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INTEGRATIVE LEGUME RESEARCH

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Genetic Components of Local and Systemic Long-Distance Signalling During Nodule Regulation in Soybean

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Plant & Animal Genome Conference 2012

Thank you to:

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Bernard Carroll, Karsten Oelkers, Da Luo, Satoshi Tabata, Shusei Sato,
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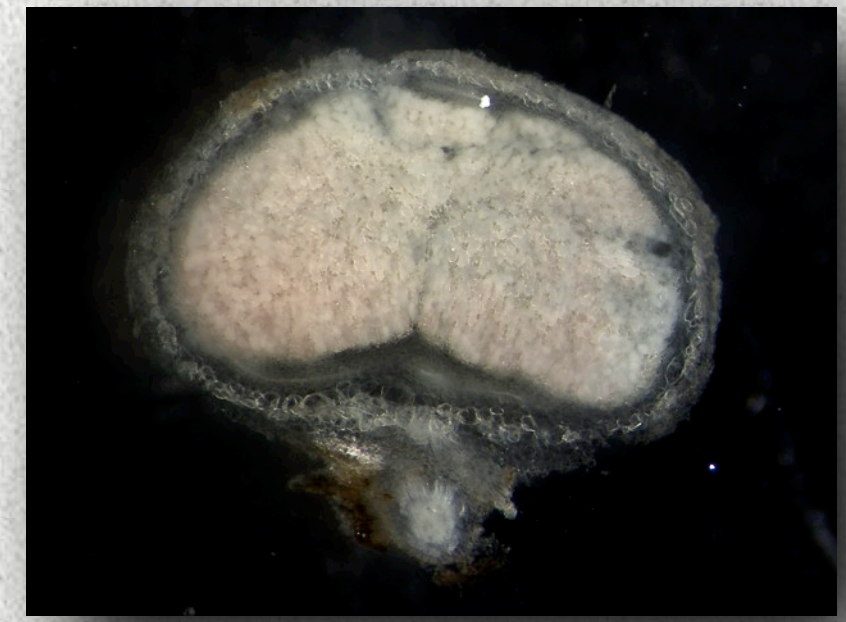
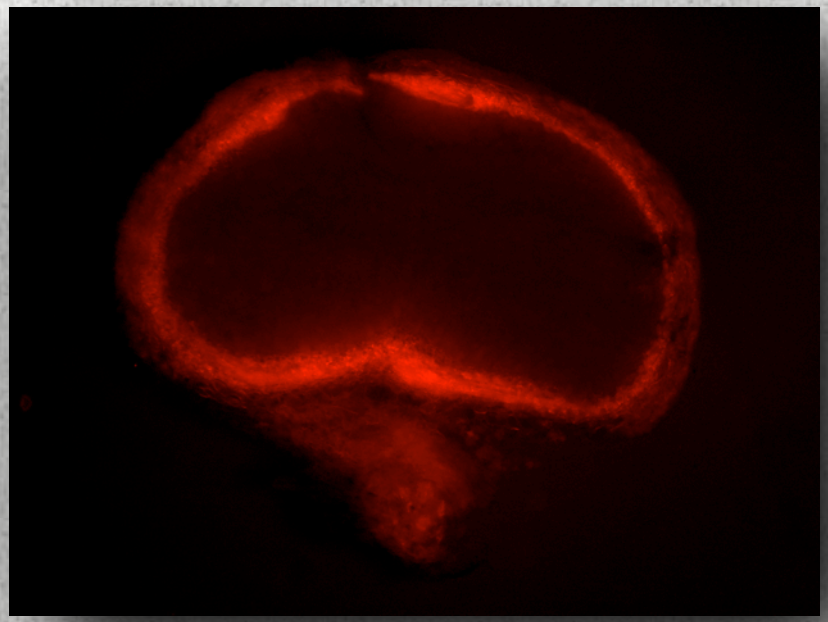
CLR ARC Centre of Excellence





Lin et al, MOLECULAR PLANT 2011

Legumes form **new** organs after symbiotic interaction:
- post-embryonically on their roots
- in response to signals from soil bacteria called “Rhizobium”
    - regulated external clues such as nitrate and pH
    - homeostatically regulated also **INTERNALLY**

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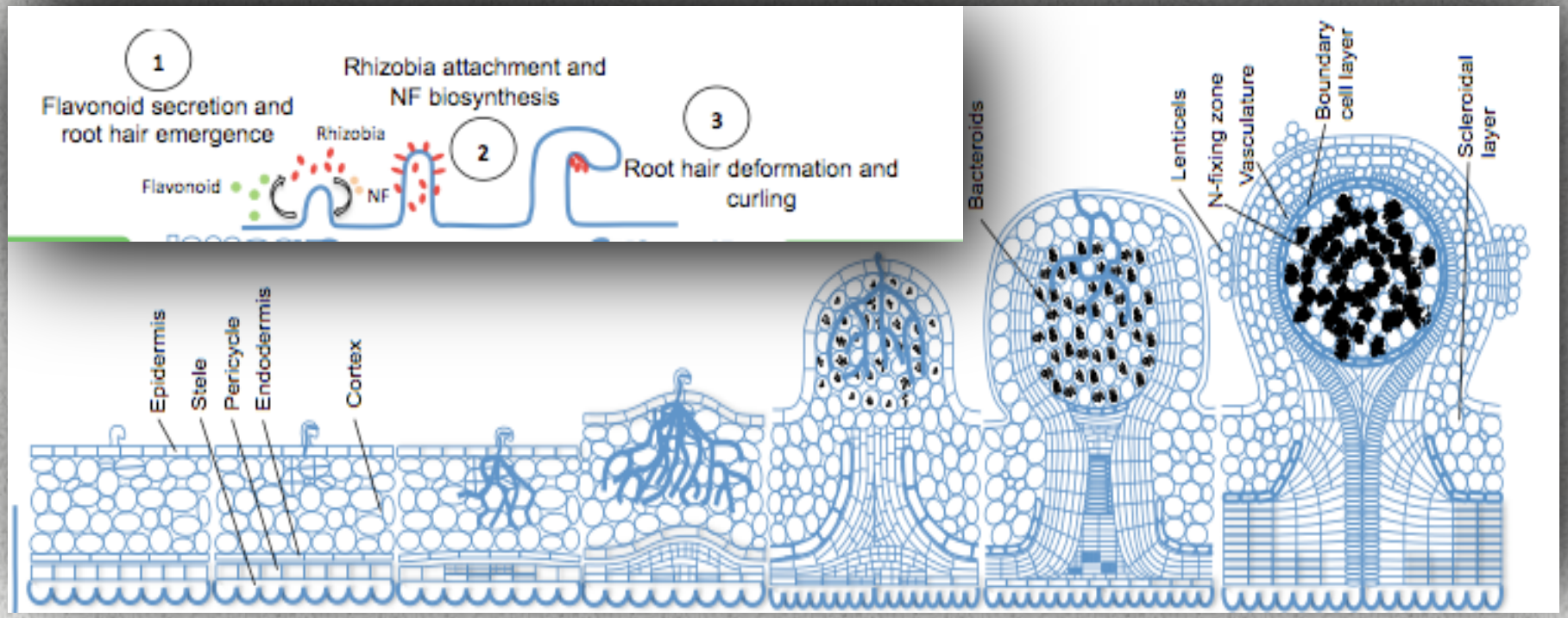
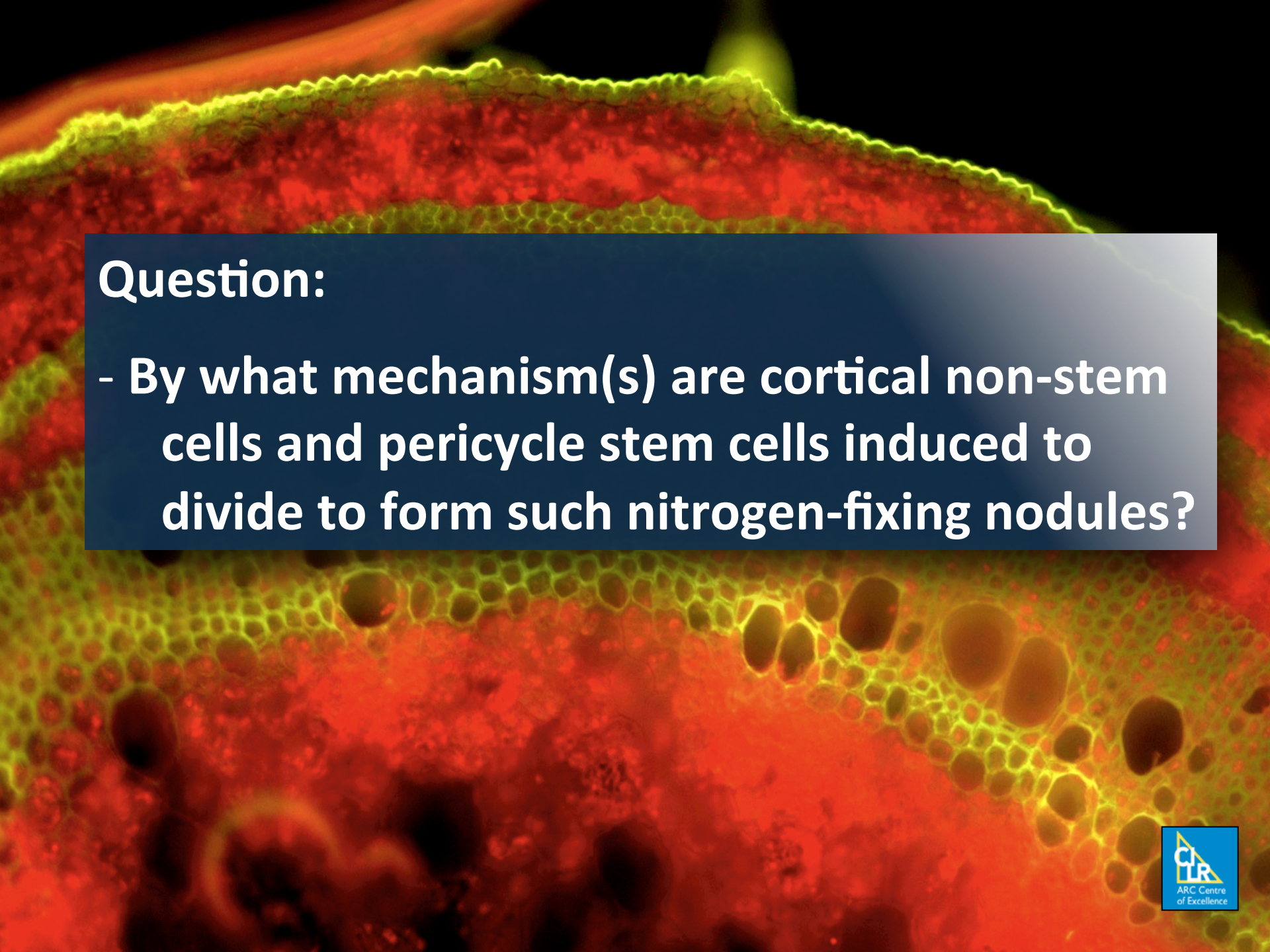
Soybean Nodule Ontogeny



Ferguson *et al* (2010)
Journal of Integrative
Plant Biology **52**: 61-76



Question:

- By what mechanism(s) are cortical non-stem cells and pericycle stem cells induced to divide to form such nitrogen-fixing nodules?

