

Nitrogen-fixing symbiosis:

3. Plant genes and cell responses in nitrogen-fixing symbiosis

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*Rhizobium-legume symbiosis:
N₂ fixation happens in root nodules*

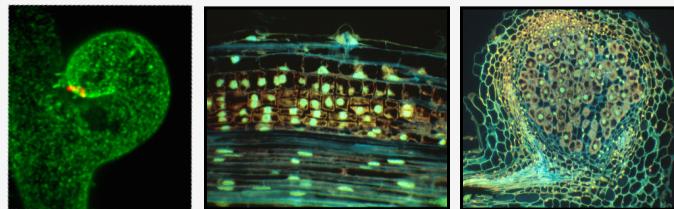
Complex developmental process requires genes of both bacteria and plant.

It is species-specific.

Bacterium and plant each respond to signals from the other

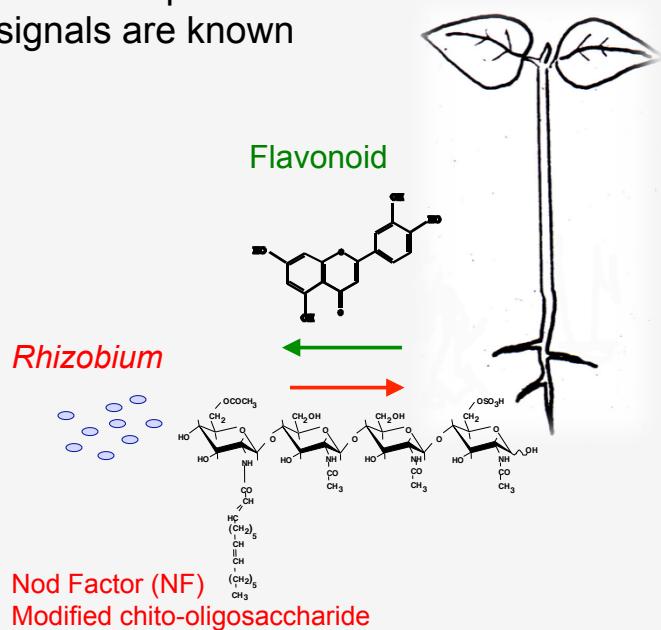


Outline



- Introduction:
 - Plant cell responses to Nod Factor
 - Genetic dissection of early events in symbiosis
 - Nod Factor signal transduction
- Infection
 - Flotillins and Nod Factor receptors
- *DNFs*: plant genes required for *nif* function
 - DNF1 signal peptidase: plant peptides control bacterial differentiation
 - DNF2, a phospholipase homolog: a role for uninfected cells?

The initial plant and bacterial signals are known



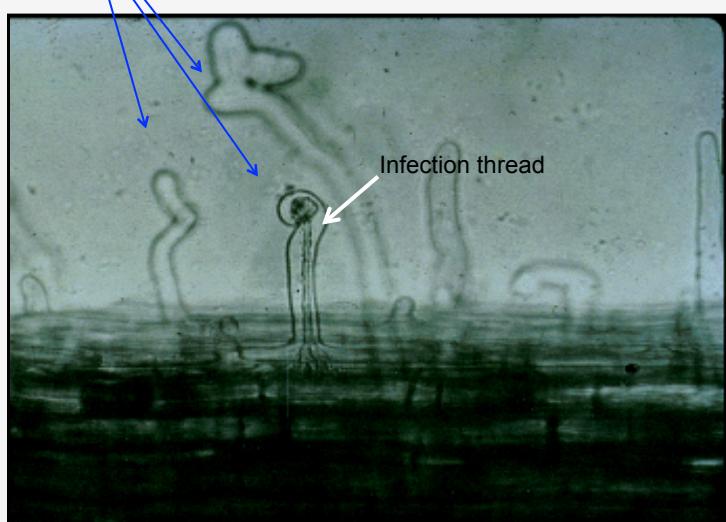
Nod factors imitate *Rhizobium*



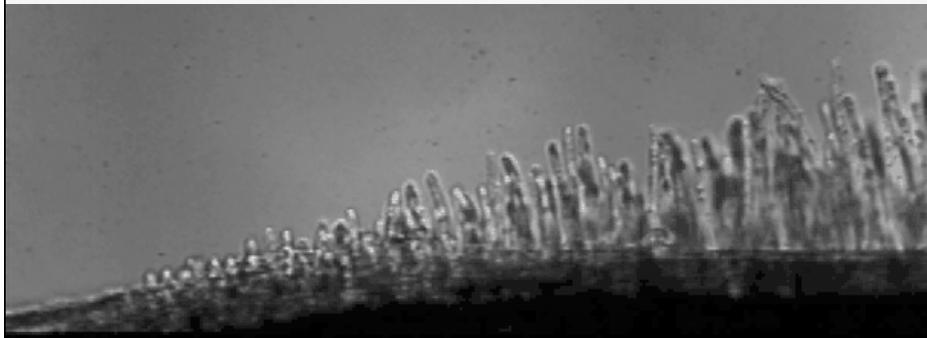
Top: A normal nodule induced by *Sinorhizobium meliloti*

Bottom: a nodule-like structure formed in response to droplet of Nod Factor

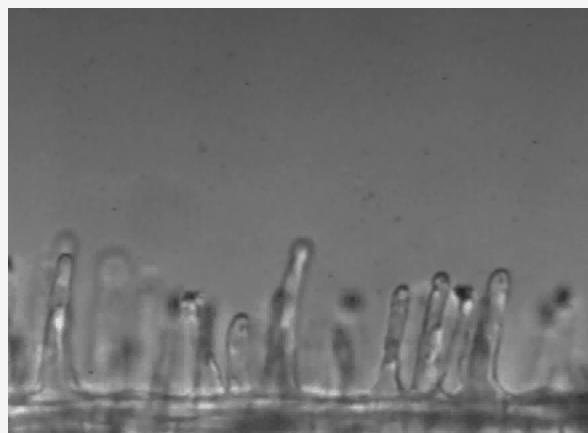
Rhizobium alters root hair growth on host:
deform, branch, form a curl / 'crozier'
form infection thread

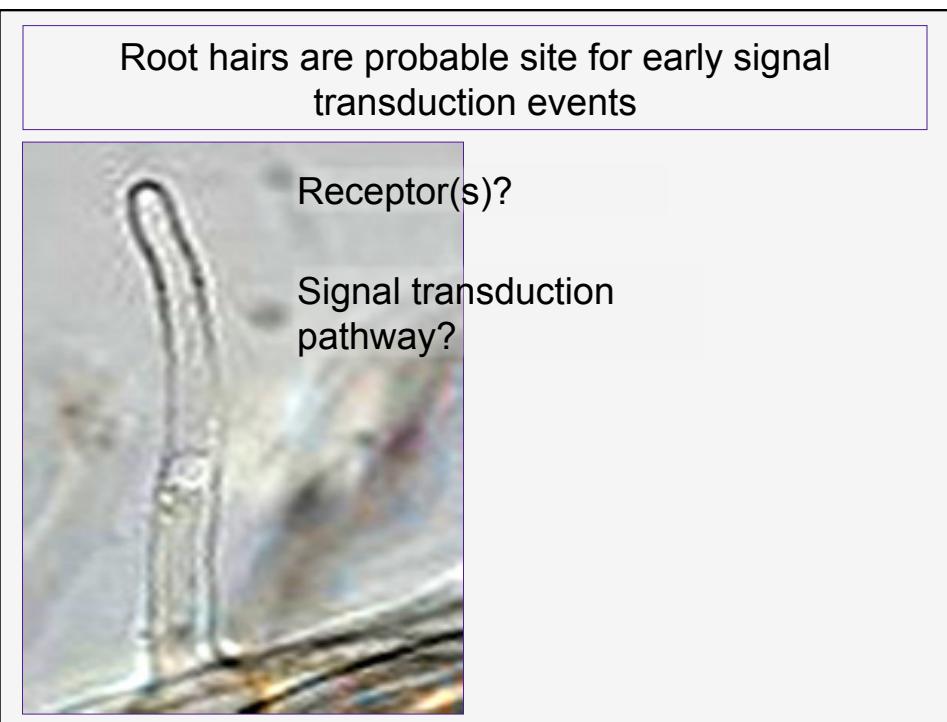
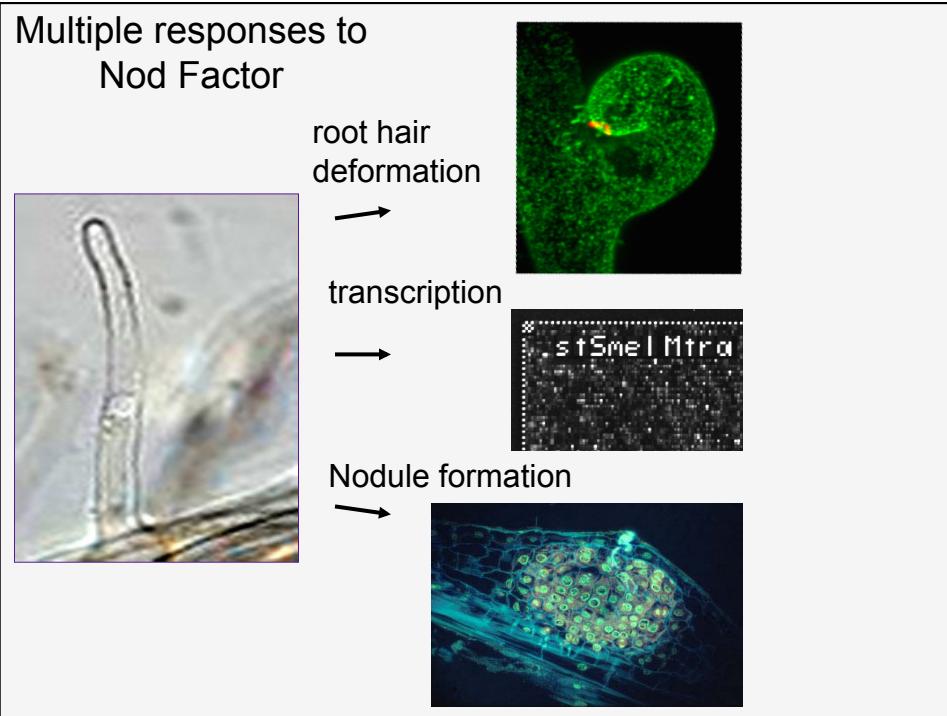


