Managing Plants as Transient Recombinant Protein Factories

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Graduate students:
Larry Joh (PhD 2005), Yik Lam (MS 2006), Christopher Simmons (MS 2007)
Collaborators: Nick Ewing (CSUS), Karen McDonald (UCD)
Staff and Postdoctoral Scholars: Hongyun Guo (UCD), Taduesz Wroblewski (UCD)

Our Initial Research Objectives

2002

- Develop expression system for mouse anti-human IgG 2a in tomato fruit (CSUS)
- Develop bench-scale extraction and purification processes
- Model process scale-up to 10 kg/yr
  - is a tomato-based process feasible?
- PhD for Larry Joh
  - Collaborators: Nicholas Ewing (CSUS) and Antibodies Inc., Davis, CA

Why Processing Tomatoes?

- Low risk of cross pollination
- Economic production in greenhouses year round
  - minimize contamination of food supply
  - processing plant can be operated year round

Our Initial Research Objectives

2002

- Develop expression system for mouse anti-human IgG 2a in tomato fruit (CSUS)
  - After >1 year attempting to transform tomato with gene encoding antibody, we learned our plant/seed source was preventing us from selecting transformants

SEEDS OF DOUBT BY THE SACRAMENTO BEE

UCD cleared in seed mix-up

Researchers unknowingly sent out altered tomato seeds for seven years.

By Mike Lee -- Bee Staff Writer

Published Wednesday, Dec. 1, 2004

Two West Coast mix-ups involving genetically engineered seeds ended with modest fines for two companies and no fault for the University of California, Davis, according to federal records made public Tuesday.

Oxnard-based Seminis Inc., the world's largest fruit and vegetable seed company, and The Scotts Co. of Marysville, Ohio, a grass seed giant, are on the hook for penalties totaling $5,625 for violations of rules set to contain biotech genes.

The fines are toward the low end of the scale for the U.S. Department of Agriculture, which oversees biotech field tests and movement of plants between states. In 2002 for in... the USDA fine...

Recombinant Protein Expression in Plants

- Transgenic plants
  - stable protein expression
  - demonstrated technology
  - easy scale-up
  - environmental issues
- Transient expression (“agroinfiltration”)
  - temporary protein expression
  - can be accomplished in post-harvest tissues
  - addresses environmental and contamination risks
  - lack of engineering data
  - requires bacterial production

What Became Our Research Objective

2003-05

- Collect engineering data to enable consistent expression of recombinant proteins via agroinfiltration
  - Identify factors and corresponding levels critical for expression
    - Vacuum application
    - *A. tumefaciens* concentration
    - Surfactant
    - Light vs. dark
    - Temperature
  - Examine kinetics of recombinant protein expression upon infiltration
  - Determine protein stability during leaf storage and upon protein extraction

Model Recombinant Protein and Plant

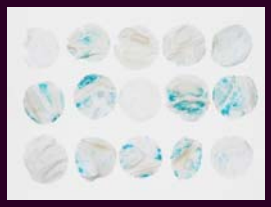
- β-glucuronidase enzyme (GUS)
  - multiple, simple detection methods
  - stable
  - not produced in plants
- Romaine lettuce
  - easy to cultivate
  - grown year-round
  - relatively inexpensive
  - high transient expression in post-harvest tissues

Recombinant Protein (GUS) Expression upon Incubation

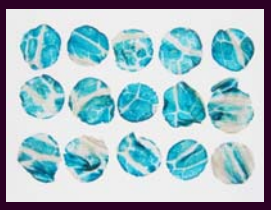
0 hr

16 hr

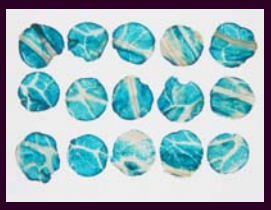
24 hr



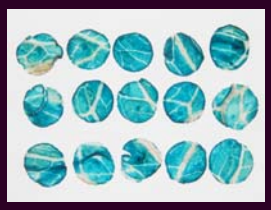
36 hr



48 hr



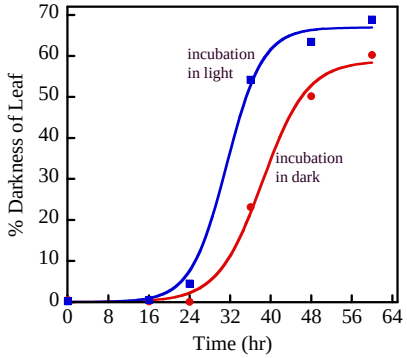
60 hr



Agroinfiltration Engineering Data

Maximum expression was observed 3 days post infiltration.

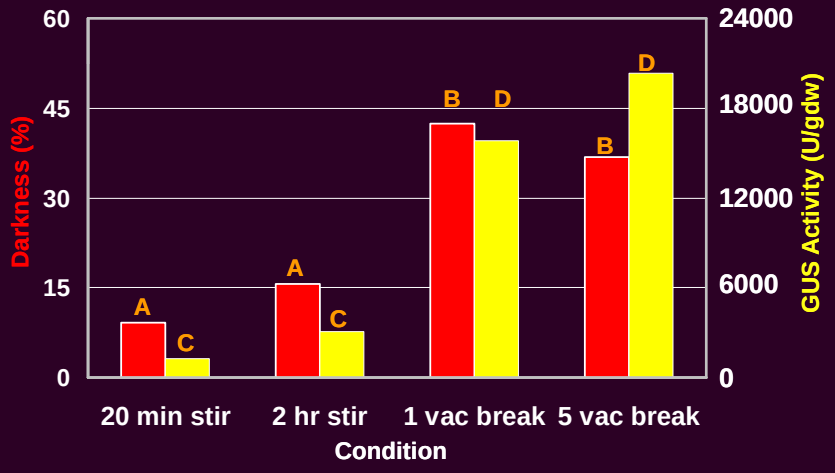
Infiltrated tissues incubated in the dark had comparable expression to tissues incubated in the light



Joh, L. et al. 2005. High-level transient expression of recombinant protein in lettuce. Biotechnology and Bioengineering. 91(7). 861-871

Agroinfiltration Engineering Data

Vacuum application during leaf and *A. tumefaciens* contact significantly improves expression

