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:

Dugald Reid, Yu-Hsiang Lin, Brett Ferguson, Arief Indrasumunar, Michael Djordjevic, Jacqui Batley, Dongxu (‘Snow’) Li, Satomi Hayashi, Saeid Mirzaei, Khalid Meksem, Paul Scott, Bandana Biswas, Fateh, Nurain, Meng-Han Lin, Qunyi Jiang, Alina Tolleneare, Attila Kereszt, Akira Miyahara, Tony Bacic, Ning Nontachaiyapoom, Liqi Han, Ning Chen, Jim Hanan, Stacey Cook, Julia Wilde, Ray Rose, Julia Kehr, Hua, Jim Reid, Malcolm R, Tancred Frickey, Steve Kazakoff, Bethany v. H., David Lightfoot, Lisette Prejelt, Peer Schenk, Bernard Carroll, Karsten Oelkers, Da Luo, Satoshi Tabata, Shusei Sato, Dave Edwards, Jiri Stiller, Sara Schaarschmidt, Mark Kinkema, Artem Men, Bostjan Kobe, Pick-Kuen Chan, Jessica, April, Bryan, Rob Capon (IMB), George Weiller (ANU). Said Ghahriel (Kentucky), Horst Vierheilig (Granada), Joachim Schulze and Bettina Hause (Germany), Gary Stacey (Missouri), Dash Lohar (BASF) ARC CoE funding, Qld Smart State Initiative, UQ Strategic Fund.
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

Lin et al, MOLECULAR PLANT 2011
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?
AND:

How is this process regulated???
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*

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 Indrasumunar1,2, Attila Kereszt1,2, Iain Searle3,5, Mikiko Miyagi1, Dongxue Li1, Cuc D.T. Nguyen1, Artem Men6, Bernard I. Carroll1,4 and Peter M. Gresshoff1,∗

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

**The Plant Journal** (2010)

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

Arief Indrasumunar1,2, Iain Searle3,4, Meng-Han Lin1, Attila Kereszt1,5, Artem Men1,6, Bernard J. Carroll1,3 and Peter M. Gresshoff1,*
Transgenic complementation confirms allele detection and gene identity.

Isolation of AON/supernodulation mutants

Wild type

EMS mutation

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†
Positional Cloning of *GmNARK* in Soybean:

**GmNARK**

- **UQC-IS5**
  - SP
  - LRRs
  - TM
  - Kinase domain
  - Q106* K115*
  - K506*
  - V837A
  - Q920*

**AtCLV1**
- 2590
- 353

**GmCLV1A**
- 2656
- 587

**GmNARK**
- 2602
- 362

**Graph:**
- X-axis: \(0.000\) to \(2.500\)
- Y-axis: \(0.000\) to \(2.500\)
- Data points for various conditions: NOD16, YNOD28, NOD28, RT16, RT28, UF16, FTF16, STF16, UF28, FTF28, STF28, TTF28, SAM16, SAM28

**Images:**
- Leaf images
- Microscopic images

**Logo:**
- ARC Centre of Excellence
- CTR
The cleft and the severity of the two mutant alleles suggest a ligand binding site.
<table>
<thead>
<tr>
<th>Line</th>
<th>Mutation</th>
<th>Phenotype</th>
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</thead>
<tbody>
<tr>
<td>nod4 (W)</td>
<td>T&gt;C = V370D</td>
<td>supernod</td>
</tr>
<tr>
<td>nod9p (W)</td>
<td>T&gt;C = V370D</td>
<td>supernod</td>
</tr>
<tr>
<td>nod1-3 (W)</td>
<td>G&gt;A = G863D</td>
<td>hypernod</td>
</tr>
<tr>
<td>nts1116 (B)</td>
<td>V837A</td>
<td>hypernod</td>
</tr>
<tr>
<td>nod3-7 (W)</td>
<td>C&gt;T = L346F</td>
<td>hypernod</td>
</tr>
<tr>
<td>F262 (F)</td>
<td>W677* + L829V</td>
<td>supernod</td>
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<tr>
<td>nod2-4 (W)</td>
<td>Het L346F</td>
<td>WT*</td>
</tr>
<tr>
<td>F23 (F)</td>
<td>H811Q and H789=</td>
<td>WT</td>
</tr>
<tr>
<td>nts1007 (B)</td>
<td>Q106*</td>
<td>supernod</td>
</tr>
<tr>
<td>PvNARK1</td>
<td></td>
<td>supernod</td>
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</table>
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

<table>
<thead>
<tr>
<th></th>
<th>NARK</th>
<th>RIC1</th>
<th>NIC1</th>
<th>RIC2</th>
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<tr>
<td>root</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>leaf</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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
Three CLE peptide genes

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

Signal peptide (25-30 aa)  
Variable region (60 aa)  
CLE peptide (12 aa)  
C terminal region (4-8 aa)

Reid et al (2011) MPMI
The Down-SIGNAL: Shoot Derived Inhibitor (SDI)

Lin et al; New Phytologist 2010; NATURE Protocols 2011
Developmental Cross-Feeding

Water Extraction & Treatment

Leaf of treated WT plant

Petiole Feed & Plant Movement

Feed into Mutant Plant

Phenocopy to WT (Suppression Assay)
Petiole-applied glucose and mannitol travel in both basi- and acropedal direction.
Glucose: 0.5, 1.0, 3.0 hr
Mannitol: 24 hr
Simple and fast

Broadly applicable

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).
**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).
Finding the active principle for SDI: **Solvent/HPLC separation**

**BRAGG (WT)**

- **Methanol-fraction**
  - -1 12.2 mg
  - -2 26.1 mg
  - -3 6.9 mg

- **Water-fraction**
  - -1-1 6.5 mg
  - -1-2 1.5 mg
  - -1-3 4.7 mg
  - -1-4 0.5 mg
  - -1-5 0.4 mg
  - -2-1 7.4 mg
  - -2-2 0.7 mg
  - -2-3 0.8 mg
  - -2-4 0.6 mg
  - -2-5 2.1 mg

**nts mutant V837A**

- **nts1116 52.3 mg**
  - -1 10.4 mg
  - -2 30.5 mg
  - -3 7.8 mg

- **Not soluble**
Finding the active principle for SDI: Separation Scheme

1. Leaf extraction (inoculated mutant and WT)
   - Suppression Bioassay

2. Water/Methanol partitioning
   - Suppression Bioassay

3. LH-20 Sephadex column separation
   - Suppression Bioassay

4. High resolution LC-ESI MS

5. Mass determination

6. Molecular structure prediction

7. Molecular structure determination by NMR
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.
Finding the active principle for SDI: Analysis

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

MW:XXX (small)

High resolution LC-ESI MS (positive and negative mode) of nts1116 (V837A)
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: YYYYYYYYYYYYYY,
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
Current Model of Legume Autoregulation of Nodulation (AON)
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.