Root hair growth: without *Rhizobium*

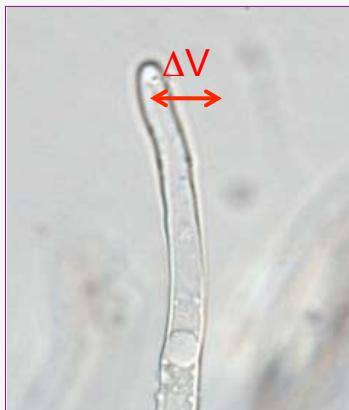


Root hair growth: with *Rhizobium*



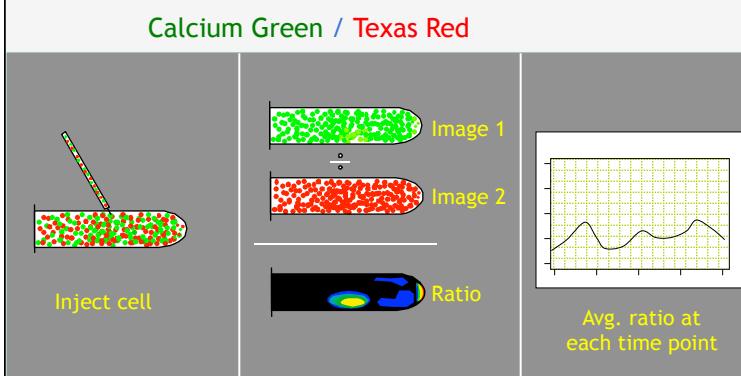


Root hair plasma membrane potential
is **depolarized** by NF

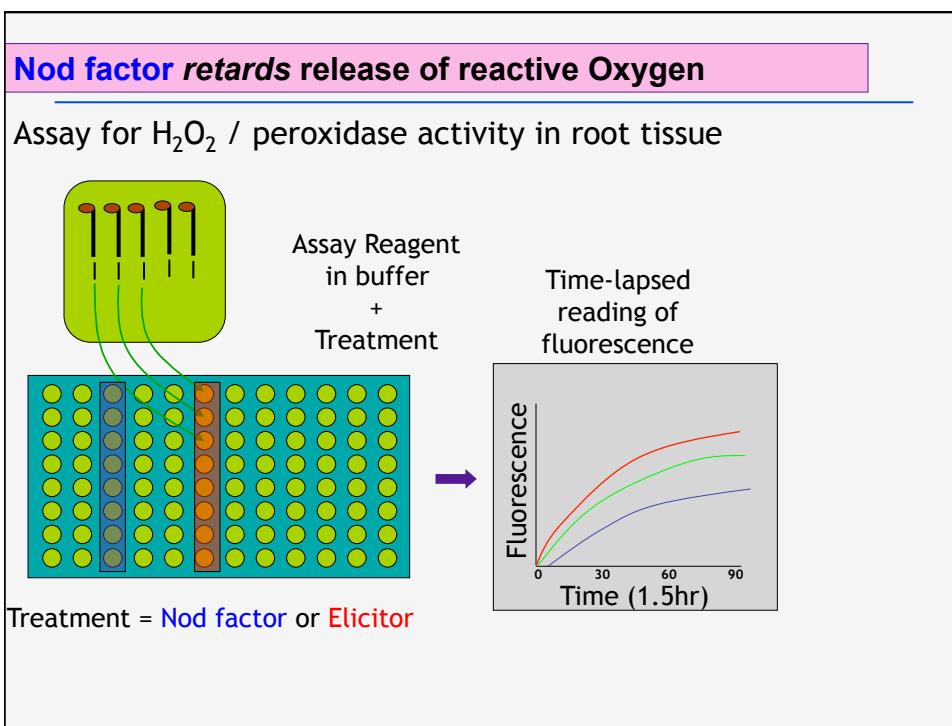
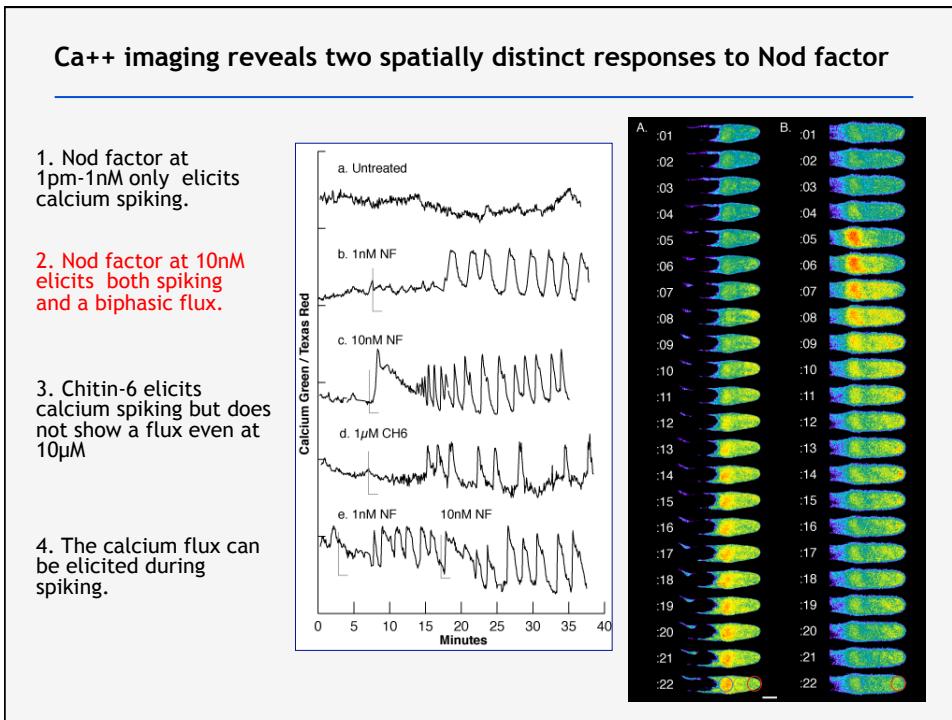


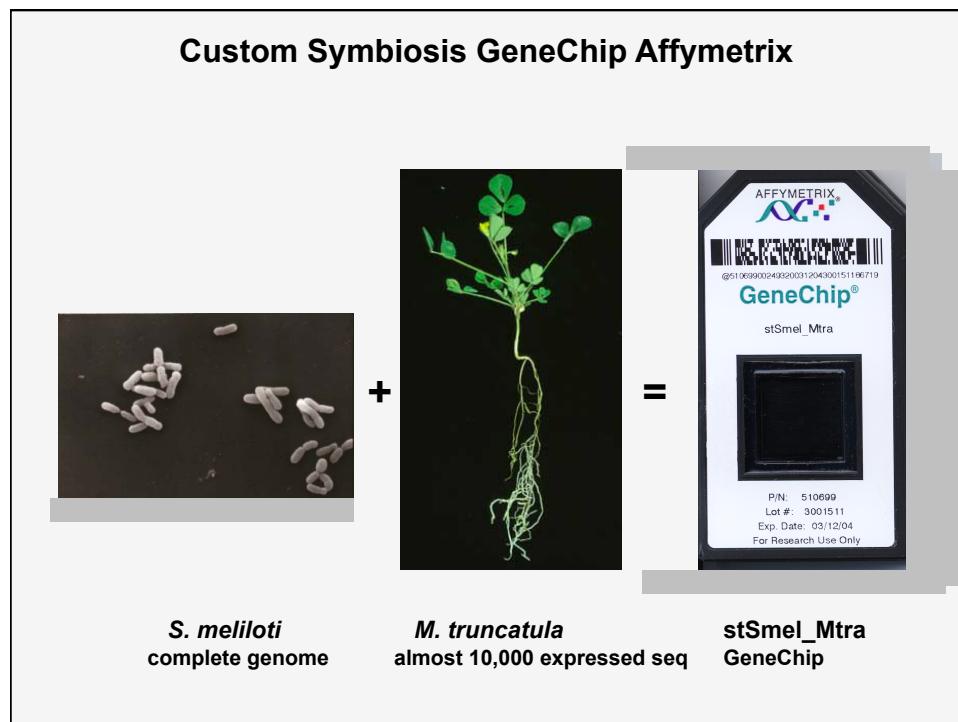
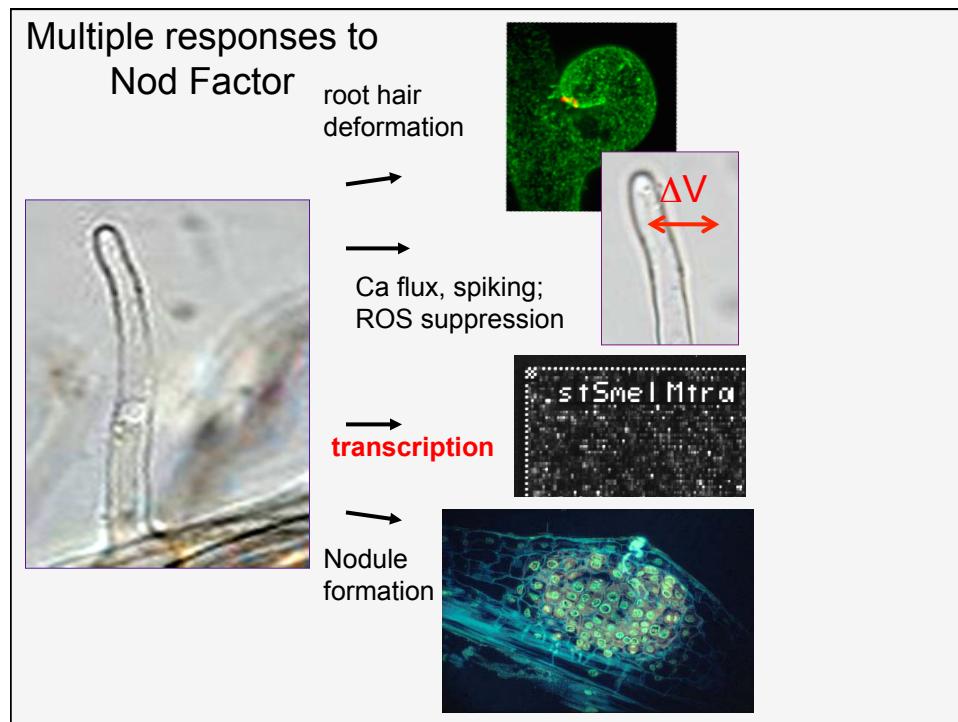
- ΔV untreated root hair: av. -134 mV
- ΔV after exposure to NF: depolarize +20mV
- Change occurs within one minute
- Accompanied by ion flux near root hair tip