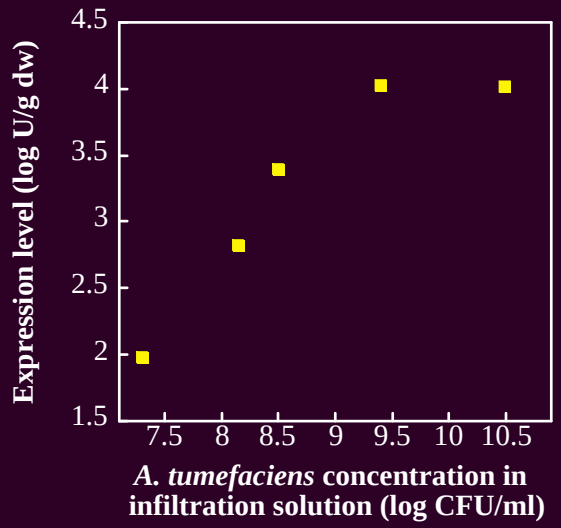


Bars with same color and letter are not significantly different at □=0.05

Joh, L. et al. 2005. High-level transient expression of recombinant protein in lettuce. Biotechnology and Bioengineering. 91(7). 861-871

Agroinfiltration Engineering Data

No increase in expression was observed for cell concentration >10^9 CFU/ml

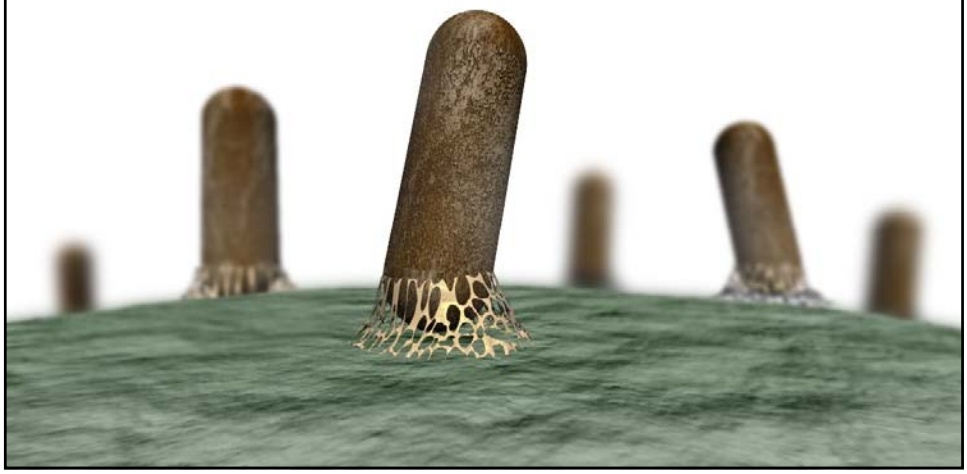


Current Research

- Elucidating key steps in agroinfiltration
  - Physical steps
    - Bringing *A. tumefaciens* into contact with the plant
  - Biological steps
    - Protein and DNA transport during conjugation

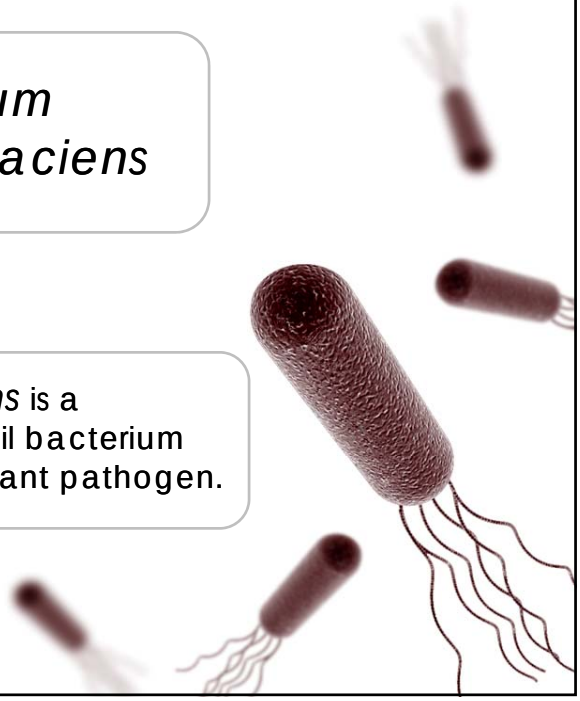
A Kinetic Model of T-Strand Transfer During Agroinfiltration

