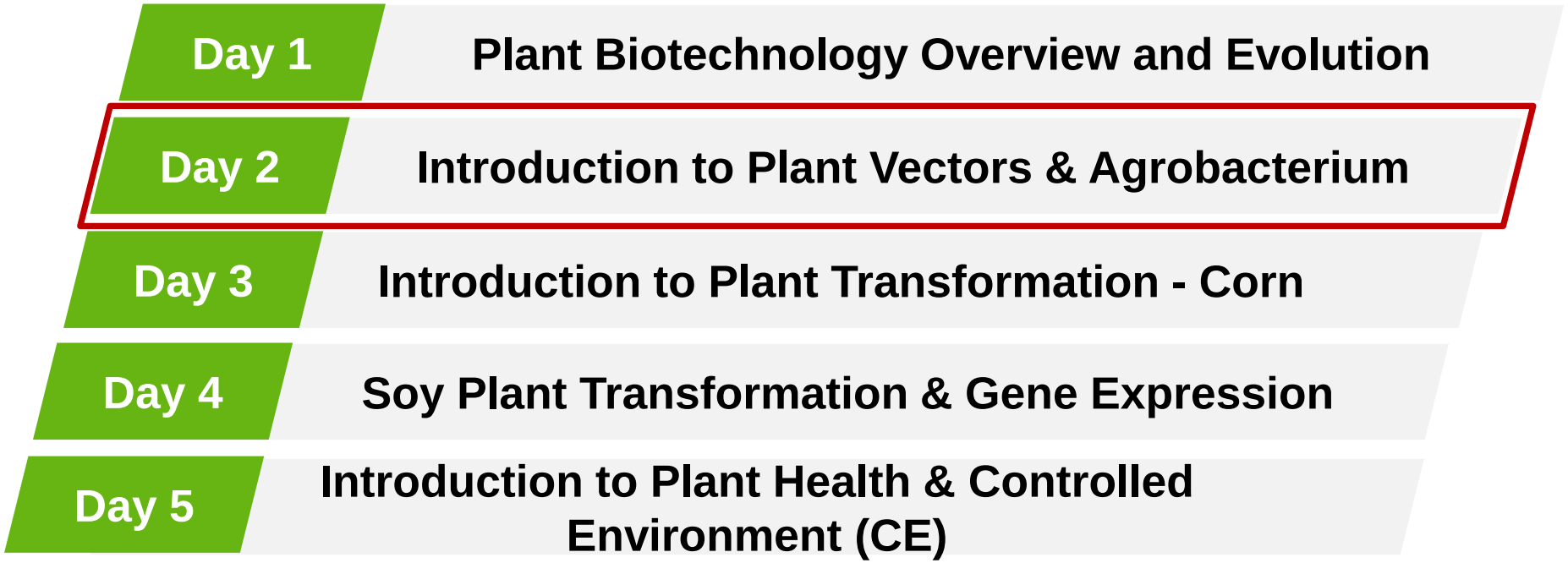
Plant Biotechnology

Bayer Russia Plant Biotechnology Conference

July 2023 – week 1

Bayer Russia Plant Biotechnology Conference:



Introduction to vectors

Xudong Ye, PhD.
Bayer Crop Science

About me:

Principal Scientist, Bayer Science Fellow

PhD	Institute of Plant Science, ETHZ, Switzerland (Prof. Ingo Potrykus)
	Ryegrass transformation, fructan metabolism
M.S.	Plant Pathology, Nanjing Agricultural University, China
B.S	Plant Protection, Zhejiang (Agricultural) University, China

My research fields

- Plant transformation (dicot and monocot, nuclear and plastid)
- *Agrobacterium* biology (strain modification, vector strategies)
- Molecular biology
- Gene editing

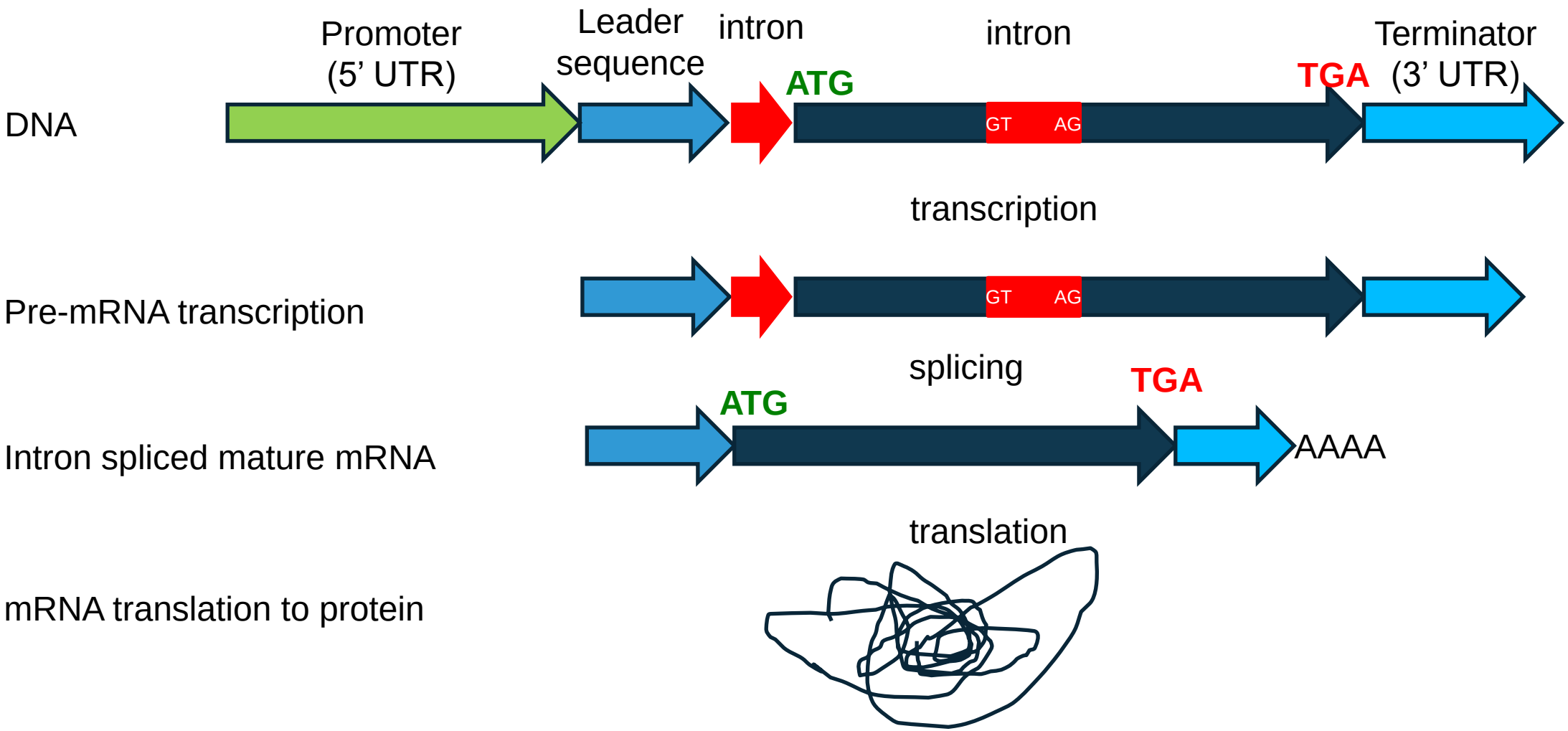
Patents

Publications

Agenda

- Vectors designs for particle gun and *Agrobacterium*
- Selectable markers
- Scorable marker genes (GFP, GUS)
- Backbone
- Main cloning strategies

Plant gene structure

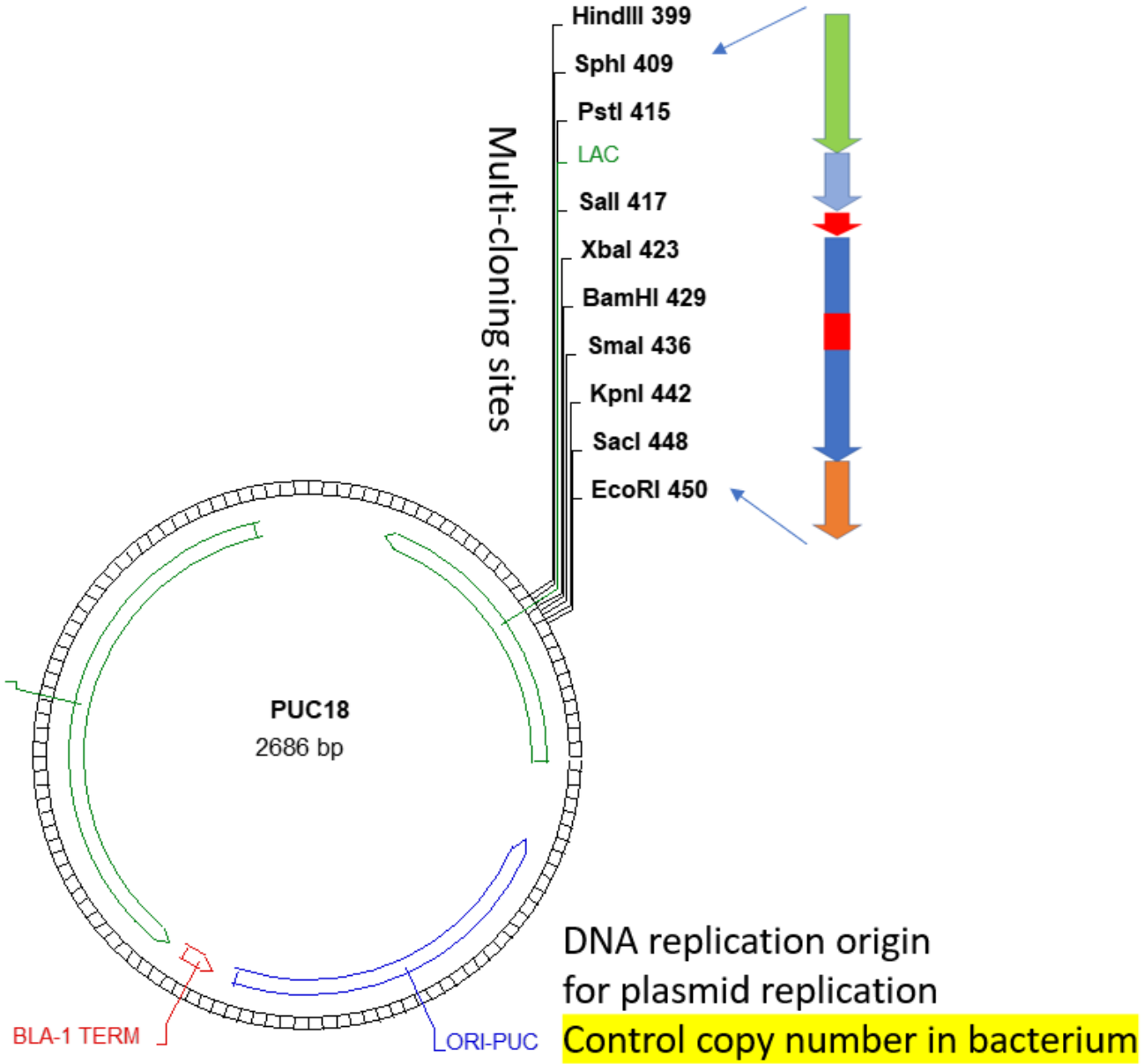


From genome to plasmid: cloning DNA fragments by in vitro manipulation

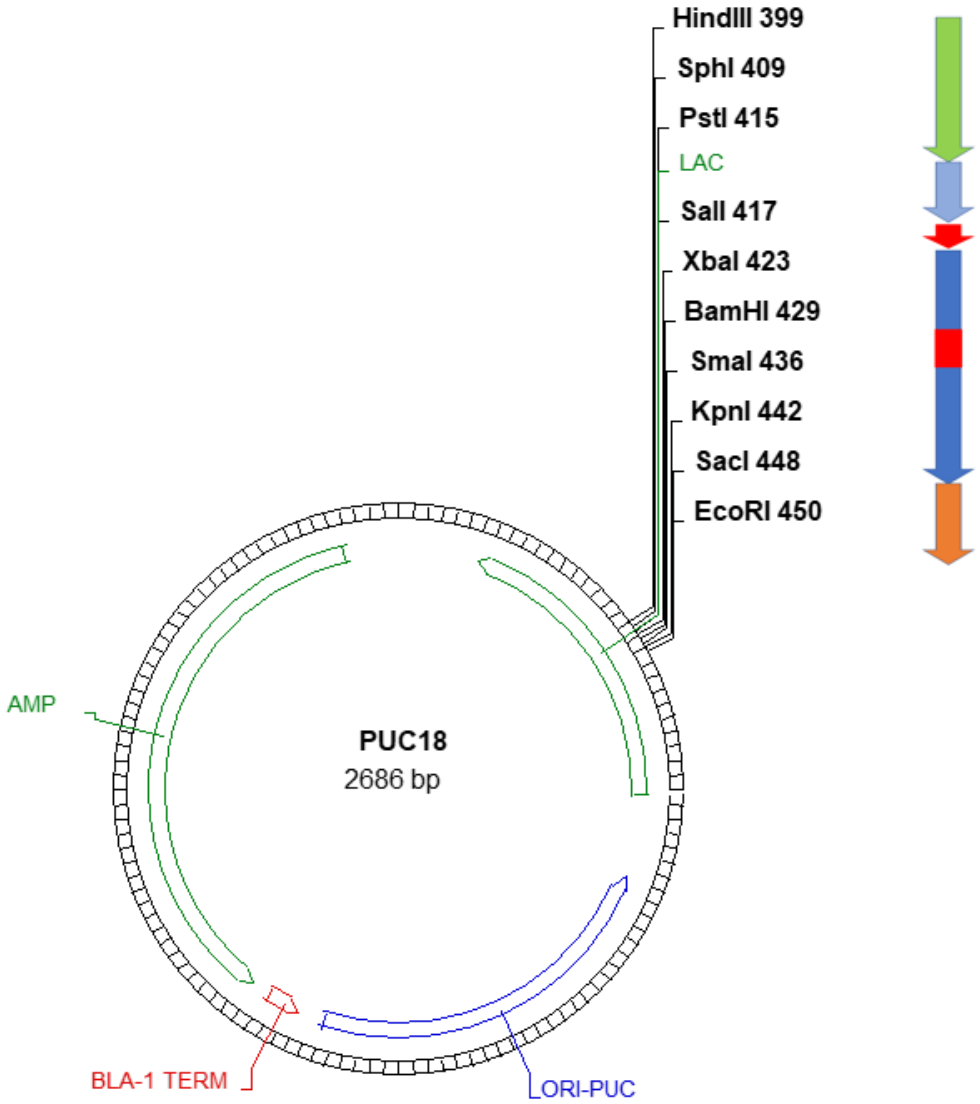
Plasmid

Vector

Construct



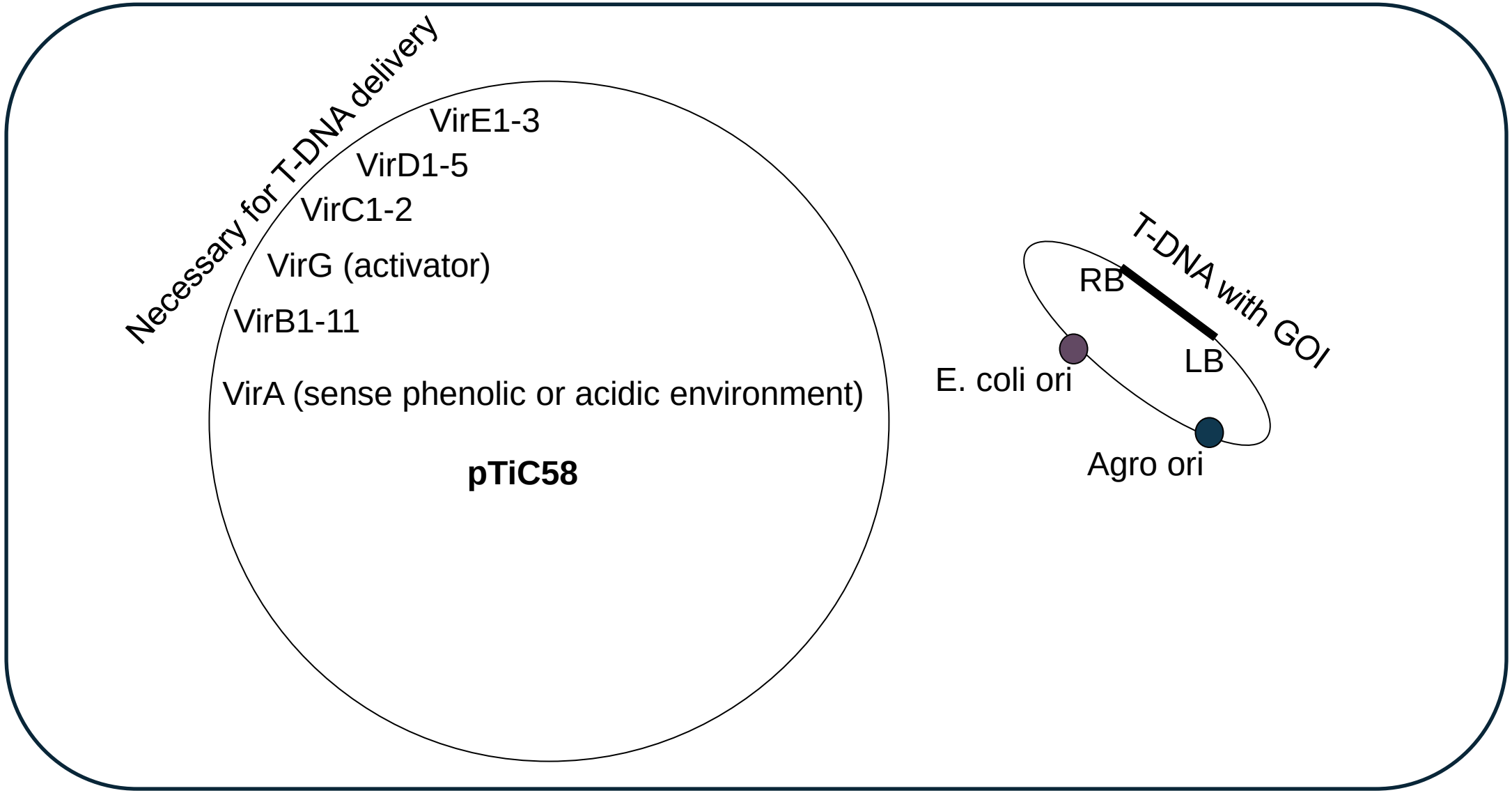
Plasmids for gun bombardment are different from Agrobacterium vectors



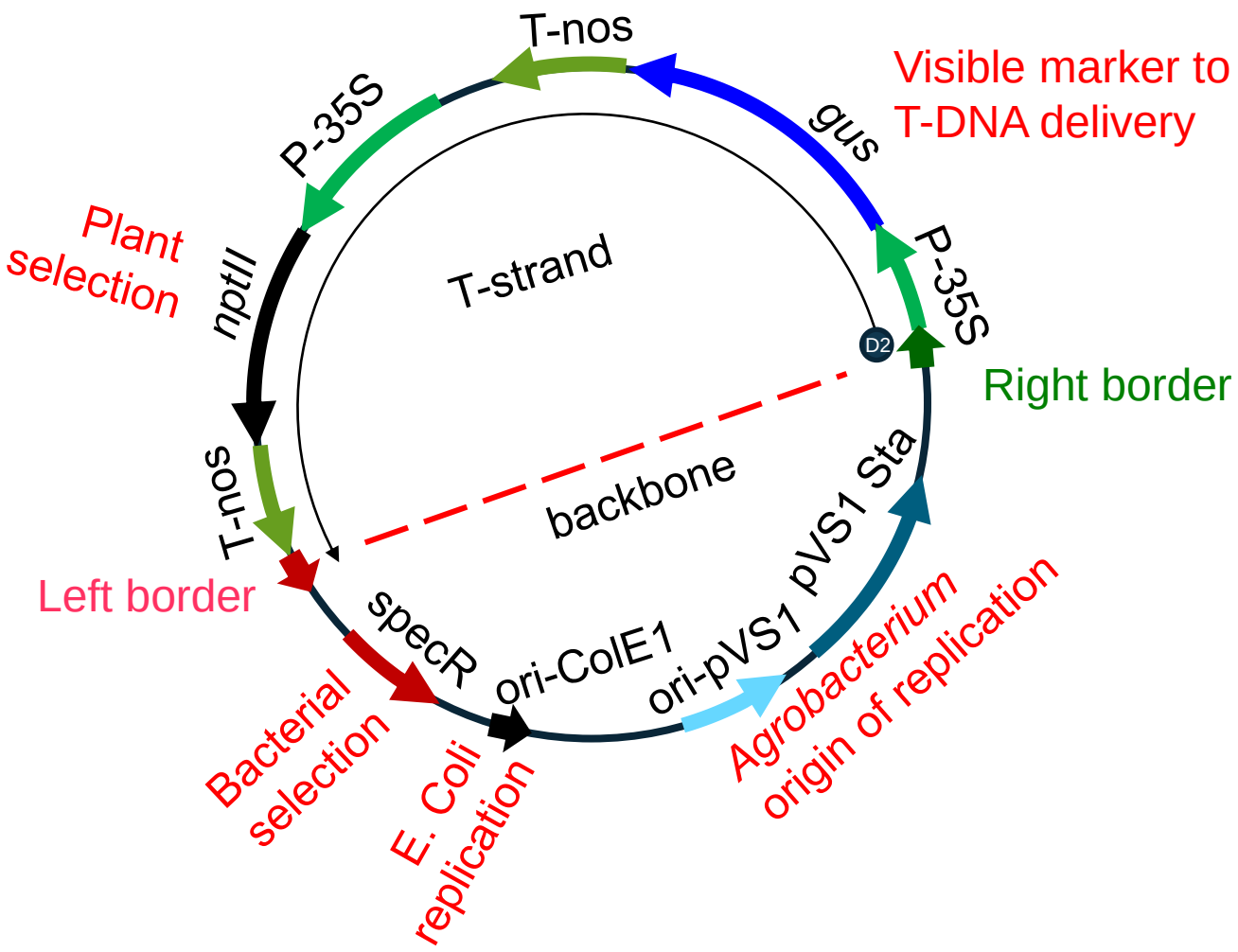
- Smaller than *Agrobacterium* binary vectors
- Single origin of replication, high copy for higher yield
- May need to release the GOI by restriction enzymes and HPLC purify the gene fragment for product development