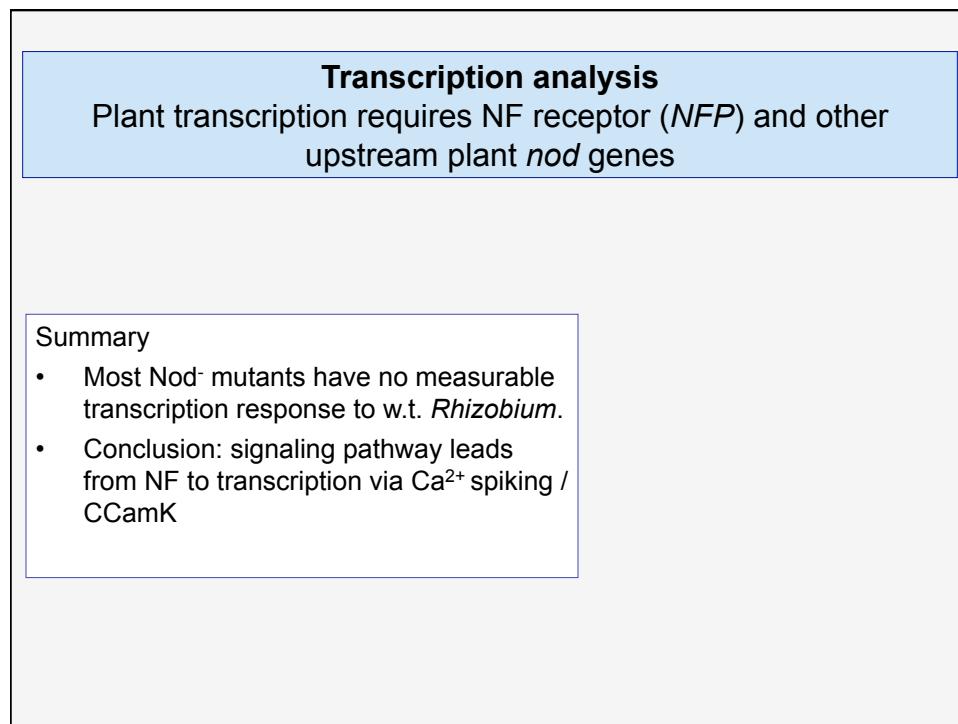
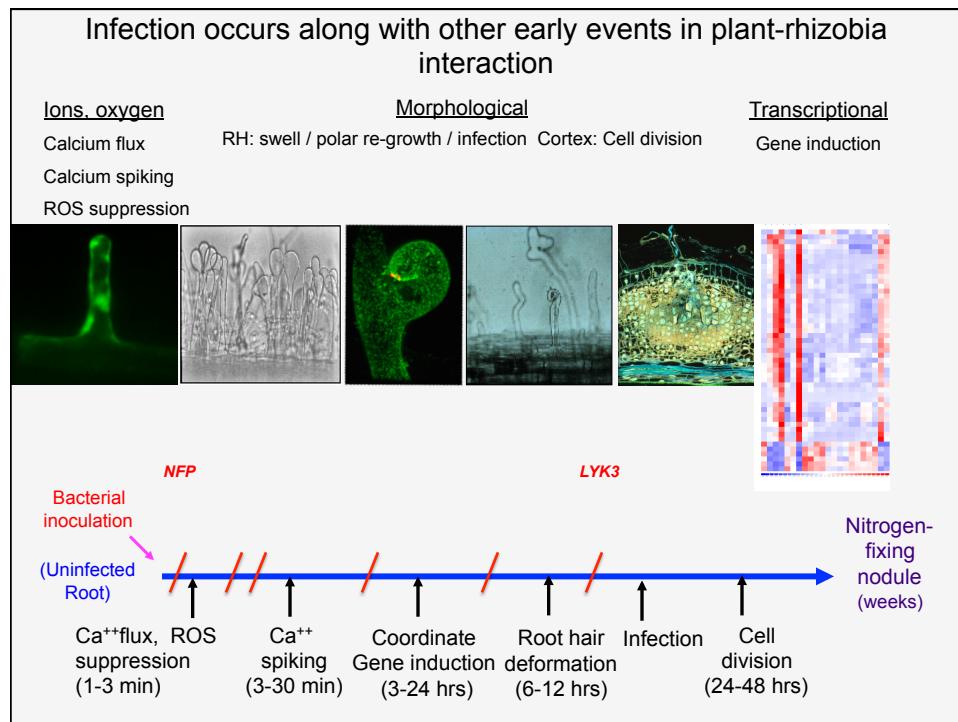
Measuring Calcium behavior in root hairs:
Ratio imaging two dextran-linked dyes

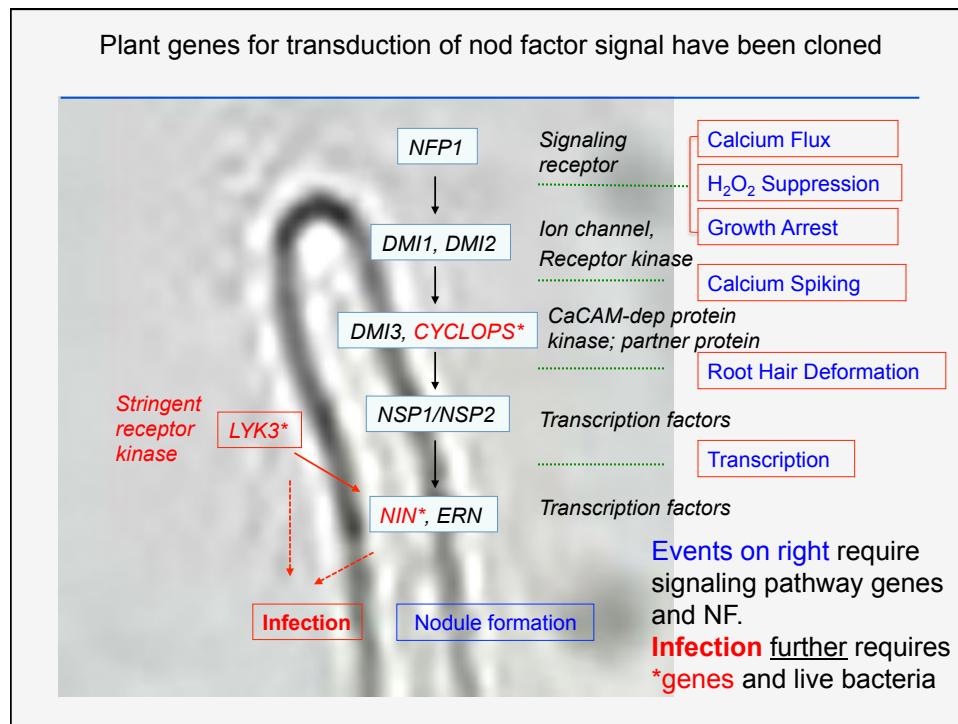


Ratio proportional to free calcium ion concentration in the cytosol

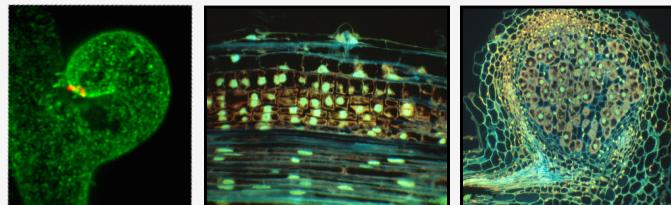




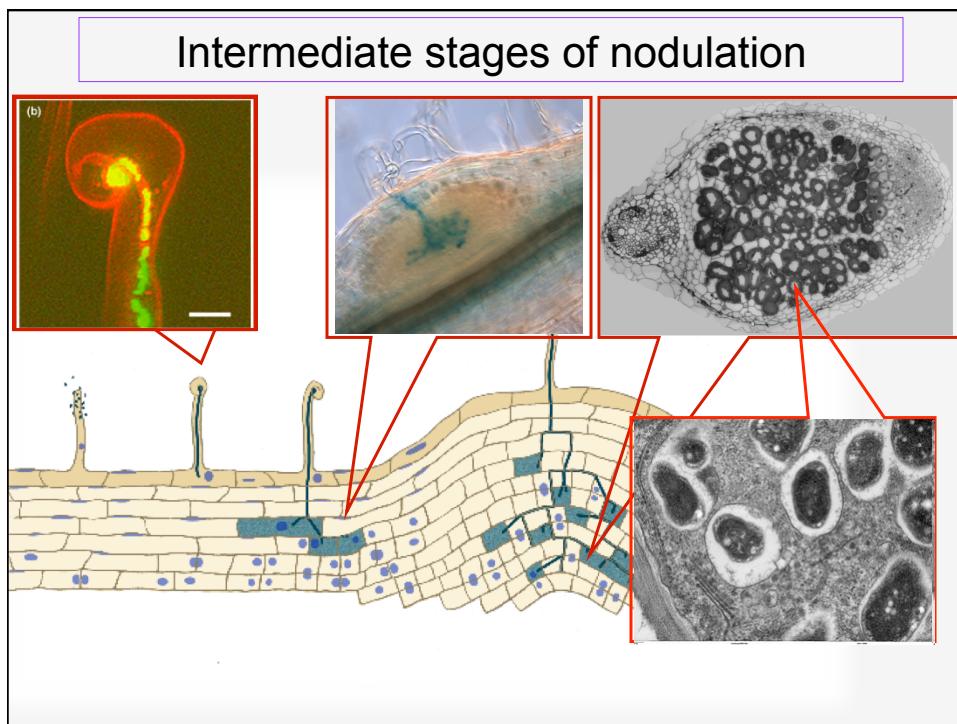
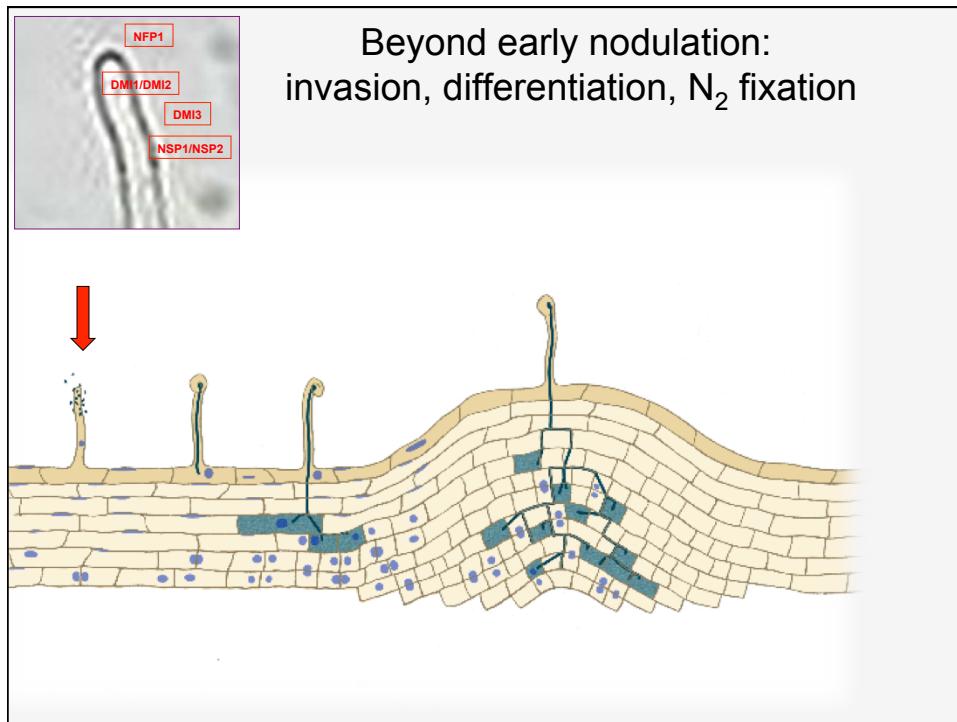




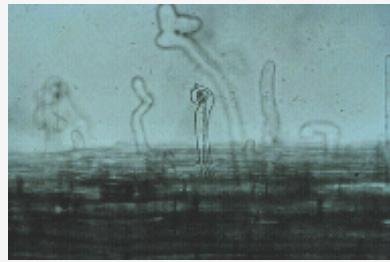
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 - Nod Factor signal transduction
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How do bacteria stimulate plants to form infection threads?