by Chris Simmons
Department of Biological & Agricultural Engineering

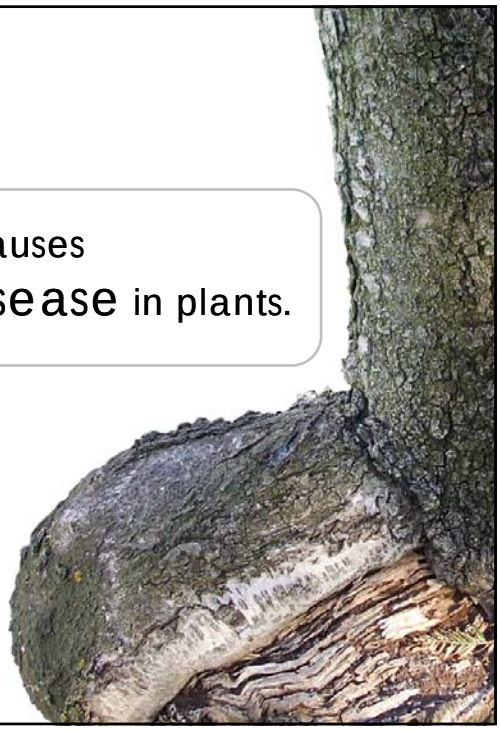


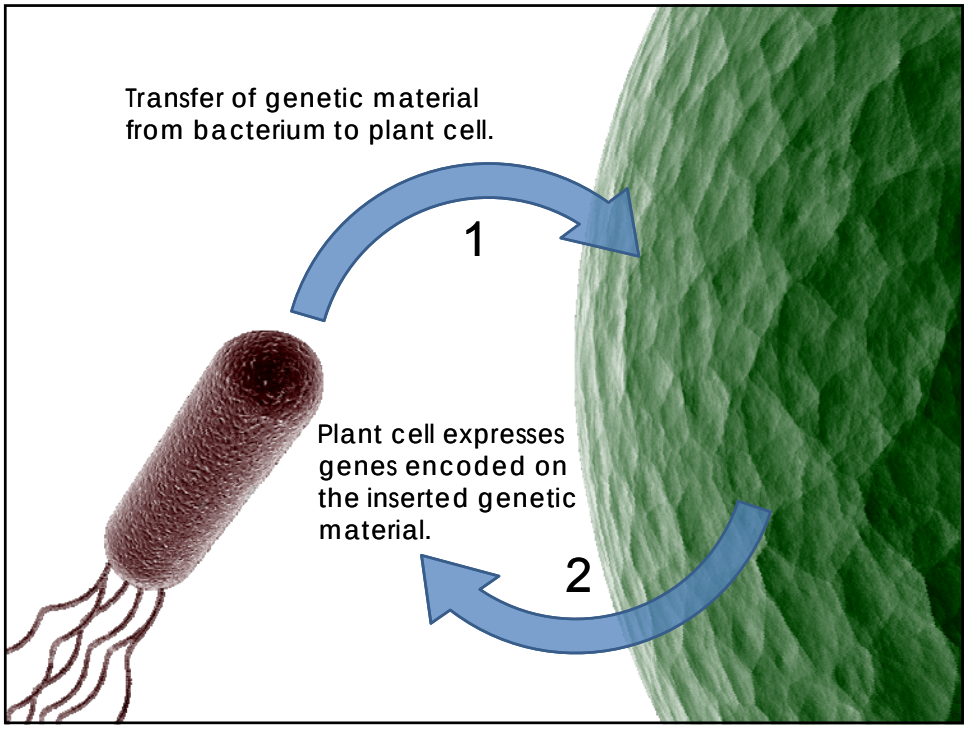
Agrobacterium tumefaciens

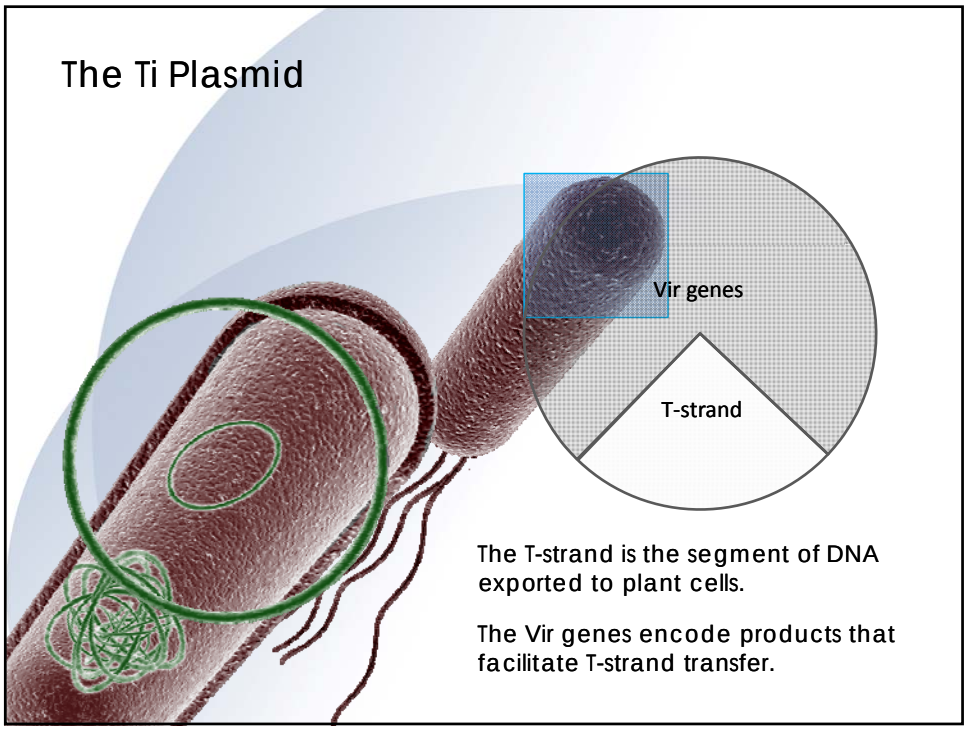
A. tumefaciens is a common soil bacterium and plant pathogen.



A. tumefaciens causes crown gall disease in plants.




Transfer of genetic material
from bacterium to plant cell.
1
Plant cell expresses
genes encoded on
the inserted genetic
material.
2

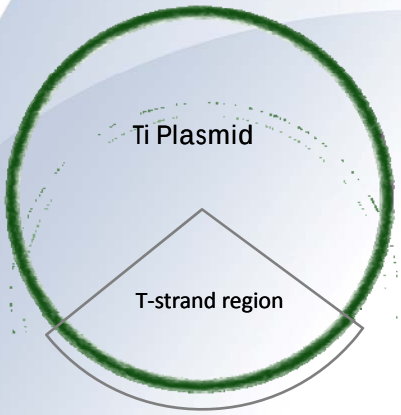

The Ti Plasmid
Vir genes
T-strand
The T-strand is the segment of DNA
exported to plant cells.
The Vir genes encode products that
facilitate T-strand transfer.

The genes on the T-strand can be modified .

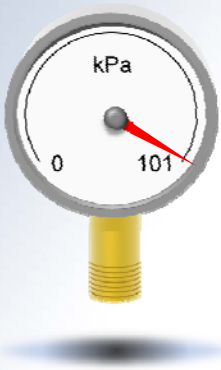
1. Excise the endogenous T-strand sequence.

2. Introduce new T-strand sequence encoding proteins of interest.

3. Transform new Ti plasmid into *A. tumefaciens.*

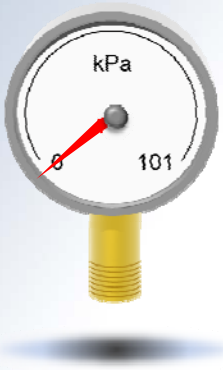


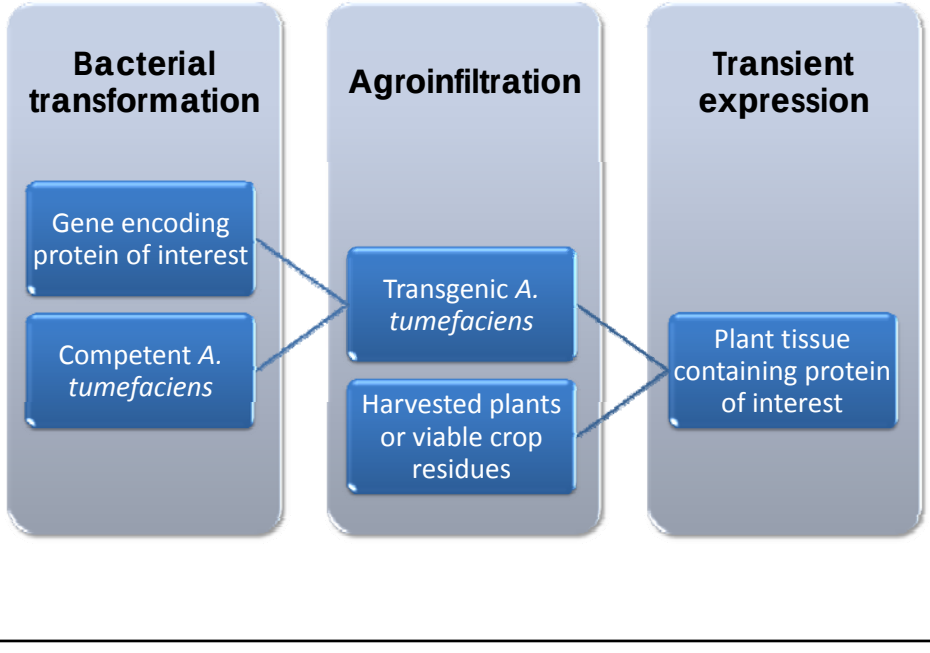
Vacuum application draws gases out of leaf tissue.

