are then loaded into a binary vector





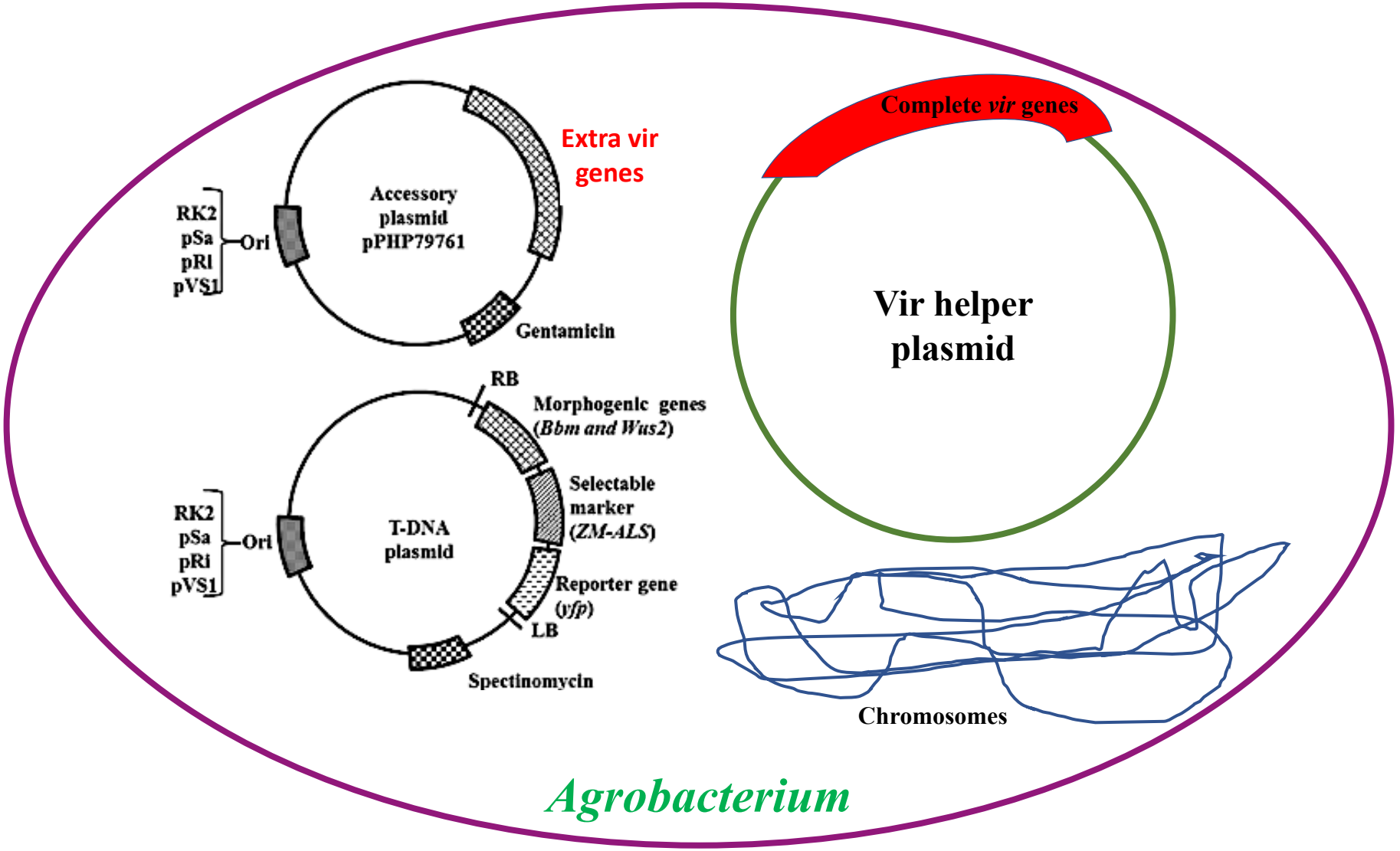
T-DNA binary vectors for the pSAT fluorescence protein tagging system