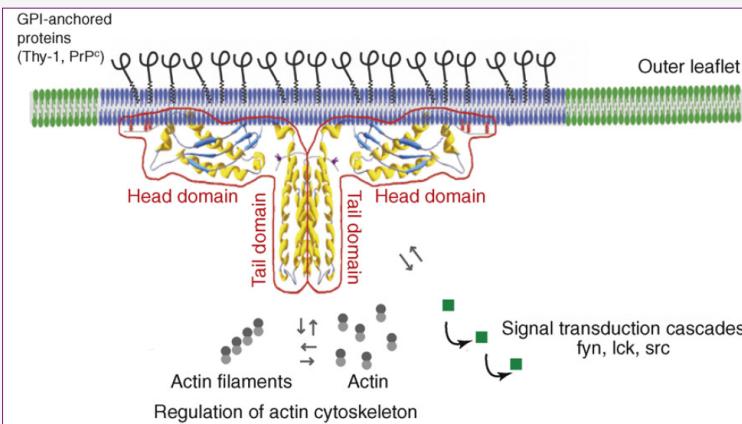


A candidate approach: plant flotillin proteins

- Two plant flotillins are selectively required for infection thread initiation (FLOT4) and extension (FLOT2).
- Transcription of FLOTS depends on signal pathway
- FLOTs re-localize upon NF treatment

Candidate genes to study root hair reactions to Nod Factor: **flotillins**.

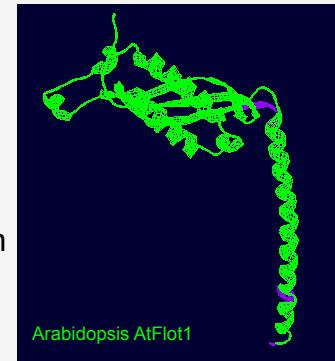
In animal cells, these proteins localize to **membrane microdomains** ("lipid rafts") and have been implicated in: endocytosis / pathogens; membrane shaping; actin polymerization



Steurmer. 2009. Trends in Cell Biology

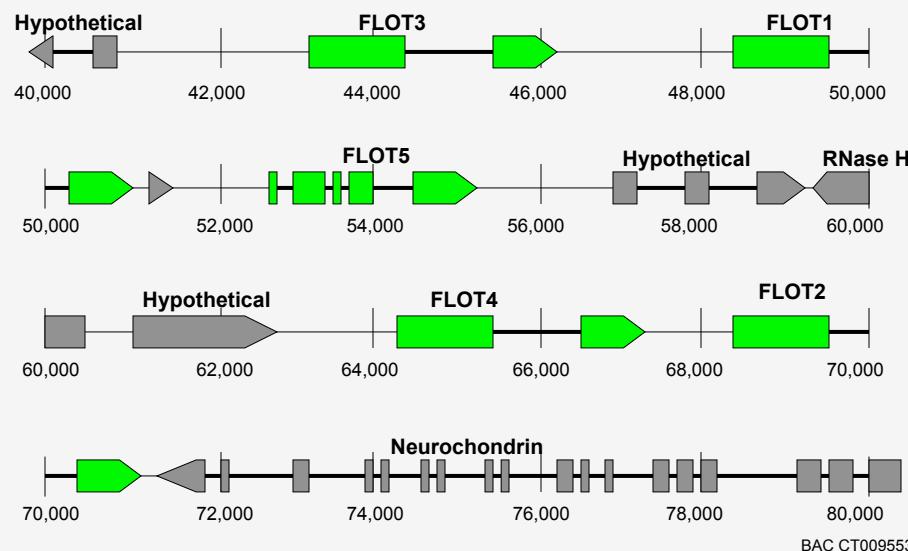
Plant Flotillins (*FLOTs*)

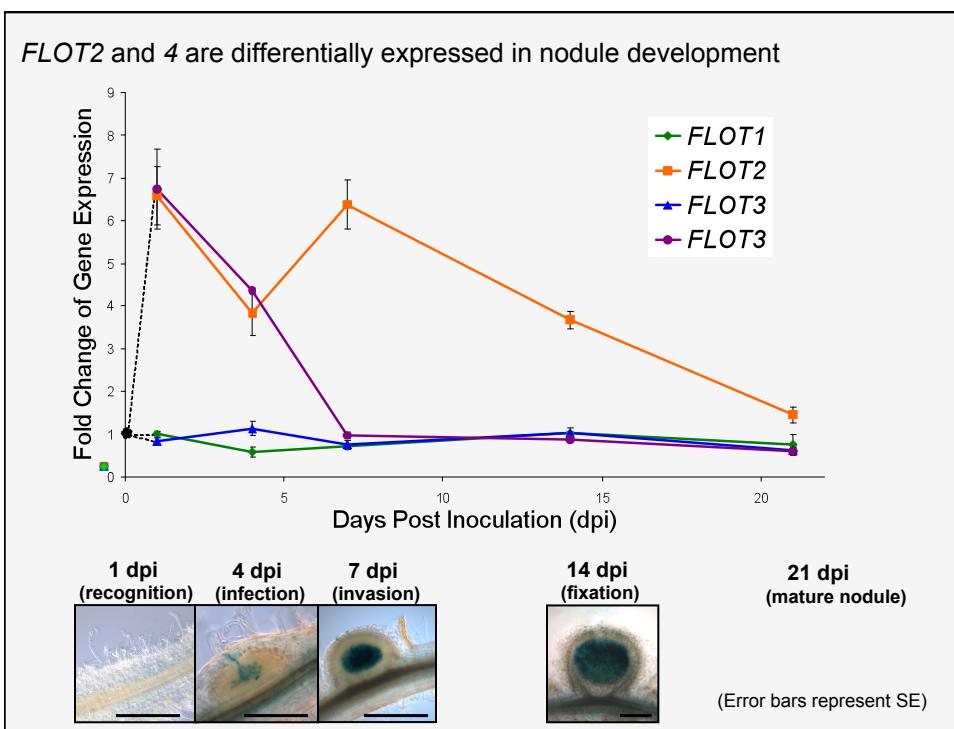
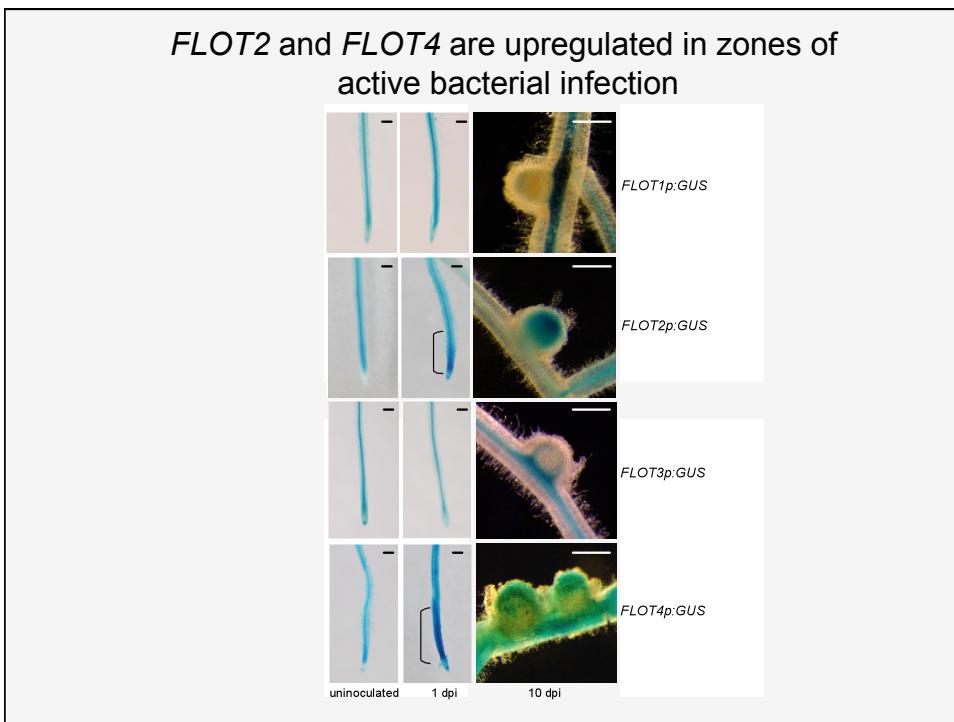
- Ubiquitous
 - 3 in *Arabidopsis*
 - 7+ in *Medicago truncatula*
- In *M. truncatula*, two genes, ***FLOT2*** and ***FLOT4*** are uniquely expressed in nodules
- Both Flot2 and Flot4 are present in **puncta** – likely membrane micro-domains
- **Flot4 relocates to root hair tip** after exposure to bacteria; associates with growing infection thread