Binary vectors for Agrobacterium-mediated transformation

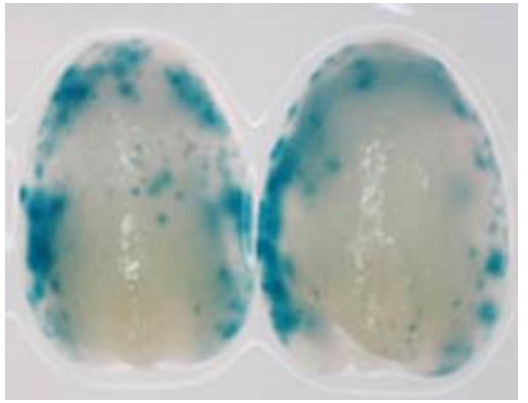
Free vector in Agrobacterium, replicate in E. coli and Agrobacterium



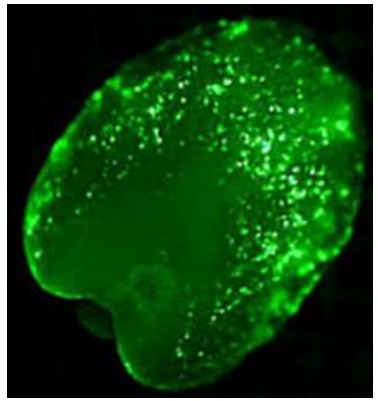
A typical binary vector for Agrobacterium-mediated transformation



Destructive assay



GFP transient expression



[Plant development inhibitory genes in binary vector backbone improve quality event efficiency in soybean transformation | SpringerLink]

[Enhanced production of single copy backbone-free transgenic plants in multiple crop species using binary vectors with a pRi replication origin in Agrobacterium tumefaciens | SpringerLink]

Frequently used selectable marker genes

Selectable marker gene	Selection reagent	Comments
nptII	Kanamycin, G418, paromomycin	Antibiotic, lethal
epsps-CP4	glyphosate	Herbicide, lethal
bar, pat	Glufosinate, phosphinothricin	Herbicide, lethal
hpt	hygromycin	Antibiotic, lethal
PMI (manA)	mannose	Non-lethal
aadA	Spectinomycin, streptomycin	Antibiotic, non-lethal

Selectable marker genes in transgenic plants: applications, alternatives and biosafety - ScienceDirect

Introduction to cloning methods

The bible of cloning:

Molecular Cloning: A Laboratory Manual (Fourth Edition) (cshlpress.com)

1) Traditional cloning method by ligation of compatible fragments with T4 DNA ligase

2) Golden-gate cloning for multiple fragment ligation using type IIs restriction enzymes

3) Assemble multiple fragments by fusion with short homologue ends
(Gibson assembly, hot fusion, In-fusion etc.)

Restriction enzymes

https://www.pnas.org/content/102/17/5905
How restriction enzymes became the workhorses of molecular biology

https://www.nature.com/scitable/topicpage/restriction-enzymes-545/
Restriction Enzymes

https://www.britannica.com/science/restriction-enzyme

Restriction enzymes (ppt)
https://capricorn.bc.edu › 11-Restriction-mapping

```
5′  GAATTC  3′
3′  CTTAAG  5′      EcoRI

5′  GGTACC  3′
3′  CCATGG  5′      KpnI

5′  AGGCCT  3′
3′  TCCGGA  5′      StuI
```

Purchase restriction enzymes
www.NEB.com
https://www.thermofisher.com/

Read user manual for instruction
No double dip when take RE

Different REs may have different requirement:

- Buffer (salts, pH)
- Temperature (37, 25, 55, 65 ^{o}C)
- Double digestion consideration
- DNA methylation
  - Dam (GATC) (e.g. XbaI)
  - Dcm (CCAGG and CCTGG)
  - Dam- E coli is available for Dam- DNA (SCS110, INV110)

https://blog.addgene.org/plasmids-101-methylation-and-restriction-enzymes

***Cloning method 1**: restriction enzyme digestion and ligation*

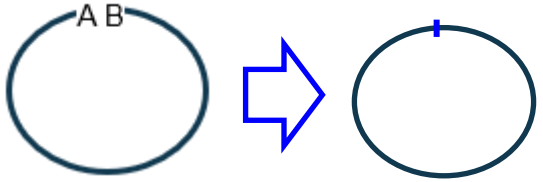
Considerations for ligation-based cloning

- Compatible cut ends between fragments, if can't find, need to blunt the fragment ends by T4 DNA polymerase

- Prevent vector backbone self-ligation
  - if vector cut ends are compatible, dephosphorylate the vector fragment (e.g. CIP, or Shrimp phosphatase)
  - select 2 incompatible restriction cut for cloning to avoid

- Always purify fragments by electrophoresis

- Adjust vector fragment/insert ration to **1:2** or **1:3**



- DNA loads: for 15-25 kb vector backbone, use 50~100 ng/20 μl reaction volume, adjust insert in proportion
  - 3~15 kb 5~50 μg/20 μl reaction volume
  - Blunt end ligations use higher end concentration
  - Stick end ligations use lower end concentration
- Ligation at 16 °C overnight is recommended

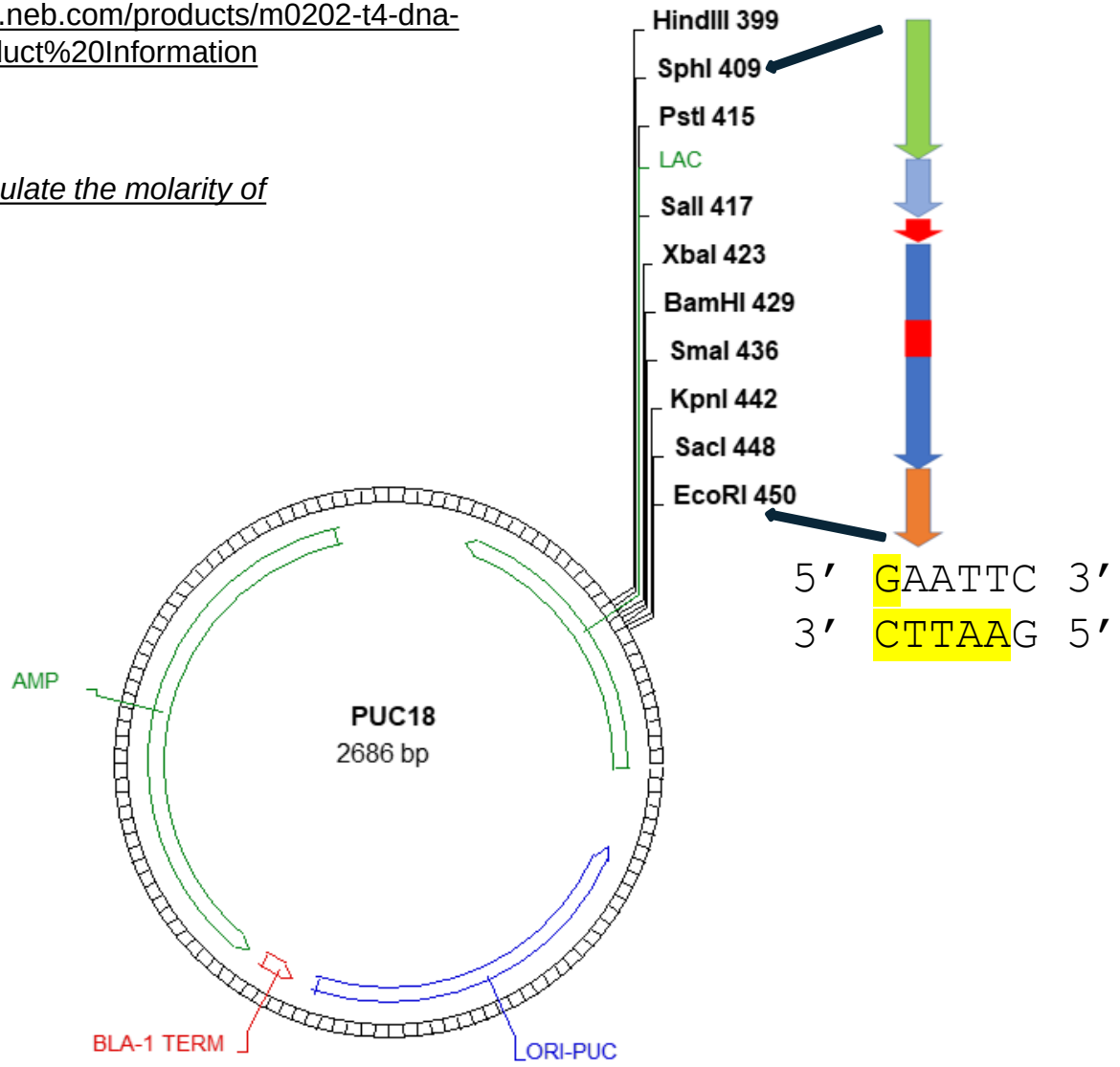
EcoRI	*EcoRI* *EcoRI*	*EcoRI* *KpnI*	*KpnI*
5′ GAATTC 3′ 3′ CTTAAG 5′	5′ p-AATTC —— G-OH 3′ 3′ HO-G —— CTTA-p 5′	5′ p-AATTC —— GGTAC-OH 3′ 3′ HO-G —— C-p 5′	5′ GGTACC 3′ 3′ CCATGG 5′

T4 DNA ligase ligates compatible DNA end together

5′ GCATGC 3′
3′ CGTACG 5′

https://www.neb.com/products/m0202-t4-dna-ligase#Product%20Information

How to calculate the molarity of DNA ends

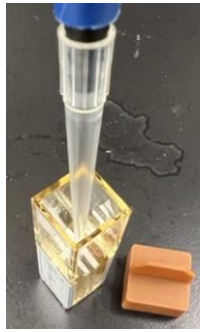


5′ GAATTC 3′
3′ CTTAAG 5′

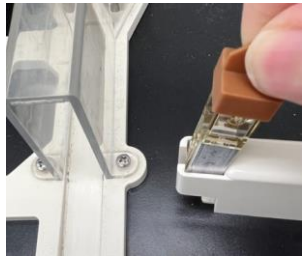
1) Digest 2 µg pUC18 with SphI/EcoRI in 50 µl volume
2) Digest the insert DNA template with the same enzymes (2~5 µg/50 µl)
3) Fractionate digested plasmids in 1% Agarose gel
4) Excise the expected size bands with a scalpel over UV light box or Darkbox
5) Use commercial kit to dissolve the Agarose and purify the DNA fragments
6) Elute the DNA with water
7) Measure the DNA concentration
8) Prepare ligation reaction:
    1) 2 µl 10x T4 ligase buffer
    2) 5 ng pUC18 *EcoRI* / *SphI*
    3) 2x or 3x end moles of the insert
    4) Add H2O to final 19 µl
    5) Add 1 µl T4 DNA ligase
    6) Mix well with pipet, incubate at 16 ^{o}C overnight

Transfer the ligate into E coli competent cells

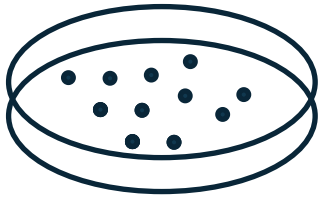
https://blog.addgene.org/plasmids-101-common-lab-e-coli-strains?gclid=EAIaIQobChMIu_-bkeDi7gIVCjiGCh0lLA-nEAAYASAAEgLy2_D_BwE

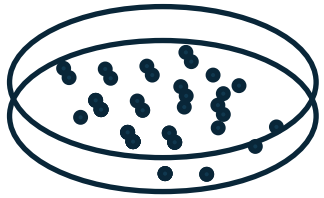


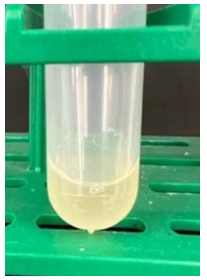
DH10B is the most common strain used by us
ElectroMAX™ DH10B Cells
https://www.thermofisher.com/order/catalog/product/18290015#/18290015



Electroporation of plasmid into the competent cells	
	1 ul ligate into 20 ul aliquot of ice-thawed competent cell in an Eppendorf tube
	Transfer into 1 mm gap BioRad cuvette
	Tap on surface to be sure the drop is spread in the cuvette (prevent spark)
	Set up 1.8 kv
	Press pulse button
	Add 500-1000 ul SOC, transfer into 14-ml tube
	shake for 45-1 hr
	Take 10 ul and 200 ul and spread into 2 plates with corresponding plasmid antibiotic
	Reverse plates, culture at 37 ^{o}C overnight



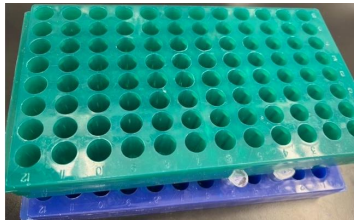


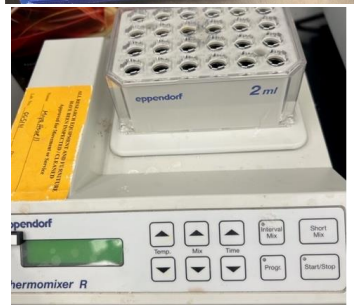


Plasmid minipreparation (alkaline lysis)

Aliquot 2 ml LB with corresponding antibiotic (kan 50 or spec 50 mg/L) for same day prep at 8~9 a.m.,
or 3 ~5 ml at 4~5 p.m. for overnight culture, in 14 ml round bottom tubes

- Pick a well separated colony with a toothpick tip, throw it into media, 8~12 individual colonies/construct
- Shake at 37 ^{o}C for 5-6 hrs (2 ml same day) or overnight (3~5 ml)
- Pour ~1.5 ml into an Eppendorf tube carefully, closed
- Spin 10~30 seconds at maximum speed
- Remove supernatant with a tip connected to a vacuum flask
- Add 200 ul P1 (with RNase added, Qiagen maxipep kit) or individual order. Vortex to suspend
- Add 200 ul P2 to lysate bacterial cells
- Add 200 ul P3 and close lid, sandwich with racks and shake to neutralize the lysates
- Spin 1~3 min at maximal speed
- Transfer the supernatants into fresh Eppendorf tubes (pre-filled with 600 ul isopropanol)
- Close lids, sandwich with racks, shake to mix well
- Spin for 5~10 min at maximum speed
- Remove the supernatants carefully with vacuumed tip without touching the pellets
- Add 1.5 ml 70% ethanol, remove with tip right away to reduce salt residues
- Dried by SpeedVac for 1-3 min, or leave open at room temperature for 30 min or overnight
- Resuspend in 50~100 ul pure water.





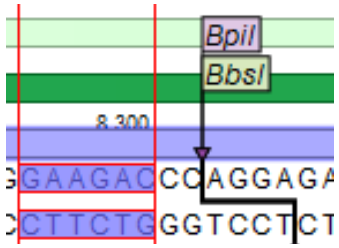
The quality is good for subcloning or direct sequencing

Miniprep kits available from Qiagen or Promega
QIAprep Spin Miniprep Kit

Cloning method 2: Golden Gate cloning

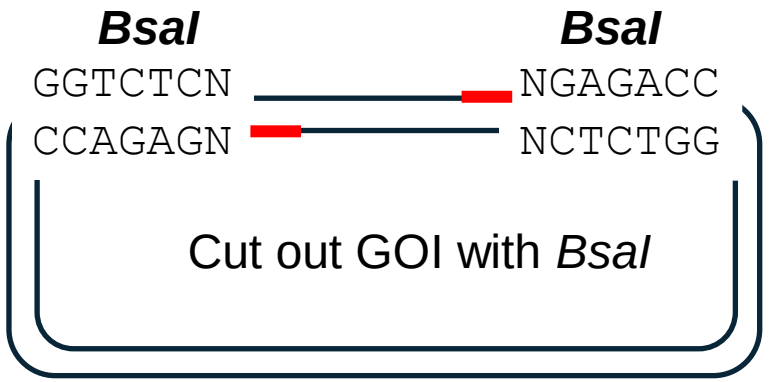
Type IIS restriction enzymes are unique from "traditional" restriction enzymes in that they cleave outside of their recognition sequence, creating four base flanking overhangs

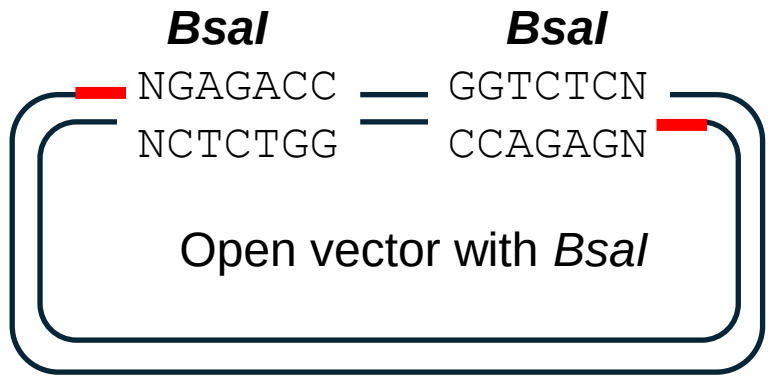
```
BbsI    gaagac12|3456
(BpiI)  cttctg123|456
```