Lee and Gelvin, 2014



Ternary vector system

(Anand et al., 2018)



Promoters commonly used to drive transgene expression

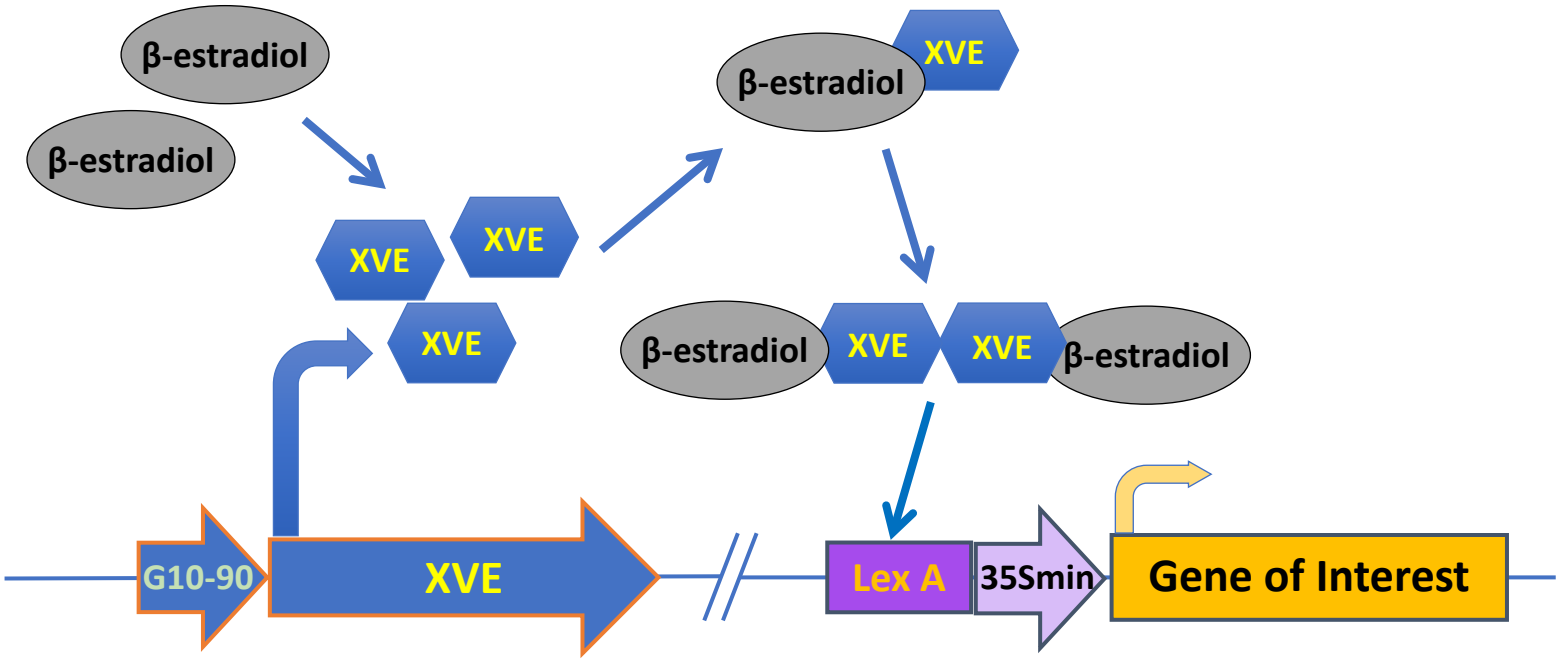
Constitutive (relative strength): Super-promoter > CaMV double 35S > mannopine synthase (*mas2'*) > nopaline synthase > octopine synthase

Regulated: Tissue/cell-specific, developmental, hormone inducible, environmentally inducible, pathogen-inducible, heat shock inducible, etc.

Chemically inducible: dexamethasone, β-estradiol, etc.

