

Engineering *Agrobacterium tumefaciens* with a Type III Secretion System to Express Type III Effectors

Vidhyavathi Raman¹ and Kirankumar S. Mysore^{2,3,*}

¹Department of Plant and Microbial Biology, University of Minnesota, St. Paul, MN 55108, USA

²Institute for Agricultural Biosciences, Oklahoma State University, Ardmore, OK 73401, USA

³Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078, USA

*For correspondence: kmysore@okstate.edu

Abstract

Plants elicit defense responses when exposed to pathogens, which partly contribute to the resistance of plants to *Agrobacterium tumefaciens*-mediated transformation. Some pathogenic bacteria have sophisticated mechanisms to counteract these defense responses by injecting Type III effectors (T3Es) through the Type III secretion system (T3SS). By engineering *A. tumefaciens* to express T3SS to deliver T3Es, we suppressed plant defense and enhanced plant genetic transformation. Here, we describe the optimized protocols for mobilization of T3SS-expressing plasmid to engineer *A. tumefaciens* to deliver proteins through T3SS and fractionation of cultures to study proteins from pellet and supernatants to determine protein secretion from engineered *A. tumefaciens*.

Keywords: Type III secretion system, Type III effectors, *Agrobacterium*, Triparental mating, Transformation

This protocol is used in: Nat. Commun. (2022), DOI: 10.1038/s41467-022-30180-3

Background

The Type III secretion system (T3SS) is a specialized complex structure that translocates Type III effectors (T3Es) into the plant cell, where they interact with the host immune system. T3SS is present in many pathogenic Gram-negative bacteria (Cornelis, 2006). Heterologous expression of the genes encoding the type III secretion apparatus is functional in *Pseudomonas fluorescens* (Huang et al., 1988) and *Escherichia coli* (Ham et al., 1998). Gene cluster coding structural and regulatory components of T3SS from *P. syringae* have been cloned and are available in a plasmid called pLN18 (Jamir et al., 2004). We have transferred this plasmid into *Agrobacterium tumefaciens* to express T3SS and deliver T3Es to suppress plant defense and enhance plant genetic transformation (Raman et al., 2022). Because of the large size, we used triparental mating to mobilize pLN18 to *A. tumefaciens* in the presence of helper plasmid (pRK2013; containing *tra* genes) containing *E. coli*, using the protocol by Wise et al. (2006) with some modifications. In addition, we optimized culture growth and fractionation of the liquid culture into pellet and supernatant fractions to avoid cell lysis that leads to false positive results.

Materials and reagents

1. 1.5 mL microcentrifuge tubes (USA Scientific)
2. 50 mL Falcon tubes (Corning, catalog number: 352070)
3. 0.45 μm Durapore PVDF membrane Millipore filter (Steriflip-HV Sterile Centrifuge Tube Top Filter Unit) (Millipore Sigma, catalog number: SE1M003M00)
4. Amicon Ultra-15 centrifugal filters (Millipore Sigma, catalog number: UFC901024)
5. Amicon Ultra-0.5 centrifugal filters (Millipore Sigma, catalog number: UFC501024)
6. *A. tumefaciens* GV3101 (Rifampicin resistance)
7. *E. coli* HB101 (pRK2013) (Kanamycin resistance)
8. Donor *E. coli* containing pLN18 (Kanamycin and Tetracycline resistance)
9. Luria Bertani (LB) medium (MP Biomedicals, catalog number: 3002011)
10. Luria Bertani (LB) medium with agar (MP Biomedicals, catalog number: 3002201)
11. PhiLOV-specific antibody (gift from Dr. John M. Christie, University of Glasgow)
12. Bacto peptone (BD, catalog number: 211677)
13. Yeast extract (BD, catalog number: 212750)
14. Bacto agar (BD, catalog number: 214010)
15. NaCl (J.T. Baker, catalog number: 3624-19)
16. K_2HPO_4 (J.T. Baker, catalog number: 4012-01)
17. NaH_2PO_4 (Fisher Scientific, catalog number: S369-500)
18. NH_4Cl (Fisher Scientific, catalog number: A661)
19. KCl (Fisher Scientific, catalog number: P217-500)
20. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich, catalog number: M1880)
21. CaCl_2 (J.T. Baker, catalog number: 1313-01)
22. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma-Aldrich, catalog number: F8263)
23. Sucrose (Invitrogen, catalog number: 15503-022)
24. Rifampicin (Sigma-Aldrich, catalog number: R3501-1G)
25. Kanamycin sulfate (Sigma-Aldrich, catalog number: 70560-51-9)
26. Tetracycline (Sigma-Aldrich, catalog number: T3258)
27. Yeast extract peptone (YEP) medium (1 L) (see Recipes)
28. 20 \times AB buffer (1 L) (see Recipes)
29. 20 \times AB salts (1 L) (see Recipes)
30. Water agar (see Recipes)
31. 25% sucrose (see Recipes)
32. *Agrobacterium* minimal (AB)-sucrose agar medium or AB medium (see Recipes)
33. 0.8% NaCl solution medium (see Recipes)

