Control of nitrogen fixation in bacteria that associate with cereals

Ryu, M.H., Zhang, J., Toth, T., Khokhani, D., Geddes, B.A., Mus, F., Garcia-Costas, A., Peters, J.W., Poole, P.S., Ané, J.M. and Voigt, C.A., 2020

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Introduction

- Based on the original article by Ryu *et.al* (2020)
- Nitrogen is a typical fertilizer in agriculture as it cannot obtained from air by cereal
- Legumes obtain N_2 via mutualism with nitrogen-fixing bacteria (rhizobia) in their root nodules
  - endophytes: live inside roots
  - epiphytes: live on root surface
- Some legume root rhizobia endophytes also found in cereal roots but unable to fix N_2 outside the nodules
  - Could they be?

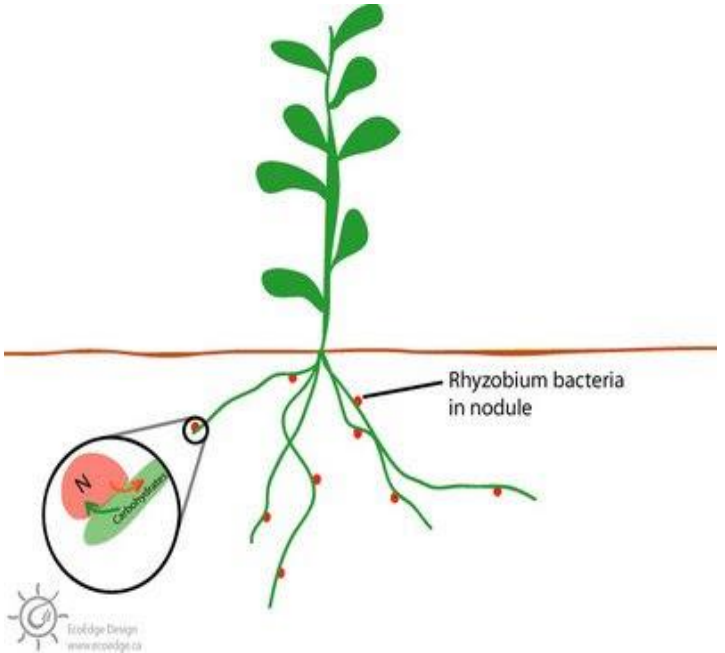

Figure https://www.permaculturereflections.com/rhizobium-symbiosis-with-woody-plants-leguminous-nitrogen-fixing-trees/

Introduction: *nif* genes

- Nitrogen fixation (*nif*) genes are organized as clusters
- Conserved genes include those encoding nitrogenase and cofactor biosynthesis
- Nif genes are under stringent regulatory control due to metabolic and energy resources
    - nitrogenase can take 20% of the cell mass
    - each ammonium requires approx. 40 ATPs to be produced
- Nitrogenase is O2 sensitive

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Motivation

- Different cereals are the main calorie source of the world’s population
- Reducing the need for N2 fertilizers would be beneficial
  - economically
  - environmentally
  - energetically
- Aim: engineering inducible nitrogenase activity in cereal root node bacteria

Strategy

- **Evaluation of native and engineered clusters** from diverse sources transferred to different species
  - side-by side comparisons of activity
- **Best candidates** of high levels of inducible nitrogenase activity and reduced oxygen sensitivity selected
- **Regulatory control replaced** by synthetic, genetically encoded on/off sensors for *nif* transcription regulation
  - sensors responding to natural root exudates
- **Plants engineered** to release chemical signals from their roots (opine, rhizopine..)

Methods

- **Bioinformatics and protein engineering from ground up (refactoring)**
  - DNA synthesis, DNA fragment amplification, yeast assembly, cloning into plasmid backbones
  - Part libraries
- **Culturing**
  - in organism appropriate conditions
  - in presence of oxygen, ammonium
- **Quantifying transfers and evaluating performance**
  - RNA-seq
  - Ribosome profiling
  - Acetylene reduction assay (ARA)

Transfer of native *nif* clusters to new hosts

- **Goal: Assessing the performance of native *nif* clusters in *E. coli*, *P. protegens* Pf-5 and symbiotic rhizobia**

What succeeded:

- **gene cluster transfer to other bacterial hosts possible**
- **Protein expression in hosts**
- ***K. oxytoca* most promising**

What failed:

- ***E. coli* as host**
- **Increasing protein expression in R. sp IRBG74 by increasing *nif* expression**