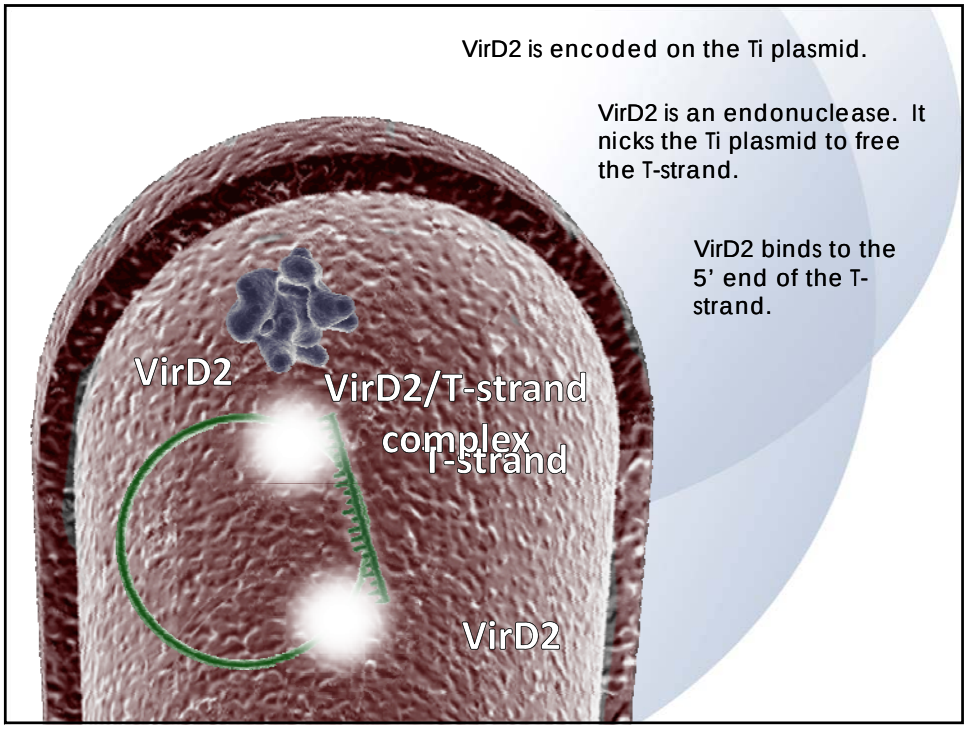


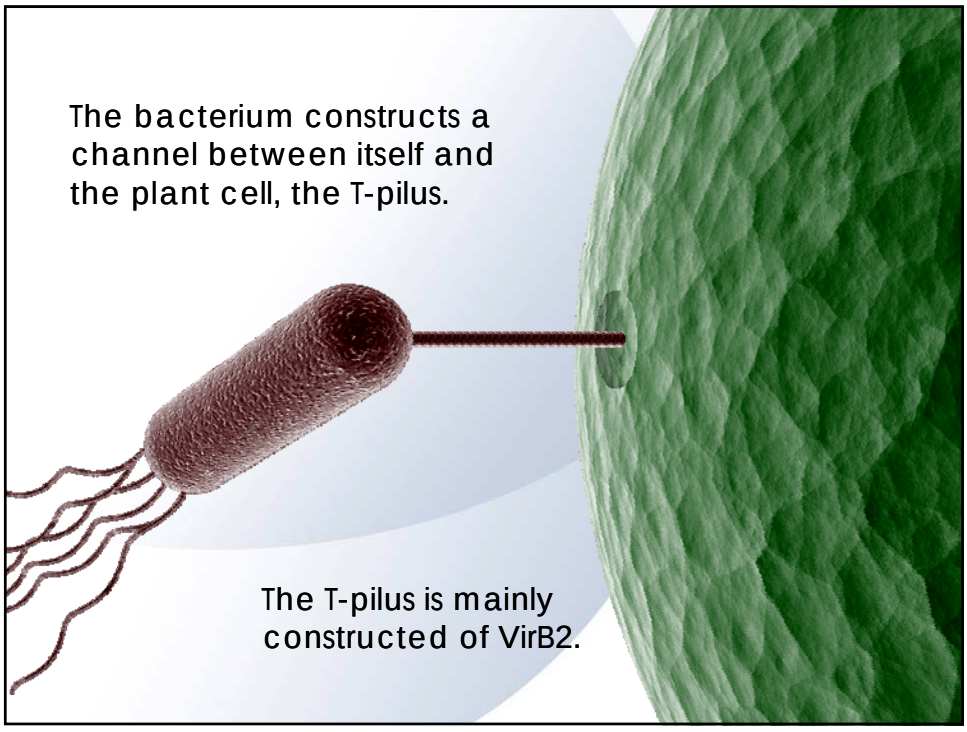
After vacuum is broken, liquid enters the leaf to replace the escaped gases.