BsaI
5′-GGTCTCN
3′-CCAGAGNNNNN-5′

SapI
5′-GCTCTTCN
3′-CGAGAAGNNN-5′





GGTCTCN**GGAG** AATGNGAGACC
CCAGAGNCCTC **TTAC**NCTCTGG

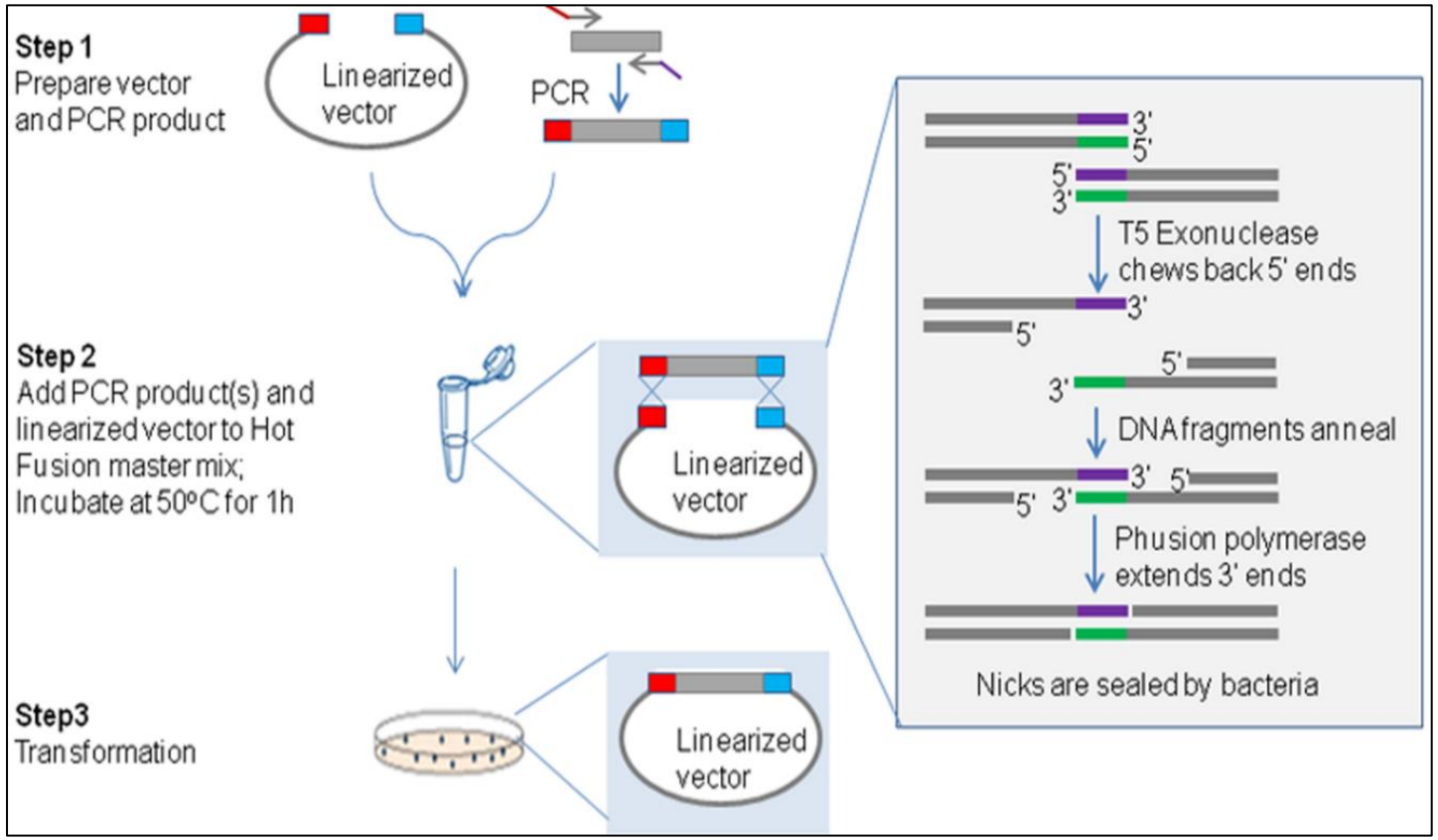
GGTCTCN**AATG** GCTTNGAGACC
CCAGAGNTTAC **CGAA**NCTCTGG

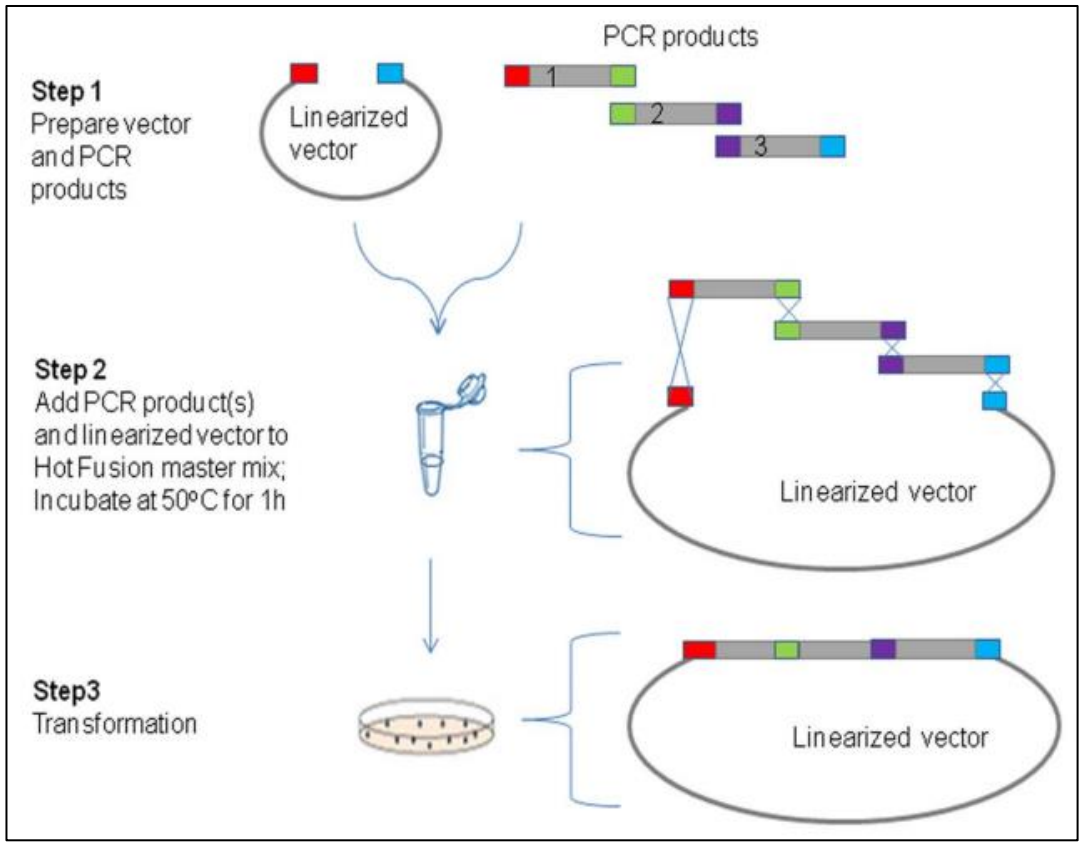
GGAG **AATG** **GCTT**
TTAC **CGAA** **TCAG**

GGAG **AATG** **GCTT**
TTAC **CGAA** **TCAG**

A User's Guide to Golden Gate Cloning Methods and Standards | ACS Synthetic Biology (Review, 2022)

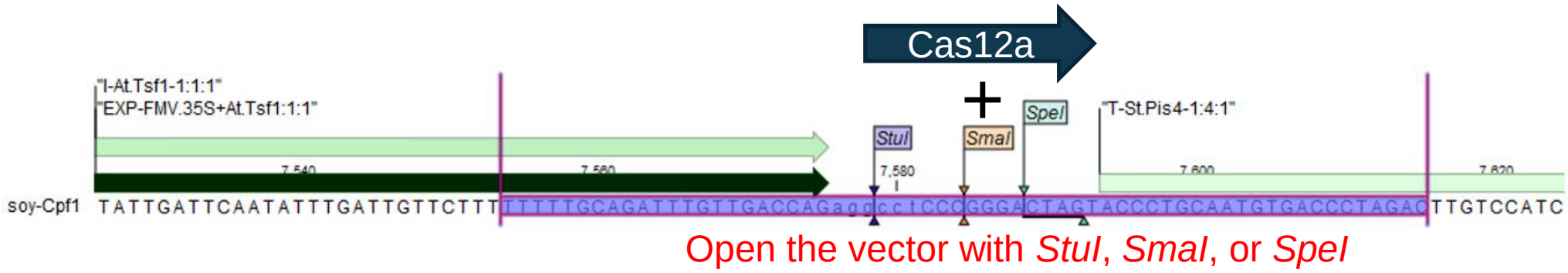
Cloning method 3: Gibson assembly or hot fusion





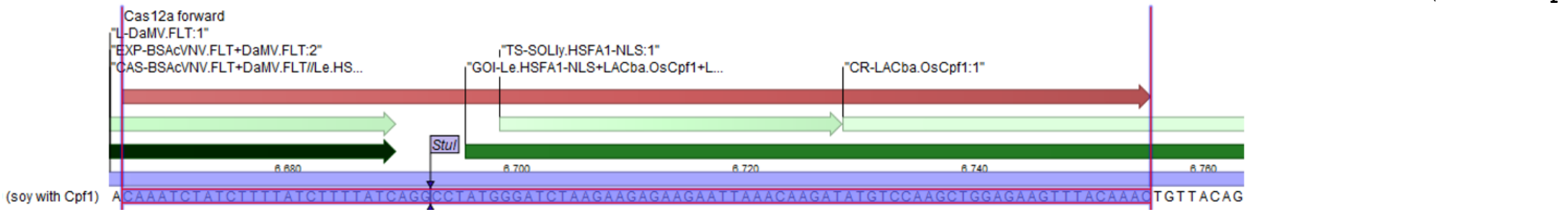
Gibson assembly https://doi.org/10.1126/science.1190719
Hot fusion https://doi.org/10.1371/journal.pone.0115318

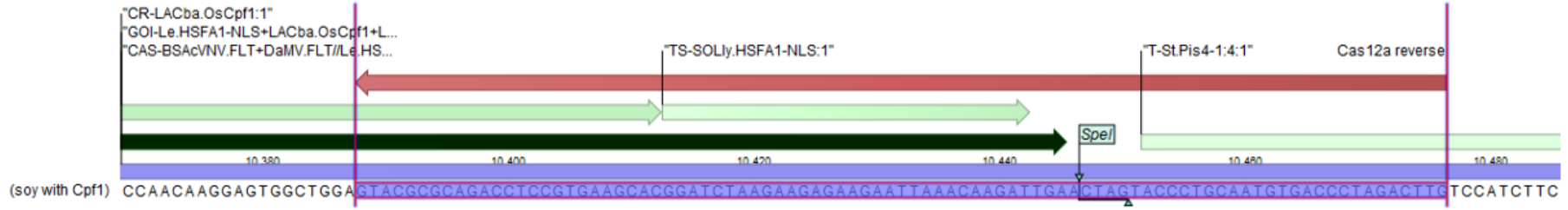
Primer design example for Gibson assembly or hot fusion



Open the vector with *StuI*, *SmaI*, or *SpeI*

5′ CAAATCTATCTTTTATCTTTTATC**AGGCCT**ATGGGATCTAAGAAGAGAAGAATTAAACAAGAT**ATG**TCCAAGCTGGAGAAGTTTACAAAC 3′ (**forward primers**)





5′ CAAGTCTAGGGTCACATTGCAGGGT**ACTAGT**TCAATCTTGTTTAATTCTTCTCTTCTTAGATCCGTGCTTCACGGAGGTCTGCGCGTAC 3′ (**reverse primers**)

Use 1~100 ng template DNA, amplify the 3.8 kb fragment for 25~30 cycles, gel purified. Mix 2 fragment at 1:2~3 ratio for fusion

Cloning by hot fusion method

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0115318

- Open recipient vector with restriction enzymes
- Prepare insertion fragment with PCR amplification which should have 20-25 bp overlap sequence in the vector
- Measure DNA concentration by Nanodrop
- Calculate DNA end mole (calculator is available from NEB or Promega)

- Vector DNA 30-60 ng (~20 kb) + insert = 1:2 OR 1:3 end concentration in 10 ul
- Add 10 ul 2x hot fusion buffer, mix
- Place on PCR machine to complete the reaction cycles (50 ^{o}C for 1 hr, 20 ^{o}C for 10 min, 4 ^{o}C forever)
- Transfer 1 μl into 20 μl *E coli* competent cells on ice
- Electroporation
- Shake 1 hr
- Plate
- 37 ^{o}C overnight
- Pick single colonies
- Miniprep
- Sequencing verify

How to calculate the molarity of ends

https://nebiocalculator.neb.com/#!/ligation

https://www.promega.com/resources/tools/biomath/

Example to calculate end molarity

DNA	Size (kb)	Weight (ng)
Vector	15	60
Fragment 1	6	72
Fragment 2	1	12

required mass insert (g) = desired insert/vector molar ratio x mass of vector (g) x ratio of insert to vector lengths

6/15 x 60 x3=72 ng (1:3 ratio)

1/15 x 60 x3=12 ng (1:3 ratio)

A typical flowchart for vector construction

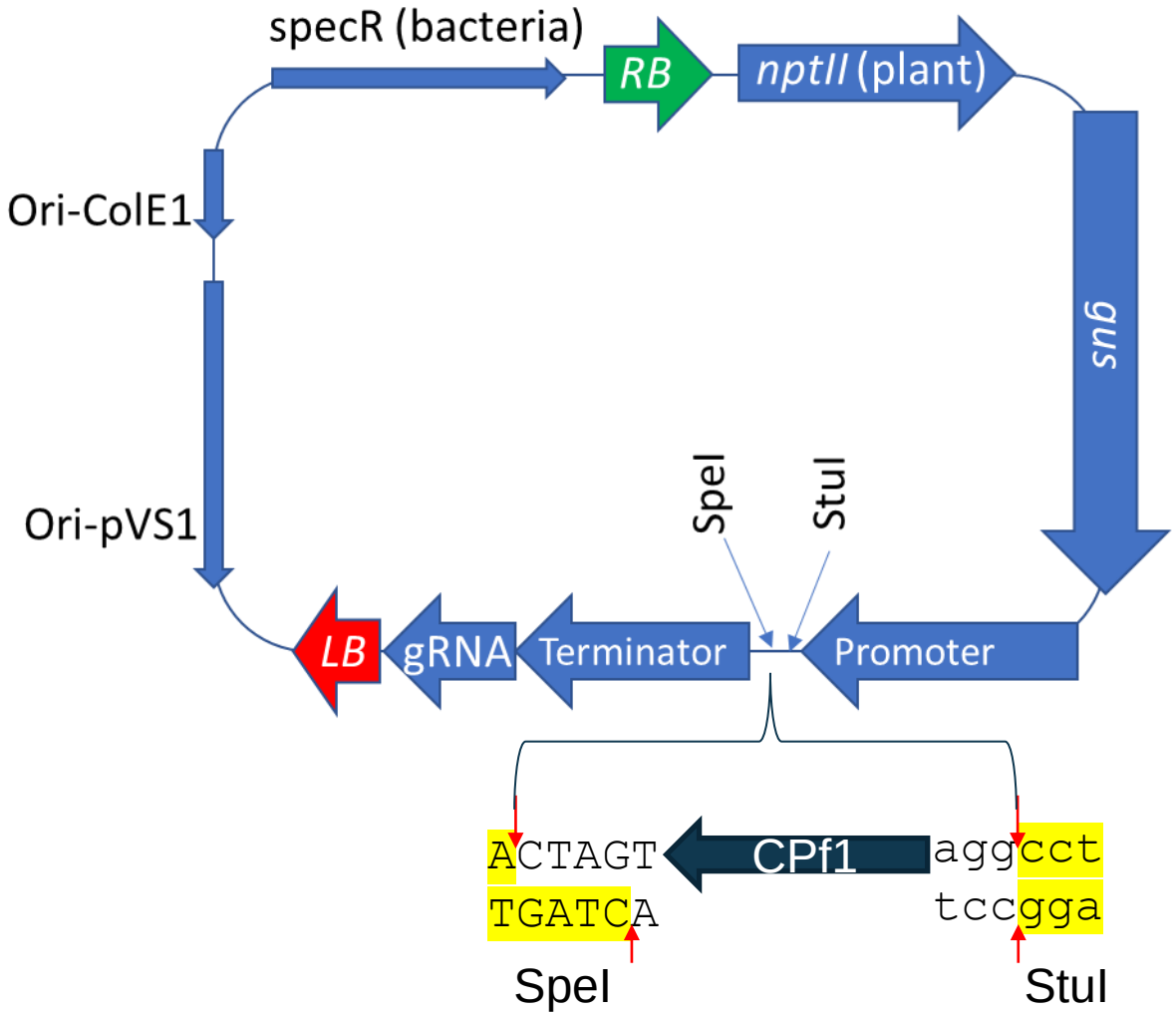
- Design vector map
- Request base vector and templates
- Primer design and order
- Digest base vector, gel purify, measure concentration
- PCR insert fragments or restrict cut out fragments to be cloned
- Gel purify fragments, measure concentration
- Hot fusion PCR fragments with the base vector fragment
- Electroporate 1 μl to *E. coli* competent cells
- Plate overnight
- Pick 8~12 single colonies, miniprep
- Sequence verification
- Re-prep a verified clone/construct
- Archive the plasmid
- Register workplan for plant transformation
- Design molecular assay (copy #, backbone, edits, etc.)
- Data collection

Protocol training:

Cpf1 fragment cloning into an Agrobacterium binary vector

Xudong Ye

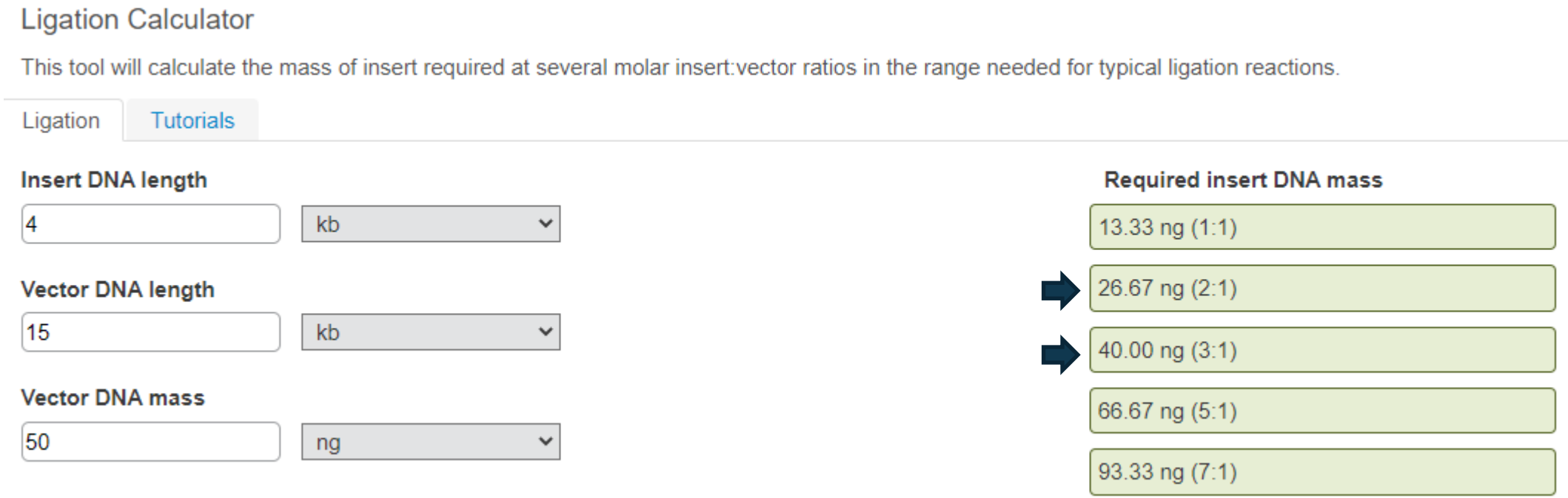
Corn and soybean base vector for LbCpf1 cloning



Cpf1 is synthesized

How to calculate the molarity of DNA ends

The best ratio for insert 1 fragment in a vector may be 1:2 or 1:3



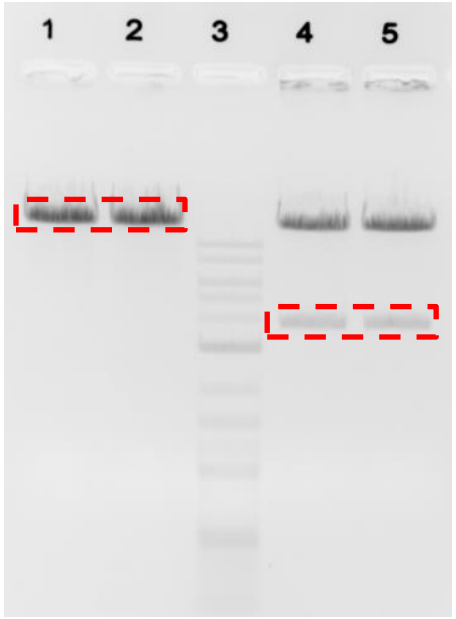
Vector SpeI/StuI: ~15 kb
Cpf1 fragment: ~4 kb

Recommend using 50~100 ng/20 μl reaction volume of vector backbone fragment when cloning a 15~25 kb vector

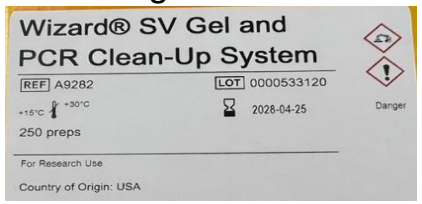
Digestion and ligation mixtures

SpeI/StuI	Backbone size	Cpf1 size	
Base vector	14.3 kb	--	
Cpf1 plasmid	14.3 kb	3.75kb	

SpeI/StuI 1 hr 37 ^{O}C, run gel



Cut gel, purify with Promega column



4 μg/50 μl Base vector

5 μg/50 μl Cpf1 plasmid

Yield: 30 ng/μl in 75 μl =2.25 ug (recovery rate =56% (2.25 μl /4 μg)

Backbone fragment 25 ng/ μl in 75 μl =1.87 μg

Cpf1 fragment 6.8 ng/ μl in 75 ul=0.51 μg

How to calculate the molarity of ends

(Cpf1 fragment size proportion: 3.75 kb/14.3 kb=0.26)

Example: 1:2 ratio: 60 ng vector backbone : 31.2 ng (60x0.26 (3.75/14.3 kb) x2) Cpf1 fragment

1:3 ratio: 60 ng vector backbone : 46.8 ng (60x0.26 x3) Cpf1 fragment

Ligation in a 1.5 ml Eppendorf tube or 200 μl PCR tube:

1) 2 μl (x 30 ng) Base vector SpeI/StuI in **20 μl**, control
2) 5 μl (x 6.8 ng) *Cpf1* fragment SpeI/StuI (~34 ng) in 20 μl (~1:2) OR
3) 7 μl (x 6.8 ng) *Cpf1* fragment SpeI/StuI (~48 ng) in 20 μl (~1:3)
4) 2 μl 10x T4 DNA ligase buffer
5) 1 μl 10x T4 DNA ligase water to final 20 μl