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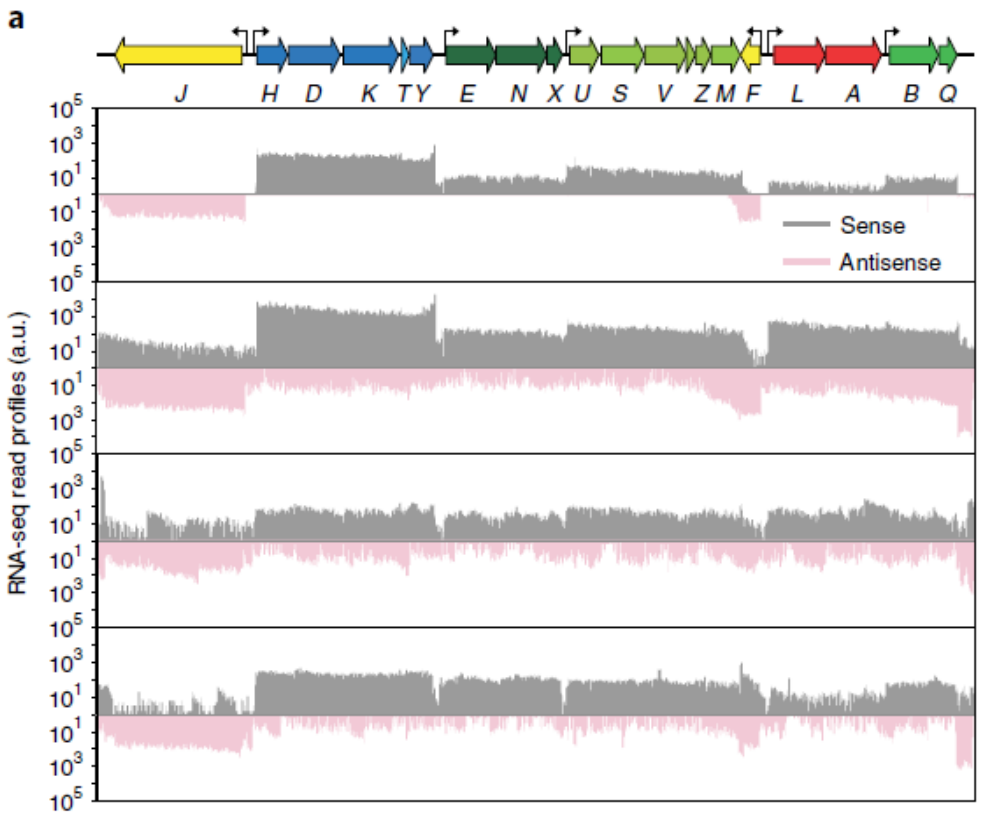