34. *Hrp*-derepressing (HDM) medium (see Recipes)

Equipment

1. 28 °C incubator with shaker (New Brunswick Scientific)
2. 37 °C incubator with shaker (New Brunswick Scientific)
3. Spectrophotometer (Bio-Rad, catalog number: 170-2525)
4. Eppendorf tabletop centrifuge (Eppendorf, catalog number: 5424)
5. Eppendorf tabletop refrigerated centrifuge (Eppendorf, catalog number: 5424R)
6. Eppendorf centrifuge with swing-bucket rotor (Eppendorf, catalog number: 5810R)

Procedure

A. Mobilization of T3SS to *A. tumefaciens* through triparental mating

Triparental mating is used to mobilize a large plasmid from *E. coli* to *A. tumefaciens* by using another *E. coli* carrying helper plasmid—in this case, HB101 (pRK2013).

1. Streak out the recipient *A. tumefaciens* strain (e.g., GV3101) from the -80 °C freezer onto YEP agar plates containing antibiotic [rifampicin (Rif); 10 µg/mL] and allow it to grow in a 28 °C incubator for two days. Streak out donor (pLN18, Tet^R) and helper (pRK2013, Km^R) plasmid-containing *E. coli* strains onto LB agar plates containing appropriate antibiotics [kanamycin (Km), 50 µg/mL; and tetracycline (Tet), 5 µg/mL] and allow them to grow in a 37 °C incubator for a day.
2. The day before triparental mating, culture a single colony of *A. tumefaciens* in 5 mL of YEP medium supplemented with the appropriate antibiotics [rifampicin (10 µg/mL)] and leave overnight with shaking at 250 rpm and 28 °C. Culture a single colony of *E. coli* strains carrying donor and helper plasmids in 5 mL of LB liquid medium supplemented with the appropriate antibiotics, shaking at 250 rpm and 37 °C overnight.
3. On the day of mating, from the overnight cultures, inoculate 300 µL of *A. tumefaciens* to 3 mL of YEP liquid medium with rifampicin (10 µg/mL) and grow for 3–5 h at 250 rpm and 28 °C; inoculate 30 µL of *E. coli* to 3 mL of LB liquid medium with appropriate antibiotics and grow for 3–5 h at 250 rpm and 37 °C. Grow all the cultures until A₆₀₀ reaches 0.3–0.5.