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AND:

How is this process regulated???

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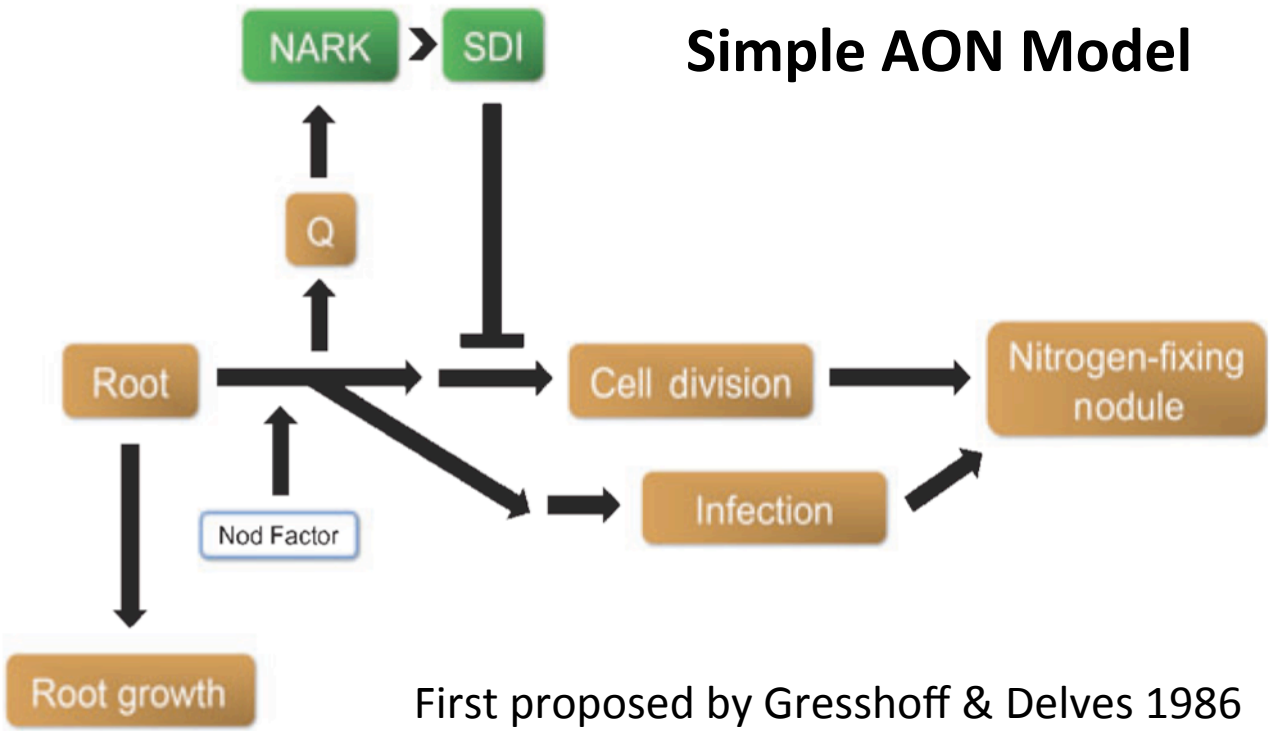
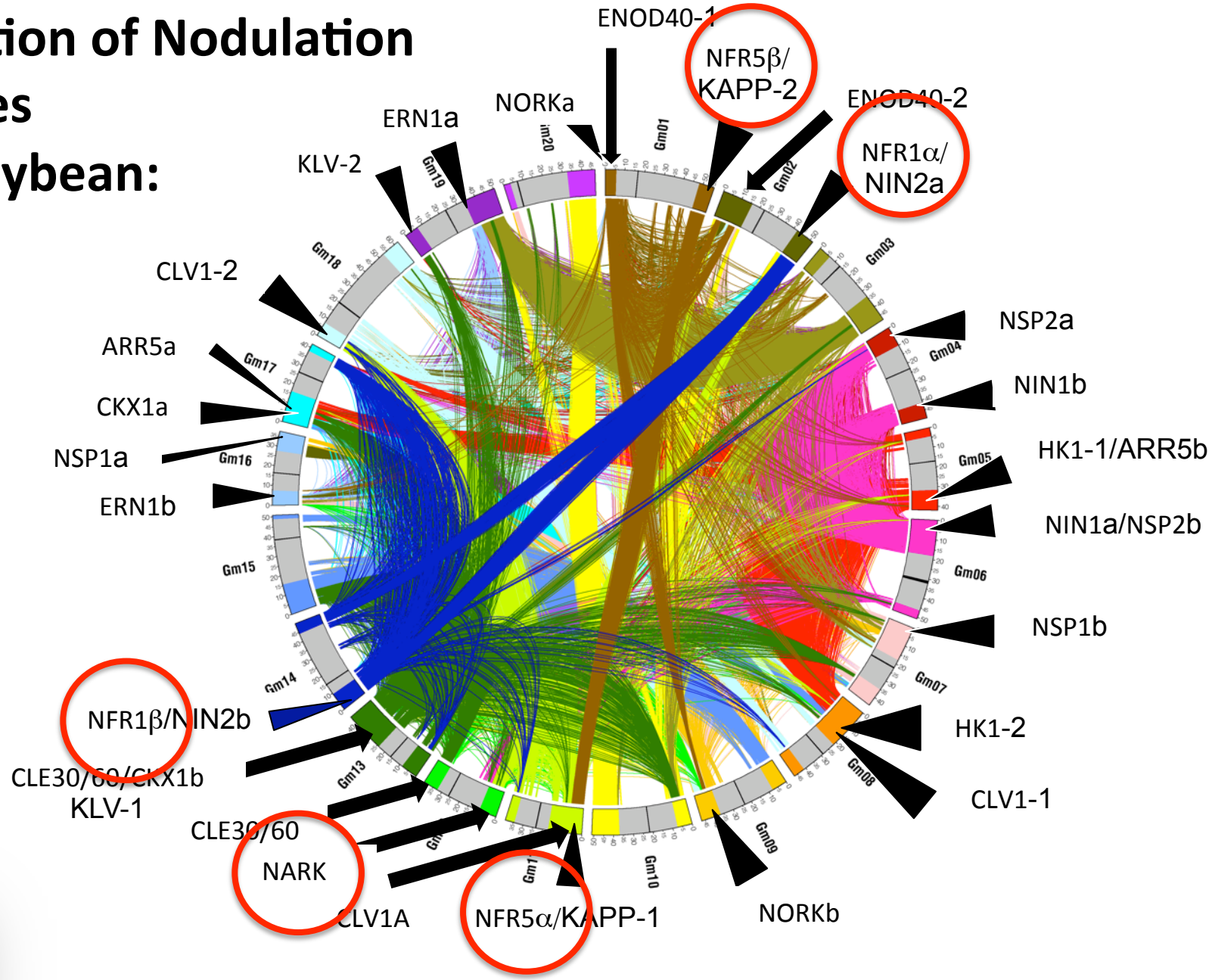
Simple AON Model



First proposed by Gresshoff & Delves 1986

Position of Nodulation Genes in Soybean:



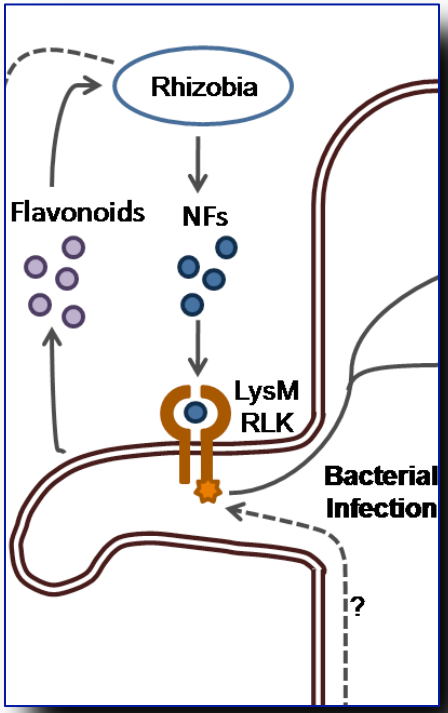
Soybean 'clock map' kindly supplied by Prof. S. Jackson (c.f., Schmutz et al, 2010)

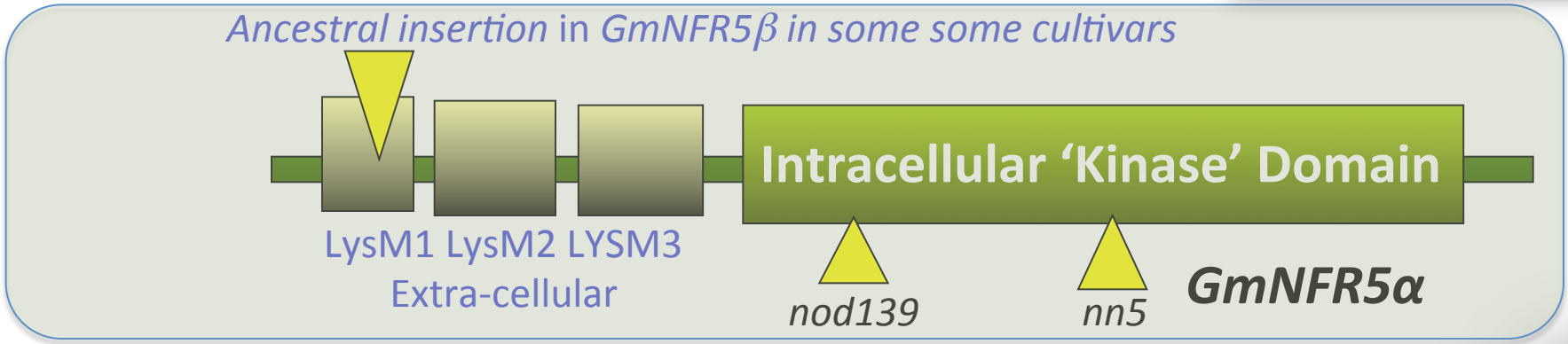
Cloning of soybean mutant *nod139* (= *GmNFR5*) and *nn5*

PCP
PLANT & CELL PHYSIOLOGY

Inactivation of Duplicated Nod Factor Receptor 5 (NFR5) Genes in Recessive Loss-of-Function Non-Nodulation Mutants of Allotetraploid Soybean (*Glycine max* L. Merr.)