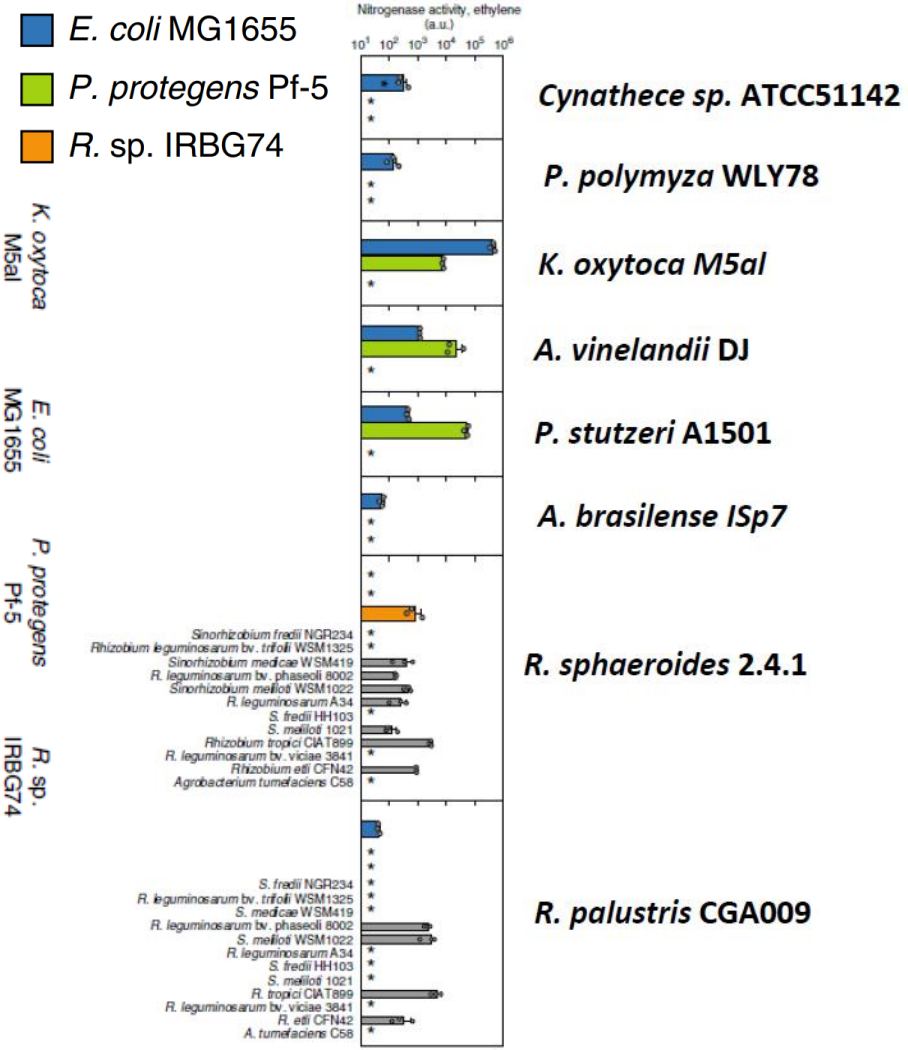

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Transfer of *K. oxytoca nif* to *R. sp* IRBG74

- **Goal: genetic refactoring → eliminate native regulation and placing the system under the control of synthetic sensors and circuits**

What was achieved:

- **good promoter induction in v2.1**
- **v3.2 active in R. sp**
- **translation rate close to *K. oxytoca***

What failed:

- ***nif* activity in v2.1**
- **v2.1 terminators**
- **v2.1 not active in R. sp**
- **low activity with v3.2**
- **induction had an optimum**

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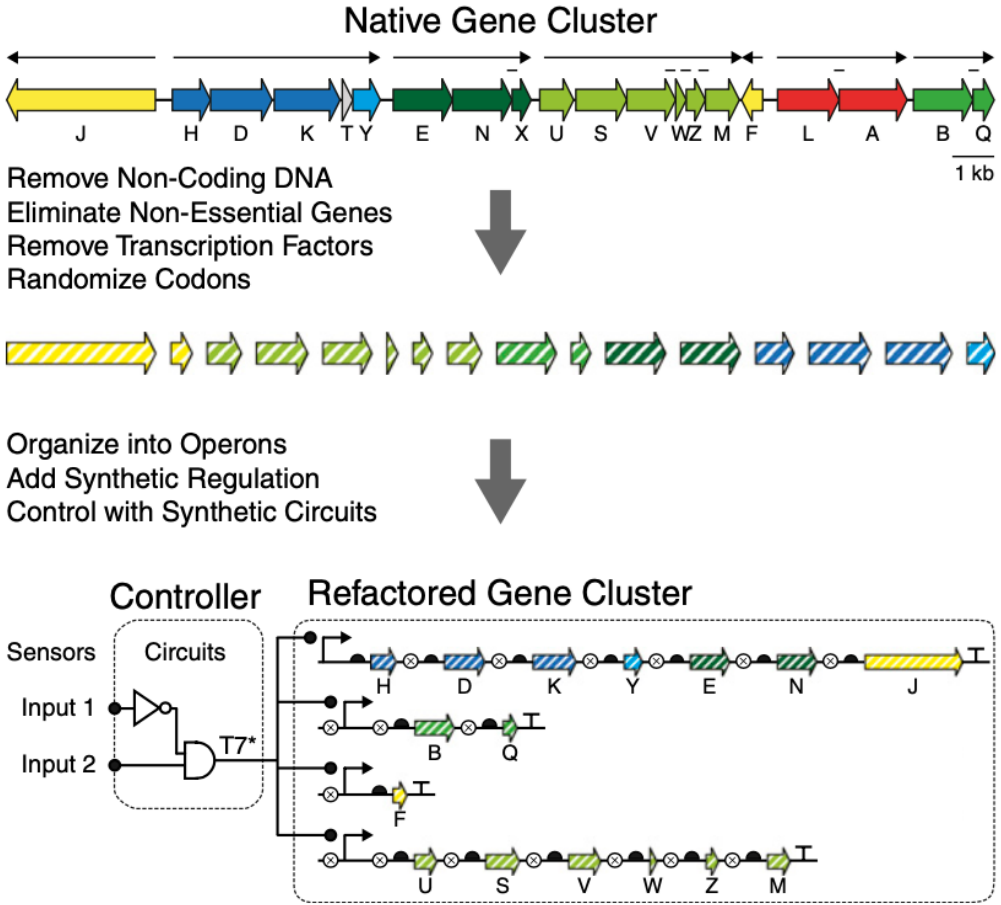
Refactoring a gene cluster