B. Triparental mating (Figure 1 and Figure 2)

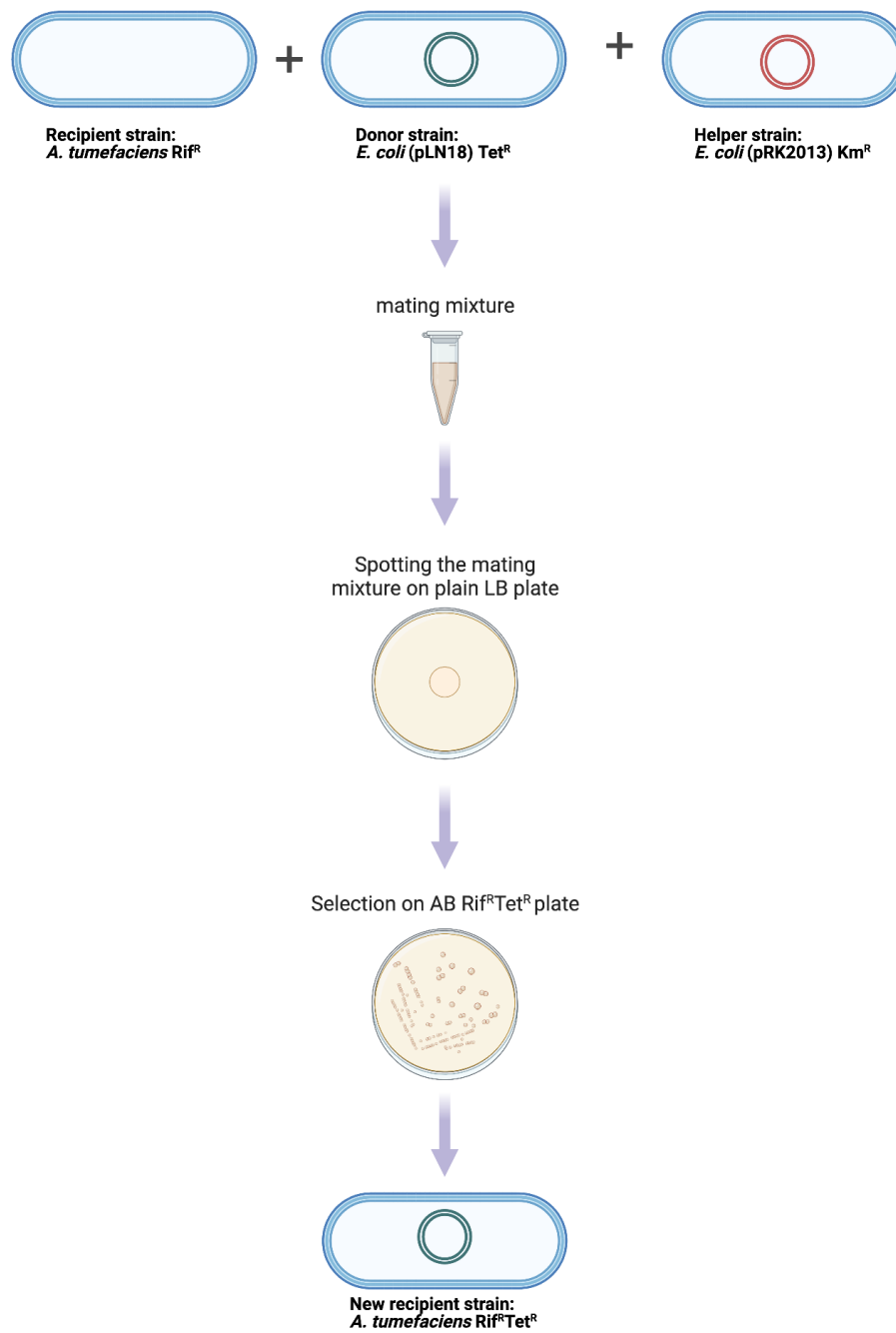


Figure 1. Experimental procedure for mobilizing pLN18 to *A. tumefaciens*

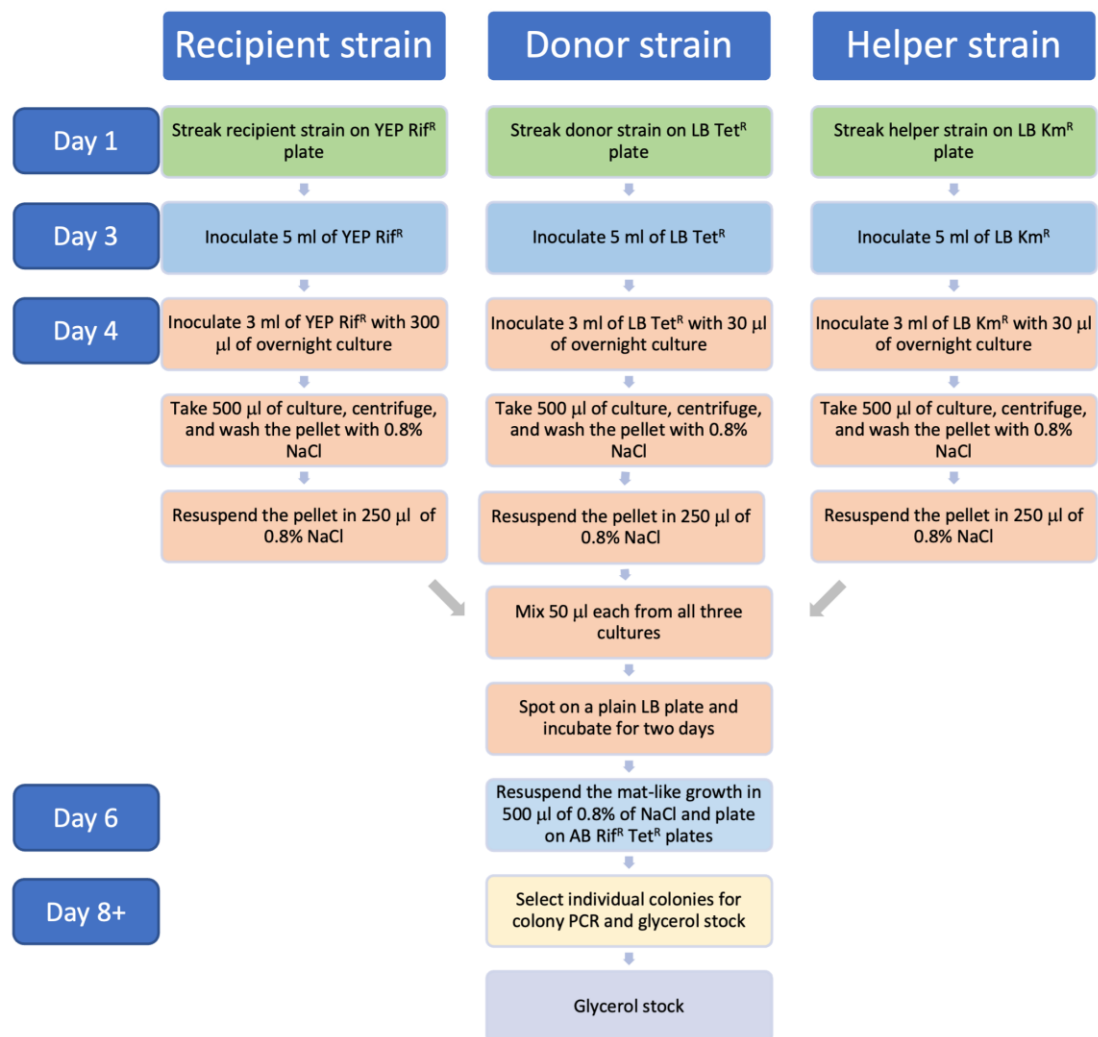


Figure 2. Flowchart describing timeline for the triparental mating protocol

1. Aliquot 500 µL each for all cultures in a 1.5 mL microcentrifuge tube separately and spin at 9,391× g for 2 min at room temperature (RT). Discard the supernatants. Wash once by resuspending the pellets in 500 µL of 0.8% NaCl and spin again.
2. Resuspend the pellet in 250 µL of 0.8% NaCl.
3. Mix 50 µL each of the three cultures in a microcentrifuge tube and spot it on a plain LB plate; allow it to dry for a few minutes and incubate the plate at 28 °C for two days.
4. Scrape off the mat-like growth (Figure 3) and resuspend in 500 µL of 0.8% NaCl in a 1.5 mL microcentrifuge tube. Spread 10 and 50 µL and the rest of the suspension in three separate AB (Gelvin, 2006) plates with Rif and Tet and incubate the plates at 28 °C for 2–4 days.