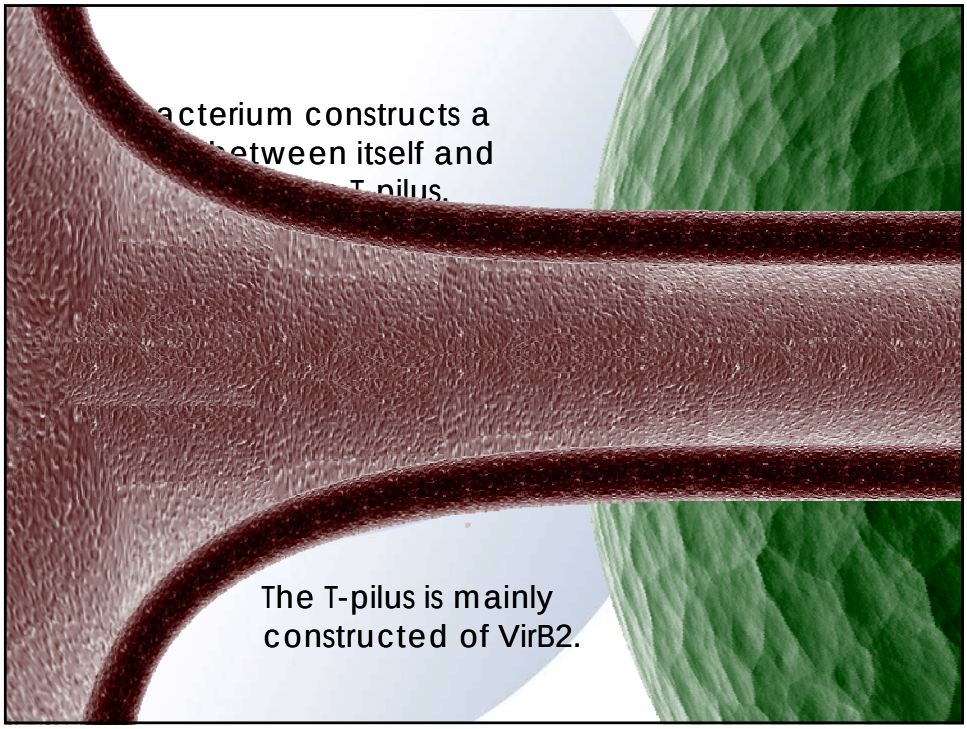
Simmons C, J VanderGheynst, and S Upadhyaya. 2008. A model of *A. tumefaciens* vacuum infiltration into harvested leaf tissue and subsequent *in planta* transgene transient expression. Biotechnology & Bioengineering. In press.






VirD2 is encoded on the Ti plasmid.
VirD2 is an endonuclease. It nicks the Ti plasmid to free the T-strand.
VirD2 binds to the 5' end of the T-strand.
VirD2
VirD2/T-strand complex
T-strand
VirD2


The bacterium constructs a channel between itself and the plant cell, the T-pilus.
The T-pilus is mainly constructed of VirB2.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T-pilus.
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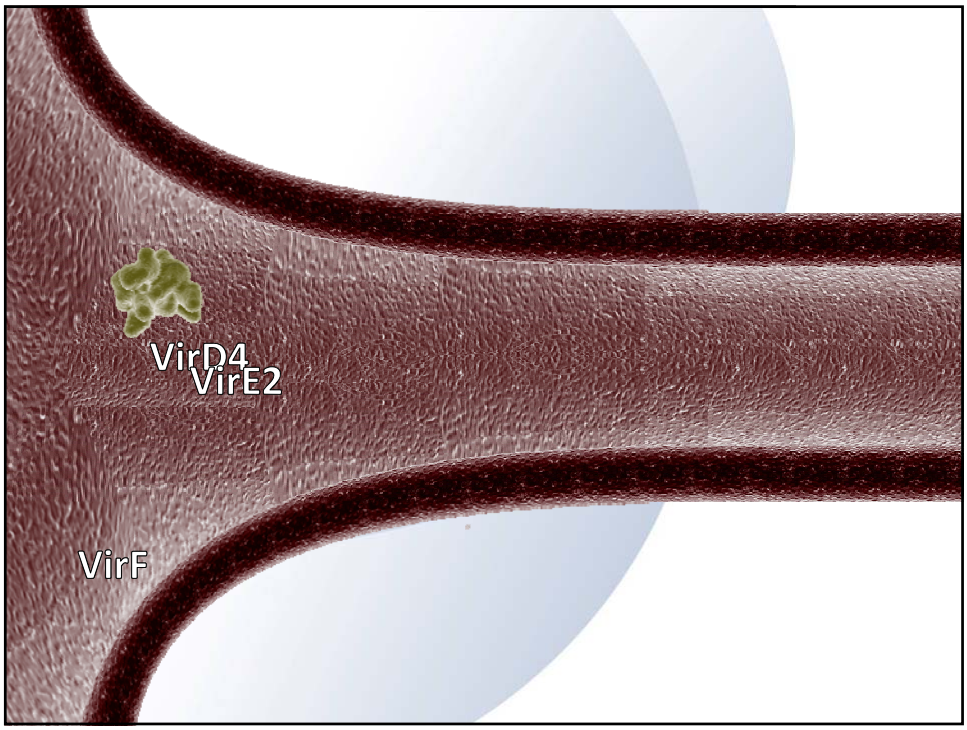
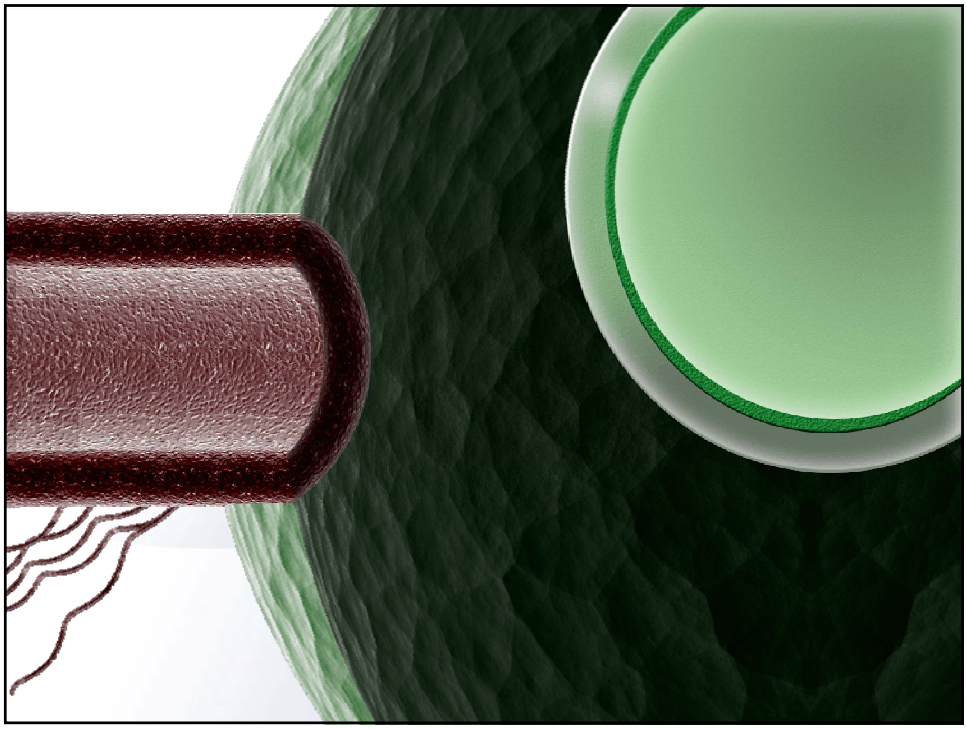
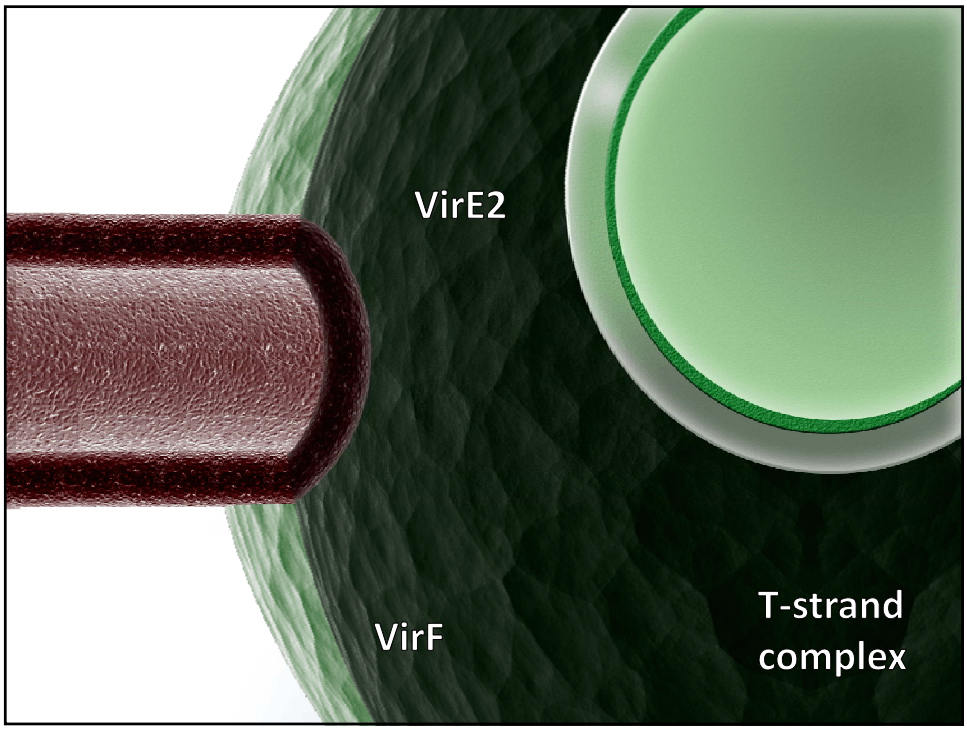