Nif gene cluster From *K. oxytoca*

- The genes are colored by function:
  - blue: nitrogenase
  - green: cofactor biosynthesis (shading corresponds to operons)
  - yellow: e- transport
  - gray: unknown



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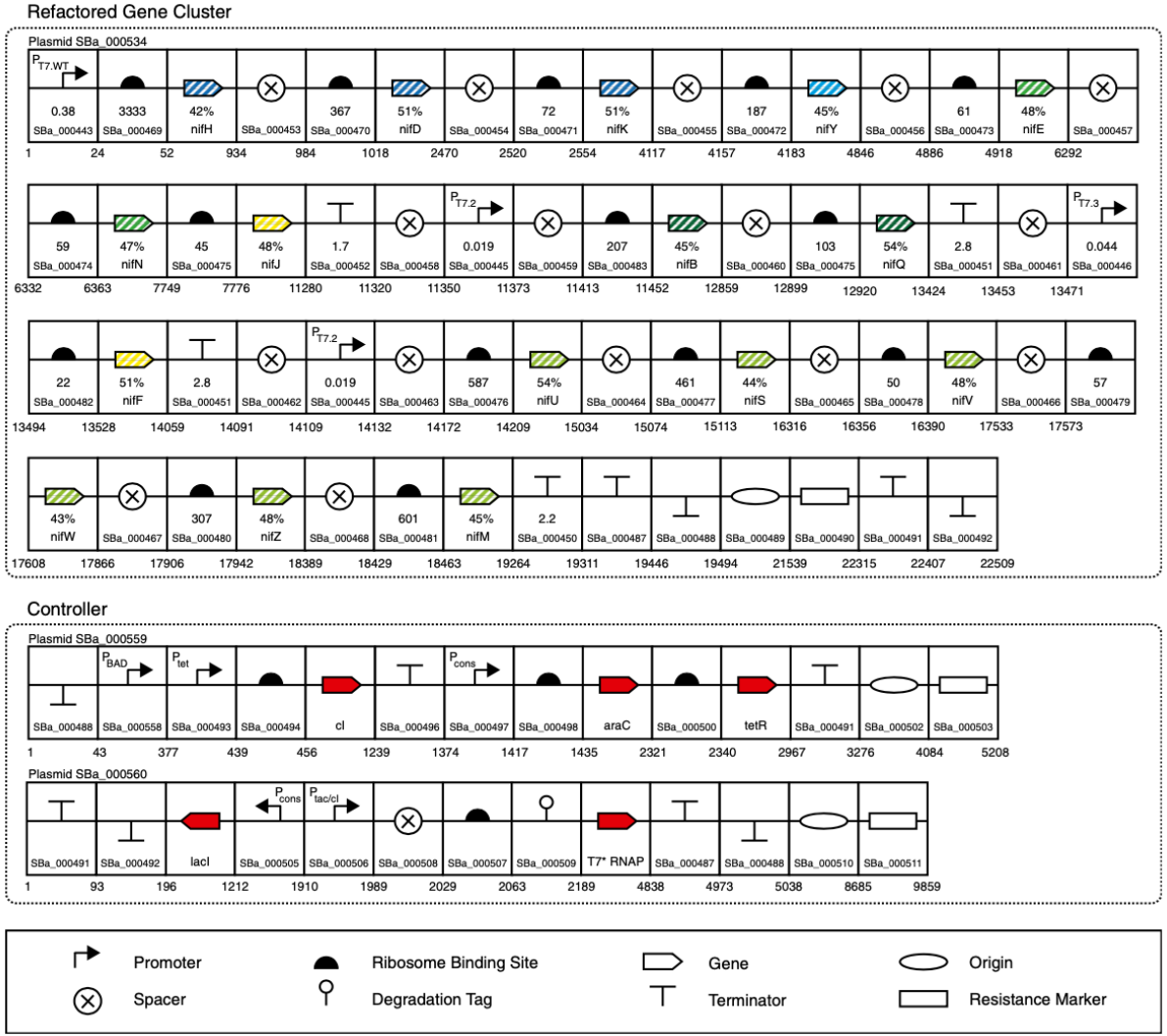