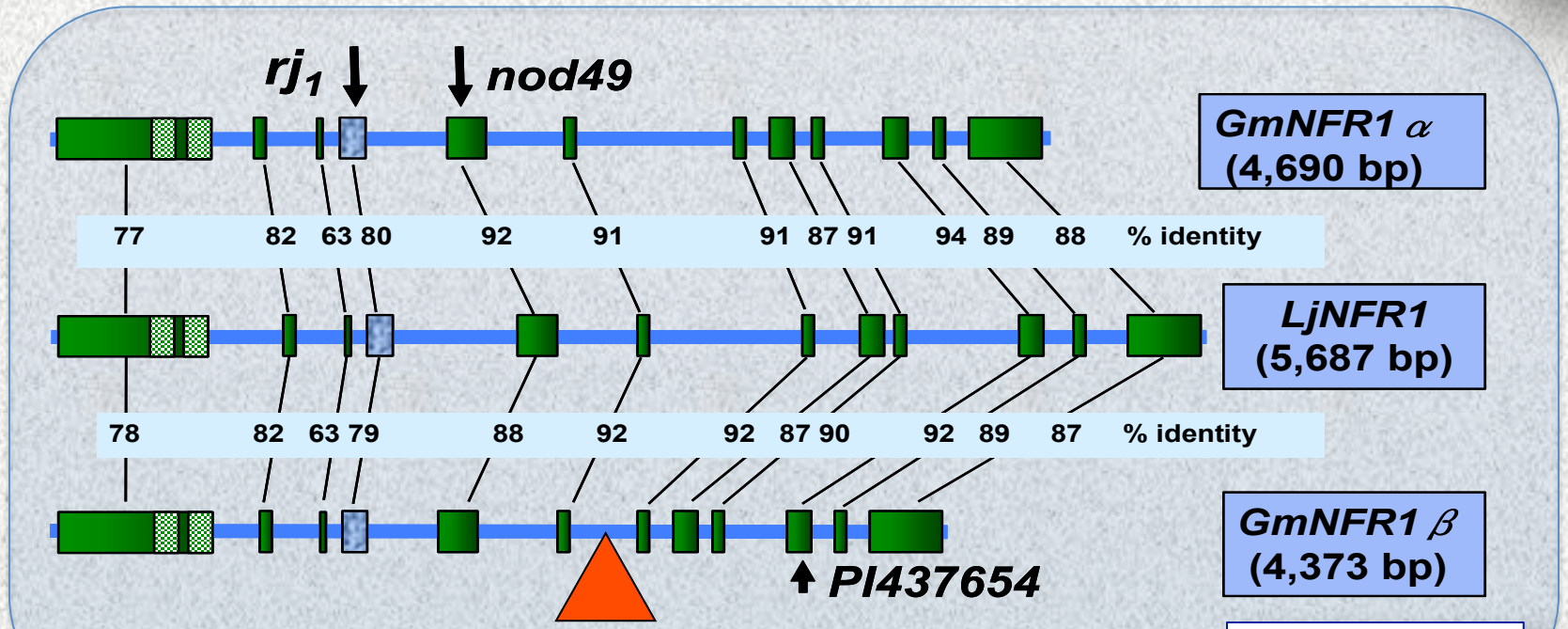
Arief Indrasumunar[1,2], Attila Kereszt[1,3], Iain Searle[4,5], Mikiko Miyagi[1], Dongxue Li[1], Cuc D.T. Nguyen[1], Artem Men[6], Bernard I. Carroll[1,4] and Peter M. Gresshoff[1,*]





Indrasumunar et al (2010) *Plant & Cell Physiol.* **51**: 201-214.

Cloning of a second Nod factor component gene in soybean (*GmNFR1*) using mutants *nod49* and *rj1*



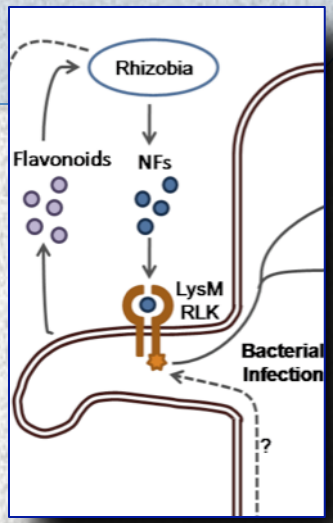
the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

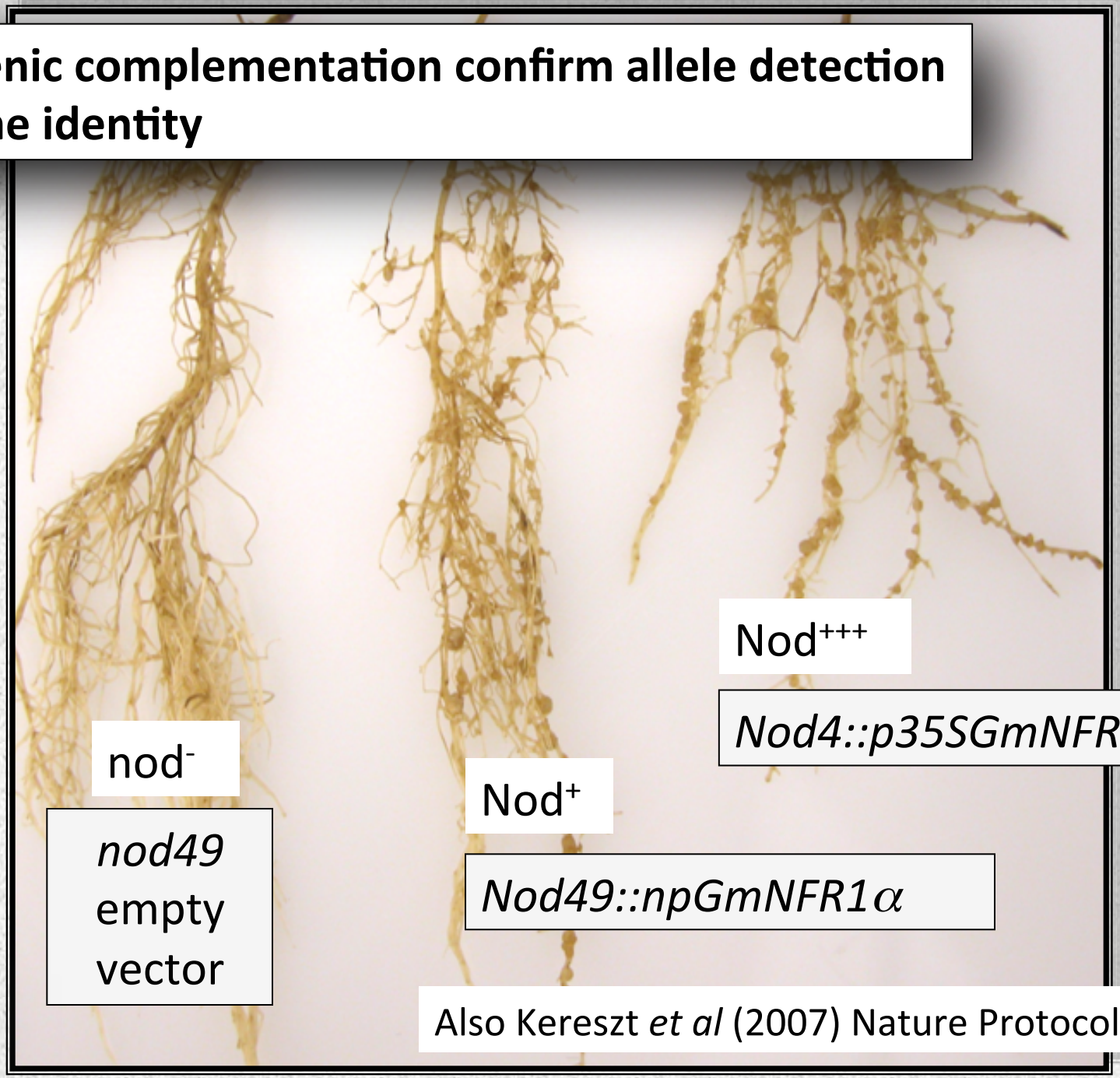
The Plant Journal (2010) doi: 10.1111/j.1365-313X.2010.04398.x

Nodulation factor receptor kinase 1α controls nodule organ number in soybean (*Glycine max* L. Merr)

Arief Indrasumunar[1,2], Iain Searle[3,4], Meng-Han Lin[1], Attila Kereszt[1,5], Artem Men[1,6], Bernard J. Carroll[1,3] and Peter M. Gresshoff[1,*]



Transgenic complementation confirm allele detection and gene identity



Also Kereszt *et al* (2007) Nature Protocols

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Isolation of AON/supernodulation mutants

EMS mutation

Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4162–4166, June 1985
Genetics

Isolation and properties of soybean [*Glycine max* (L.)] mutants that nodulate in the presence of high nitrate concentrations

(nitrate inhibition/*nts* mutants/nitrate-tolerant symbiosis/ethyl methanesulfonate mutagenesis/nitrogen fixation)