VirD4
VirE2
VirF




VirE2
VirF
T-strand
complex

Objective 1

Develop a quantitative model describing the kinetics of T-strand transfer through the pathway.

Objective 2

Develop a methodology for studying T-strand movement through key steps of the transfer pathway over time.

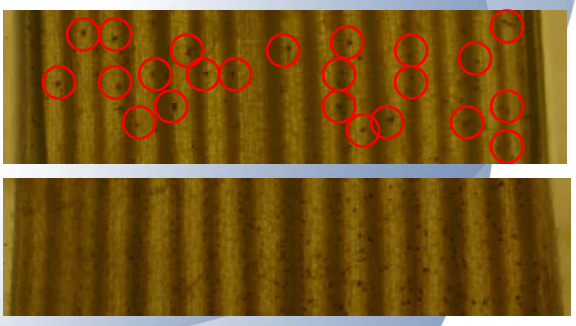
Objective 3

Apply methodology to study T-strand transfer during the agroinfiltration of a commercially relevant plant.

Romaine lettuce (dicot)

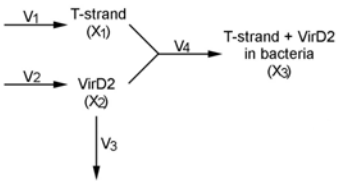
Joh et al. (2005) Biotechnology and Bioengineering 91(7):861-871

Switchgrass (monocot)



VanderGheynst et al. (2008) Biomass and Bioenergy 32(4):372-379

Objective 1:
T-strand Transfer Model



X_1 = [T-strands]

X_2 = [VirD2]

X_3 = [VirD2-T-strand complexes]

X_4 = [T-pili]

X_5 = [VirD2-T-strand complexes in T-pili]

X_6 = [VirD2-VirE2-T-strand complexes]

X_7 = [VirD2-T-strand complexes in plant]

X_8 = [VirD4]

X_9 = [VirE2]

X_{10} = [VirF]

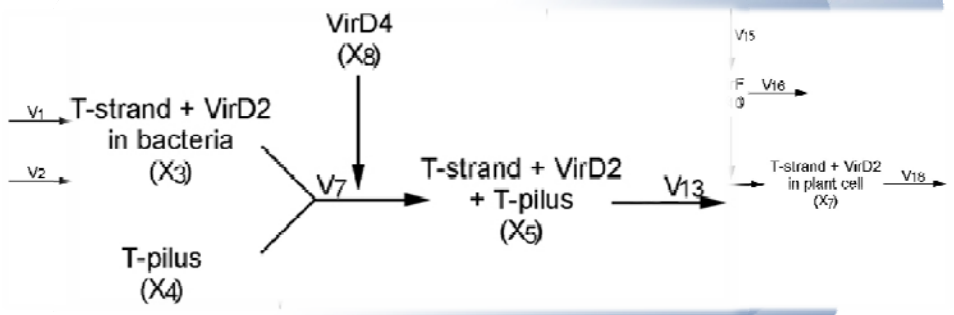
Mass balance for each pool modeled using power law representations and S-system formalism

$$dX_i / dt = \alpha_i \prod_{j=1}^{n+m} X_j^{g_{ij}} - \beta_i \prod_{j=1}^{n+m} X_j^{h_{ij}}$$

Based on the rate law from chemistry

$$nA + mB \rightarrow C$$

$$\mathrm{dC/dt} = kA^n B^m$$



Identify factors that act to increase X_5:

X_3, X_4, and X_8

Identify factors that act to decrease X_5:

synthesis of X_5 $\frac{X_5}{\alpha_5 X_3^{g_{53}} X_4^{g_{54}} X_8^{g_{58}}}$

$\frac{dX_5}{dt}$ degradation of X_5 $\beta_5 X_5^{h_{55}}$

$$\frac{dX_1}{dt} = \alpha_1 - \beta_1 X_1^{h_{11}} X_2^{h_{12}}$$

$$\frac{dX_2}{dt} = \alpha_2 - \beta_2 X_1^{h_{21}} X_2^{h_{22}}$$

$$\frac{dX_3}{dt} = \alpha_3 X_1^{g_{31}} X_2^{g_{32}} - \beta_3 X_3^{h_{33}} X_4^{h_{34}} X_8^{h_{38}}$$

$$\frac{dX_4}{dt} = \alpha_4 X_5^{g_{45}} - \beta_4 X_3^{h_{43}} X_4^{h_{44}} X_8^{h_{48}}$$

$$\frac{dX_5}{dt} = \alpha_5 X_3^{g_{53}} X_4^{g_{54}} X_8^{g_{58}} - \beta_5 X_5^{h_{55}}$$

$$\frac{dX_6}{dt} = \alpha_6 X_5^{g_{65}} X_9^{g_{69}} - \beta_6 X_6^{h_{66}} X_{10}^{h_{610}}$$

$$\frac{dX_7}{dt} = \alpha_7 X_6^{g_{76}} X_{10}^{g_{710}} - \beta_7 X_7^{h_{77}}$$

$$\frac{dX_8}{dt} = \alpha_8 - \beta_8 X_8^{h_{88}}$$

$$\frac{dX_9}{dt} = \alpha_9 - \beta_9 X_9^{h_{99}}$$

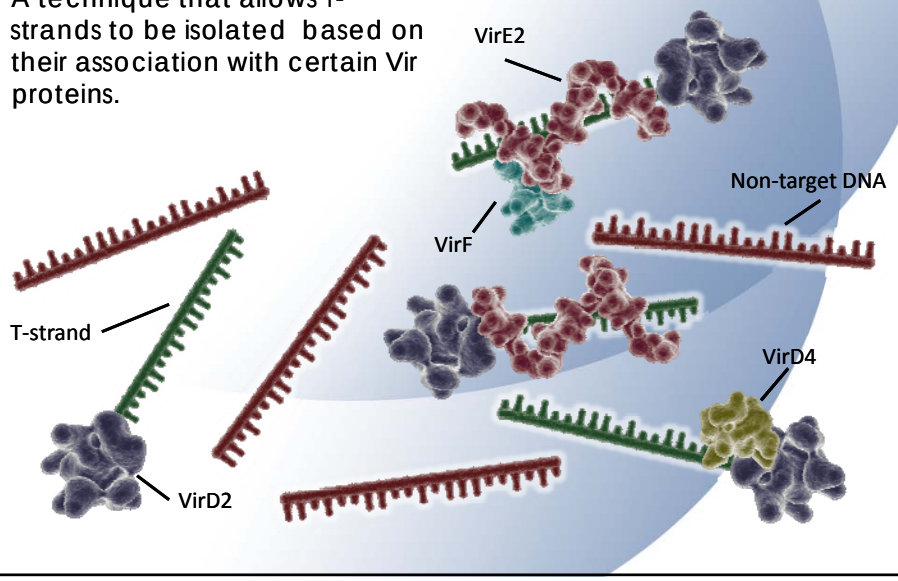
$$\frac{dX_{10}}{dt} = \alpha_{10} - \beta_{10} X_{10}^{h_{1010}}$$

Objective 2:

T-strand Complex and Vir Protein Isolation

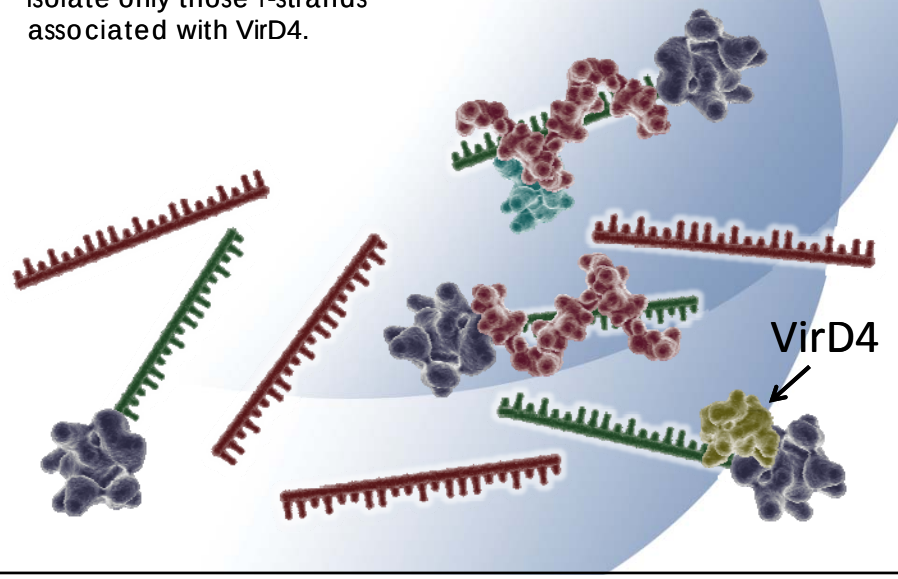
TrIP

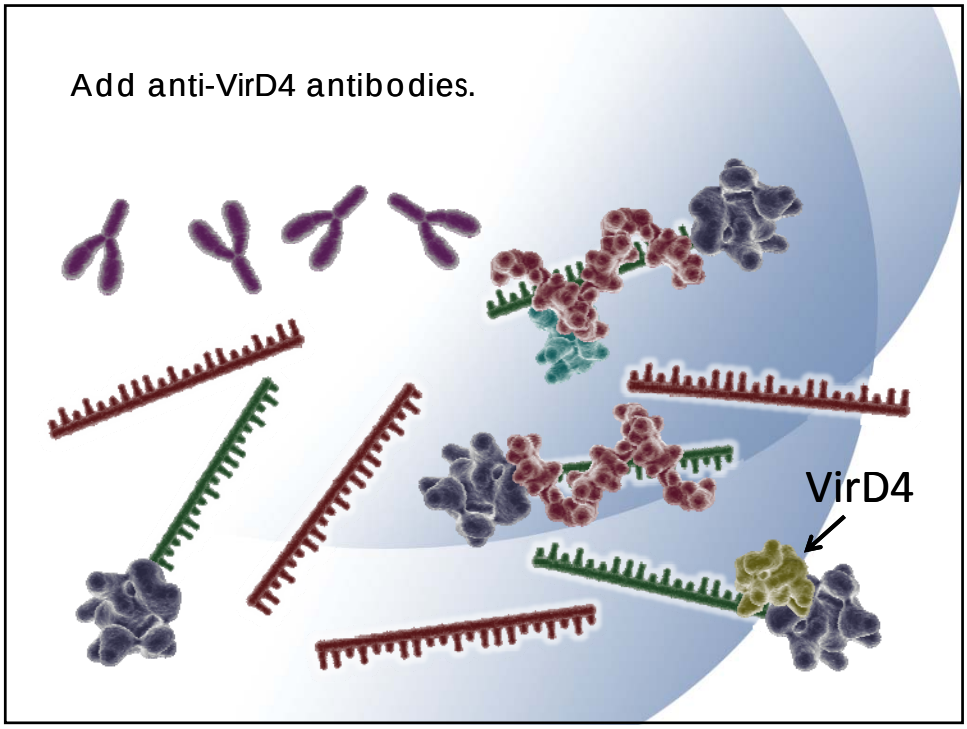
A technique that allows T-strands to be isolated based on their association with certain Vir proteins.