Ligate overnight, in a centrifuge setting at 16^{o}C OR use PCR tube for reaction overnight in a PCR machine

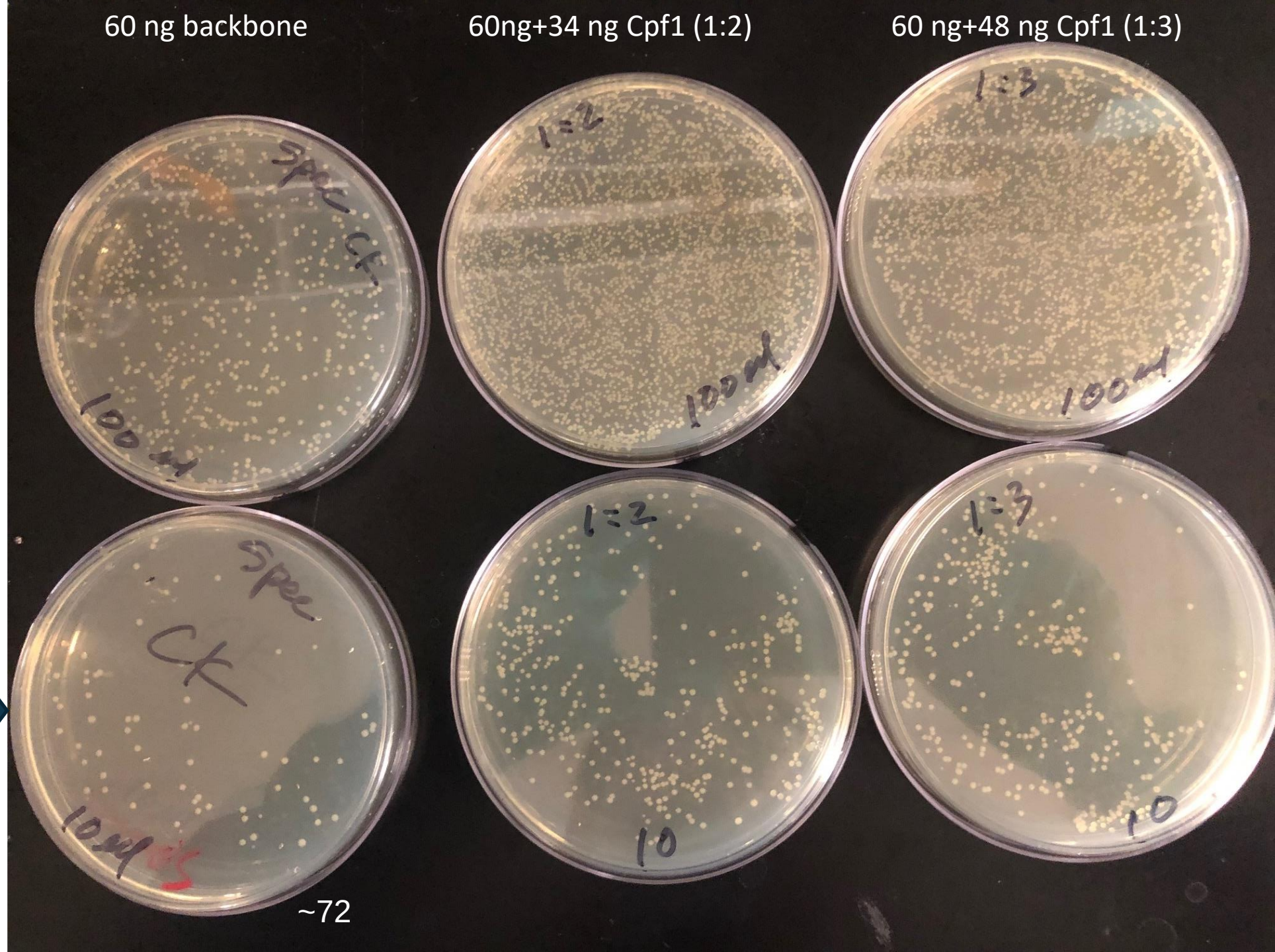
1 μl is transferred into 20 μl DH10B cells with electroporation, add 1 ml SOC, shake 1 hr at 37^{o}C

plate 20 and 200 μl into 2 plates, respectively.

Forgot in shaker for >3 hours before plating, therefore, plate less volume to avoid crowdy colonies

Upper plates: 100 ul
Lower plates: 10 ul

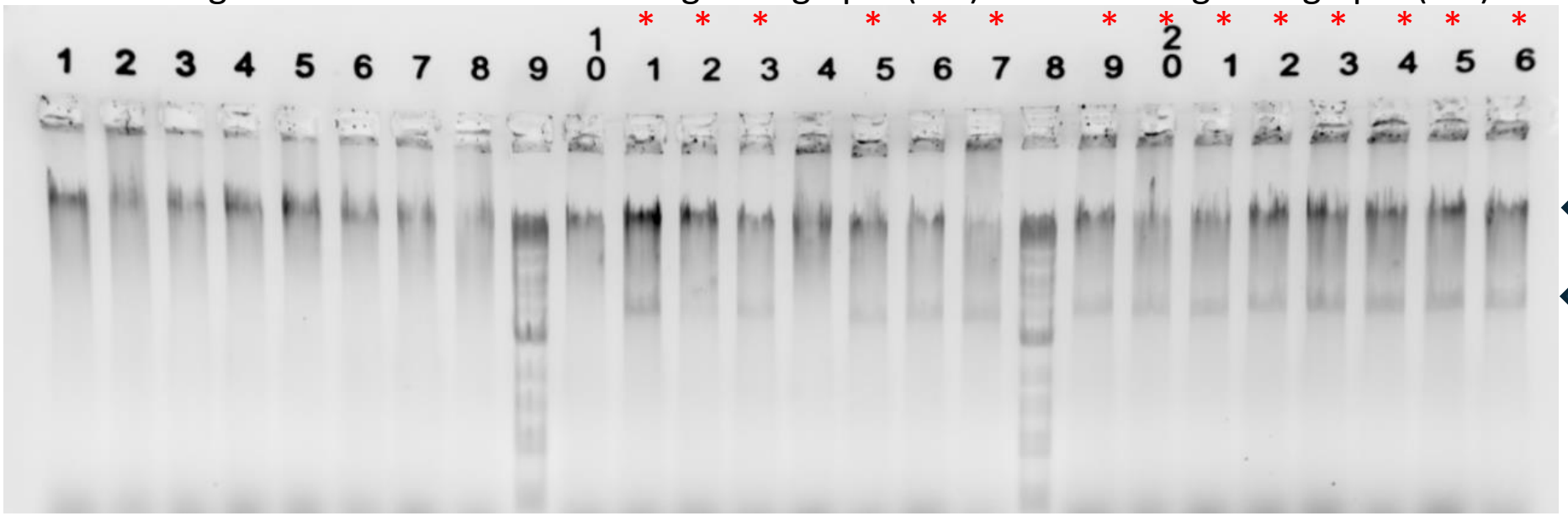
Longer digestion or CIP treat backbone fragment may decrease background but not necessary



Checking 8 colonies for Cpf1 inserts with StuI/SpeI digestion

8 colonies in 2 ml LB with spec 75 mg/L, shook for 6 hrs, pour into 1.5 ml tube, miniprepped with 200 ul P1+P2+P3, wash with 70% EtOH, dried and suspended in 100 ul water. Take 10 ul suspension, digest with StuI/SpeI in 20 ul final volume for 30 min

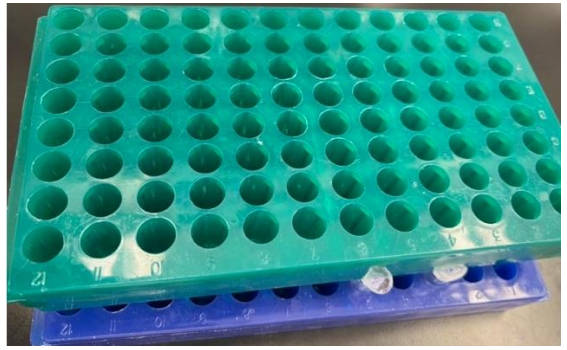
60 ng backbone | 60ng+34 ng Cpf1 (1:2) | 60 ng+48 ng Cpf1 (1:3)



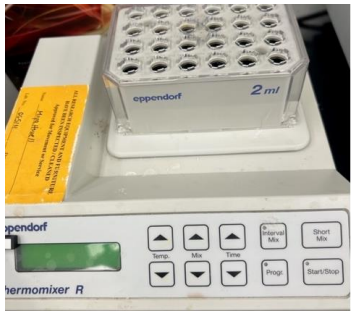
Sheared *E. coli* genomic DNA because of using the mixer

3.7 kb Cpf1 *StuI/SpeI*

Mixture method



Invert the sandwiched plates 3~6 times before centrifuge



Good for suspending pellets

Often too much for mixing, shearing DNA

What's next

- Bulk up plasmid DNA by inoculating the verified clones in large volume of LB medium with 75 mg/L spec selection
- Purify Plasmid DNA using commercial kit
- Suspend in water or 1x TE buffer, measure DNA concentration
- **Submit samples for sequence verification**
- Transfer the plasmid into *Agrobacterium* competent cells for plant transformation

Introduction to Agrobacterium

Outline

Agrobacterium tumefaciens and crown gall disease

Discover bacterium pathogenicity

Bacteria-free tumors

Ti plasmid discovery

Virulence gene induction

The mechanism: Type IV secretion system (T4SS) and T-strand transfer

Agrobacterium as a vector for plant transformation

Breakthroughs in plant genetic engineering

Disarmed *Agrobacterium* strains

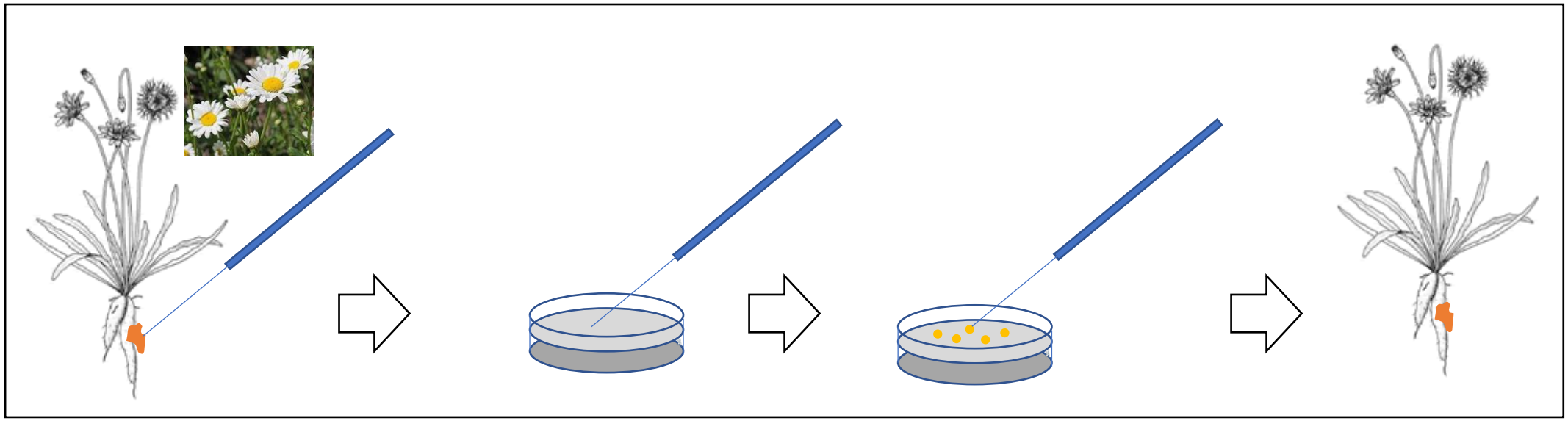
Co-integration vector, binary vector, 2T-DNA vector

Discover crown gall disease and Agrobacterium

- The first written record of crown gall disease, on grape, dates from 1853
  Over-growths at wound sites and severely limits crop yields and growth vigor

- Fridiano Cavara (1897) found that a bacterium causes crown gall in grape

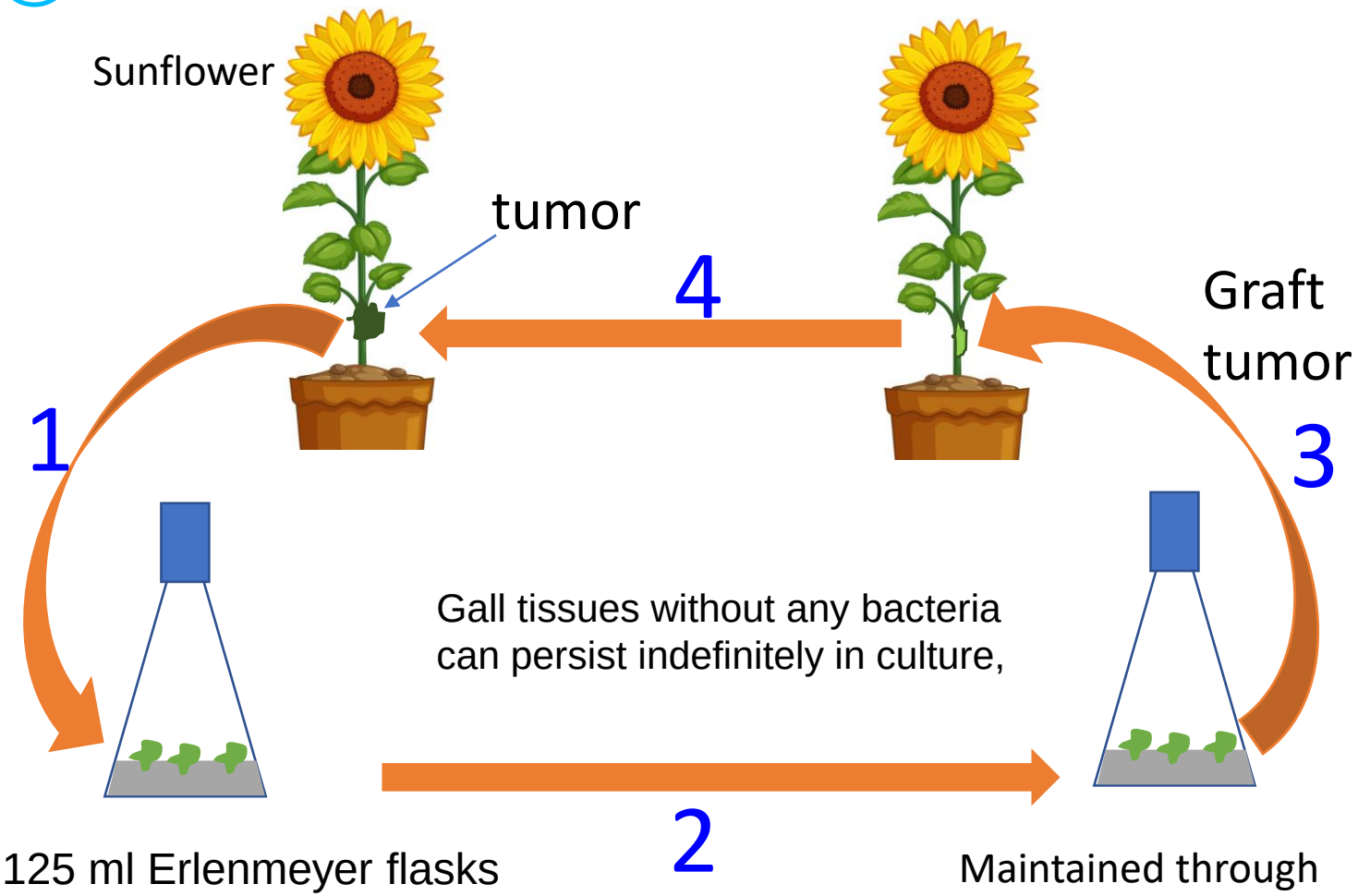
- 1907 - Erwin Smith and C.O. Townsend isolated a bacterium from galls on daisy. When inoculated the bacterium onto healthy plants, galls were reproduced



Smith, E.F. and Townsend, C.O. (1907). A plant-tumor of bacterial origin. Science. 25: 671-673.

Crown gall production by bacteria-free tumor tissues

White, P.R. and Braun, A.C. (**1941**). Crown gall production by bacteria-free tumor tissues. Science. 94**: 239-241**;



Armin C. Braun
1911 - 1986

125 ml Erlenmeyer flasks
50 ml of White's standard glycine thiamin nutrient 4 solidified with 0.6% agar.

Maintained through
13 successive passages

Braun made fundamental discoveries about how *Agrobacterium* transforms plant cells

Braun, A.C. (1958) A physiological basis for autonomous growth of the crown-gall tumor cell. Proc Natl Acad Sci U S A. 44: 344–349.

(Photo from Wood, H.N., and Kelman, A. (1987) Phytopathology 77: 991.)

A few days after inoculation, tumors become independent of Agrobacteria

Periwinkle stem inoculated by *Agrobacterium tumefaciens* at RT for a few days, followed by 5 days at 47°C to kill the bacteria

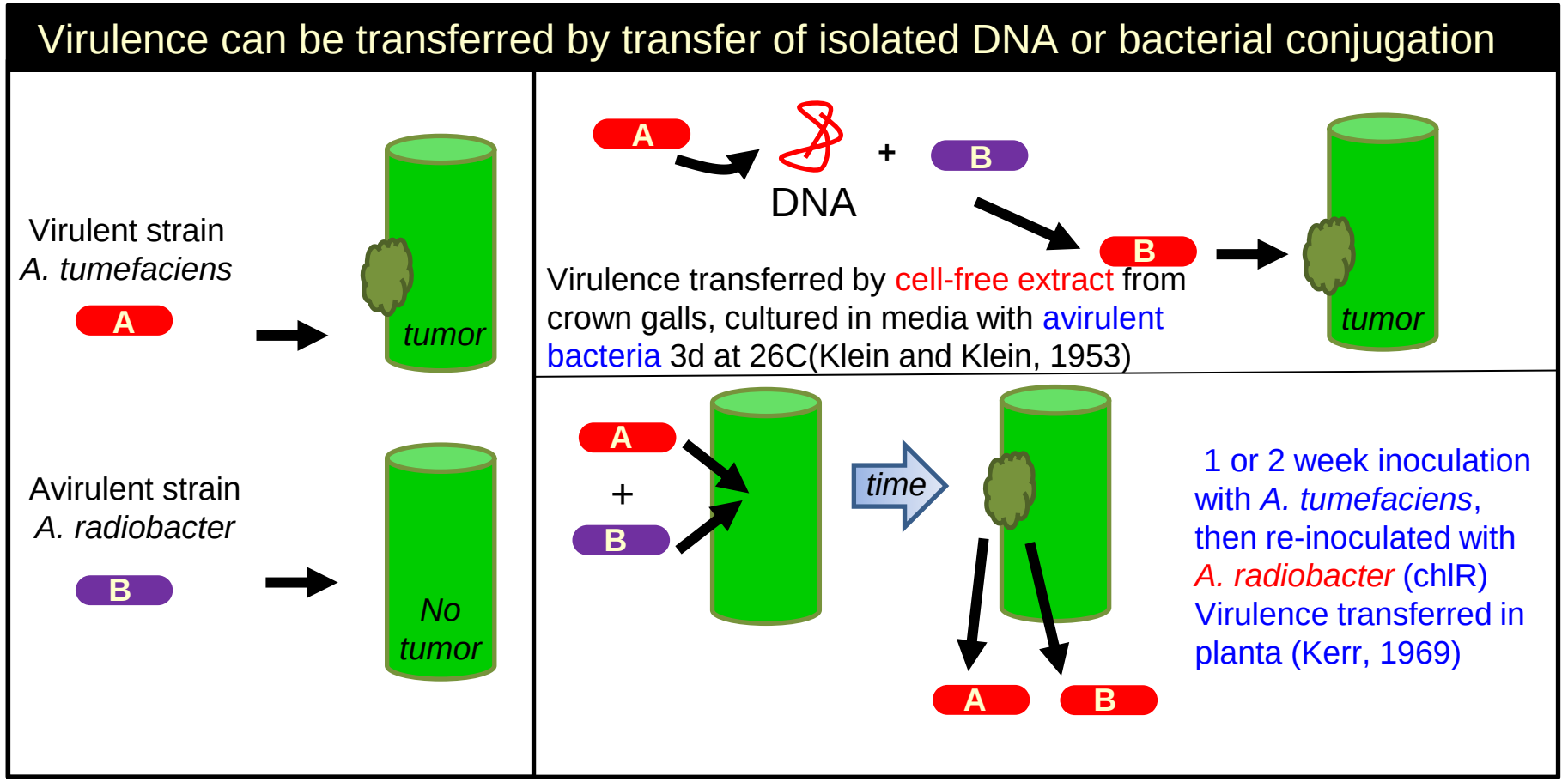
When the tissue was incubated at RT for ***one* day** before heat-killing the bacteria, no tumors were formed

When the tissue was incubated at room temperature for ***four* days** before heat-killing the bacteria, many tumors were formed

Conclusion: Viable bacteria are no longer necessary beyond two days post-inoculation. After this period, tumors become independent of the bacteria, because the bacteria have altered the host cells, by transferring some ***factors*** into them.