BERNARD J. CARROLL, DAVID L. MCNEIL*, AND PETER M. GRESSHOFF†

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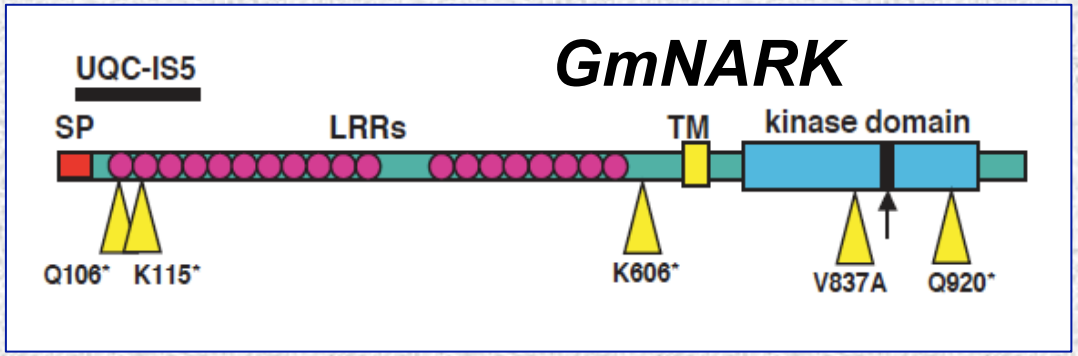
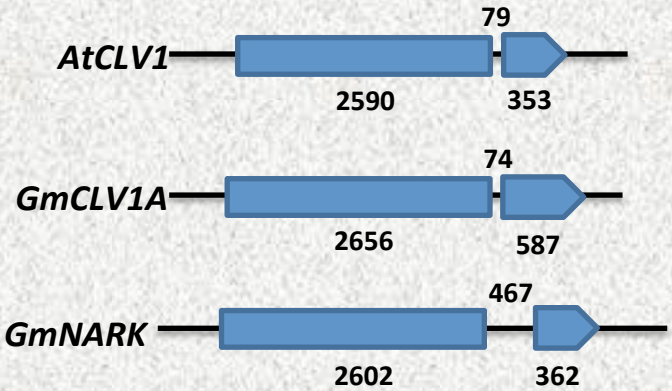
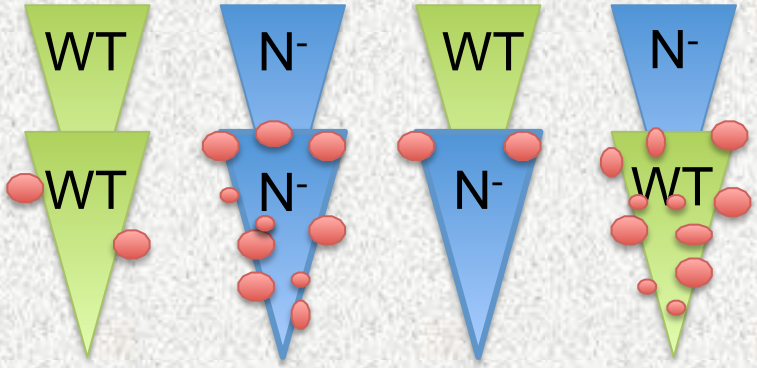
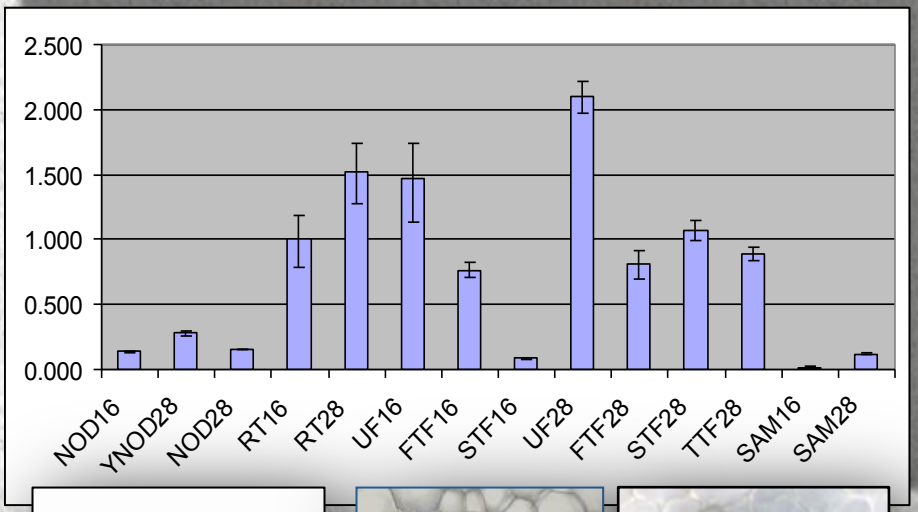
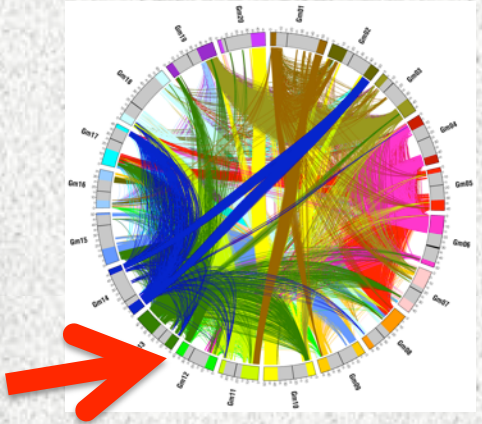
Positional Cloning of *GmNARK* in Soybean:

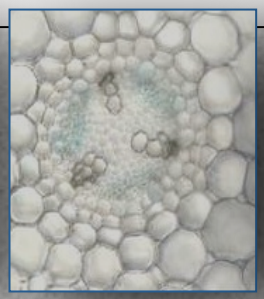


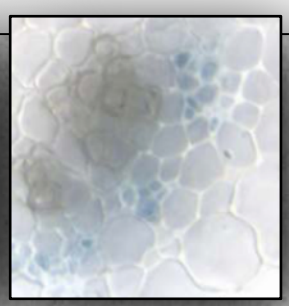












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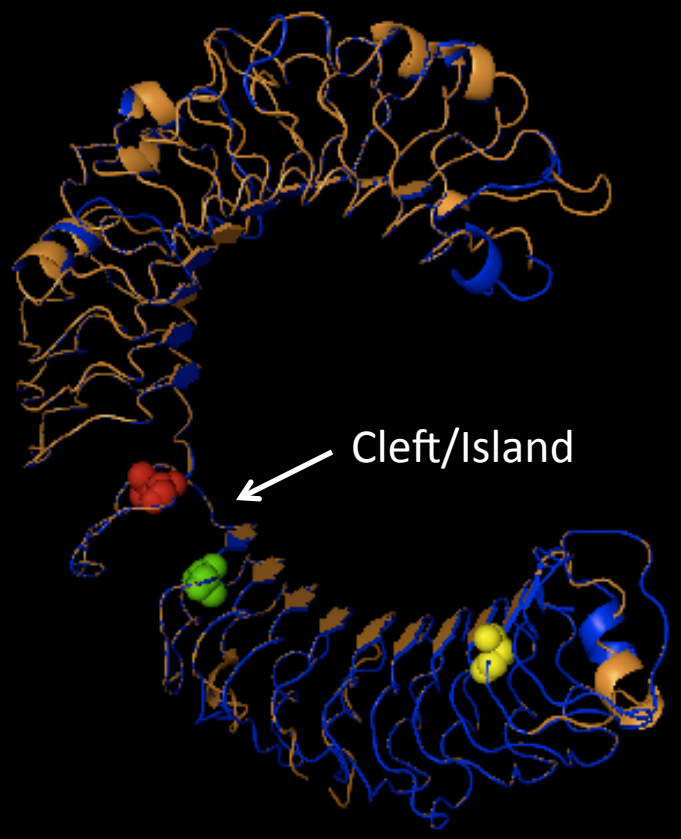
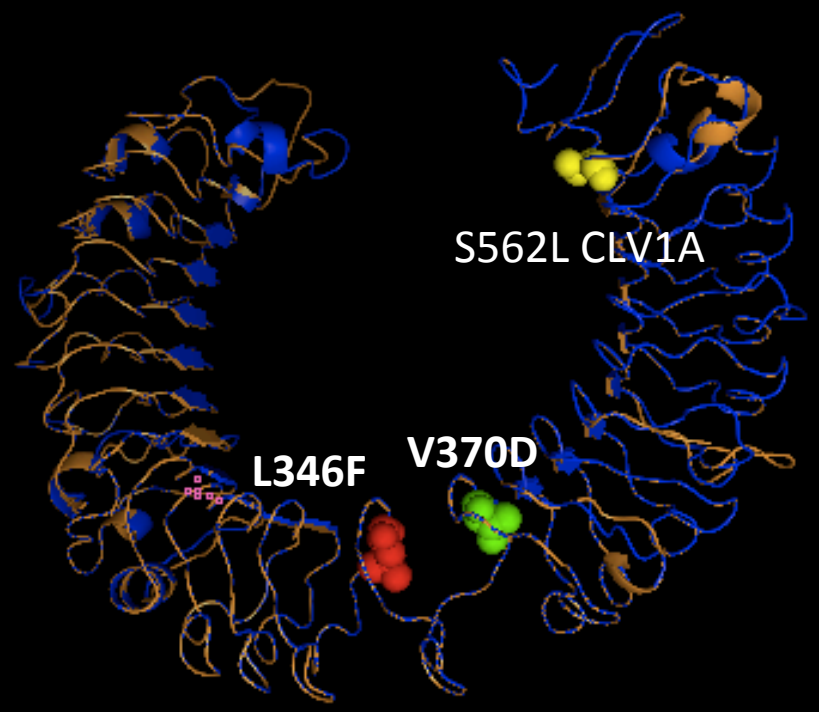
GmNARK/GmCLV1A LRR structural model





The cleft and the severity of the two mutant alleles suggest a ligand binding site

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Line	Mutation	Phenotype
nod4 (W)	T>C = V370D	supernod
nod9p (W)	T>C = V370D	supernod
nod1-3 (W)	G>A = G863D	hypernod
nts1116 (B)	V837A	hypernod
nod3-7 (W)	C>T = L346F	hypernod
F262 (F)	W677* + L829V	supernod
nod2-4 (W)	Het L346F	WT*
F23 (F)	H811Q and H789=	WT
nts1007 (B)	Q106*	supernod
PvNARK1		supernod

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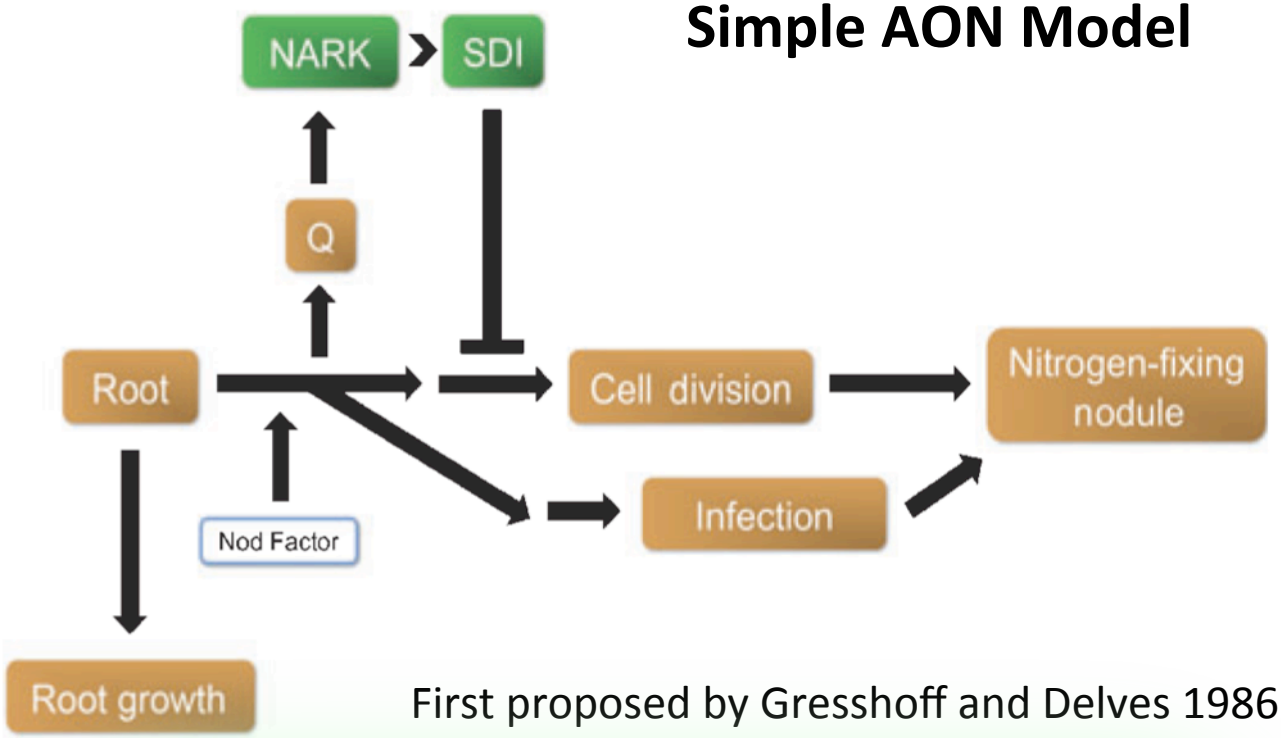
Simple AON Model



First proposed by Gresshoff and Delves 1986

So what are the signals?