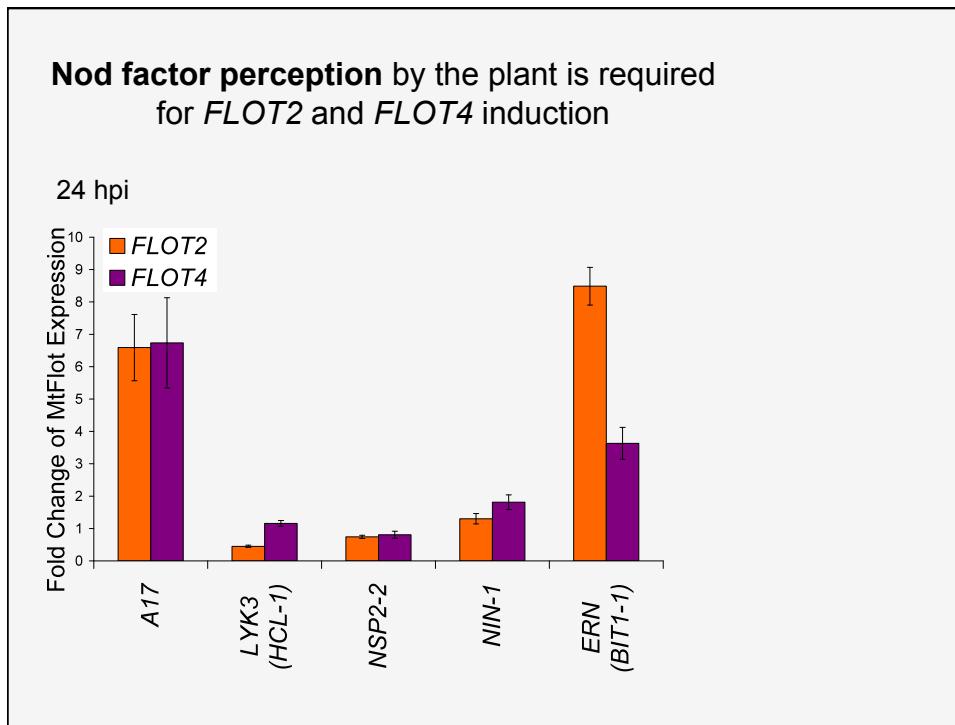
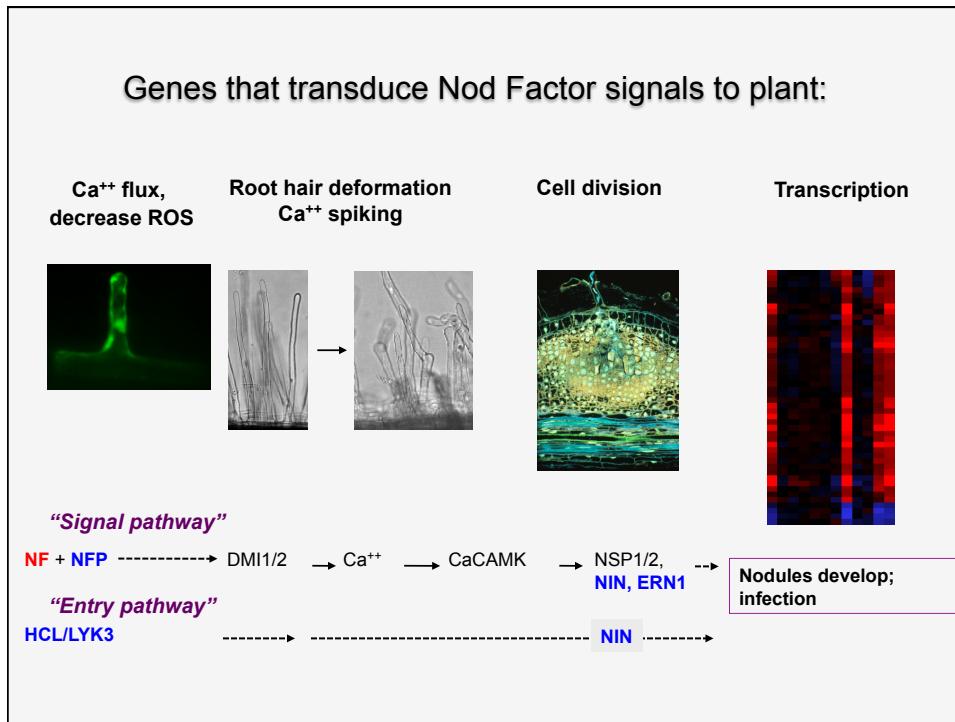


M. truncatula encodes 8 flotillin-like proteins (*FLOTs*)

Five are linked within 30 kb on chromosome 3.





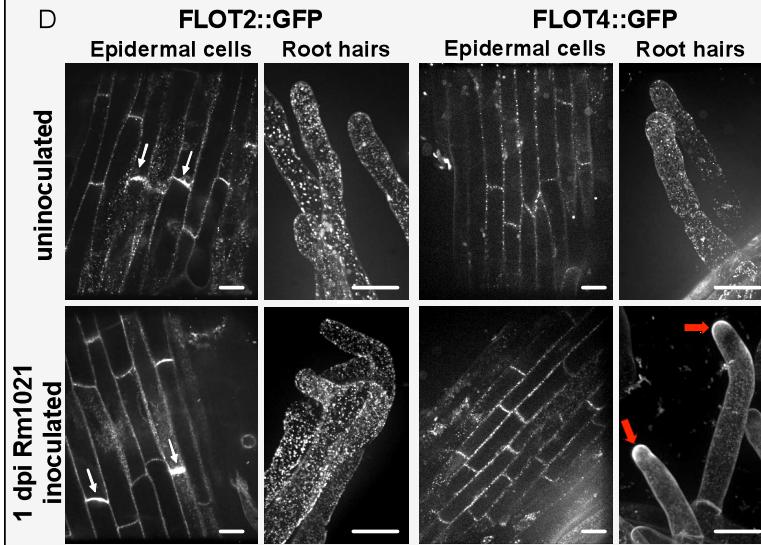


Role for flotillins in nodulation?

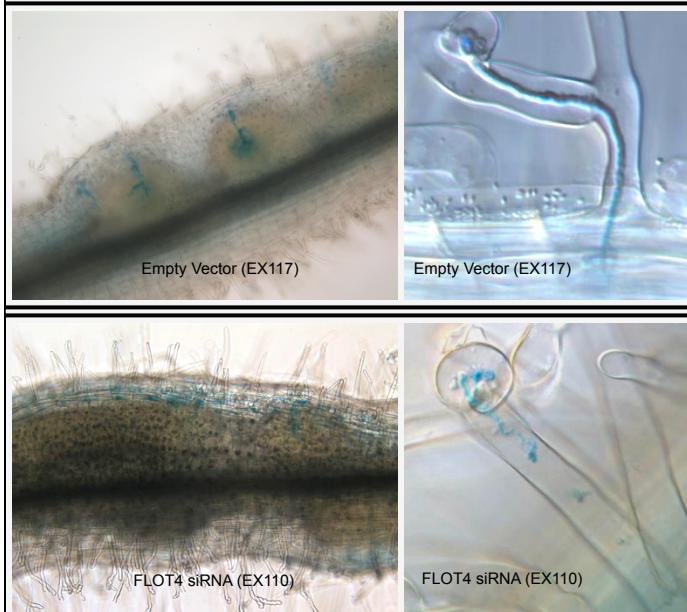
Plant genotype:	Average Nodule #	% plants with nodules	% nods that are pink (Fix ⁺)
Wild type	6.0	12%	28.4
<i>flot1-3-</i>	6.1	17.9	27.4
<i>flot2-</i>	2.5	37.3	5.2
<i>flot4-</i>	5.5	12.7	15.9
<i>flot2- flot4-</i>	2.0	50.4	2.5

➔ Flotillin 2 and 4 are both required for normal nodulation, and are non-redundant

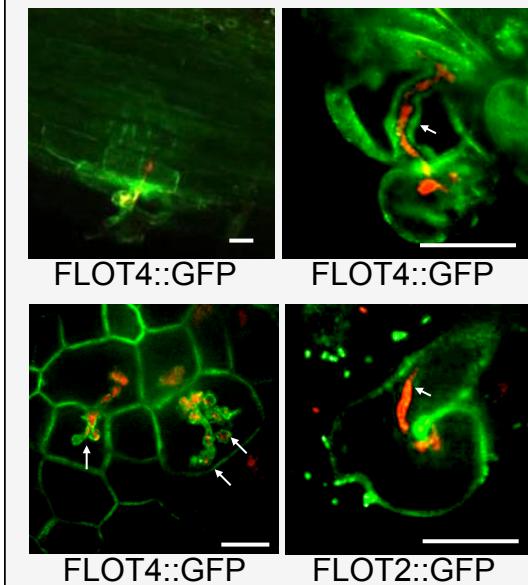
FLOT2 and FLOT4 show punctate localization.
Flot4 migrates to root hair tip upon inoculation

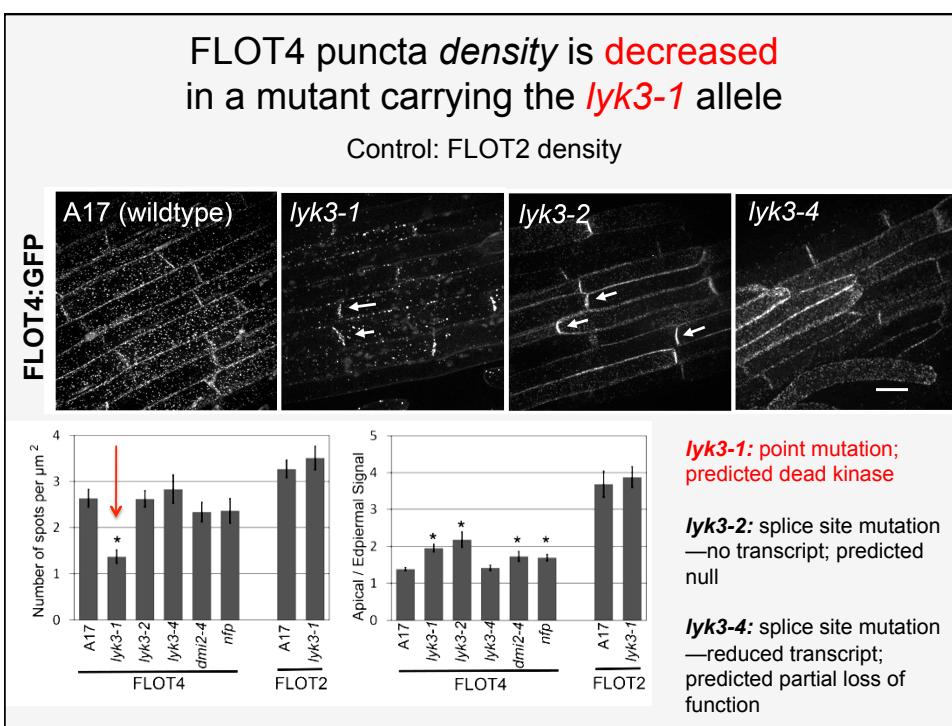
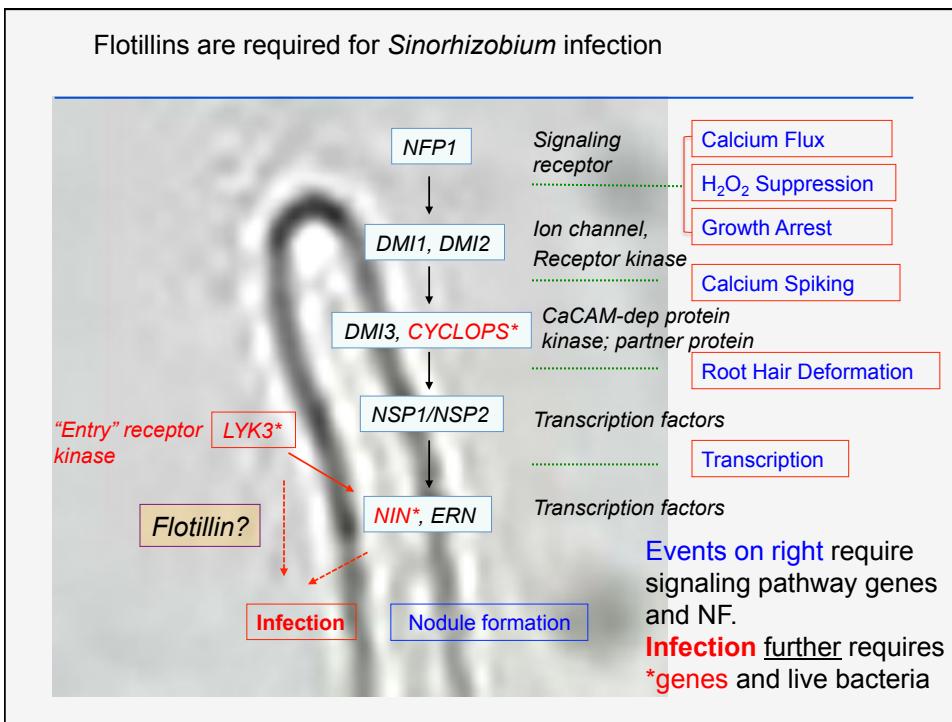