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Removing ammonium repression of *A. caulinodans*

- **Goal 1: transfer *A. caulinodans nif* to *R. sp.* IRBG74**
- **Goal 2: modify the regulation controlling *nif* to be placed under the control of synthetic sensors**

What was achieved:

- **controller co-expressing NifA and RpoN recovers activity**
- **50% activity recovered in presence of ammonium**

What failed:

- **transfer of A. caulinodans *nif***
- **WT strain 95% repressed by ammonium**

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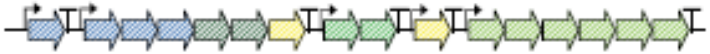
Refactored *nif* cluster v2.1



Promoters	P_6	P_5							P_2			P_3	P_2						
Terminators		T_{T7}							$T_{T7.2}$			$T_{T7.3}$	$T_{T7.3}$						T_{T7}
nif genes (recoded)	*H*	*D*	*K*	*Y*	*E*	*N*	*J*	*B*	*Q*	*F*	*U*	*S*	*V*	*W*	*Z*	*M*			

h

Refactored *nif* cluster v3.2

Promoters	P_{WT}				P_2			P_2	P_2		P_2	P_2							
Terminators					T_{23}			T_{12}	T_{19}		T_{14}	T_{24}							T_{11}
nif genes (native)	*H*	*D*	*K*	*T*	*Y*	*E*	*N*	*X*	*J*	*B*	*Q*	*F*	*U*	*S*	*V*	*W*	*Z*	*M*	

Controlling *nif* in *P. protegens*

- **Goal: to remove ammonium repression of the cluster or it being constitutively on by placing cluster under synthetic control**

What succeeded:

- **DAPG, aTc, 3OC6HSL and cuminic acid sensors functional**
- **inducible clusters showed little ammonium repression**
- **P. stutzeri and A. vinelandii clusters showed tolerance for oxygen (0,5-1%)**

What failed:

- ***K. oxytoca* cluster sensitive to oxygen**

Control of N fixation with agriculturally relevant sensors

- **Goal: to test induction of the cluster with agriculturally relevant substances**
  - sugars, hormones, flavonoids, antimicrobials, chemical

What succeeded:

- **salicylic acid sensor for A. caulinodans had a 1,000-fold induction of nitrogenase**
- **Arabinose and naringenin sensors for P. protegens Pf-5 led to nitrogenase activity**
- **Sensors in *A. caulinodans* for octopine and nopaline produced highly inducible nitrogenase activity**

What failed:

- **DAPG sensor for R. sp. had weak induction of nitrogenase**

Discussion

- **Comparison of diverse species, natural nif clusters and engineering strategies**
  - can be used towards designing a bacterium that can deliver fixed nitrogen to a cereal crops

- **The goal was to obtain inducible nitrogenase activity in a strain that can associate with cereals**

- **RNA sequencing and ribosome profiling were used to compare the function of nif parts in their native and new hosts**

Discussion

- **Most promising endophyte:**
  - variant of *A. caulinodans*: *nifA* knocked out of the genome and a mutant NifA and RpoN are supplemented on a plasmid
- **Most promising epiphyte:**
  - *P. protegens Pf-5*: transfer of *A. vinelandii nif* cluster and placement of *nifA* of *P. stuzeri* under inducible control

- **In both: nitrogenase can be placed under inducible control in response to cereal-root exudates, phytohormones and putative signaling molecules that could be released by genetically modified plants**

Discussion

- native *K. oxytoca nif* cluster performs similarly when transferred

- refactored cluster that uses codon optimization and disrupts operons and translational coupling had varying expression levels

→ disrupting operons and translational coupling does not impact their function in native host but affects the activity after transfer