The UP Signal 'Q'

Dugald Reid

- **Reid *et al.* (2011) *Annals of Botany***
- **Reid et al (2011) *MPMI***
- **See talk in Functional genomics Workshop**

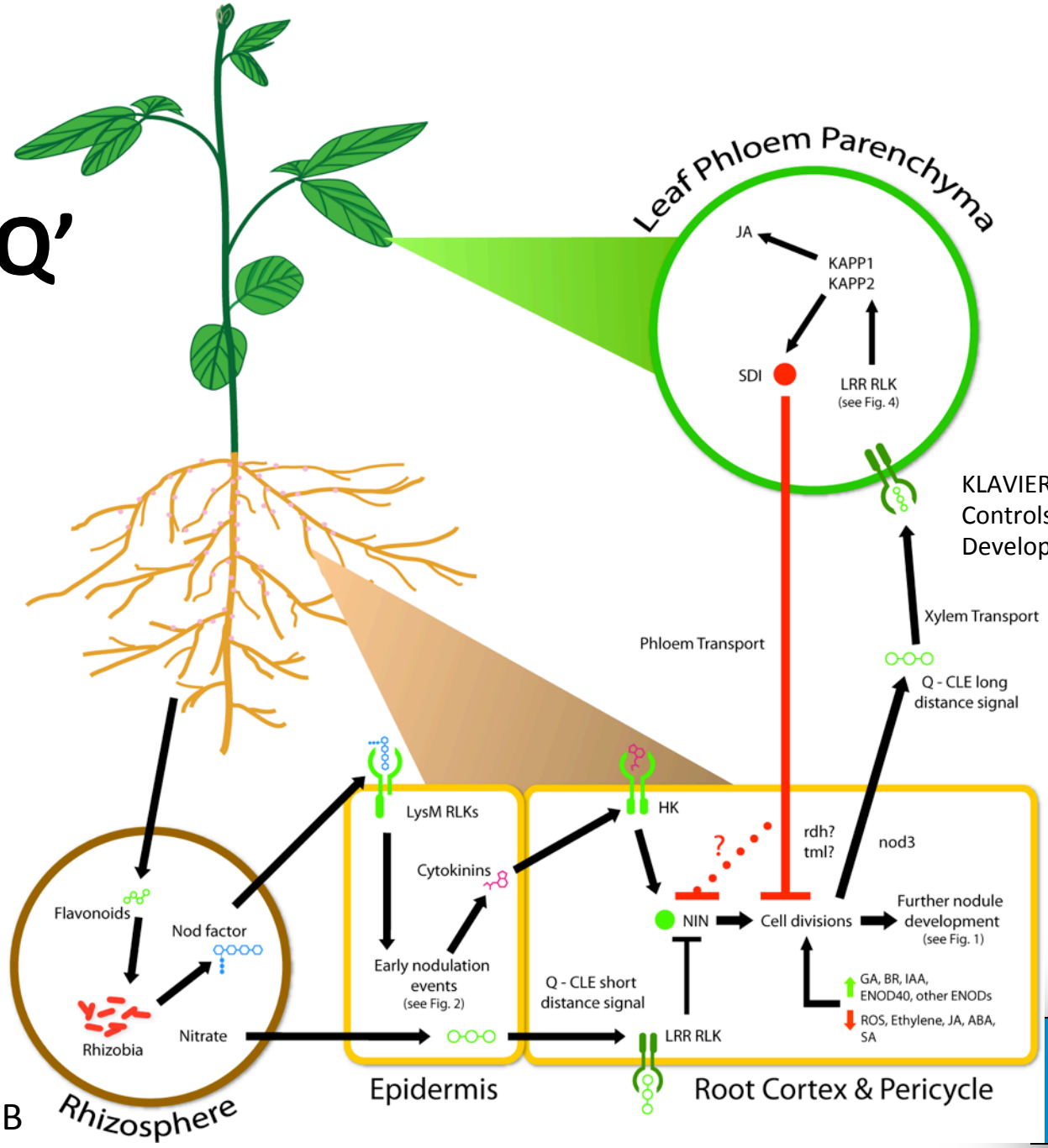


Diagram from Ferguson *et al* (2010) JIPB

RIC n' NIC, NARK RNA expression in soybean

	NARK	*RIC1*	*NIC1*	*RIC2*
root	++	+	+	+
leaf	++	-	-	-

NARK transcription in all tissues, except SAM and RAM
NARK-OX in Lotus inhibits hypocotyl transformation
NARK mutants have normal leaf, floral and SAM development
NARK mutants segregate as single recessive mutation

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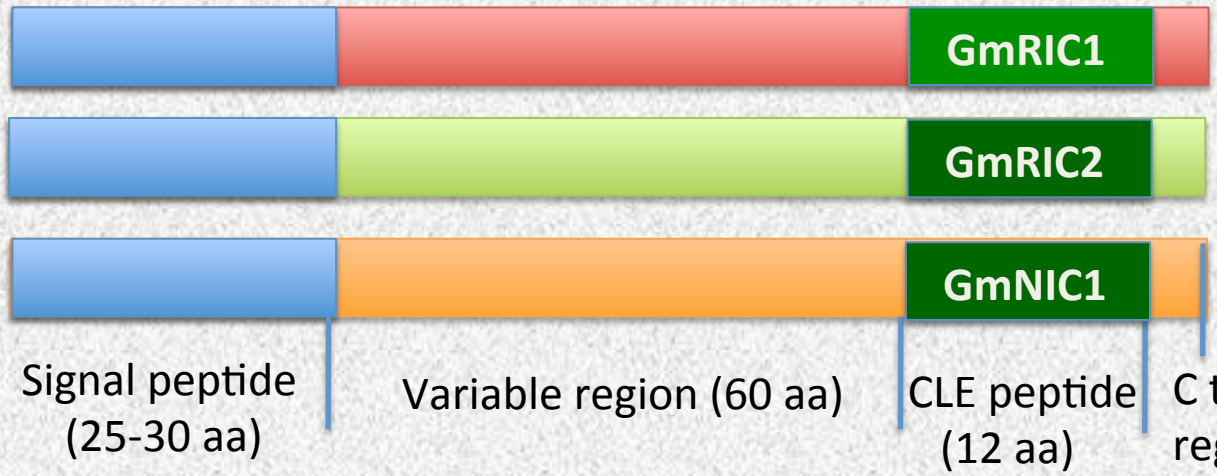

Three CLE peptide genes

GmRIC1 about 90-100 aa
GmRIC2 no introns
GmNIC1 duplicated with inactive copies

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Reid et al (2011) MPMI

The Down-SIGNAL: Shoot Derived Inhibitor (SDI)

Lin et al; *New Phytologist 2010*; *NATURE Protocols* 2011

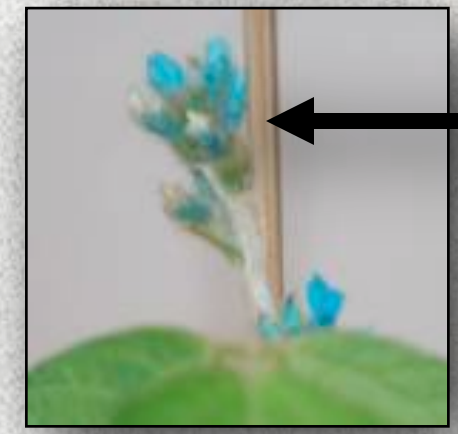
Developmental Cross-Feeding

Leaf of treated WT plant

Water Extraction & Treatment

Petiole Feed & Plant Movement

Feed into Mutant Plant



Phenocopy to WT (Suppression Assay)

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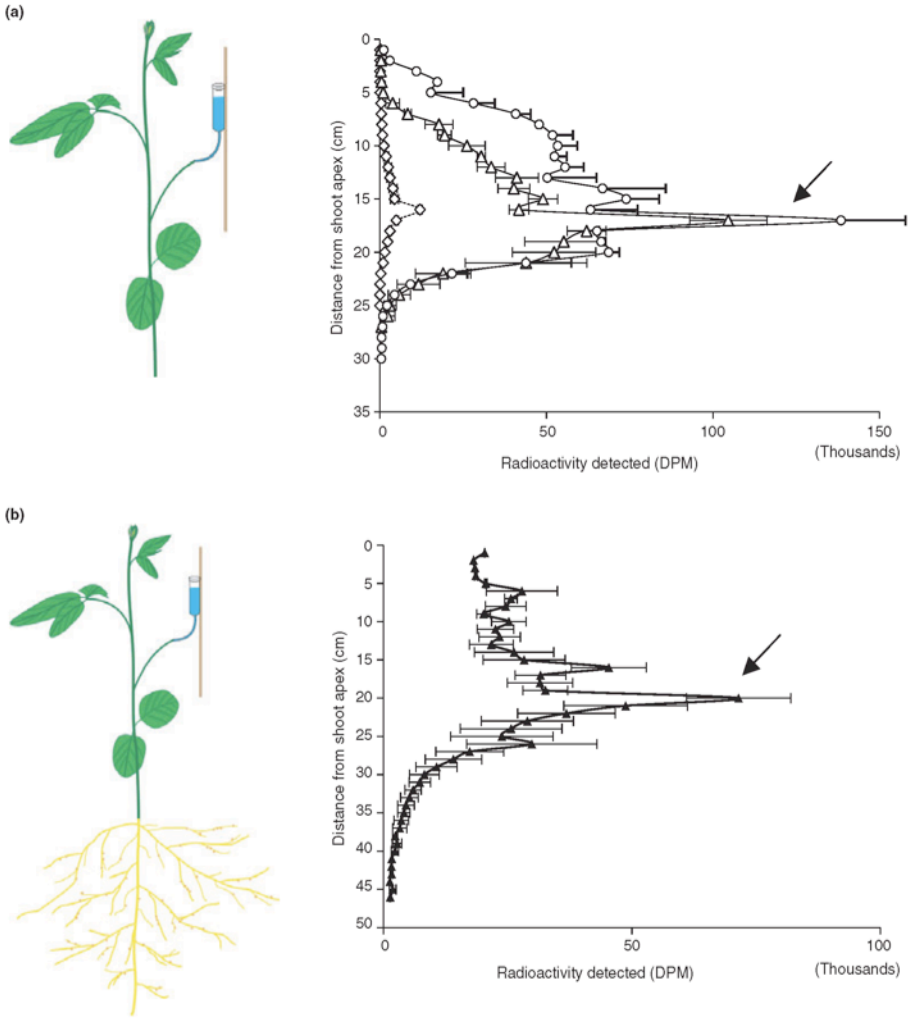

Fig. 2 Translocation of radioactivity after petiole feeding in soybean plants. Fed at the petiole of the first trifoliate with either (a) [^{3}H]-glucose at 0.5 (diamonds), 1.5 (triangles) and 3 h (circles) time-points or (b) [^{3}H]-mannitol for 24 h. The arrow represents the location of the petiole feeding site. A stylized soybean plant illustrates the developmental positions of the sampled 1-cm plant segments.