Silencing *FLOT4* results in infection threads
that lack well-defined structure



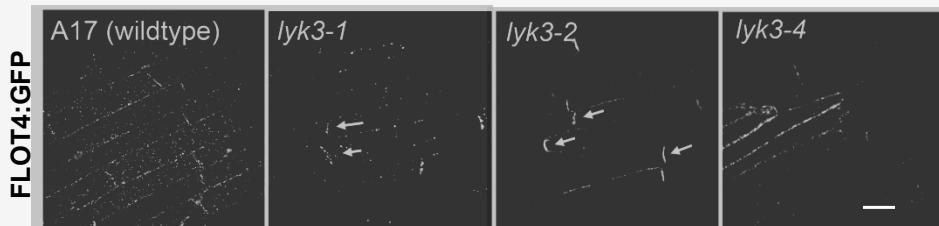
FLOT4::GFP localizes to infection thread membranes
while *FLOT2::GFP* does not





FLOT4 puncta density is decreased in a mutant carrying the *lyk3-1* allele

Do LYK3 and FLOT4 interact?

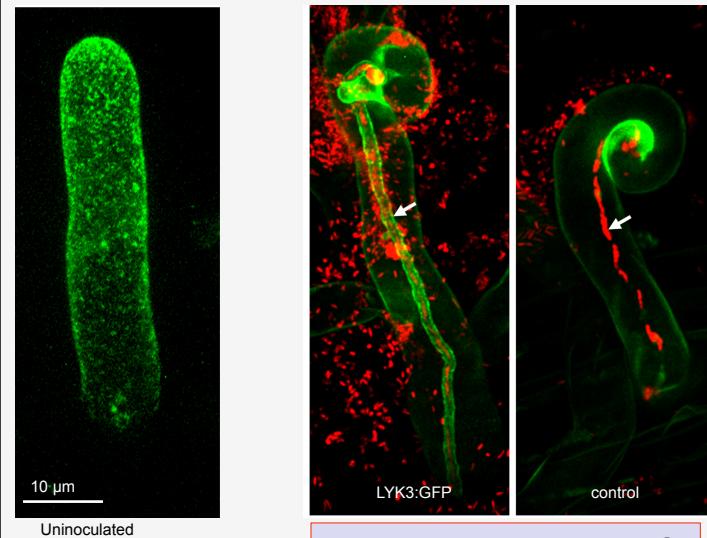


Tool for study

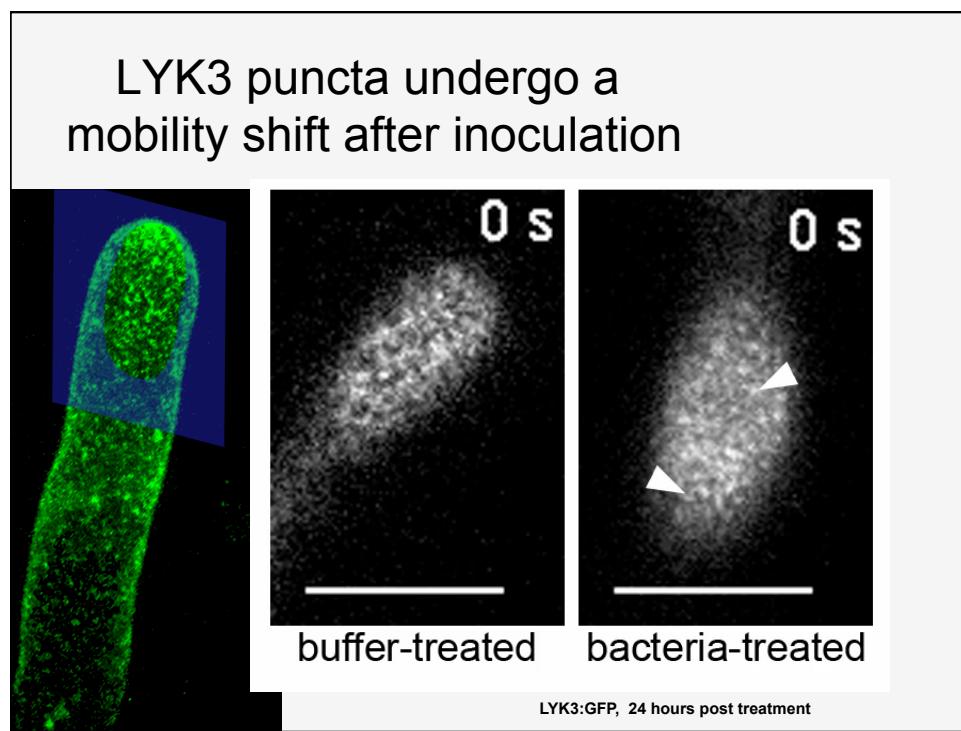
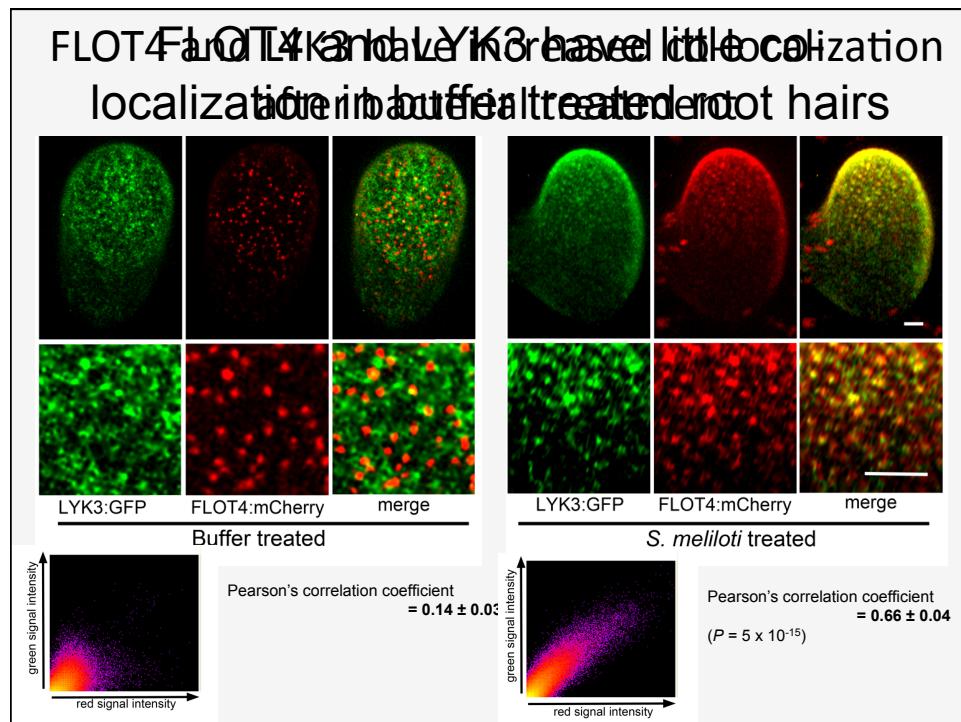
- Stable transgenic *M. truncatula* carrying LYK3-GFP fusion
 - *pLYK3:gLYK3:GFP*, native LYK3 promoter
 - Functional – complements *lyk3-1* mutant
- Hairy-root *pFLOT4:gFLOT4:mCherry* transgenics on LYK3-GFP plant
- Spinning disk confocal, long working distance objective

LYK3 localizes to membrane microdomains
and to infection thread membrane

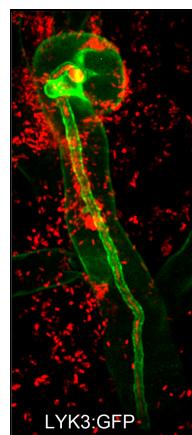
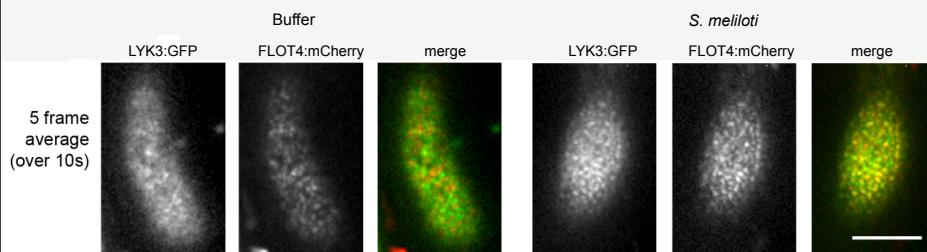
(*lyk3-1* complemented by *pLYK3:gLYK3:GFP* fusions)



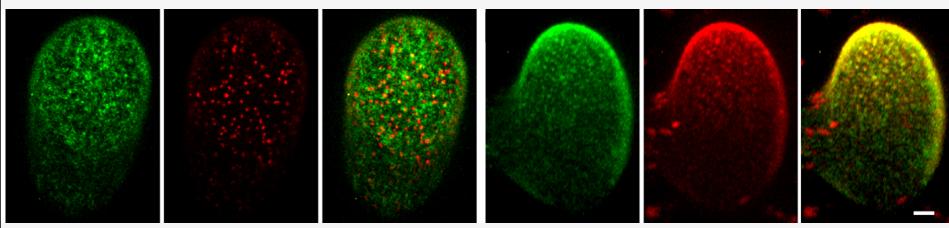
What about Flotillins?