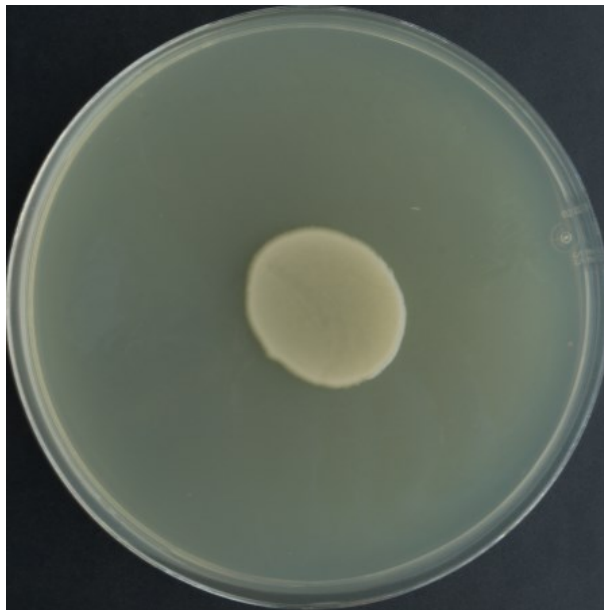


Figure 3. Representative image showing mat-like growth on plain LB plate

5. Isolate single colonies for glycerol stocks and colony PCR.

Assays using *A. tumefaciens* expressing T3SS and T3Es

Either electroporation or the freeze-thaw method is used to transform GV2260 (pLN18) with a broad host range plasmid containing T3E (e.g., pBBR1MCS5-AvrPto-PhiLOV). Western blot and microscopy can be used to demonstrate effector secretion and delivery, respectively. In this protocol, we use western blot hybridization to show that T3Es are expressed and delivered out of the cell.

1. *A. tumefaciens* strains expressing T3SS and T3E are streaked onto YEP agar plates along with appropriate controls and incubated at 28 °C for two days. GV2260, GV2260 (pLN18), and GV2260 (pBBR1MCS5-AvrPto-PhiLOV) are used as controls.
2. Inoculate two colonies into 20 mL of HDM medium (Huynh et al., 1989) in 50 mL screw-cap tubes and incubate at 28 °C for 16 h shaking at 220 rpm.
3. Aliquot a small amount of culture ($A_{600} = 0.25$) in a 1.5 mL microfuge tube and centrifuge in a tabletop centrifuge at $9,391\times g$ at RT for 2 min. Use the resulting pellet for pellet fraction analysis.
4. Centrifuge remaining cultures at $3,220\times g$ for 15 min at 21 °C.
5. Using 25 mL pipettes, carefully remove the top 15 mL of the supernatant solutions without disturbing the pellet, by lowering the pipette slowly into the supernatant.
6. Transfer the supernatant fraction to 50 mL tubes and filter using a 0.45 μm Durapore PVDF membrane Millipore filter with lab vacuum connection.
7. Transfer 13 mL of the filtrate to Amicon Ultra-15 centrifugal filters and concentrate using a swinging bucket rotor at $3,220\times g$ for 30 min at 4 °C.
8. Add the remaining 12 mL of the filtrate to the same Amicon Ultra-15 centrifugal filter and concentrate at 4 °C for another 30 min at $3,220\times g$.
9. Recover the concentrated filtrate from the bottom of the filter device and again concentrate to $\sim 30\ \mu\text{L}$ using Amicon Ultra-0.5 centrifugal filters at $14,000\times g$ for 40–60 min at 4 °C.
10. Recover the concentrated sample by keeping the filter device upside down in a new microcentrifuge tube and spin for 2 min at $1,000\times g$ in a refrigerated centrifuge. Store the concentrated sample at -80 °C until analysis.

- Proteins from the pellet and supernatant fractions were subjected to electrophoresis through an SDS-PAGE gel using standard protocols, and immunoblot analysis was carried out using PhiLOV-specific antibody (dilution 1:5,000) (Figure 4).

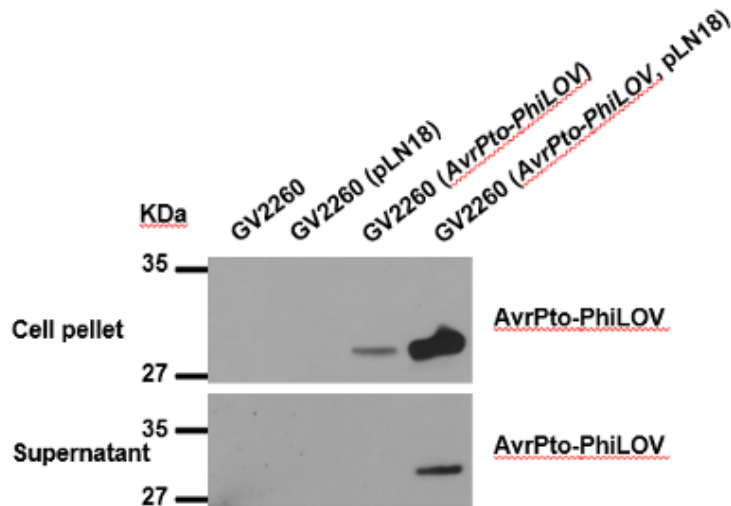


Figure 4. Western blot analysis of AvrPto expression and secretion in cell pellet and supernatant fractions

