ARTICLE:
Control of nitrogen fixation in bacteria that associate with cereals

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Glossary

Legumes (beans, peas…) obtain nitrogen from air through rhizobia residing in root nodules.

Cereals (any grass cultivated for its grain) cannot obtain nitrogen from the atmosphere and gets it from fertilizer.

Rhizobia are diastrophic bacteria is **bacteria that fix atmospheric nitrogen gas** into a more usable form such as ammonia. They do this after infecting the roots of legumes to form root nodules.

Endophytes are a bacterium that lives within the roots of a plant without causing apparent disease.

Epiphytes are a bacterium that lives on the root surface and does not affect the host negatively.

Inoculants microbes such as rhizospheric bacteria that either fix nitrogen naturally or are potential hosts into which the capability could be transferred.

Root exudates are a suite of substances in the rhizosphere that are secreted by the roots to e.g. prevent excessive dehydration.

Biocontrol agents prevent infection of the host plant by the pathogen.

Phytohormones (aka plant hormones) control all aspects of plant growth and development.

Nif cluster is a cluster of genes that encodes enzymes involved in the fixation of atmospheric nitrogen.

Introduction

-Nitrogen can either be obtained by plants from the air (legumes) or from fertilizer (cereals)

-If this ability could be transferred to cereals it could be a game changer as it reduces the need for nitrogenous fertilizer

→ economical, environmental and energical benefits

-The article presents ways to engineer the bacteria that associate with cereals either on the root surface (epiphytes) or living inside the roots (endophytes) to fix nitrogen

Main aim

-The article presents ideas for how inducible nitrogenase activity in cereal can be engineered

- The main approach is to eliminate ammonium repression and to add nif clusters so that nitrogen fixation can be controlled in the bacteria

-For example in the endophytes Azorhizobium caulinodans ORS571 and Rhizobium sp. IRBG74, as well as the epiphyte Pseudomonas protegens Pf-5

-Different strategies were taken to do this under the control of relevant signals: root exudates, biocontrol agents and phyto hormones

- Rhodobacter sphaeroides/Klebsiella oxytoca → R. sp. IRBG74

-Pseudomonas stutzeri/Azotobacter vinelandii → P. protegens Pf-5

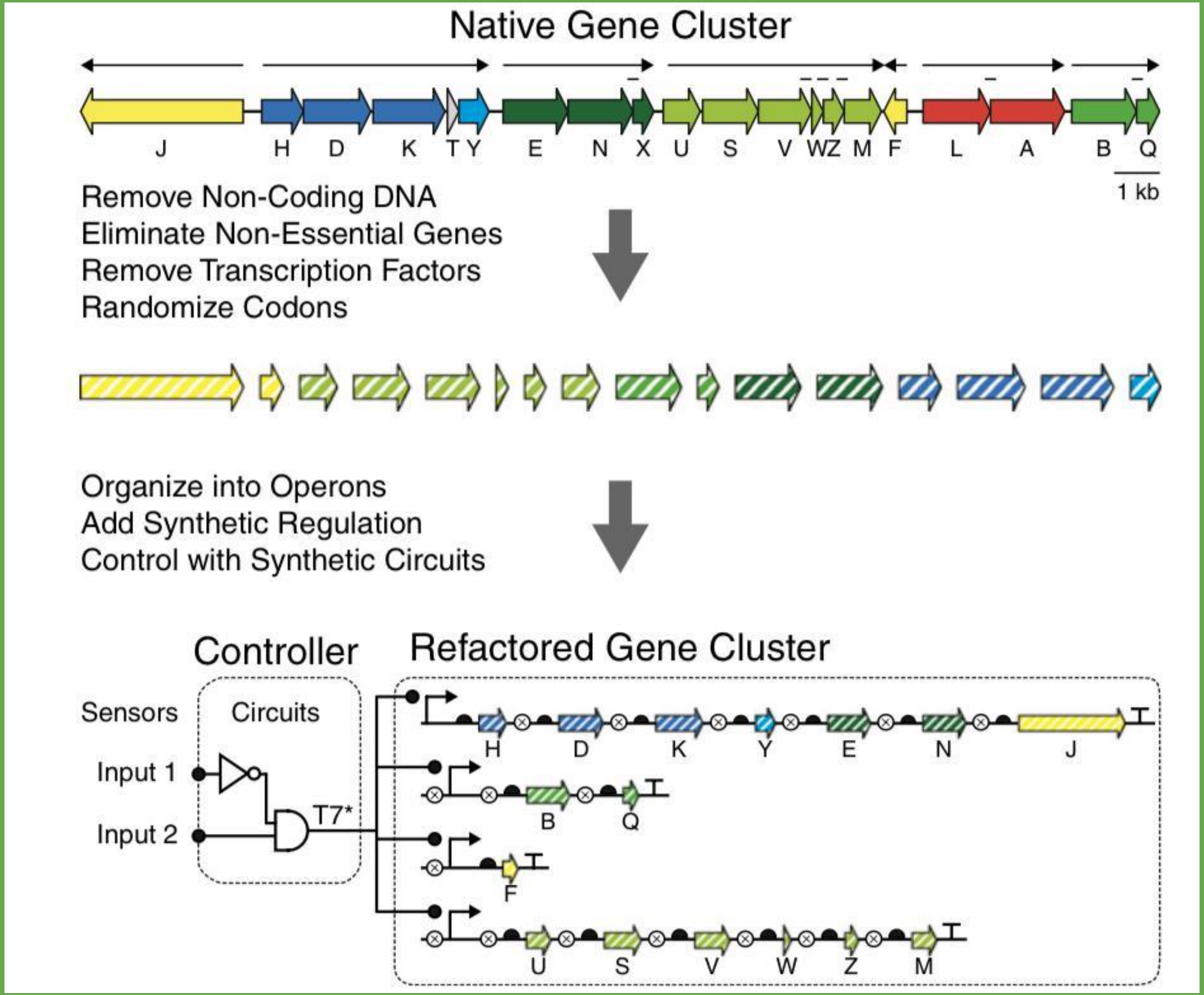
-Ideally the synthetic regulatory control could make the plants release chemical signals from their roots that keep nif transcription off at undesirable times and turn it on when needed