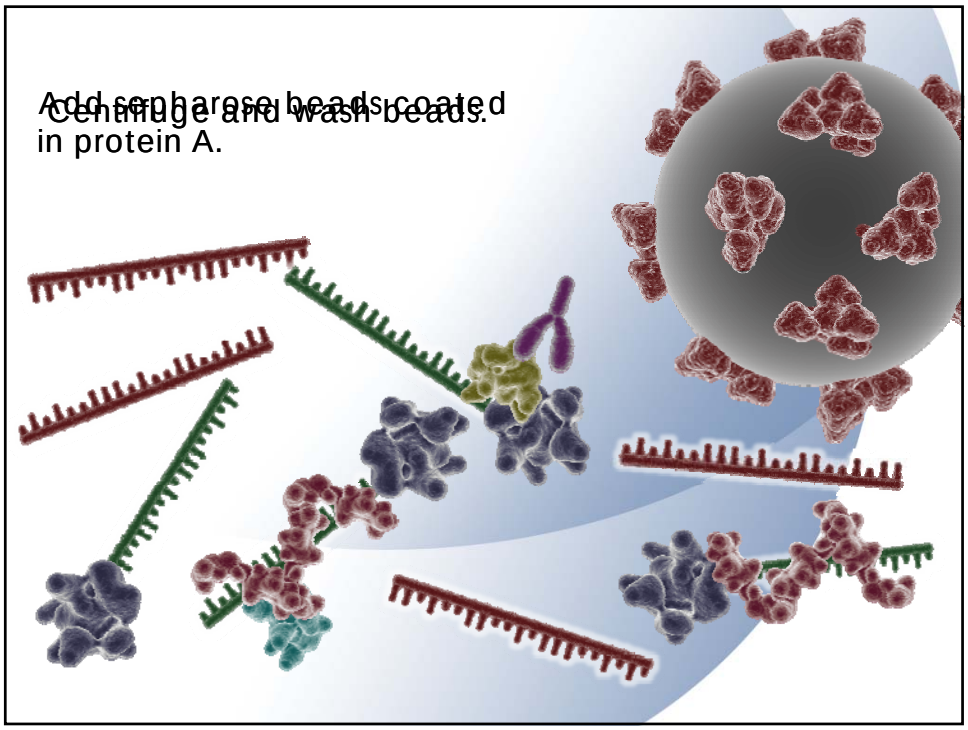


Example:

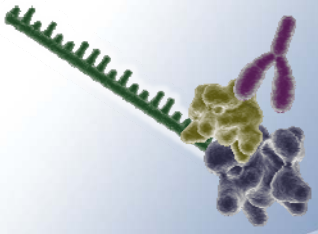
Isolate only those T-strands associated with VirD4.



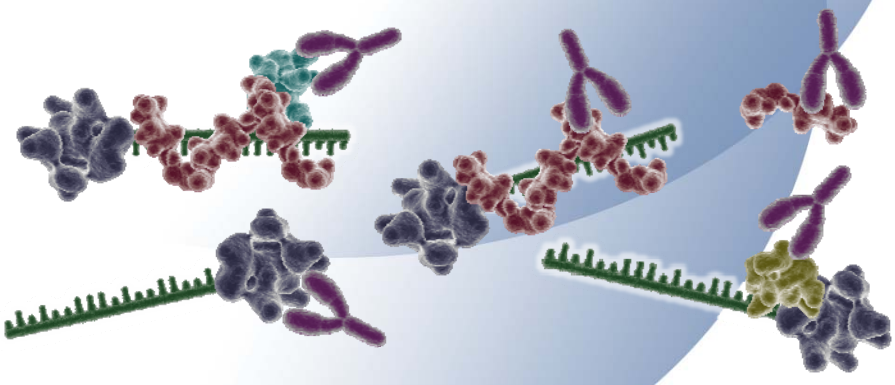

Add anti-VirD4 antibodies.
VirD4


Add sepharose beads coated in protein A.
Centrifuge and wash beads.

Purify DNA and quantify using qPCR



- Use different antibodies to isolate different Vir protein complexes via TrIP.
- Repeat TrIP over time.
- Obtain *X* vs *t* data needed to fit model.



Objective 3:
Extension of Model to Switchgrass

L. sativa valmaine is a line of romaine lettuce.

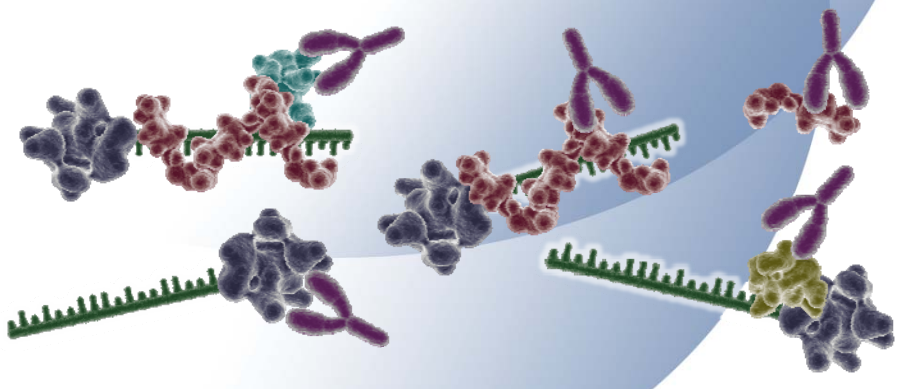
It is readily agroinfiltrated.

Alamo switchgrass (*Panicum virgatum*) is a commercially relevant crop.

It can not be efficiently agroinfiltrated.

Currently

We are working to produce antibodies to Vir proteins we are interested in.

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