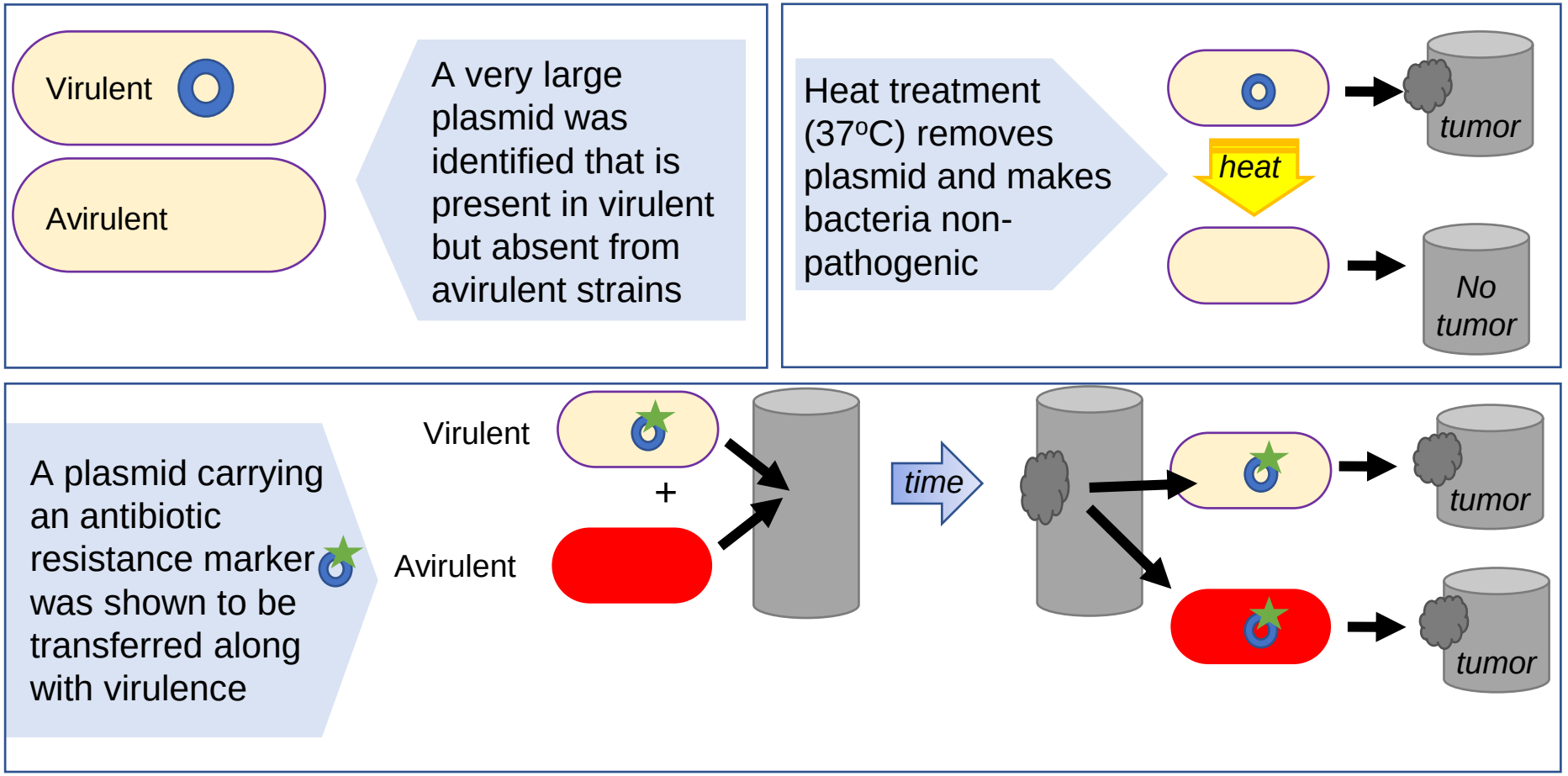
Braun, A.C. (**1943**) Studies on tumor inception in the crown-gall disease. Am. J. Bot. 30: 674-677

Virulence can be transferred from one Agrobacterium to another



Klein, D.T. and Klein, R.M. (1953). Transmittance of tumor-inducing ability to avirulent crown-gall and related bacteria. J. Bacteriol. 66: 220-228.;
Kerr, A. (1969). transfer of virulence between isolates of Agrobacterium. Nature. 223: 1175-1176.

A large plasmid in gall-inducing Agrobacterium transfers virulence in 1974



Zaenen, I., van Larebeke, N., Teuchy, H., van Montagu, M. and Schell, J. (1974). **Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains**. Journal of Molecular Biology. 86: 109-127.

Larebeke, N.V., Engler, G., Holsters, M., Den Elsacker, S.V., Zaenen, I., Schilperoort, R.A. and Schell, J. (1974). Large plasmid in *Agrobacterium tumefaciens* essential for crown gall-inducing ability. Nature. 252: 169-170.

Van Larebeke, N., Genetello, C.H., Schell, J., Schilperoort, R.A., Hermans, A.K., Hernalsteens, J.P. and Van Montagu, M. (1975). Acquisition of tumour-inducing ability by non-oncogenic agrobacteria as a result of plasmid transfer. Nature. 255: 742-743.

T-DNA transfer from the Ti plasmid into the plant cells (1977)

*Sma*I digestion

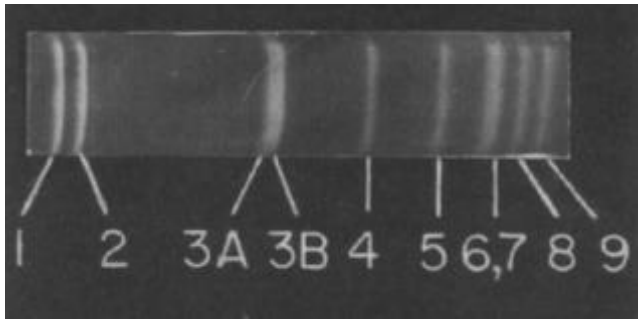
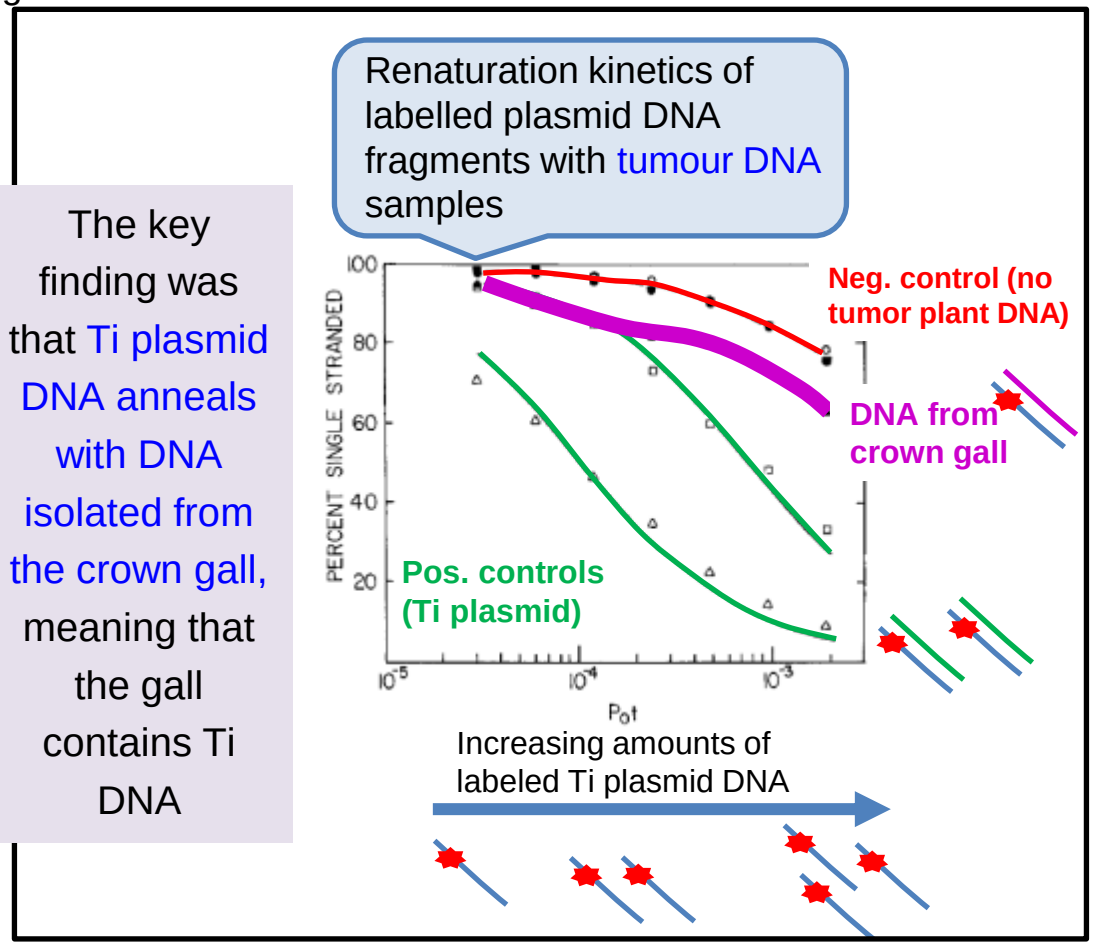


Figure 7. Preparative Horizontal Agarose Slab Gel Electrophoretic Separation of A277 Plasmid Fragments A277 plasmid was cleaved with *Sma* I

Figure 5. Renaturation Kinetics of ***A. tumefaciens*** A277 Plasmid *Sma*I Digest Band 3 DNA in the Presence of Tumor DNA and Control DNAs

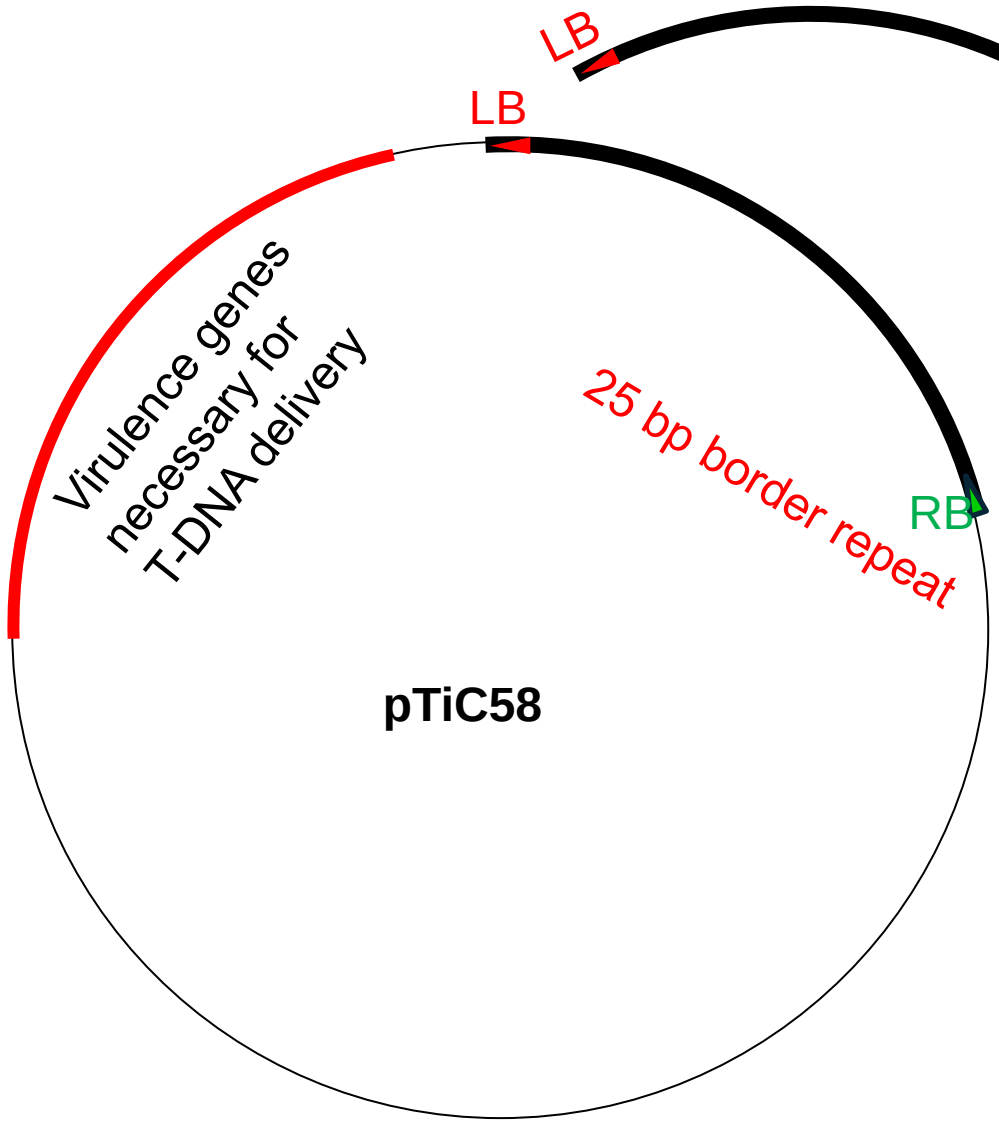
The key finding was that Ti plasmid DNA anneals with DNA isolated from the crown gall, meaning that the gall contains Ti DNA

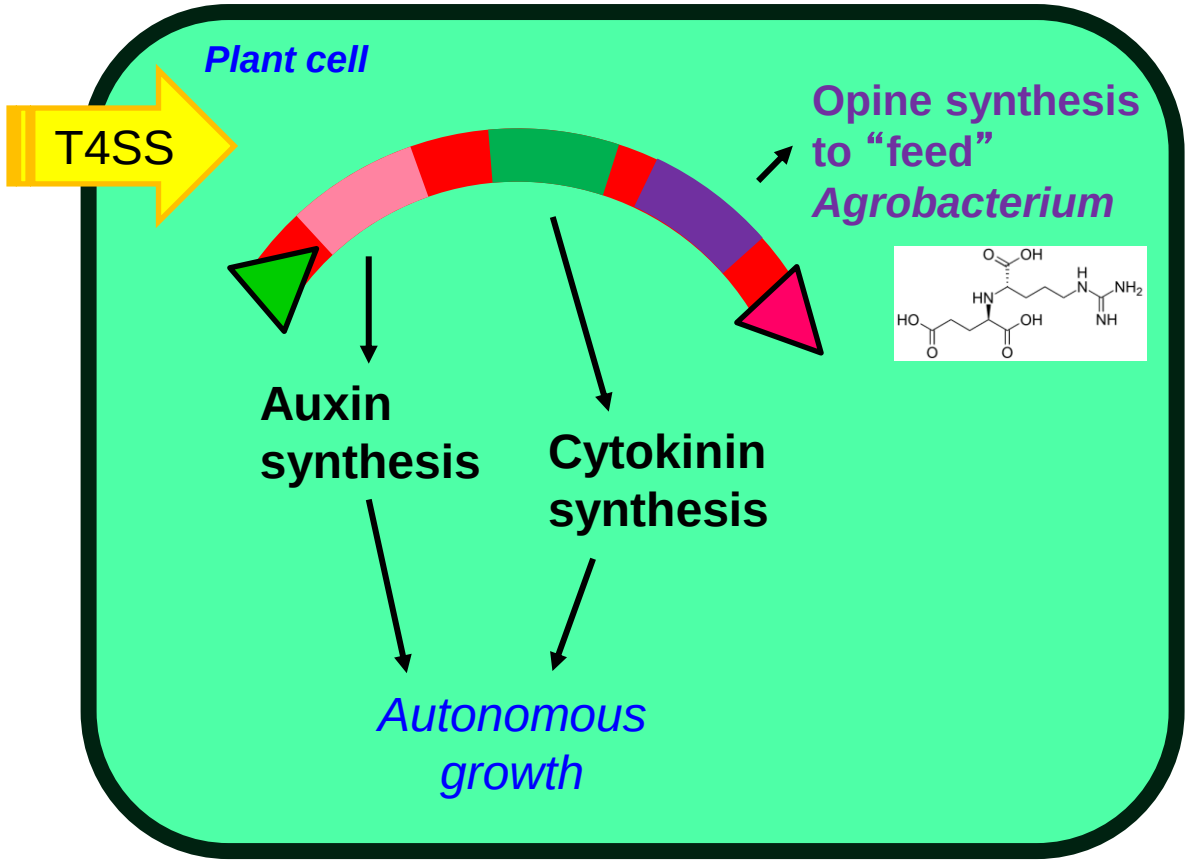


- Extract tumor DNA
- Hybridize with Ti plasmid

Chilton, M.-D., Drummond, M.H., Merlo, D.J., Sciaky, D., Montoya, A.L., Gordon, M.P. and **Nester, E.W.** (**1977**). **Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis**. Cell. 11: 263-271.
Yadav, N.S., Postle, K., Saiki, R.K., Thomashow, M.F. and **Chilton, M.D**. (1980). T-DNA of a crown gall teratoma is covalently joined to host plant DNA. Nature**.** 287: 458-461.

Tumor-inducing and opine synthesis genes on T-DNA imported into plant cells (1977-1985)



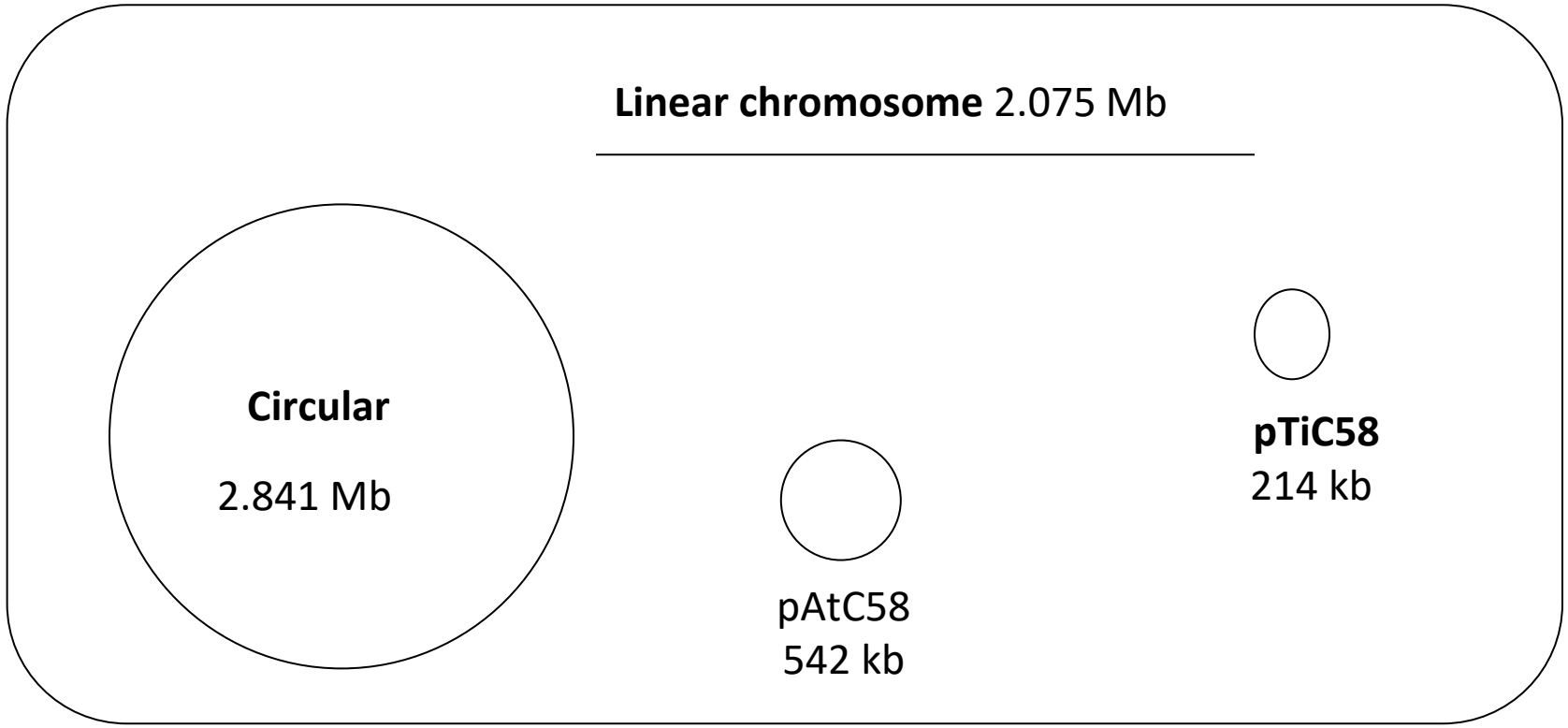


T4SS = Type IV Secretion System

Wang K, Herrera-Estrella L, Van Montagu M, Zambryski P (1982) Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from agrobacterium to the plant genome. Cell. 38(2):455-62.