RNA polymerase III-type to drive expression of guide RNAs for CRISPR engineering: U6, U3

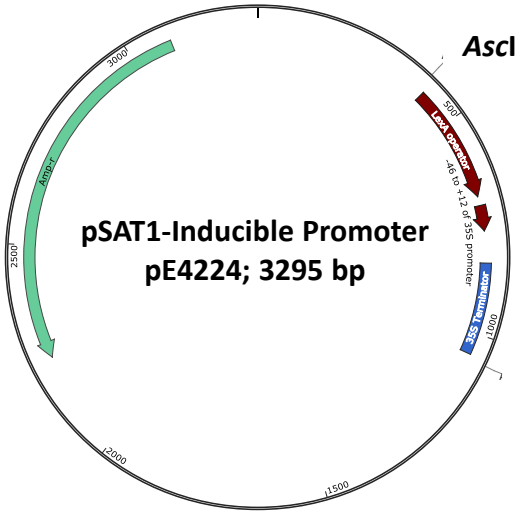
Use a β-estradiol inducible XVE system to express genes inducibly in transgenic plants

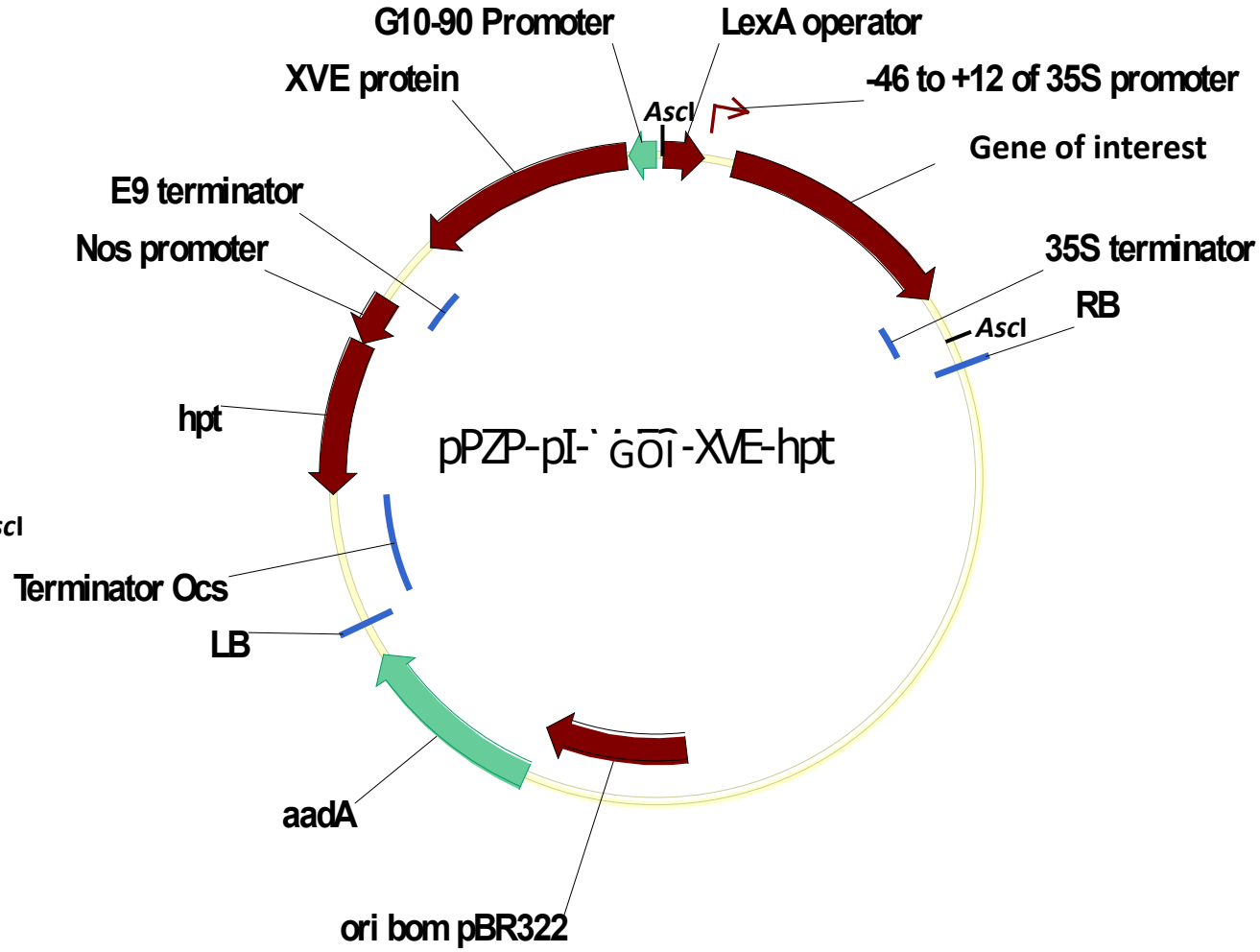


XVE: a chimeric transcription activator
X: DNA-binding domain of bacterial repressor LexA
V: the transcription activation domain of VP16
E: carboxyl region of the human estrogen receptor
Target promoter: 8 copies of LexA operator plus CaMV 35S minimal promoter

Zuo et al. 2000

pSAT system to make T-DNA binary vectors to express genes using the β-estradiol inducible system





Lan-Ying Lee

Virulence genes

The Virulence (*vir*) regulon is composed of a number of operons:

- ***VirA*** **(single gene): Sensor of phenolic inducers**
- ***VirG*** **(single gene): Response/transcriptional regulator of *vir* promoters**
- ***VirB*** **(11 genes): Proteins form a Type IV Protein Secretion System (T4SS) with VirD4**
- ***VirC*** **(2 genes): T-DNA processing, overdrive binding proteins**
- ***VirD*** **(4 genes/VirD3 not present in many strains):**
  - **VirD1—helicase**
  - **VirD2—T-DNA border-specific endonuclease, T4SS export, nuclear targeting**
  - **VirD4—Coupling factor for T4SS**
  - **VirD5—Proposed to function in Vir protein stability, chromosome segregation, plant transcriptional regulation**
- ***VirE*** **(3 genes):**
  - **VirE1—Chaperone for VirE2**
  - **VirE2—ssDNA binding protein, regulates plant gene expression**
  - **VirE3—Regulates plant gene expression**
- ***VirF*** **(single gene): F-box protein involved in proteosomal degradation**

Other non-essential *vir* genes: *VirH, J, K*

- ***GALLS*** **(single gene, two proteins): Found instead of *virE1* and *virE2* in many *A. rhizogenes* strains; can complement an *A. tumefaciens virE2* mutant , but protein does not resemble VirE2**

Virulence gene induction

Virulence gene induction is mediated by the VirA/VirG proteins, with help from ChvE.