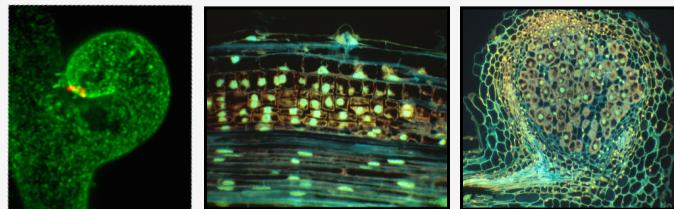
When LYK3 puncta stabilize after inoculation, they co-localize with FLOT4



- FLOT4 puncta density is altered in the *lyk3-1* mutant
- LYK3 localizes to membrane microdomains and infection threads
- LYK3 and FLOT4 microdomains have little overlap and have distinct dynamics in buffer-treated roots
- After inoculation with *S. meliloti* LYK3 and FLOT4 microdomains co-localize and have similar dynamics
- **Remaining questions:** nature of protein-protein interactions

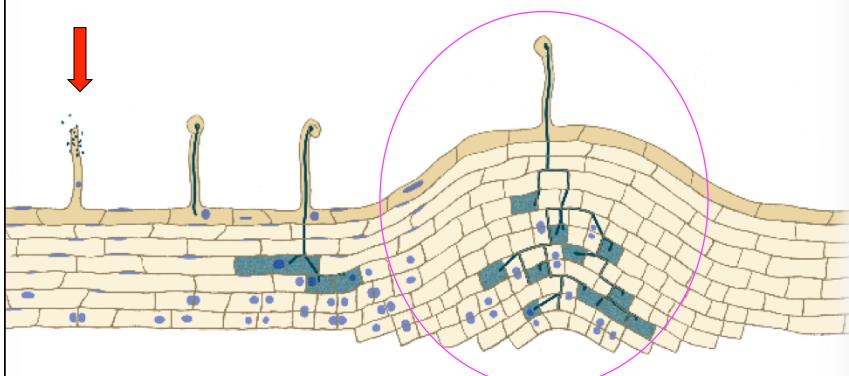
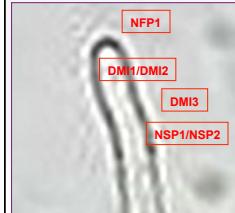


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Beyond early nodulation:
invasion, differentiation, N_2 fixation



***dnf* mutants**

- Generated by fast neutron radiation (often deletions);
- Form white nodules (wild type is pink);
- Nitrogenase activity abolished

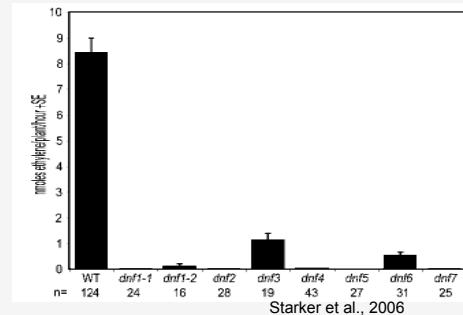
White nodules



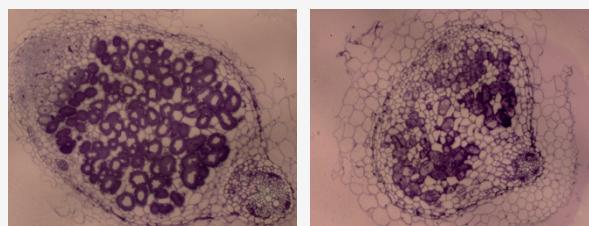
Wild type

(Colby Starker & Joel Griffitts)

Nitrogen fixation defect



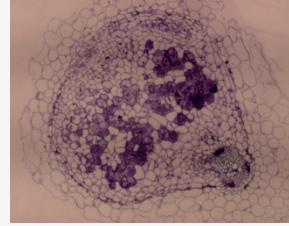
***dnf1* nodules show abnormal development.**



Wild Type

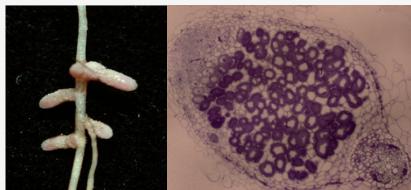
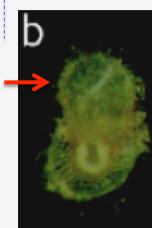
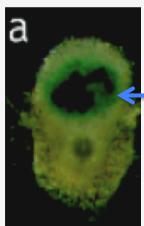
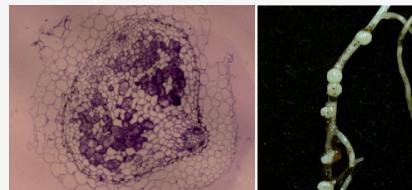
dnf1-1

dnf1-2



Plants control bacterial gene expression during late stages of symbiosis

normal nodules

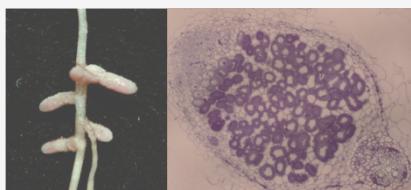
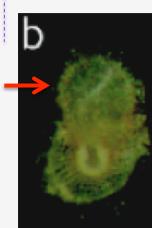
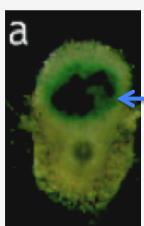
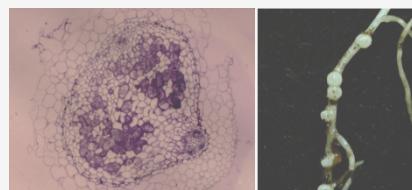
bacteria express N₂ase genes*dnf1* mutant plantbacteria do not express N₂ase genes

Photos: Colby Starker, Joel Griffits

Plants control bacterial gene expression

Bacteria need “DNF1” plant function to support N₂ fixation

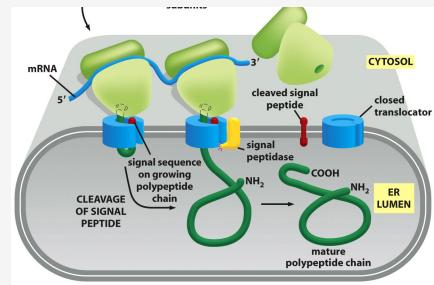
Normal nodule

bacteria express N₂ase genes*dnf1* mutant plantbacteria do not express N₂ase genes

Photos: Colby Starker, Joel Griffits

Bacteria need “DNF1” plant function to support N₂ fixation

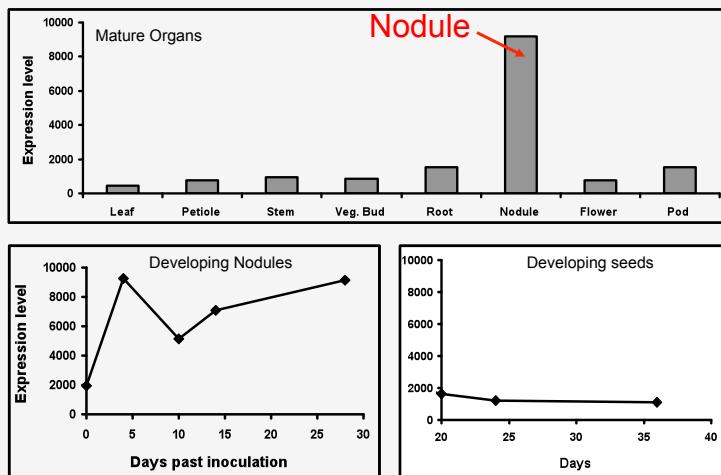
DNF1 encodes a nodule-specific Signal Peptidase



- Signal peptidase cleaves a signal peptide from precursor protein delivered into the Endoplasmic Reticulum.
- The mature protein may then traffic into vesicles for delivery elsewhere in the cell

Wang et al. Science 327 (Feb 26, 2010)

Is DNF1 expressed in nodules?



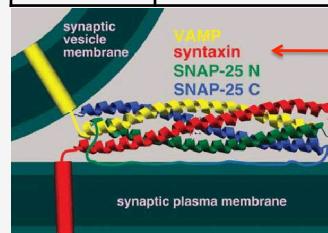
- *DNF1* is most highly expressed in early nodules.

Wang et al. Science (2010)

Source: *Medicago truncatula* Gene Expression Atlas

What functions are co-regulated with DNF1?

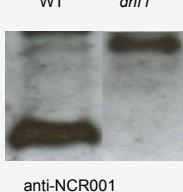
Name	Annotation/Function	Correlation
<i>SPC25</i>	Signal Peptidase Complex subunit	0.97
<i>SPC12</i>	Signal Peptidase Complex subunit	0.96
<i>SPC18</i>	Signal Peptidase Complex subunit	0.95
<i>SPP</i>	Signal Peptide Peptidase Degrades cleaved signal peptides.	0.95 0.92
<i>SYP132</i>	Syntaxin 132 (target-membrane localized SNARE protein)	0.93



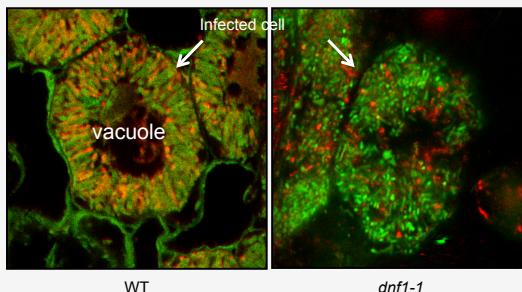
SYP132 marks the last step of protein secretion.
It has been observed on both plasma membrane and the symbosome membrane.

What are the substrates for DNF1 signal peptidase complex? *NCR Peptides* in WT and *dnf1*

Western



Co-localization



Red: anti-NCR001
Green: SYTO13 (indicates bacteroid)

- NCR peptides are substrates of the DNF1 complex.
- In WT, the NCR peptides are delivered to the symbosome.
- In *dnf1*, they are retained in the ER.

Van de Velde *et al.* Science (2010)

What are the substrates (precursor proteins) for DNF1 complex?

The plant encodes nodule specific “**NCR**” proteins*, processed by DNF1⁺, that cause bacterial differentiation.