Recipes

1. YEP medium (1 L)

Bacto peptone 10 g
 NaCl 5 g
 Yeast extract 10 g
 Bacto agar 15 g
 Sterilize by autoclave at 121 °C for 15 min

2. 20× AB buffer (1 L)

K₂HPO₄ 60 g
 NaH₂PO₄ 20 g
 Adjust the pH to 7.0

3. 20× AB salts (1 L)

NH₄Cl 20 g
 KCl 3 g
 MgSO₄·7H₂O 6 g
 CaCl₂ 0.2 g
 FeSO₄·7H₂O 50 mg

4. Water agar

Bacto agar 3.75 g in 220 mL of water
 Autoclave 20× AB buffer, 20× AB salts, and water agar separately at 121 °C for 15 min

5. 25% sucrose

12.5 g sucrose/50 mL water, mix well, and filter sterilize using 0.22 µm filter

6. AB-sucrose agar medium or AB medium

Add 20× AB buffer, 20× AB salts, and 25% sucrose when the water agar is warm

Water agar 220 mL

20× AB salts 12.5 mL

20× AB buffer 12.5 mL

25% sucrose 5 mL

7. 0.8% NaCl solution medium (100 mL)

NaCl 0.8 g

Water 100 mL

Sterilize by autoclave at 121 °C for 15 min

8. HDM medium (1 L)

Fructose 1.801 g

(NH₄)₂SO₄ 1.004 g

MgCl₂·6H₂O 0.345 g

NaCl 0.099 g

1 M K₂HPO₄ 6.6 mL

1 M KH₂PO₄ 43.4 mL

Water 950 mL

pH 6.0 (No need to adjust the pH)

Sterilize by autoclave at 121 °C for 15 min

Acknowledgments

This work was supported by the National Science Foundation (grant # IOS-1725122 and IOS-2219792 to K.S.M.) and the Noble Research Institute, LLC. Media recipes described here are adapted from Gelvin (2006) and Huynh et al. (1989), and triparental mating procedure was modified from Wise et al. (2006). Protocols for fractionation of *A. tumefaciens* culture and western blot are from our published paper Raman et al. (2022). Figure 1 was generated with the help of Biorender (<https://biorender.com/>).

Competing interests

The authors declare no competing interests.

References

- Cornelis, G. R. (2006). [The type III secretion injectisome](#). *Nat rev Microbiol* 4(11): 811-825.
- Gelvin, S. B. (2006). [Agrobacterium virulence gene induction](#). *Methods Mol Biol* 343: 77-84.
- Ham, J. H., Bauer, D. W., Fouts, D. E. and Collmer, A. (1998). [A cloned Erwinia chrysanthemi Hrp \(type III protein secretion\) system functions in Escherichia coli to deliver Pseudomonas syringae Avr signals to plant cells and to secrete Avr proteins in culture](#). *Proc Natl Acad Sci U S A* 95(17): 10206-10211.

- Huang, H. C., Schuurink, R., Denny, T. P., Atkinson, M. M., Baker, C. J., Yucel, I., Hutcheson, S. W. and Collmer, A. (1988). [Molecular cloning of a *Pseudomonas syringae* pv. *syringae* gene cluster that enables *Pseudomonas fluorescens* to elicit the hypersensitive response in tobacco plants.](#) *J Bacteriol* 170(10): 4748-4756.
- Huynh, T., Dahlbeck, D. and Staskawicz, B. (1989). [Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity.](#) *Science* 245(4924): 1374-1377.
- Jamir, Y., Guo, M., Oh, H. S., Petnicki-Ocwieja, T., Chen, S., Tang, X., Dickman, M. B., Collmer, A. and Alfano, J. R. (2004). [Identification of *Pseudomonas syringae* type III effectors that can suppress programmed cell death in plants and yeast.](#) *Plant J* 37(4): 554-565.
- Raman, V., Rojas, C. M., Vasudevan, B., Dunning, K., Kolape, J., Oh, S., Yun, J., Yang, L., Li, G., Pant, B. D., et al. (2022). [Agrobacterium expressing a type III secretion system delivers *Pseudomonas* effectors into plant cells to enhance transformation.](#) *Nat Commun* 13(1): 2581.
- Wise, A. A., Liu, Z. and Binns, A. N. (2006). [Three methods for the introduction of foreign DNA into *Agrobacterium*.](#) *Methods Mol Biol* 343: 43-54.