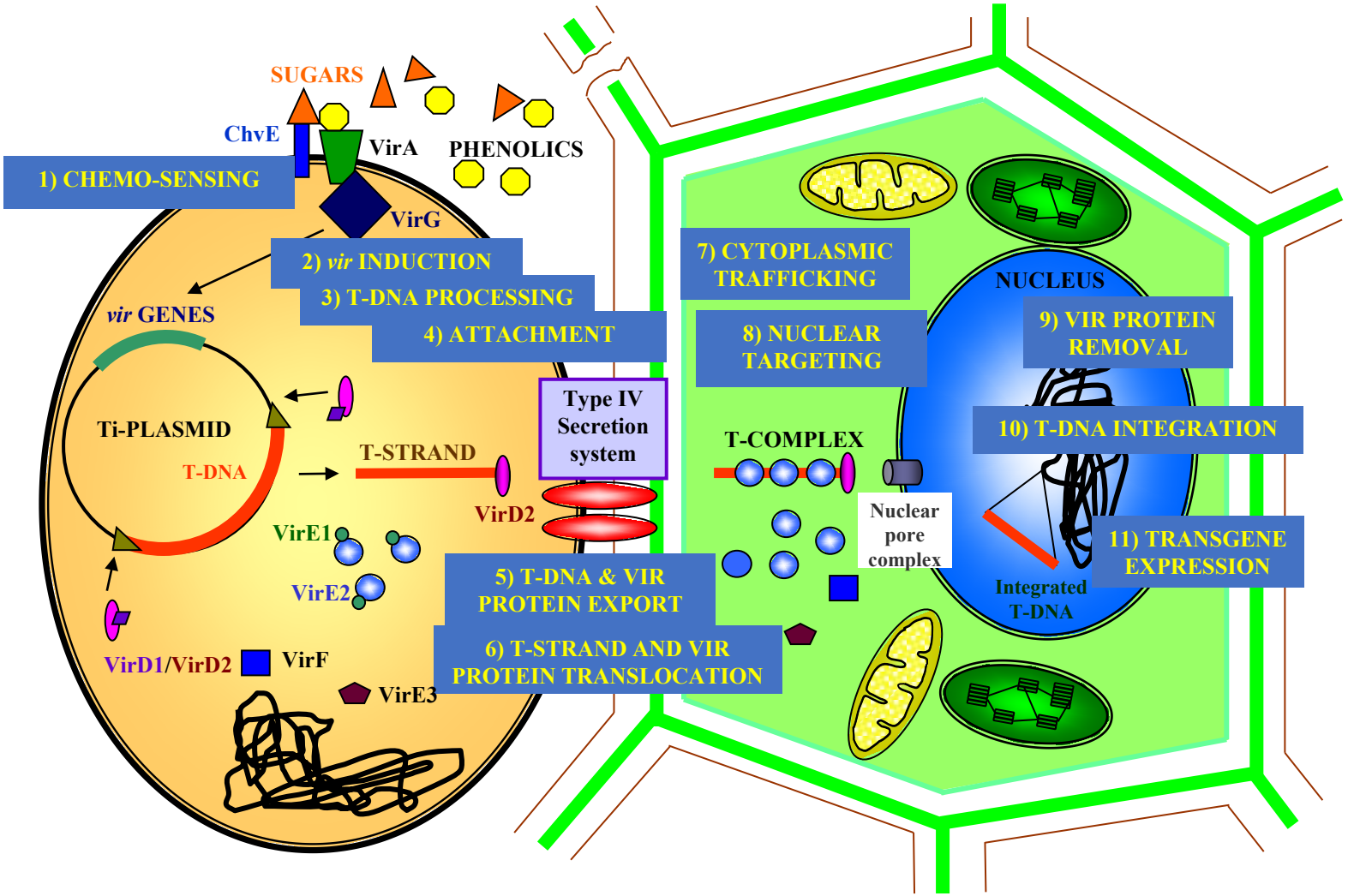
VirA senses phenolic molecules; ChvE is a sugar transporter.

Phenolic molecules, such as acetosyringone (AS) are recognized by VirA.

Optimal *vir* gene induction requires:

1. The phenolic compound
2. Certain neutral or acidic sugars
3. Low pH (5-6.5)
4. Low phosphate
5. Moderate temperatures
6. Certain chromosomal virulence (*chv*) genes: *chvG,I*

The *Agrobacterium* – plant cell interaction is complex and consists of numerous steps



Agrobacterium tumefaciens PLANT CELL

Virulence proteins can be manipulated to alter transformation characteristics

A mutant VirD2 protein can mediate efficient transient transformation and genome editing but extremely poor T-DNA integration

Cas9 cleavage site

VirD2 allele	286	287	288	289	290	291	292	293	294
Day 4									
Wild-type	0.94	1.07	1.14	0.92	1.70	2.28	0.44	0.48	0.32
Mutant	0.45	0.61	0.66	0.59	1.43	1.54	0.30	0.45	0.32
Day 5									
Wild-type	2.34	2.44	2.73	2.36	3.78	4.10	0.49	0.50	0.40
Mutant	0.90	0.84	1.23	1.02	2.36	2.20	0.42	0.44	0.42

An N. benthamiana PDS2 gene was targeted for CRISPR/Cas9 mutagenesis by a wild-type or a *virD2* mutant *Agrobacterium* strain. Shown are the % of mutant nucleotides at each position surrounding the Cas9 cleavage site at 4 and 5 days after Agroinfiltration.

Work of Lan-Ying Lee