Possible scenario might be a variant of this:

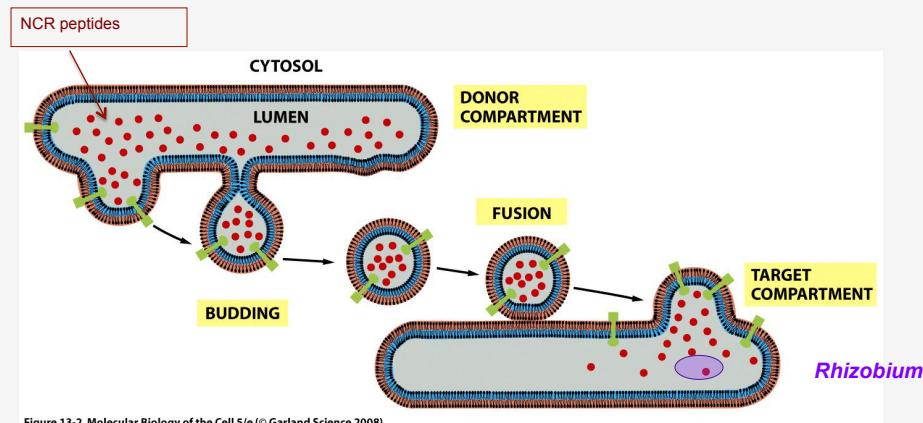


Figure 13-2 Molecular Biology of the Cell 5/e (© Garland Science 2008)

*Van de Velde et al. and †Wang et al. Science 327 (Feb 26, 2010)

dnf mutants

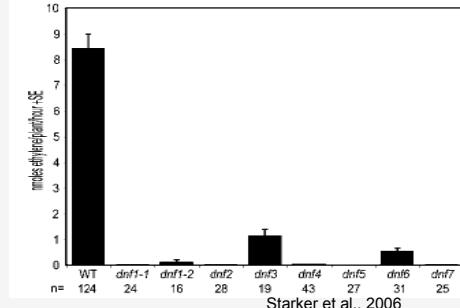
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- Form white nodules (wild type is pink);
- Nitrogenase activity abolished

White nodules



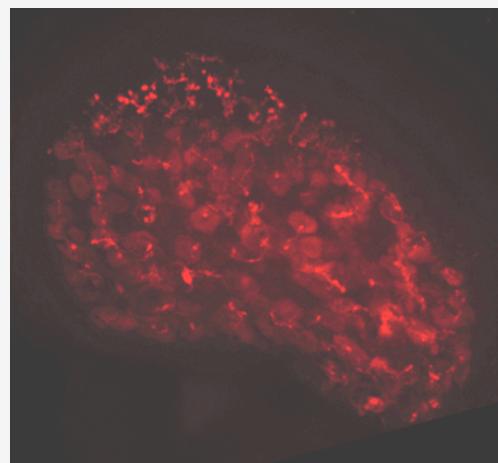
(Colby Starker & Joel Griffitts)

Nitrogen fixation defect



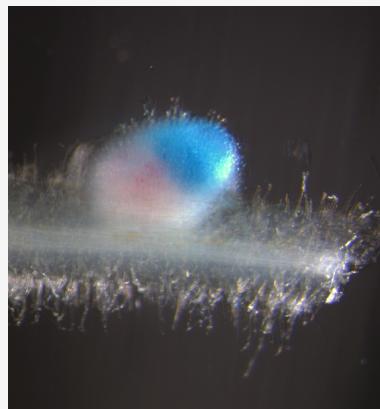
DNF2 mutants show bacterial infection but fail to differentiate
DNF2 does not express *nifH* in bacteroids

Distal



Fluorescently labeled bacteria in *dnf2* nodules

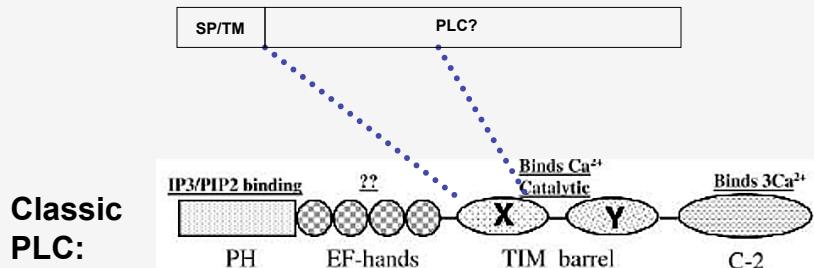
pDNF2::GUS



Other data:

- DNF2 expressed uniquely in nodules
- expression peaks at 10 days, then decreases slightly
- upstream cis-acting element has been defined

DNF2 sequence shows moderate similarity to phospholipase C (PLC).



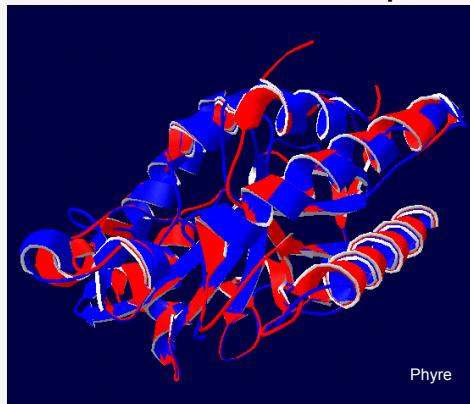
Concerns:

E-values high;

Predicted DNF2 only matches the **X** half of the catalytic domain

No match of DNF2 to other domains of classic PLC

The predicted structure of DNF2 fits well with a known phospholipase C.

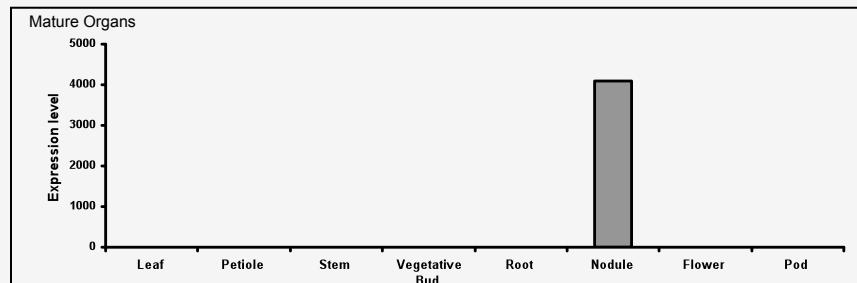


Red: PLC of *Listeria monocytogenes*

Blue: DNF2

Underway: the biochemical activity of DNF2

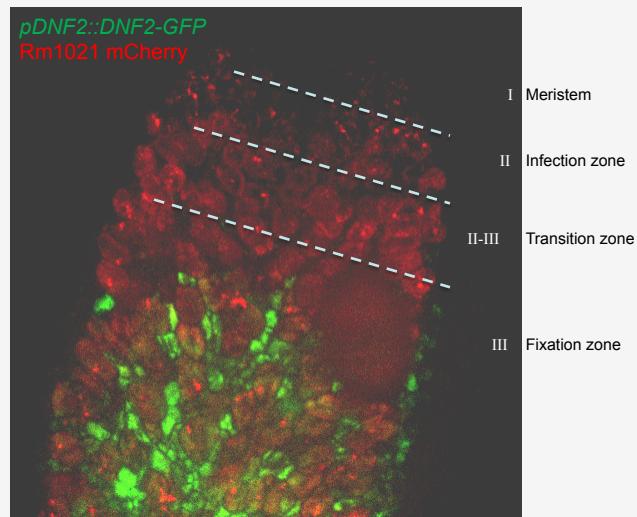
DNF2 is expressed exclusively in nodules.



Source: *Medicago truncatula* Gene Expression Atlas

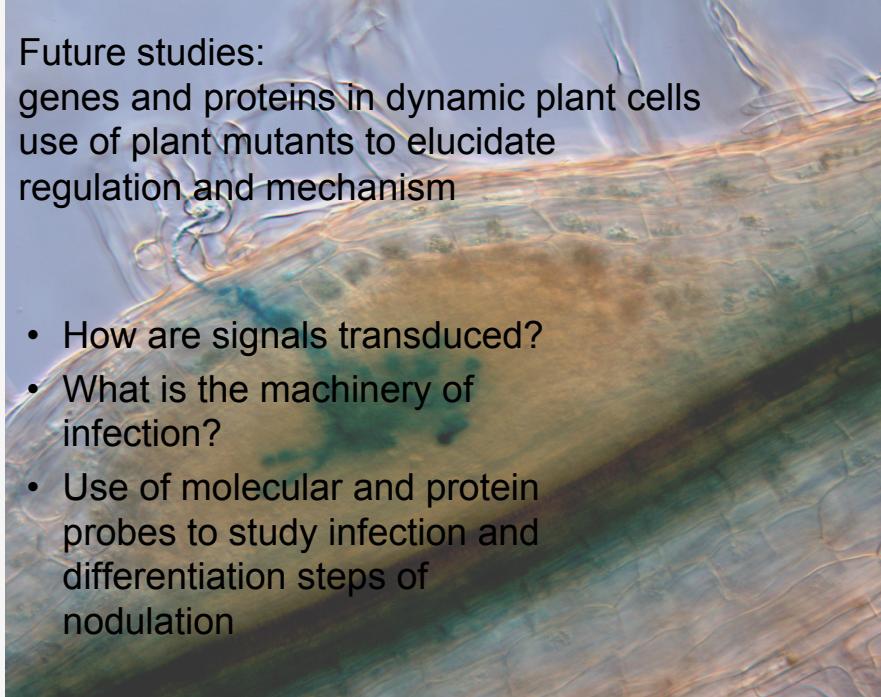
DNF2 transcriptional control may be critical to its biological function.

DNF2 exists in uninfected cells.



- DNF2 may mediate the infection process indirectly.

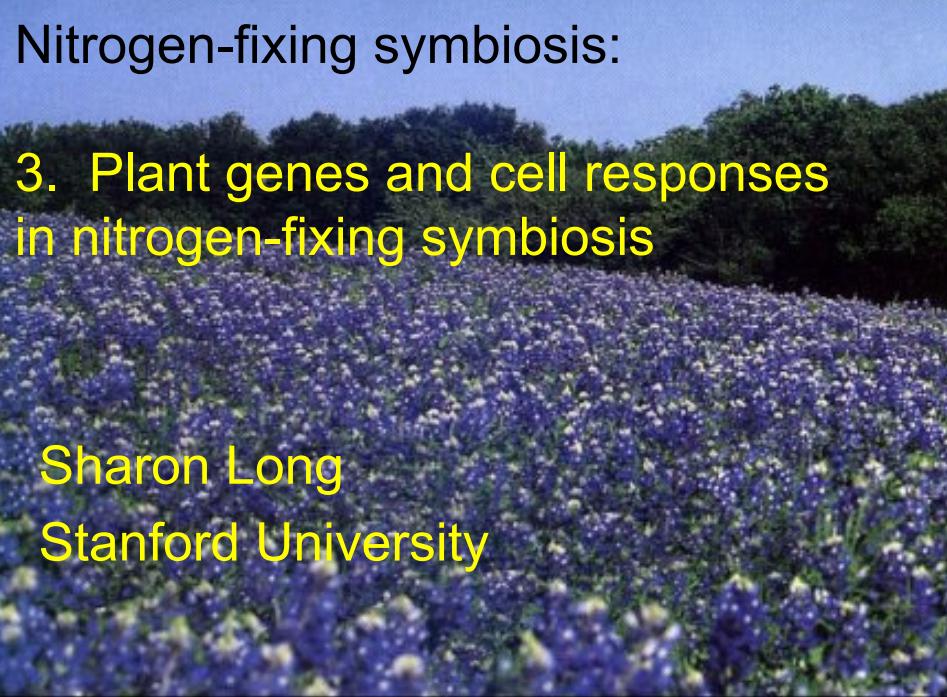
Future studies:
genes and proteins in dynamic plant cells
use of plant mutants to elucidate
regulation and mechanism



- How are signals transduced?
- What is the machinery of infection?
- Use of molecular and protein probes to study infection and differentiation steps of nodulation

Nitrogen-fixing symbiosis:

3. Plant genes and cell responses
in nitrogen-fixing symbiosis



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