Path forward

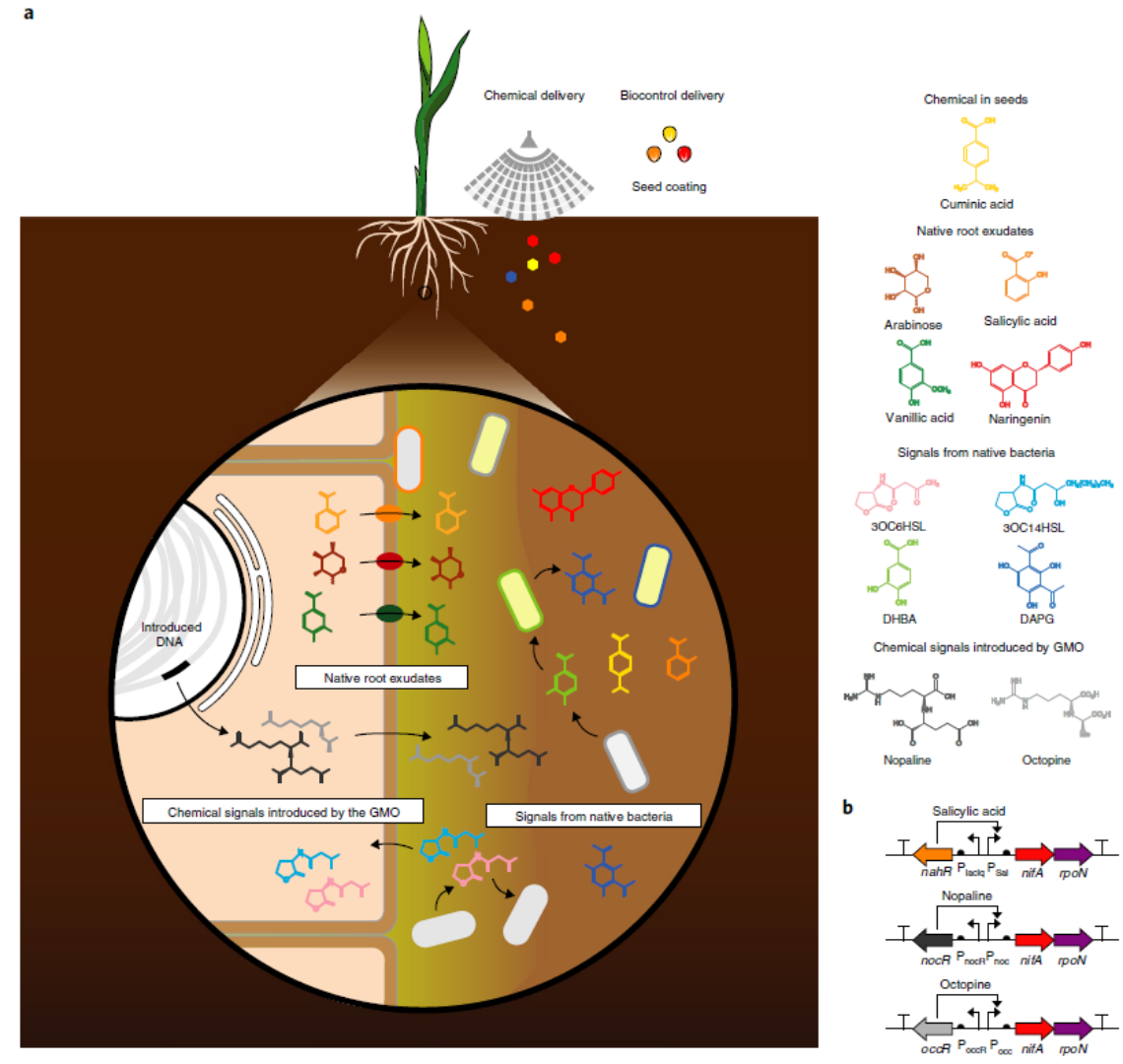
- **First step towards building strains that can efficiently deliver fixed nitrogen to cereals**

- **Additional genetic engineering required to:**
  - maximize the ability of the microorganism to catabolize carbon sources from the plant
  - increase the flux of fixed nitrogen delivery
    - redirection of metabolism
    - introducing transporters
    - optimization of electron transfer

- **Other possibility: genetically engineer the plant to produce orthogonal carbon sources and then transfer the corresponding catabolism pathway into bacterium**

  → synthetic symbiosis

Thank you!
Questions?


a
Chemical delivery
Biocontrol delivery
Seed coating
Introduced DNA
Native root exudates
Chemical signals introduced by the GMO
Signals from native bacteria
Chemical in seeds
Cuminic acid
Native root exudates
Arabinose
Salicylic acid
Vanillic acid
Naringenin
Signals from native bacteria
3OC6HSL
3OC14HSL
DHBA
DAPG
Chemical signals introduced by GMO
Nopaline
Octopine
b
Salicylic acid
nahR P lacIq P Sal nifA rpoN
Nopaline
nocR P nocR P noc nifA rpoN
Octopine
occR P occR P occ nifA rpoN