Toothman P. (1982) Octopine Accumulation Early in Crown Gall Development is Progressive. Plant Physiol. 69(1):214-9.

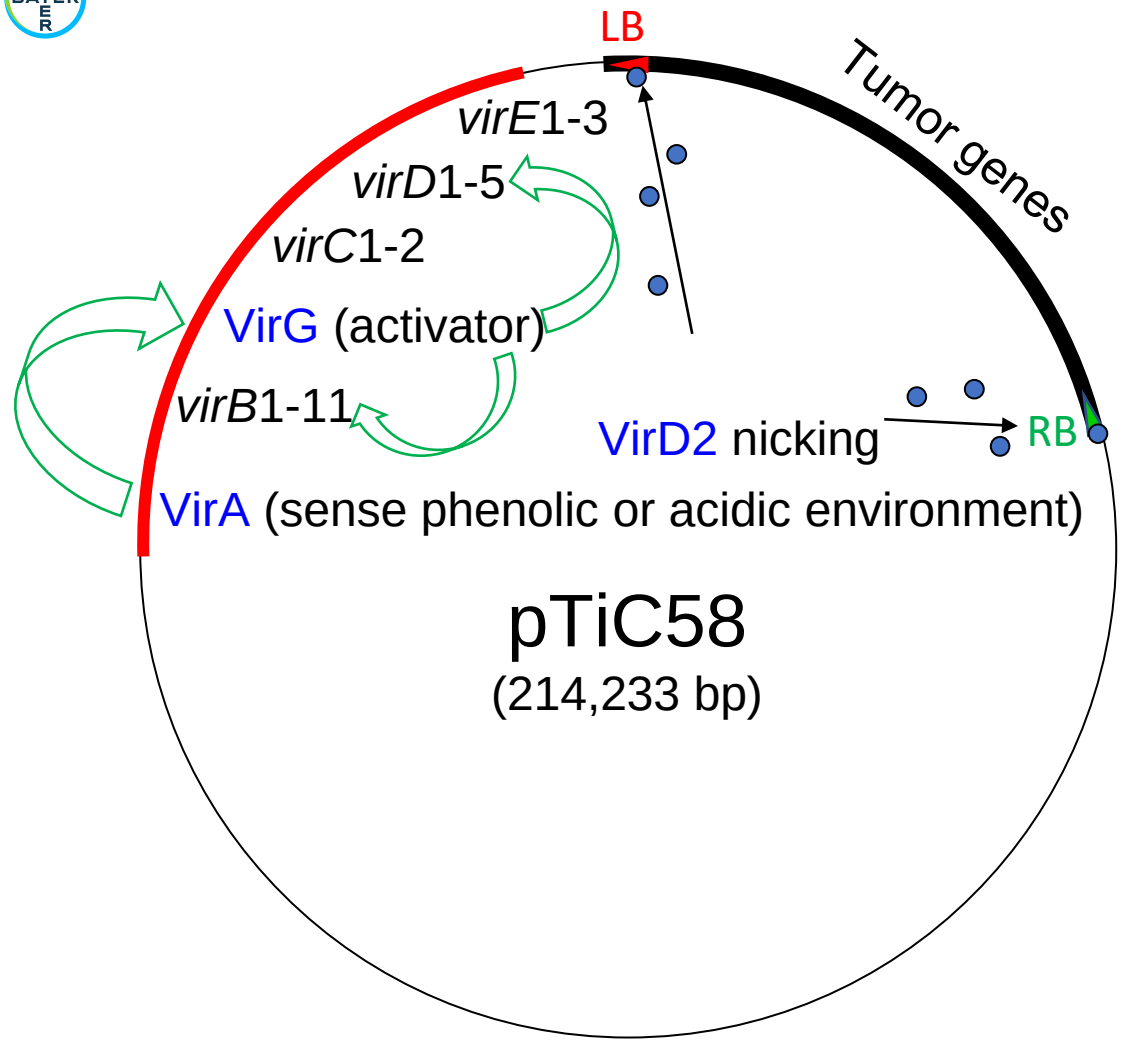
Agrobacterium genome components



Agrobacteriurn tumefaciens C58 strain

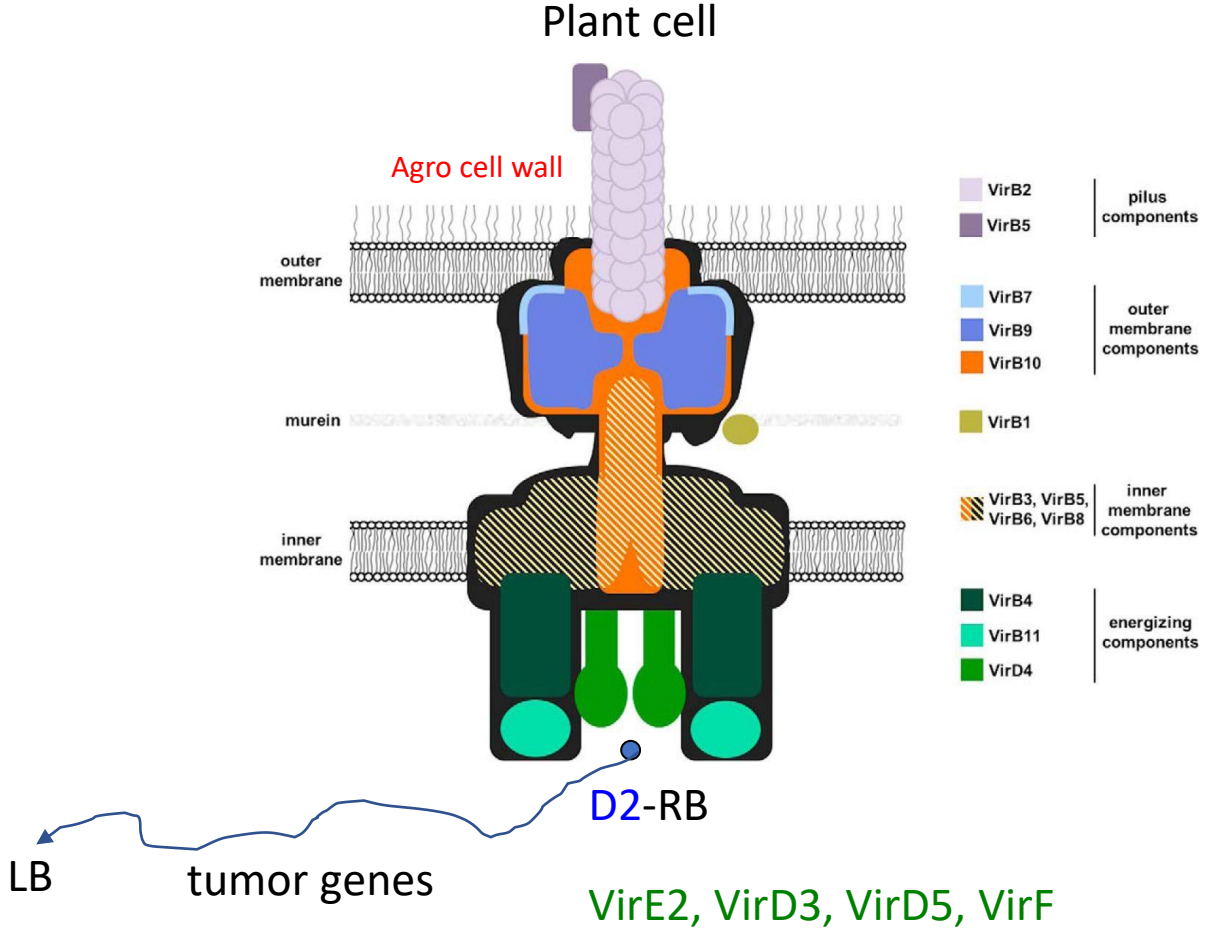
Goodner et al (2001) Genome sequence of the plant pathogen and biotechnology agent Agrobacterium tumefaciens C58. Science. 294(5550):2323-8.
Wood et al (2001) The genome of the natural genetic engineer Agrobacterium tumefaciens C58. Science. 294(5550):2317-23.

Ti plasmid: key components for *Agrobacterium T-DNA transfer*



Jin SG, Prusti RK, Roitsch T, Ankenbauer RG, Nester EW (1990) Phosphorylation of the VirG protein of *Agrobacterium* tumefaciens by the autophosphorylated VirA protein: essential role in biological activity of VirG. J Bacteriol. 172(9):4945-50.
Jin SG, Roitsch T, Christie PJ, Nester EW (1990) The regulatory VirG protein specifically binds to a cis-acting regulatory sequence involved in transcriptional activation of Agrobacterium tumefaciens virulence genes. J Bacteriol. 1990 Feb;172(2):531-7. (identified **vir box**)

Model of the type IV secretion system with the known or suspected localization of its 12 components



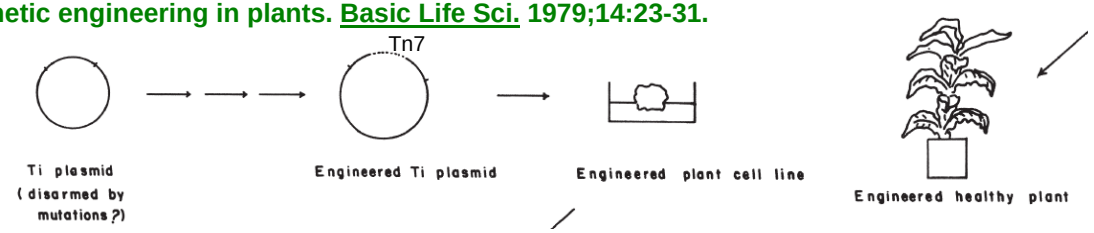
Mary C, Fouillen A, Bessette B, Nanci A, Baron C (2018) Interaction via the N terminus of the type IV secretion system (T4SS) protein VirB6 with VirB10 is required for VirB2 and VirB5 incorporation into T-pili and for T4SS function. J Biol Chem.. 293(35):13415-13426.

Agrobacterium as a vector for plant transformation

The idea of plant transformation was brewing in late 1970's

Schell J, Van Montagu M. (1977) **Transfer, maintenance, and expression of bacterial Ti-plasmid DNA in plant cells transformed with *A. tumefaciens***. Brookhaven Symp Biol. (29):36-49.

Chilton MD. (1979) *Agrobacterium* Ti plasmids as a tool for genetic engineering in plants. Basic Life Sci. 1979;14:23-31.



Schell J, Van Montagu M, De Beuckeleer M, De Block M, Depicker A, De Wilde M, Engler G, Genetello C, Hernalsteens JP, Holsters M, Seurinck J, Silva B, Van Vliet F, Villarroel R (1979) **Interactions and DNA transfer between *Agrobacterium tumefaciens*, the Ti-plasmid and the plant host.** Proc R Soc Lond B Biol Sci. 204(1155):251-66.

Zambryski P, Holsters M, Kruger K, Depicker A, Schell J, Van Montagu M, Goodman HM. (1980) **Tumor DNA structure in plant cells transformed by *A. tumefaciens*.** Science. 209(4463):1385-91.

Cloned T-DNA, direct evidence that a specific piece of the Ti plasmid, the T region, can be transferred and integrated into plant cell DNA during tumor formation.

Wullems GJ, Molendijk L, Ooms G, **Schilperoort RA**. (**1981**) Retention of tumor markers in **F1 progeny** plants from in vitro induced octopine and nopaline tumor tissues. Cell. 24(3):719-27.

Tumorous tobacco shoots
Grafted to non-transformed tobacco

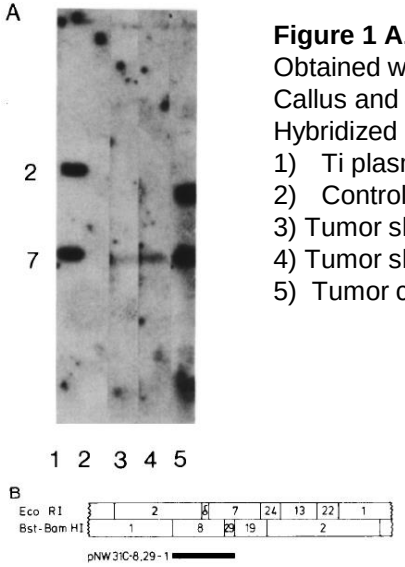


Figure 1 A. Autoradiograph of **Southern Blots** Obtained with EcoRIRI digested Transformant Callus and Shoot DNA and Normal Tobacco DNA Hybridized to a 32P-labeled T-Region Clone

1) Ti plasmid
2) Control
3) Tumor shoot 1
4) Tumor shoot 2
5) Tumor callus

Figure 3. Mature, Flowering Plants Obtained Following Grafting of Tumorous Shoots and Normal SRI Shoots

Plant transformation by Agrobacterium in 1983

Caplan A, Herrera-Estrella L, Inzé D, Van Haute E, Van Montagu M, Schell J, Zambryski P. (1983) Science. 222(4625):815-21.

Partially disarmed pGV3850
pNos-kanR, tobacco protoplast
Co-integration vector

Barton, K.A., Binns, A.N., Matzke, A.J.M. and Chilton, M.-D. (1983). Regeneration of **intact tobacco plants** containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. **Cell**. 32: 1033-1043.

Wide host range plasmid
pRK290:pBR325 kanR (36 kb)

Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL, Woo SC. (1983) Expression of bacterial genes in plant cells. Proc Natl Acad Sci U S A. 1983 Aug;80(15):4803-7.

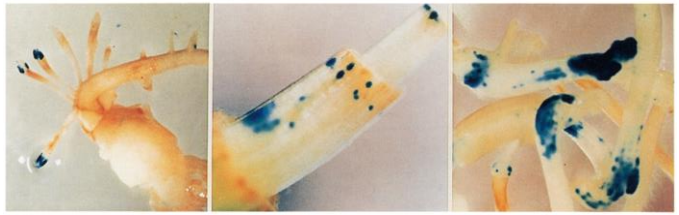
Agrobacterium GV3111
Petunia/npt2
co-integration vector

2013 World Food Prize

Monocot transformation by Agrobacterium requires induction signals

Wang K, Stachel SE, Timmerman B, VAN Montagu M, Zambryski PC (1987) Site-Specific Nick in the T-DNA Border Sequence as a Result of *Agrobacterium* vir Gene Expression. Science. 235(4788):587-91. (**acetosyringone-induced border cleavage**)

Li XQ, **Liu CN**, Ritchie SW, Peng JY, Gelvin SB, Hodges TK. (**1992**) Factors influencing *Agrobacterium*-mediated transient expression of *gusA* in rice. Plant Mol Biol. 20(6):1037-48. (AB salts, 1% glucose, 20 mM MES buffer pH 5.6, 2 mM sodium phosphate buffer pH 5.6, 10 mM octopine and 100/μM acetosyringone)



Chan MT, Chang HH, Ho SL, Tong WF, Yu SM (**1993**) *Agrobacterium*-mediated production of transgenic **rice** plants expressing a chimeric alpha-amylase promoter/beta-glucuronidase gene. Plant Mol Biol. 22(3):491-506.
(**Co-incubation of potato suspension culture (PSC) with the *Agrobacterium* inoculum**)

Hiei Y, Ohta S, Komari T, Kumashiro T. (**1994**) Efficient transformation of **rice** (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J. 6(2):271-82.

Nat Biotechnol. **1996** Jun;14(6):745-50. (maize)

Plant Physiol. **1997** Nov;115(3):971-980. (wheat)

Agrobacterium suspension+200 μM acetosyringone induction + immature embryos

Agrobacterium and strain classification

Order: *Rhizobiales*

Family: *Rhizobiaceae*

Genus: *Agrobacterium*

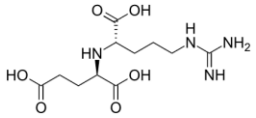
Flores-Félix JD, Menéndez E, Peix A, García-Fraile P, Velázquez E. (2020) History and current taxonomic status of genus Agrobacterium. Syst Appl Microbiol. 43(1):126046.

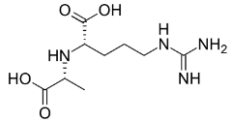
Species	Tumor or root inducing	Common strain
Agrobacteriurn tumefaciens	Ti plasmid	A6, C58, Ach5, T37
Agrobacteriurn rhizogenes	Ri plasmid	NCPPB2659 (K599), A4
Agrobacteriurn rubi	Ti	
Agrobacteriurn vitis	Ti	

Traditionally call *Agrobacterium* strain type based on opine synthesis

A. tumefaciens:

nopaline	pTiC58, pTiT37
octopine	pTiA6, pTiAch5
agrocinopine	pTiBo542

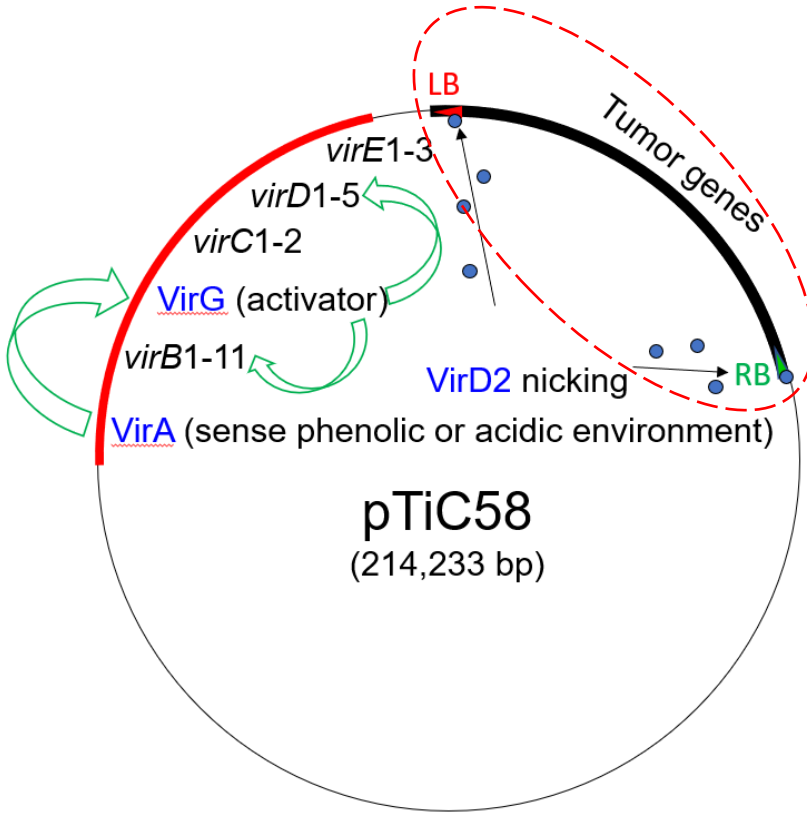


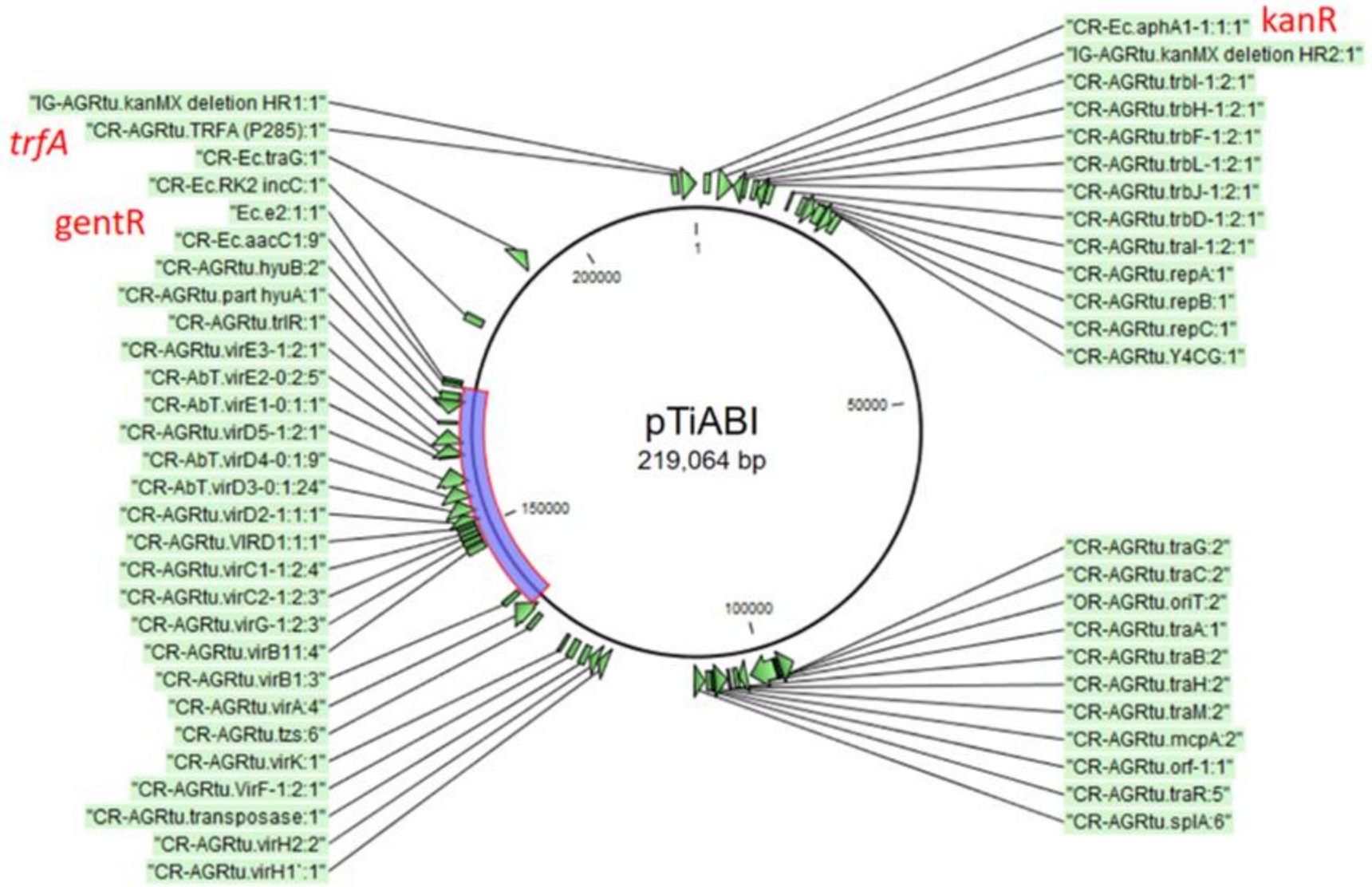


A. rhizogenes (Rhizobium rhizogenes, new*):*

Cucumopine	pRi2659
mikimopine	pRi1724

Disarmed ABI strain Ti plasmid





trfA and kanR were integrated into GV3101 Ti plasmid (GenBank NZ_KY000036)
(TrfA is required for maintaining RK2 *oriV* binary vector)