Petiole-applied glucose and mannitol travel in both basi- and acropedal direction.

Glucose: 0.5, 1.0, 3.0 hr

Mannitol: 24 hr

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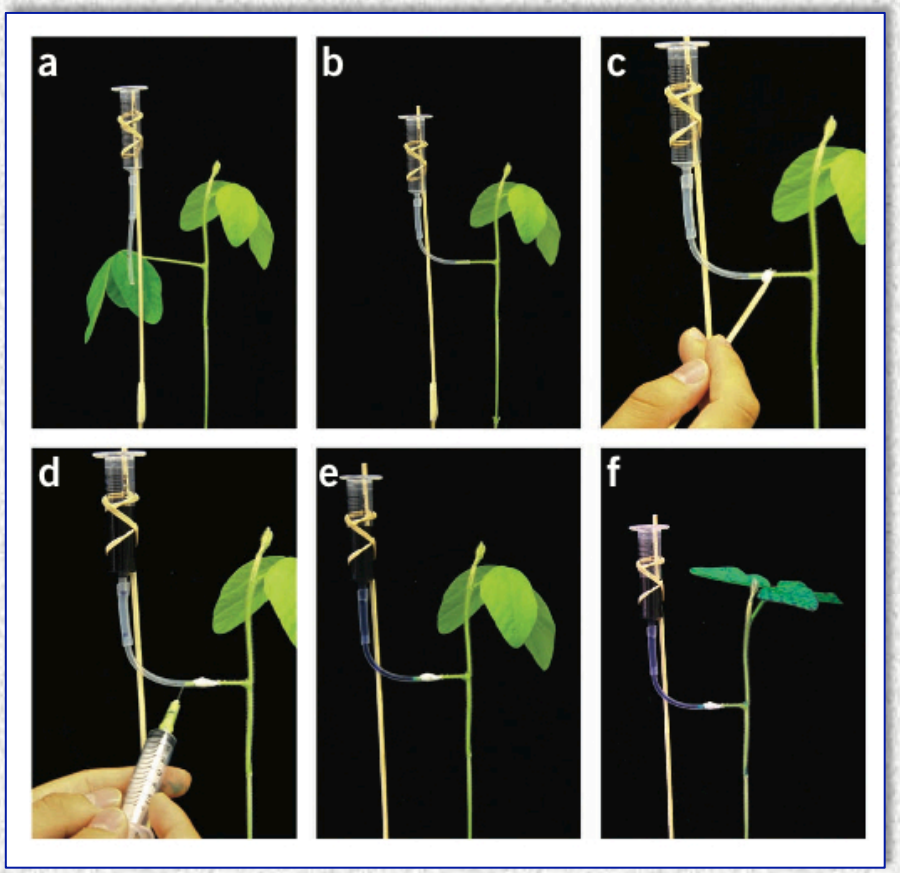

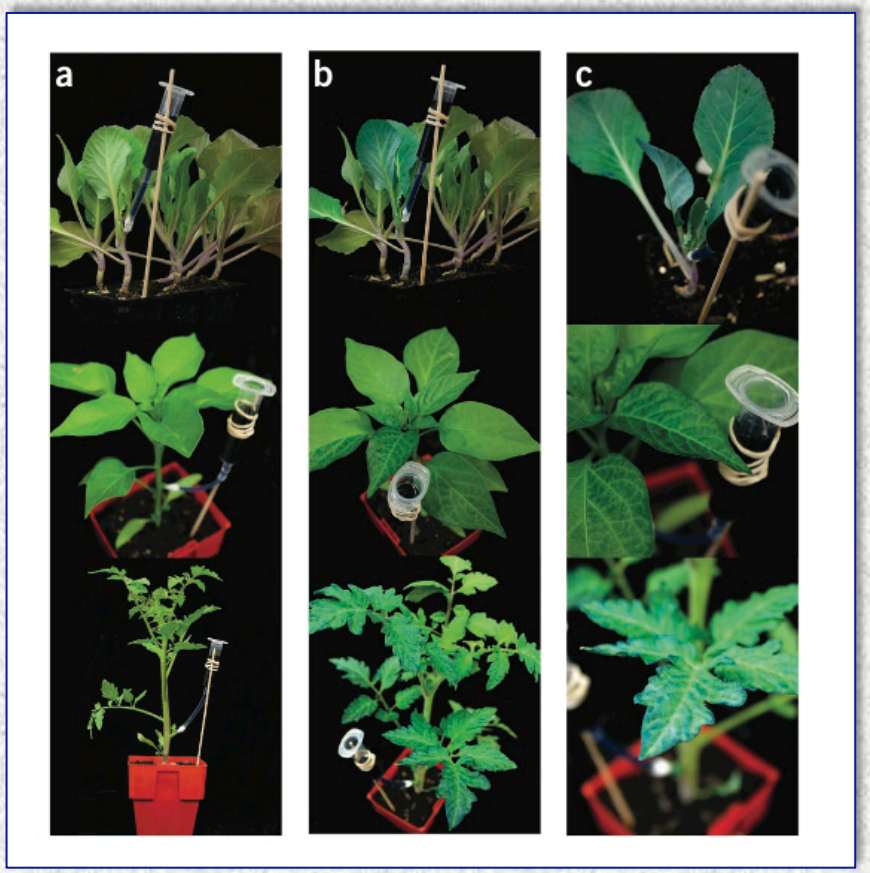

Simple and fast

Broadly applicable

nature protocols
Recipes for Researchers
www.natureprotocols.com

An efficient petiole-feeding bioassay for introducing aqueous solutions into dicotyledonous plants

Yu-Hsiang Lin, Meng-Han Lin, Peter M Gresshoff & Brett J Ferguson

Australian Research Council Centre of Excellence for Integrative Legume Research, The University of Queensland, Brisbane, Queensland, Australia. Correspondence should be addressed to P.M.G. (p.gresshoff@uq.edu.au).

CILR
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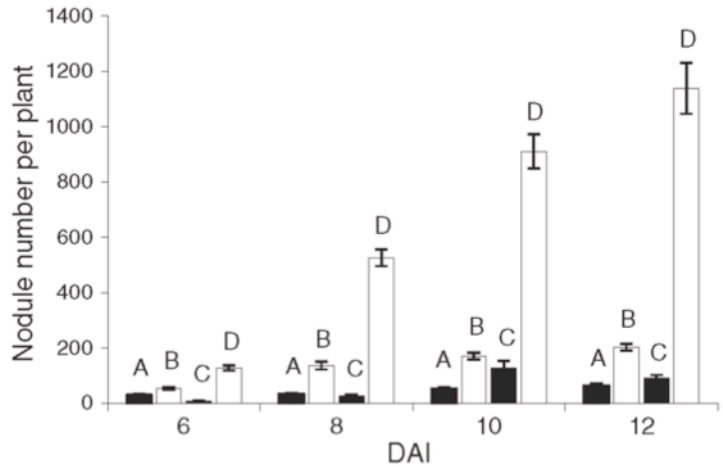


Fig. 3 Nodulation time-course of *Bradyrhizobium japonicum*-inoculated Bragg (A, WT), hypernodulating *nts1116* (B, V837A) mutant, Williams 82 (C, WT) and supernodulating *NOD4* (D, V370D) mutant soybean plants. To mimic the nodulation response in petiole-fed plants, inoculation occurred at 4 wk after germination. Values are means ± SE (n = 5); DAI, d after inoculation.

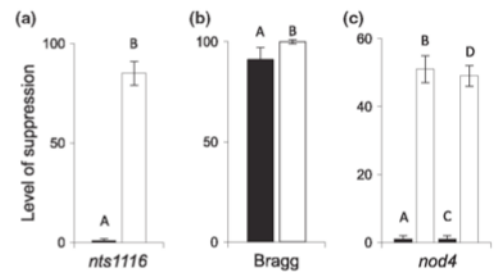
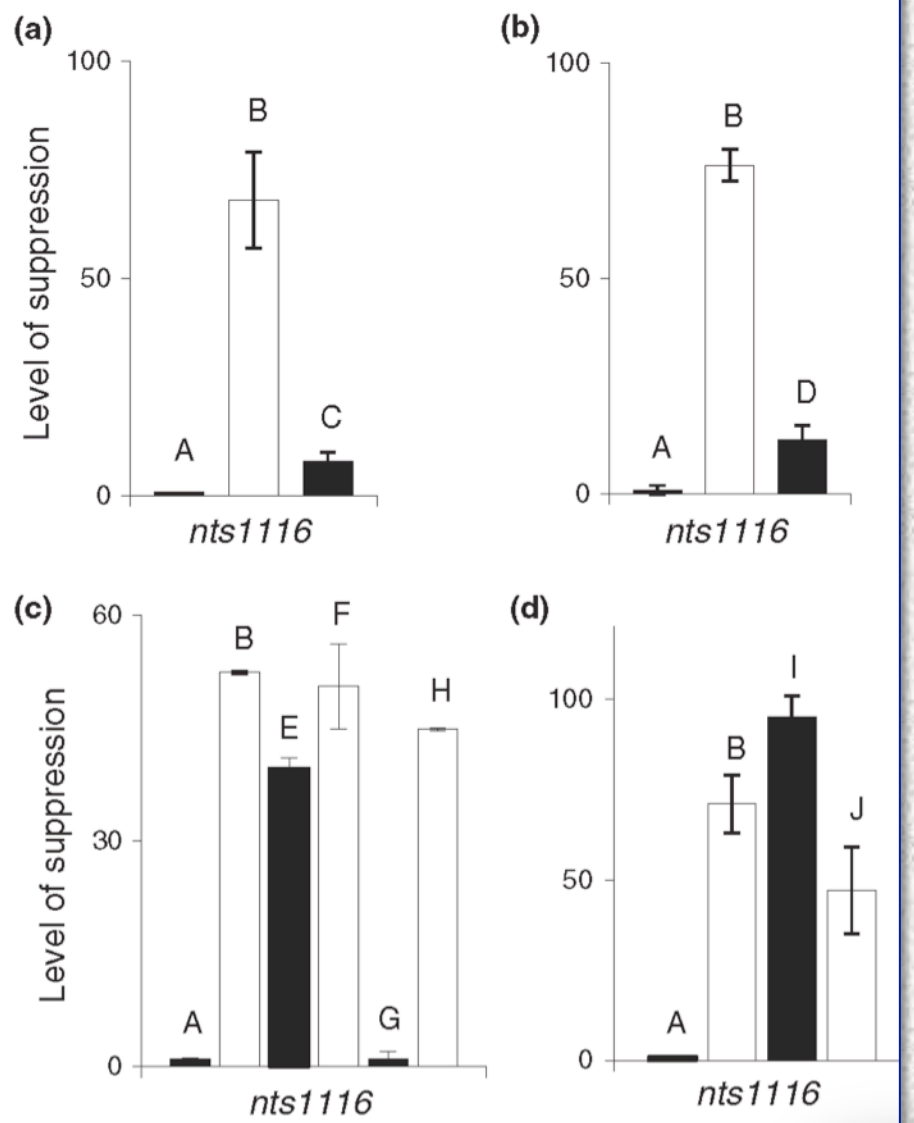


Fig. 4 Differential nodulation suppression in hypernodulation and supernodulation genotypes of soybean by petiole-fed leaf extracts: 4-wk-old (a) hypernodulating *nts1116*, (b) Bragg (WT), (c) supernodulating *NOD4* plants inoculated with *Bradyrhizobium japonicum* CB1809 at 24 h after commencement of petiole-feeding using extracts from (A) *B. japonicum*-inoculated *nts1116*, (B) Bragg, (C) *NOD4*, (D) Williams 82 leaves. The level of suppression was calculated as (a) the percentage of total nodules relative to those found on *nts1116* extract-fed *nts1116* plants (negative control), (b) percentage of total nodules relative to Bragg extract-fed Bragg plants, and (c) percentage of total nodules relative to *NOD4* extract-fed *NOD4* plants. Total nodule numbers per plant are shown in the Supporting Information Figs S2, S3 and S4. Values are means ± SE (n = 5).