Frequently used disarmed Agrobacterium strains

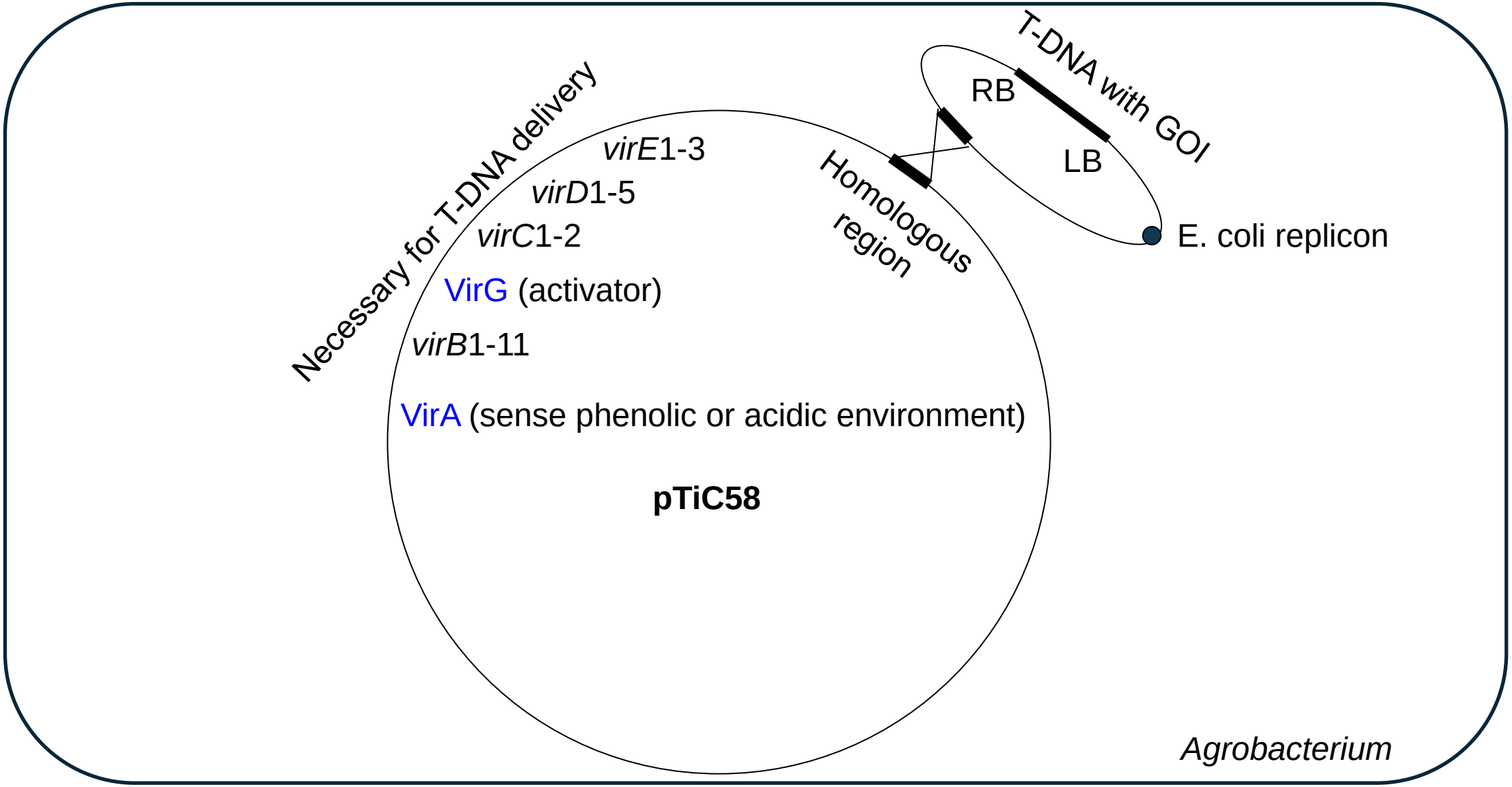
Strain Name	Chromosome background	Ti-plasmid types	Antibiotic Resistance*	Reference
LBA4404	Ach5	a disarmed octopine-type Ti plasmid pAL4404	Rm^R	Hoekema et al., 1983
EHA101	C58	a disarmed agropine-type Ti plasmid pEHA101 (pTiBo542ΔT-DNA)	Rm^R, Km^R	Hood et al., 1986
EHA105	C58	a disarmed agropine-type Ti plasmid pEHA105 (pTiBo542ΔT-DNA)	Rm^R	Hood et al., 1993
A136	C58	Cured of Ti plasmid	Rm^R	Watson et al., 1975
AGL0	C58	a disarmed pTiBo542ΔT-DNA	Rm^R, Cb^R	Lazo et al., 1991
AGL1	C58	a disarmed pTiBo542ΔT-DNA	Rm^R, Cb^R	Lazo et al., 1991
C58C1	C58	Cured of Ti plasmid	Rm^R	Deblaere et al., 1985
C58C1(pTiB6S3ΔT, pCH32)	C58	a disarmed octopine-type Ti plasmid pTiB6S3ΔT-DNA and a helper plasmid pCH32	Rm^R, Cb^R, Tc^R	McBride and Summerfelt, 1990
GV3101	C58	Cured of Ti plasmid	Rm^R	Holsters et al., 1980
GV3101(pMP90)**	C58	a disarmed nopaline-type pTiC58ΔT-DNA	Rm^R, Gm^R	Koncz and Schell, 1986
GV3101(pMP90RK)**	C58	a disarmed nopaline-type pTiC58ΔT-DNA+trfA	RmR, GmR, KmR	Koncz and Schell, 1986
ABI	C58	a disarmed nopaline-type pTiT37ΔT-DNA+trfA	Rm^R, Gm^R, Km^R	Legacy Monsanto

*Cb, carbenicillin; Em, erythromycin; Gm, gentamicin; Km, kanamycin; Rm, rifampicin, Tc: tetracyclin

** Many commercial sources and publications labeled as GV3101 without properly indicating pMP90 helper plasmid. The strain should contain disarmed pTiT37 as we sequenced.

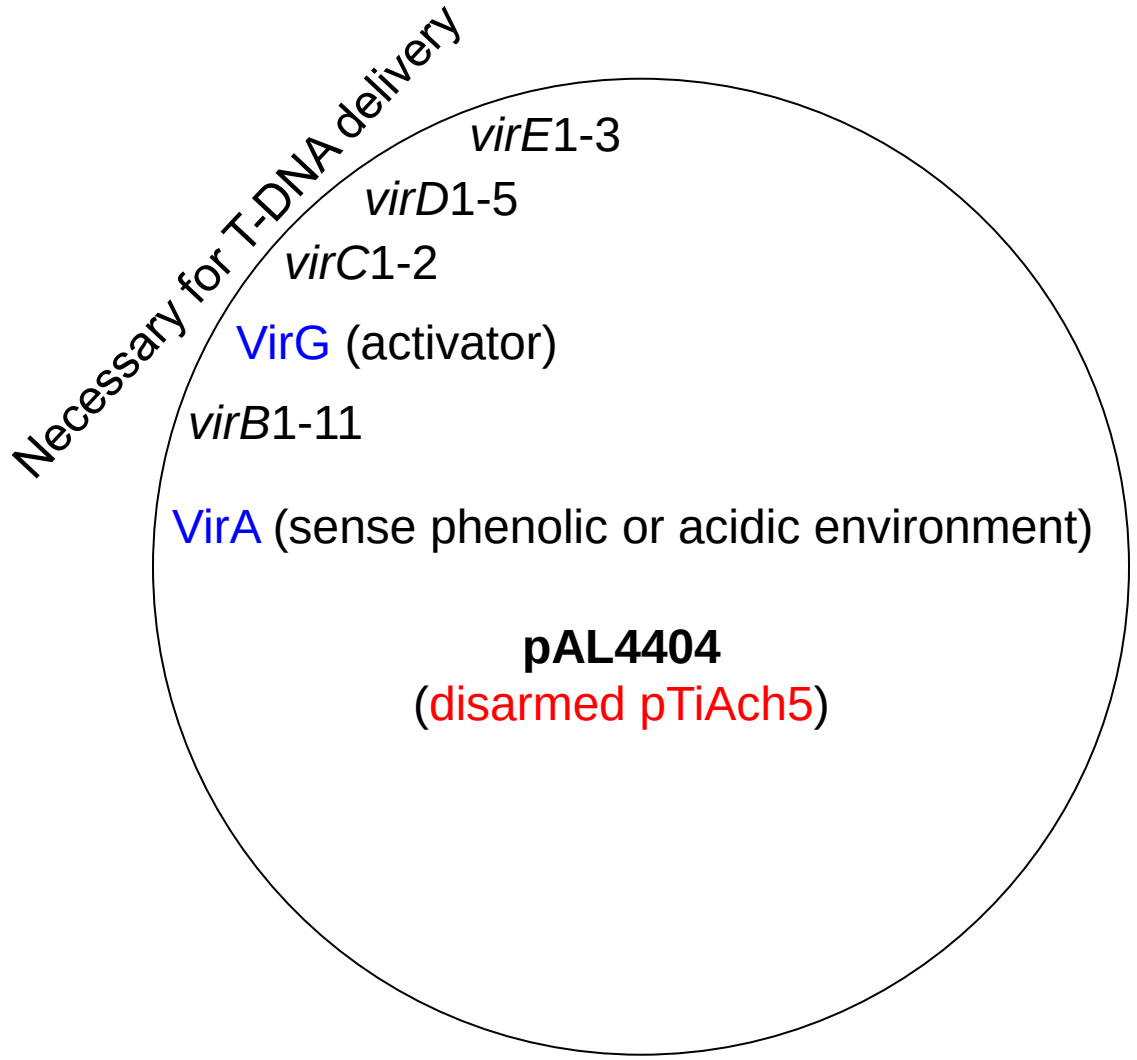
Source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6501860/

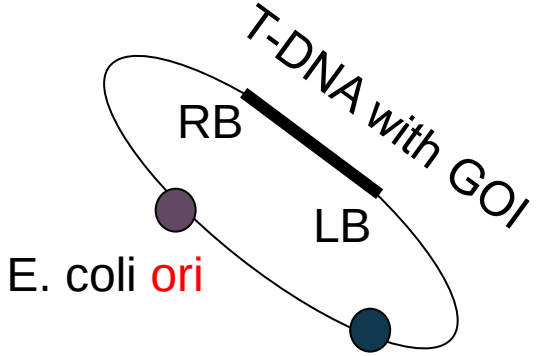
Co-integrate Vector



Binary Vector

Free vector in *Agrobacterium*, replicate in both *E. coli* and *Agrobacterium*





1~2 copies/cell
7~10 copies
~10 copies

Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. (1983). A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature. 303: 179-180. Disarmed *Agrobacterium* LBA4404
Bevan M. (1984) Binary Agrobacterium vectors for plant transformation. Nucleic Acids Res. 12(22):8711-21.

Ye X, Williams EJ, Shen J, Johnson S, Lowe B, Radke S, Strickland S, Esser JA, Petersen MW, Gilbertson LA. (2011)Enhanced production of single copy backbone-free transgenic plants in multiple crop species using binary vectors with a pRi replication origin in *Agrobacterium tumefaciens*. Transgenic Res. 20(4):773-86.

2T-DNA strategies for marker-free segregation in progeny

Conventional 2T vector

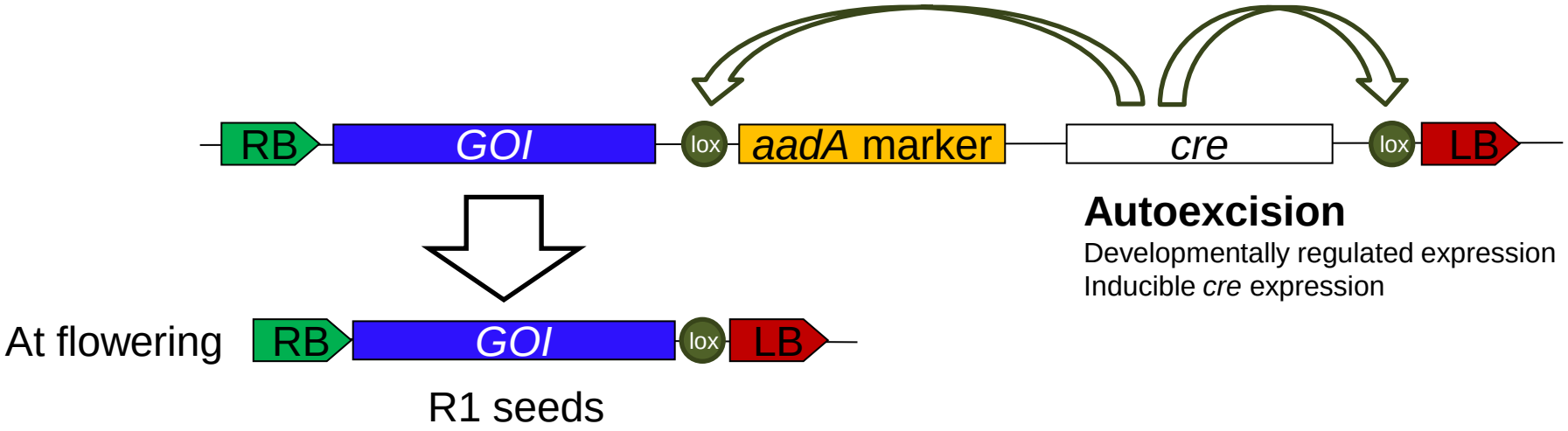
2 right border 2T vector

Tandem 2T vector

2 binary vector

2 *Agrobacterium* co-transformation

Marker gene autoexcision



Autoexcision

Developmentally regulated expression
Inducible *cre* expression

Cre-mediated autoexcision of selectable marker genes in soybean, cotton, canola and maize transgenic plants | SpringerLink

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Thank you!

Any questions?

Pioneers in Agrobacterium biology

Marc Van Montagu (left) and Jeff Schell (right), in 1993.

Rob Schilperoort (1938–2012), Leiden U

Eugene Nester

Pat Zmbryski

Paul Hooykaas

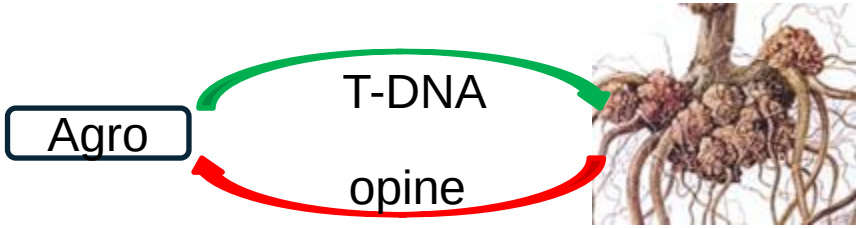
Mary-Dell Chilton

Soil bacterium, *Agrobacterium,* a natural genetic engineer

- Reported in 1907 by Erwin Smith and Charles Townsend in *Science*
- Demonstrated bacteria-free tumors at the Rockefeller Institute by Armin Braun in 1940s
- Pathogenicity transferred from a tumor producing pathogenic strain to a non-pathogenic strain, speculated a plasmid but all isolation failed (Allen Kerr Australia 1969)
- Reported 0.2 Mb plasmid by Jeff Schell and Marc Van Montagu in Ghent, Belgium &

  E. Nester, M. P. Gordon, and M.-D. Chilton, Seattle in 1974
- T-DNA is flanked by 25 bp direct repeats (Narendra Yadav in Mary-Dell Chilton's group)
- Acetosyringone vir gene induction, ssDNA formation(Scott Stachel, Pat Zambryski)
- Opine genes were transferred in a Mendelian segregation (Léon Otten in Jeff Schell's group)
- Binary vector system with 25 bp border flanks (Mary-Dell Chilton, Rob Schilperoort)
- Transgenic plants in 1983 (the Ghent group, Rob Fraley's group, and Chilton's group)
- Transgenic rice by *Agrobacterium* (Hei et al. Japan Tobacco Co. 1994)

https://www.apsnet.org/edcenter/apsnetfeatures/Pages/Agrobacterium.aspx



Recent taxonomy of Agrobacterium species

Table 1

Species currently included in the genus *Agrobacterium* and species causing tumours or hairy roots currently included in other genera of Family *Rhizobiaceae*.