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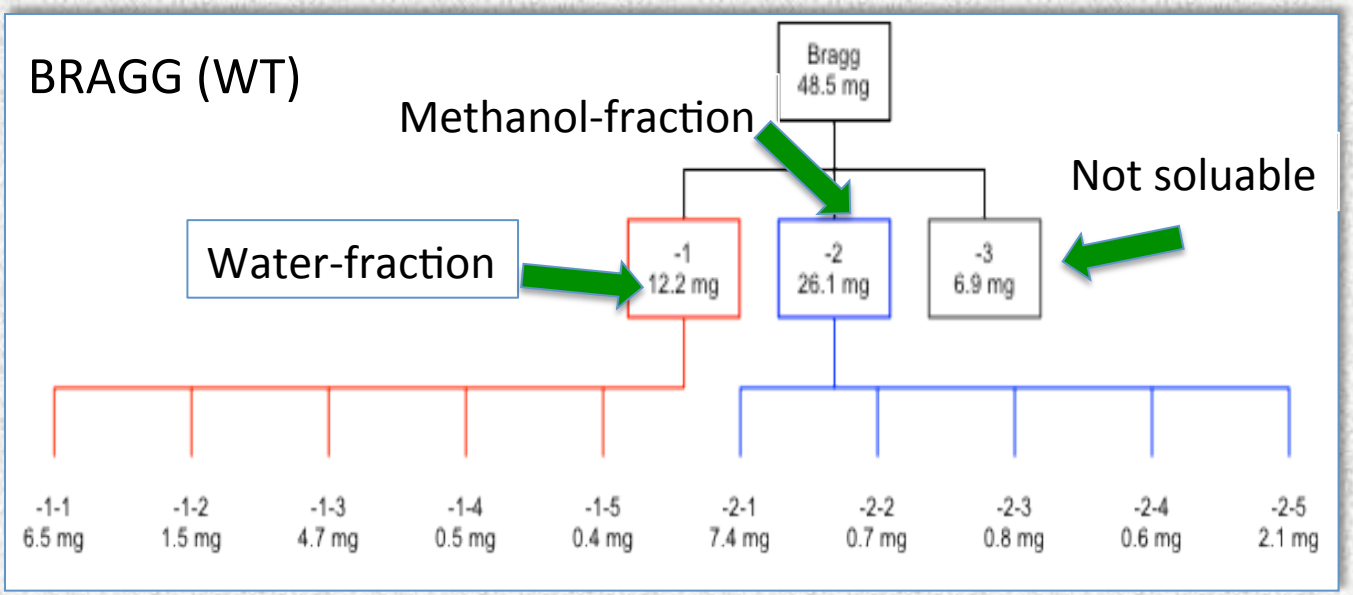
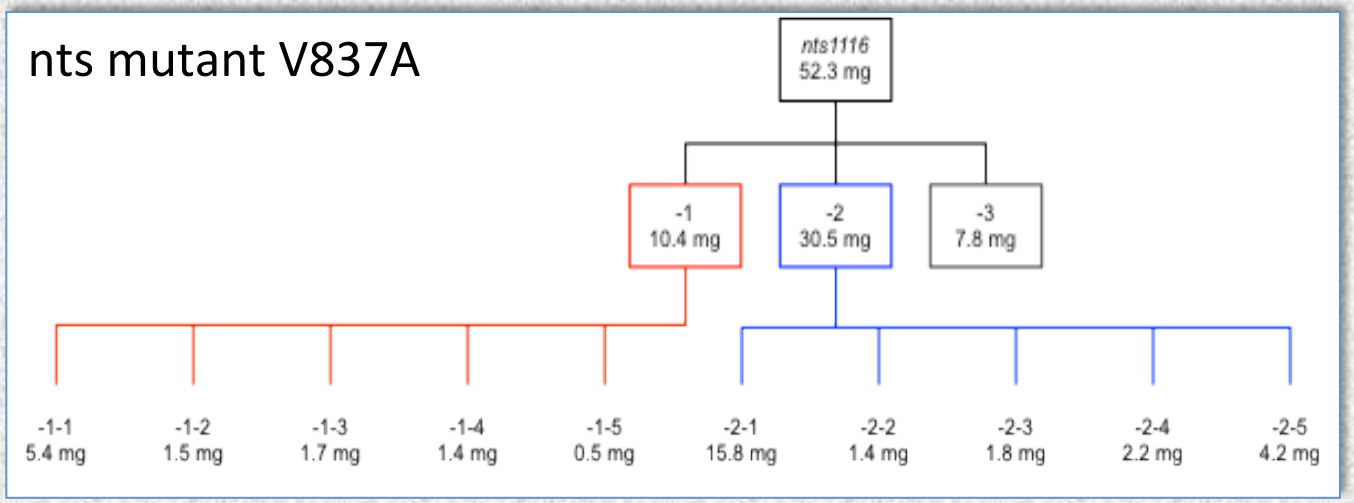
Finding the active principle for SDI: Solvent/HPLC separation





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Finding the active principle for SDI: Separation Scheme

Leaf extraction (inoculated mutant and WT) → Suppression Bioassay

Water/Methanol partitioning → Suppression Bioassay

LH-20 Sephadex column separation → Suppression Bioassay

High resolution LC-ESI MS

Mass determination

Molecular structure prediction

Molecular structure determination by NMR

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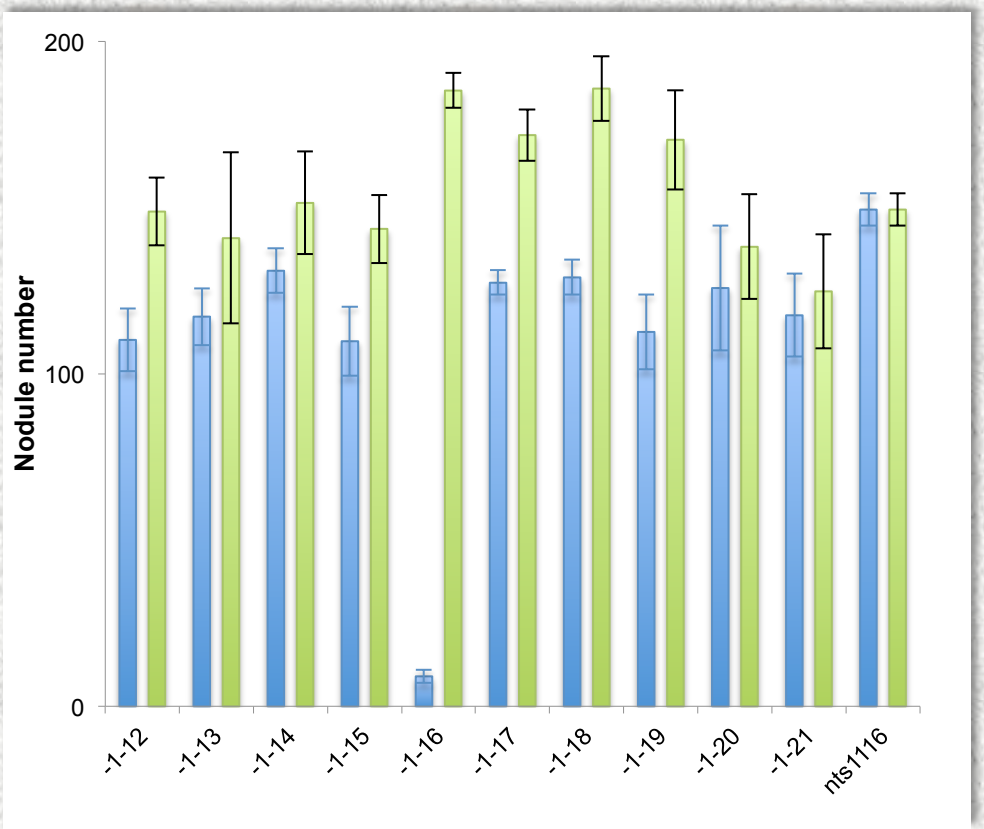
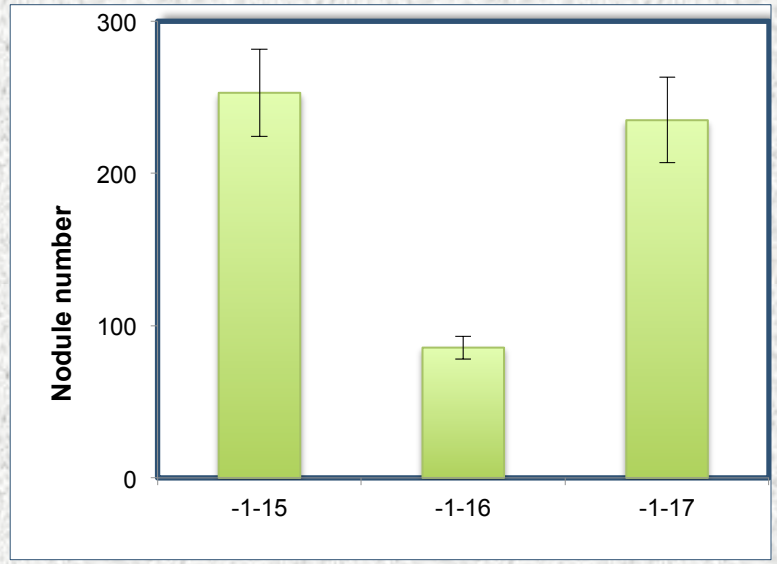
Finding the active principle for SDI: **Bioassay**





**nts1116* plants were fed with Bragg fractions -1-15 to -1-17. N=5 ± SE

* separated via a Sephadex LH-20 size exclusion column.

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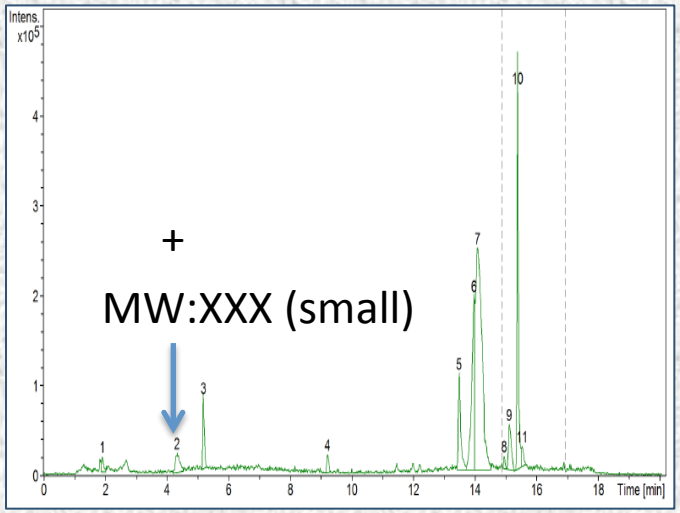
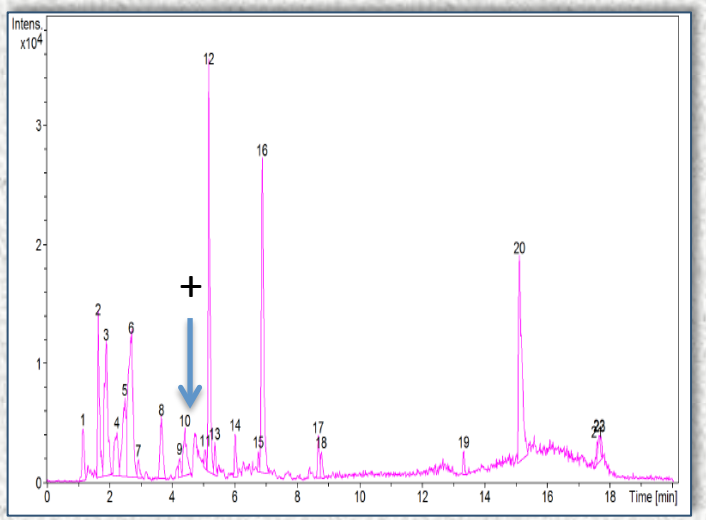
Finding the active principle for SDI: Analysis

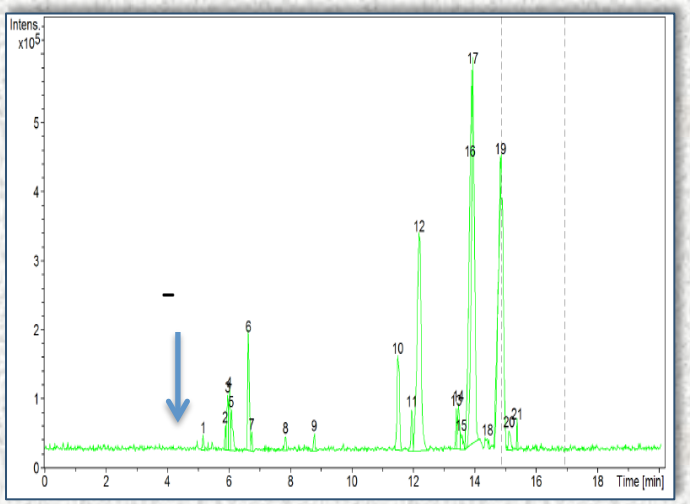
High resolution LC-ESI MS (positive and negative mode) of Bragg -1-16

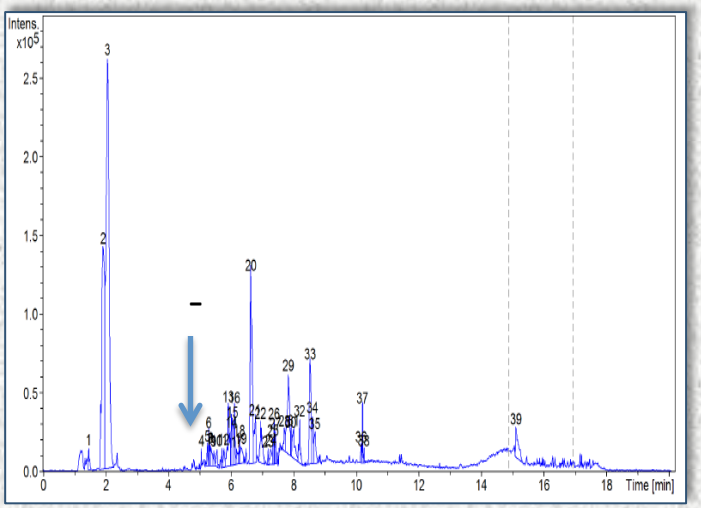


Positive mode



Negative mode





High resolution LC-ESI MS (positive and negative mode) of nts1116 (V837A)

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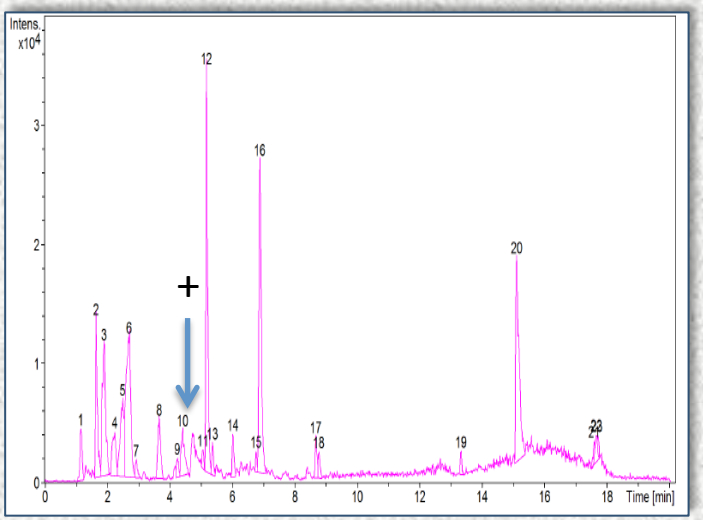
Finding the active principle for SDI: Analysis

High resolution LC-ESI MS (positive and negative mode) of Bragg -1-16



- Not present in neighboring -1-15 and -1-17
- peak (=XXXXX) (ChemCalc)
- best calculated molecular formula for the active SDI molecule was $C_{xx}H_{qq}O_{bb}$
- Scifinder (CAS, American Chemical Society)

Best **candidate:**
YYYYYYYYYYYYYYY,
a breakdown product of triterpenoid saponins.
(Structural verification is in progress)

???

- Product of mevalonate pathway: activated in AON (see Kinkema and Gresshoff 2008)
- Known to be transported in plants and inhibitory of cell divisions

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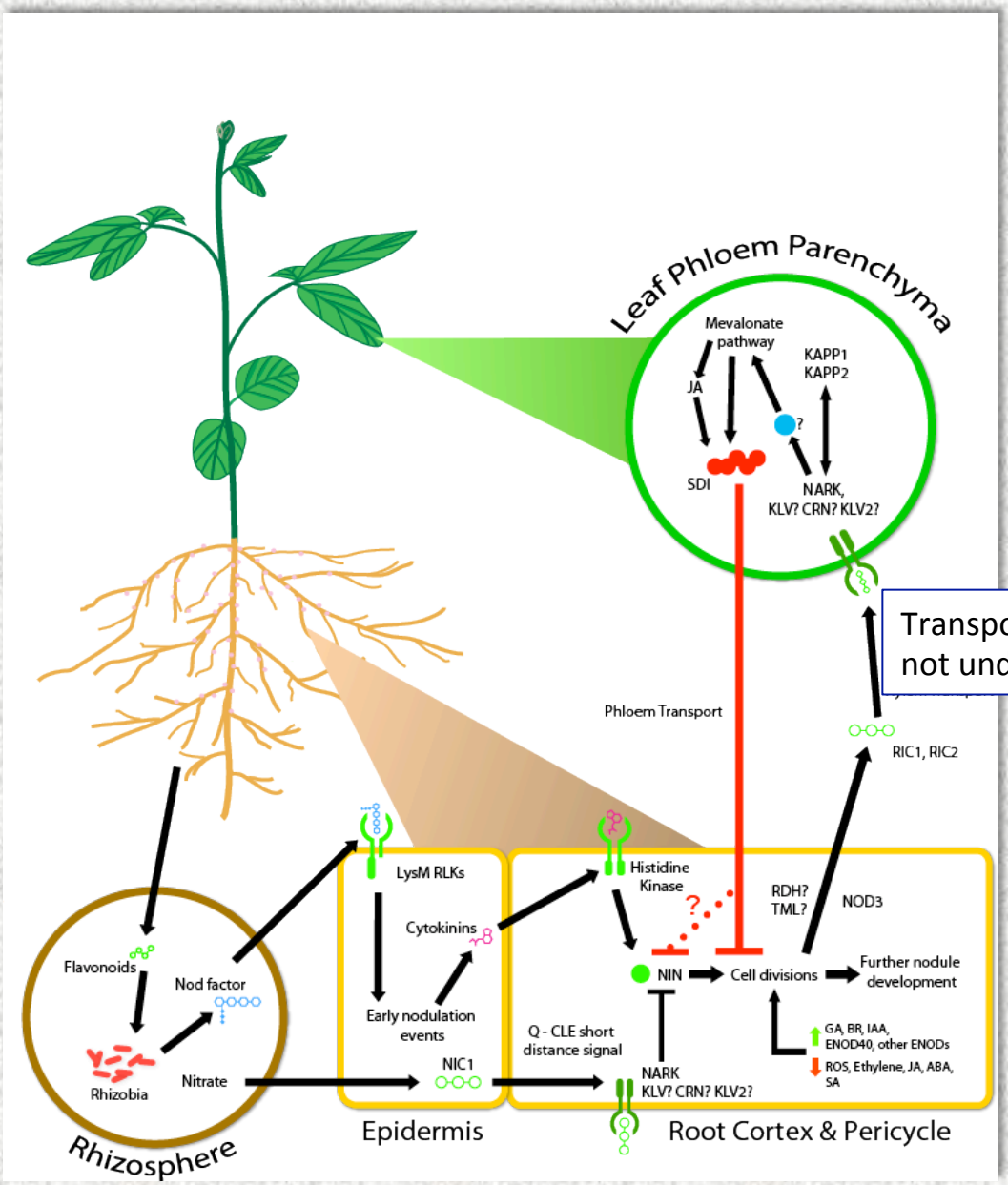
Current Model of Legume Autoregulation of Nodulation (AON)



Transport/Binding not understood

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Conclusions:

1) Coupling mutagenesis with molecular physiology and high through-put functional genomics and biochemical analysis allows establishment of causally supported molecular networks

2) Nodulation teaches us a lot about plant biology

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