Species	Source of isolation	Pathogenicity symptoms	References
Genus *Agrobacterium*			
A. radiobacter[a]	Soil and plant rhizosphere	Non-pathogenic	[4,9]
A. tumefaciens[a]	*Malus* sp. tumours	Tumours	[9,46]
A. rubi	*Rubus* sp. tumours	Tumours	[17,48]
A. larrymoorei	*Ficus benjamina* tumours	Tumours	[5]
'*A. albertimagni*'	*Potamogeton pectinatus*	No data	[42]
'*A. fabrum*'	*Prunus* sp., *Humulus lupulus*, *Euonymus alata*, *Rubus macropetalus* tumours	Tumours	[29]
A. pusense	*Cicer arietinum* rhizosphere	No data	[31,35]
A. nepotum	*Prunus*, *Vitis* and *Rubus* tumours	Tumours	[31,38]
A. skierniewicense	*Chrysanthemum* and *Prunus* tumours	Tumours	[39]
A. arsenijevicii	*Prunus* and *Rubus* tumours	Tumours	[25]
'*A. deltaense*'	*Sesbania cannabina* nodules	No data	[55]
A. salinitolerans	*Sesbania cannabina* nodules	No data	[56]
'*A. bohemicum*'	*Papaver somniferum*	Non-pathogenic	[62]
A. rosae	*Rosa x hybrida* tumours	Tumours	[26]
Genus *Allorhizobium*			
A. vitis	*Vitis vinifera* tumours	Tumours	[31,32]
Genus *Rhizobium*			
R. rhizogenes	*Malus* sp.	Hairy roots	[9,40]
'*R. tumorigenes*'	*Rubus* sp. tumours	Tumours	[27]

Flores-Félix JD, Menéndez E, Peix A, García-Fraile P, Velázquez E. (**2020**) History and current taxonomic status of genus Agrobacterium. Syst Appl Microbiol. 43(1):126046.
https://www.sciencedirect.com/science/article/pii/S0723202019303418?via%3Dihub

Protocol training: Agrobacterium

Xudong Ye

The goals of this training

- Prepare *Agrobacterium* glycerol stock for long term storage
- Prepare *Agrobacterium* competent cells
- Transform a binary vector into *Agrobacterium*
- Verify binary vector in *Agrobacterium* (quality control)
- Prepare *Agrobacterium* glycerol stock for plant transformation
- *Agrobacterium* virulence gene induction for transformation

Key points for Agrobacterium handling

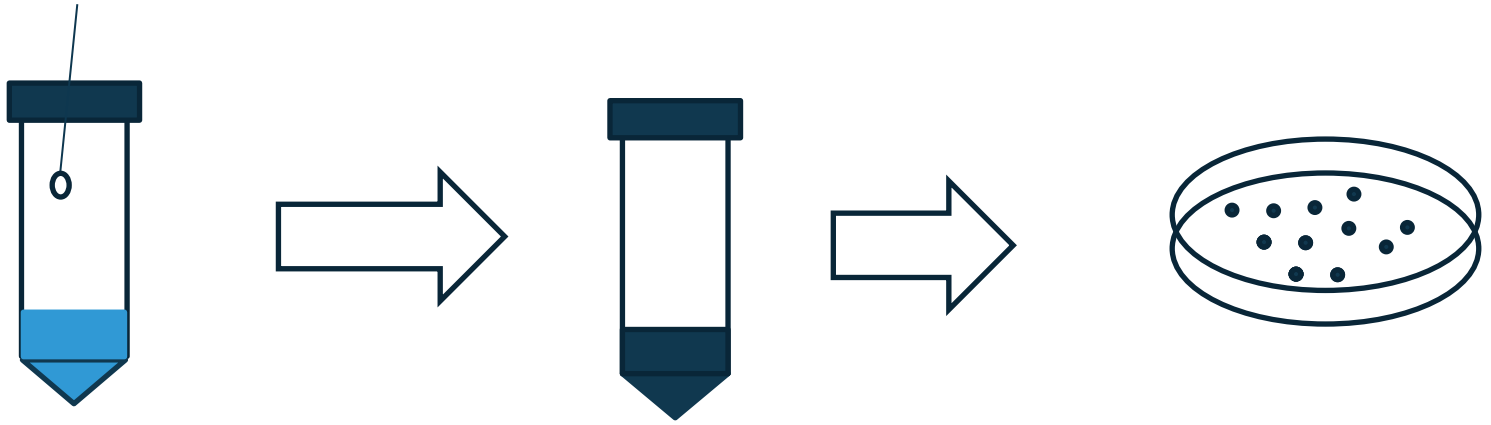
- Always culture *Agrobacterium* at 28~32 ºC. Ti plasmid may loss at 37 ºC
- Induce virulence genes between 19~28 ºC. Often 23 ºC or room temperature. Some Vir proteins are not stable at 28 ºC
- Use minimal salt media for induction. Optimized pH 5.3

Elevated Temperature Differentially Affects Virulence, VirB Protein Accumulation, and T-Pilus Formation in Different Agrobacterium tumefaciens and Agrobacterium vitis Strains - PMC (nih.gov)

Verify and check Agrobacterium strains when you receive a new Agrobacterium culture

Step 1: check antibiotic resistance on solid plates: GV3101 (pMP90): gent^{R}, spec^{S}, kan^{S}
or use specific primers for PCR detection pTiGV3101 (GenBank # NZ_KY000036)

Step 2: Purify strain by single colony



1 loop in 10 ml medium, gentamicin 30 mg/L, shake for 3~6 hours to get spread cells

1 ul culture in 10 ml LB dilution, mix well

Transfer and spread 10 ul onto LB solid plate with gent 30 mg/L, culture at 28 ^{o}C for 2~3 days for single colonies

Agrobacterium seed glycerol preparation for storage

Agrobacterium strain GV3101 (pMP90): gent^{R}, spec^{S}, kan^{S}

Pick a single colony on solid medium

10 ml LB+ gent 30 mg/L

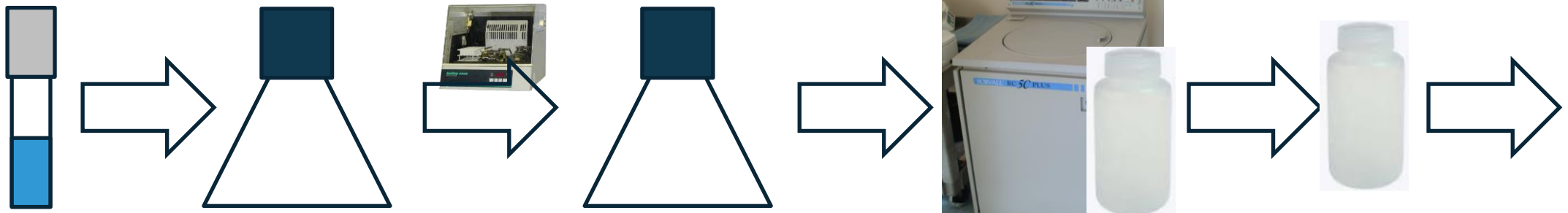
Shake at 28 ^{o}C at 225 rpm for 24-48 hrs, OD_{600}+1.0

2.5 ml 80% glycerol
7.5 ml Agrobacterium
Vortex

Aliquot 1 ml in a cryogenic vial
Store at -80 ^{o}C

Prepare Agrobacterium competent cells from glycerol stock

Agrobacterium strain GV3101 (pMP90): $gent^R$, $spec^S$, kan^S



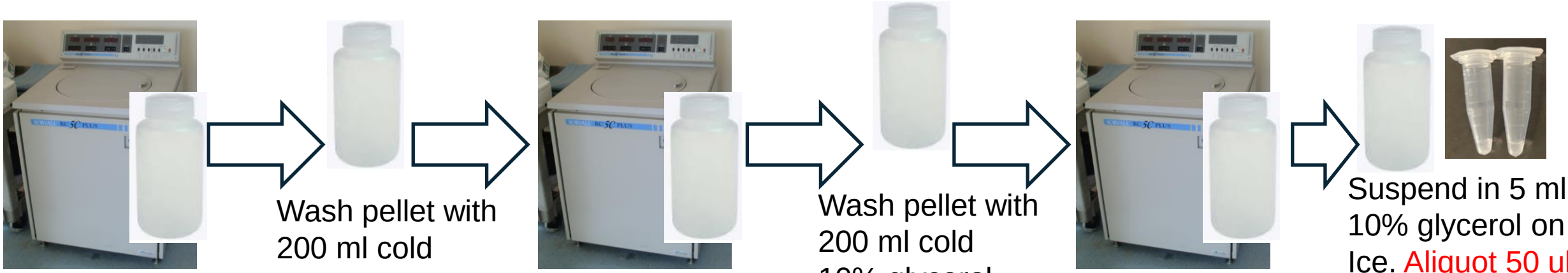
Transfer a loop or 50 ul of Agrobacterium

50 ml LB+ gent 30 mg/L Shake at 28C at 225 rpm overnight

Transfer 10 or 20 ml into 200 LB only Shake at 28C at 225 rpm until OD_{600}=~0.8

Centrifuge for 5-10 min at ~5000g on ice

Wash pellet with 200 ml Deionized/ sterilized cold water



Wash pellet with 200 ml cold 10% glycerol

Wash pellet with 200 ml cold 10% glycerol

Suspend in 5 ml 10% glycerol on Ice. Aliquot 50 ul store at -80C

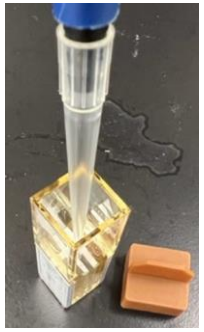
Transform a binary vector into Agrobacterium

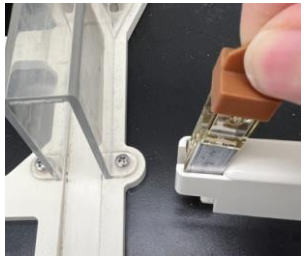
Binary vector antibiotic resistance: *aadA*/spectinomycin
Agrobacterium strain GV3101 (pMP90): $gent^R$, $spec^S$, kan^S

Thaw competent cells on ice

Add 1 ul plasmid (0.1~1 μg/μl) in to 50 ul competent cells

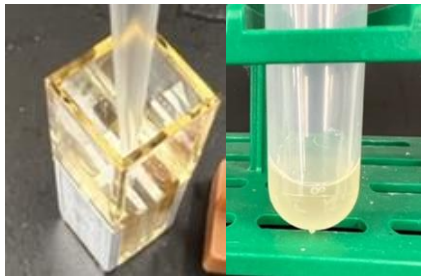
Transfer the mix (100 ul tip) into 1 mm gap cuvette on ice, covered, insert to the electroporator holder





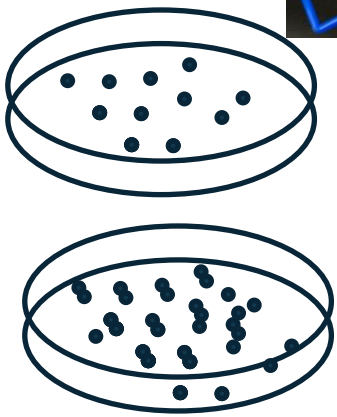
Set 2 kv for electroporation (1 mm gap cuvette)

Use 1 ml tip, add 1 ml LB to the cuvette and transfer into a labeled tube



Shake for ~2hrs at 28 °C

Transfer 10 ul and 100 ul onto 2 plates (LB gent 30, spec 75 mg/L), spread evenly



Incubate at 28 °C for 2~3 days

Three methods to verify binary vector in Agrobacterium cells (QC)

After minipreparation of plasmids from *Agrobacterium* liquid culture

1) Submit miniprep plasmid for sequencing (slow, expensive, most accurate, recommended)

2) Or transfer 1 ul miniprep plasmid into E coli competent cells by electroporation, add 3~5 ml LB with antibiotic selection, shake overnight, miniprepare 1.5 ml culture, check with restriction enzymes (less skill required)

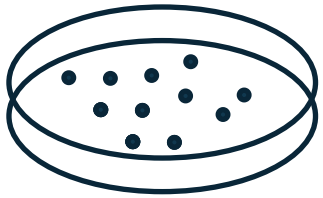
3) Direct QC Agrobacterium plasmids (more skills)

Verify binary vector in Agrobacterium

GV3101/pMON-*Cpf1* QC by restriction enzymes

Hind3	12.8, 3.6, 1.46, 0.08 kb
NcoI	11.5, 5.3, 1.2 kb
XhoI	7.1, 6.3, 4.2, 0.37 kb

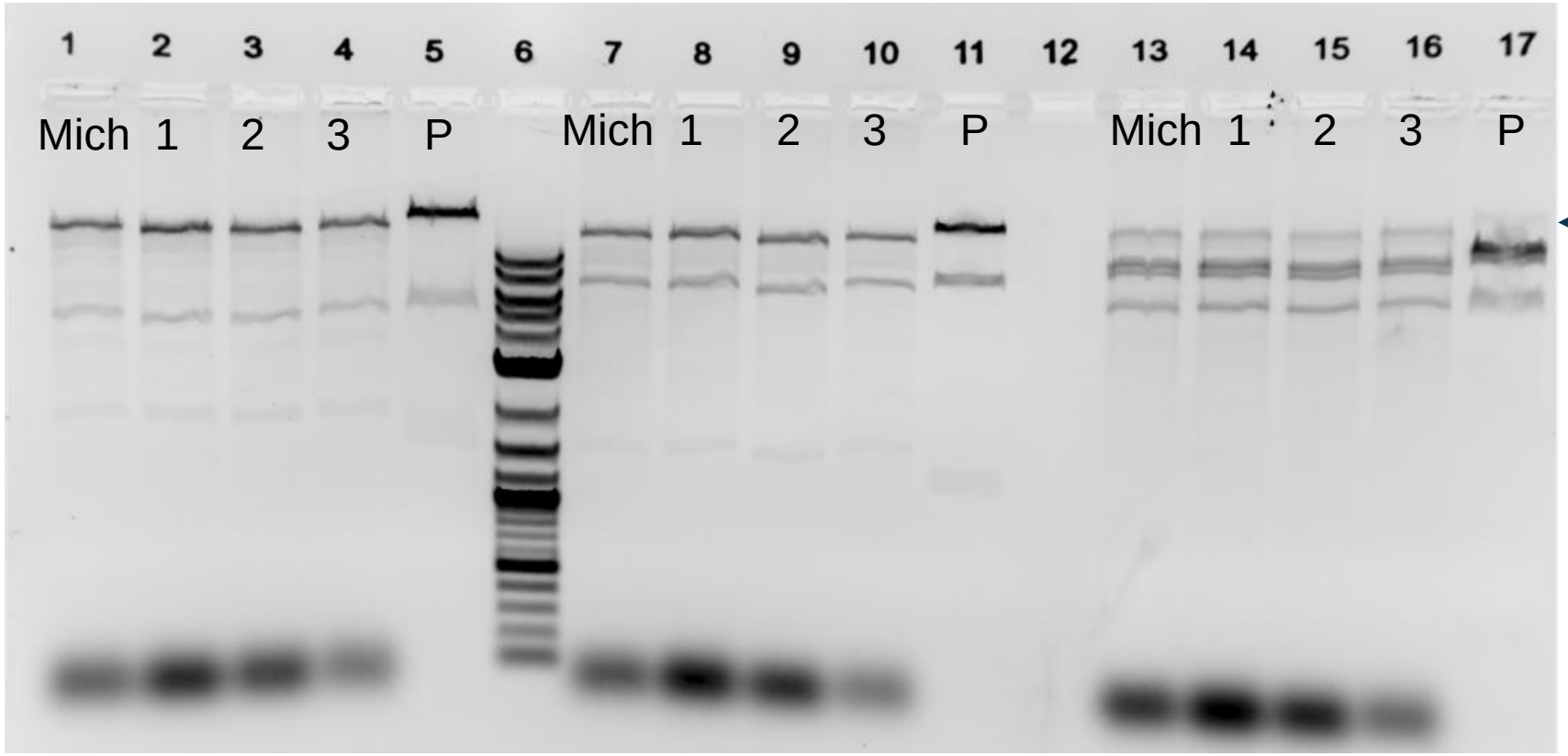
Pick 3-8 single colonies into individual tubes



Sterile a toothpick
Or plastic tip

Pick 3-8 single colonies into individual tubes

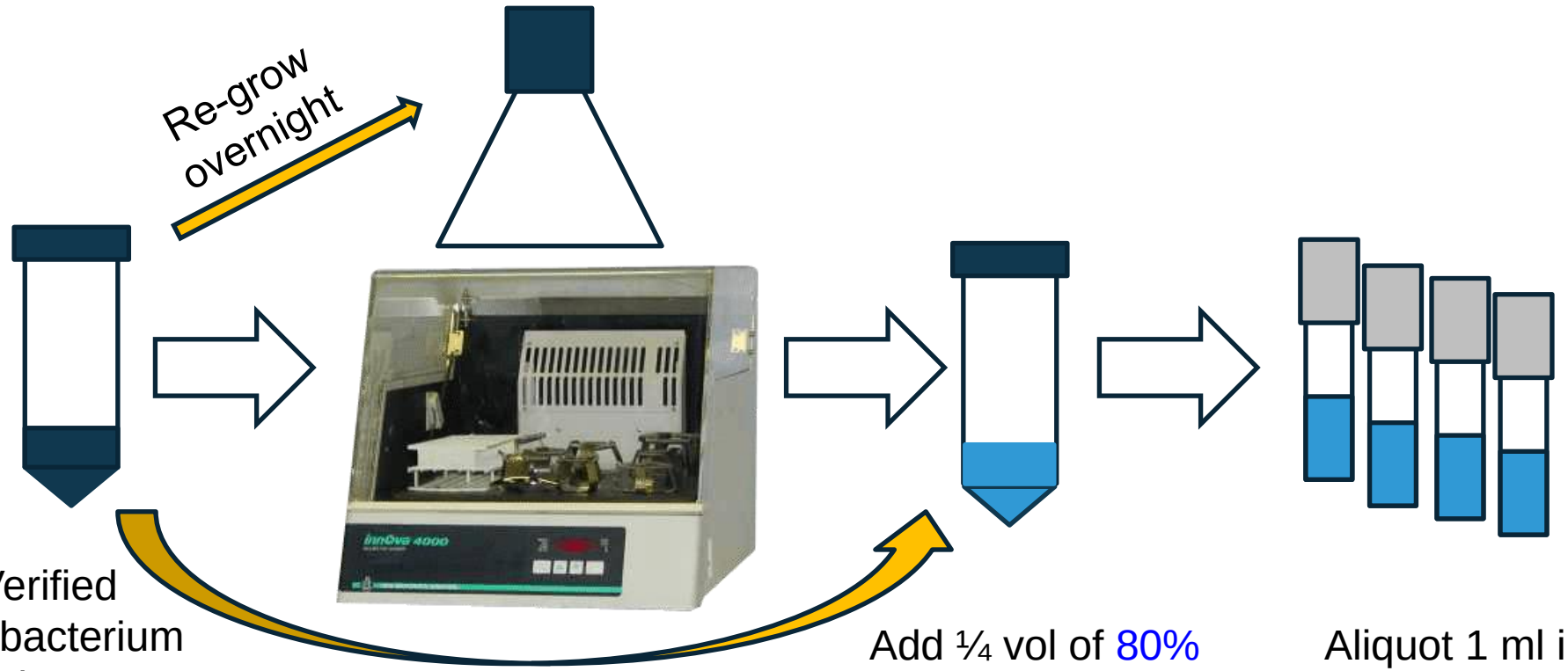
Add 10 ml LB with gent/spec selection shake at 30 °C overnight



4 ml overnight culture was purified by 300 ul P1/P2/P3 method, dried, suspended in 100 ul water. 10 ul was used for digestion

Mich: Michelle's sample; 1,2,3: 3 single colonies of GV3101 re-transformation; P: ~0.2 ug/lane purified plasmid

Prepare Agrobacterium glycerol stock for plant transformation



Virulence gene induction for maize transformation

1. Grow Agrobacterium at 30 °C overnight in 5 ml rich medium (such as LB or YEP) containing the appropriate antibiotics. (gentamicin 30 mg/L for GV3101, spectinomycin 75 mg/L for plasmid)

2. Dilute approximately 0.5 ml of the culture into 50 ml AB-sucrose minimal medium containing the appropriate antibiotics. Grow overnight at 30 °C until the bacteria are in late-log phase (OD600= ~0.8)

3. Spin down the bacteria. Resuspend in two volumes of induction medium containing 100 μM acetosyringone. Shake 14-24 hours (overnight) **at room temperature** (not at 30°C).

4. Spin down the bacteria. Resuspend in MS plant tissue culture medium. Inoculate plants.

5. After two days, rinse the plant tissue in medium containing 100 μg/ml timentin. Continue incubating the tissue on solidified medium containing timentin.

6. Stain the tissues after various periods of time (2-10 days) in X-gluc.

Agrobacterium Protocols: Volume I | SpringerLink

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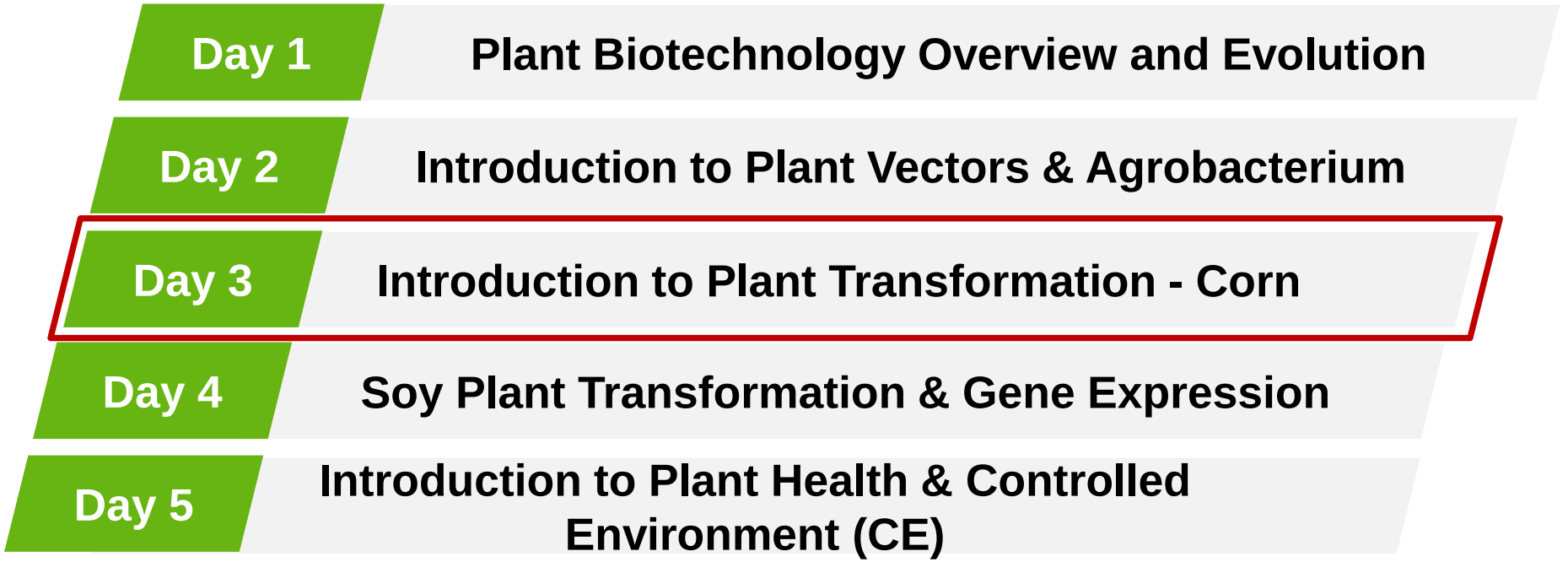
Bayer Russia Plant Biotechnology Conference:



Thank you!

Any questions?