-There are also problems that can arise (regulation, missing genes, intracellular conditions)

-It has not been shown that a Rhizobium strain can be engineered to fix nitrogen under free-living conditions.

Method

- Refactoring process
- Klebsiella oxytoca
- Rewritten DNA
- Reduced complexity, maintained functionality
- "Systematically eliminate the native regulation of a gene cluster and replace it with synthetic genetic parts and circuits"
- Haber Bosch



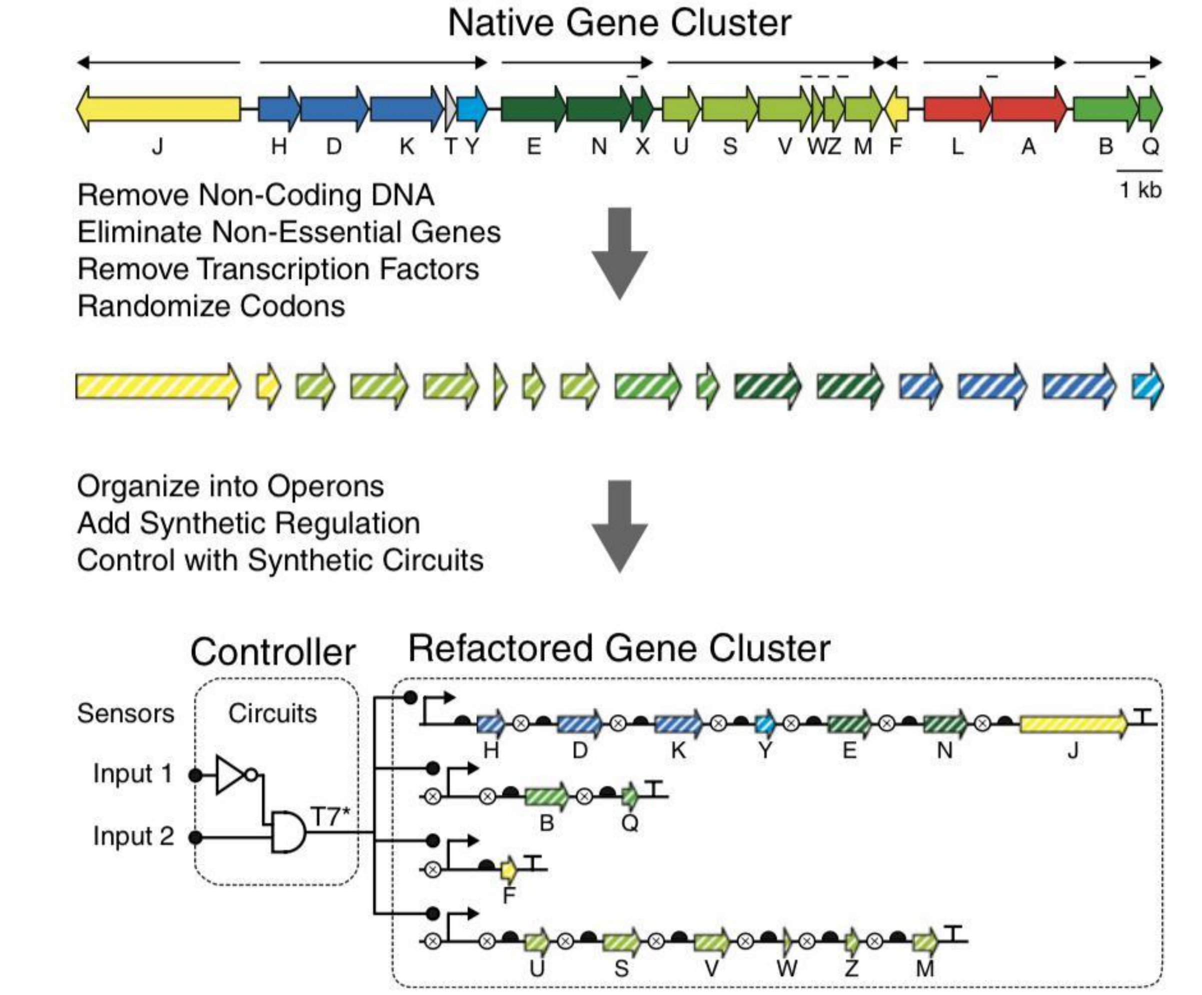
Method Refactoring

Process Steps:

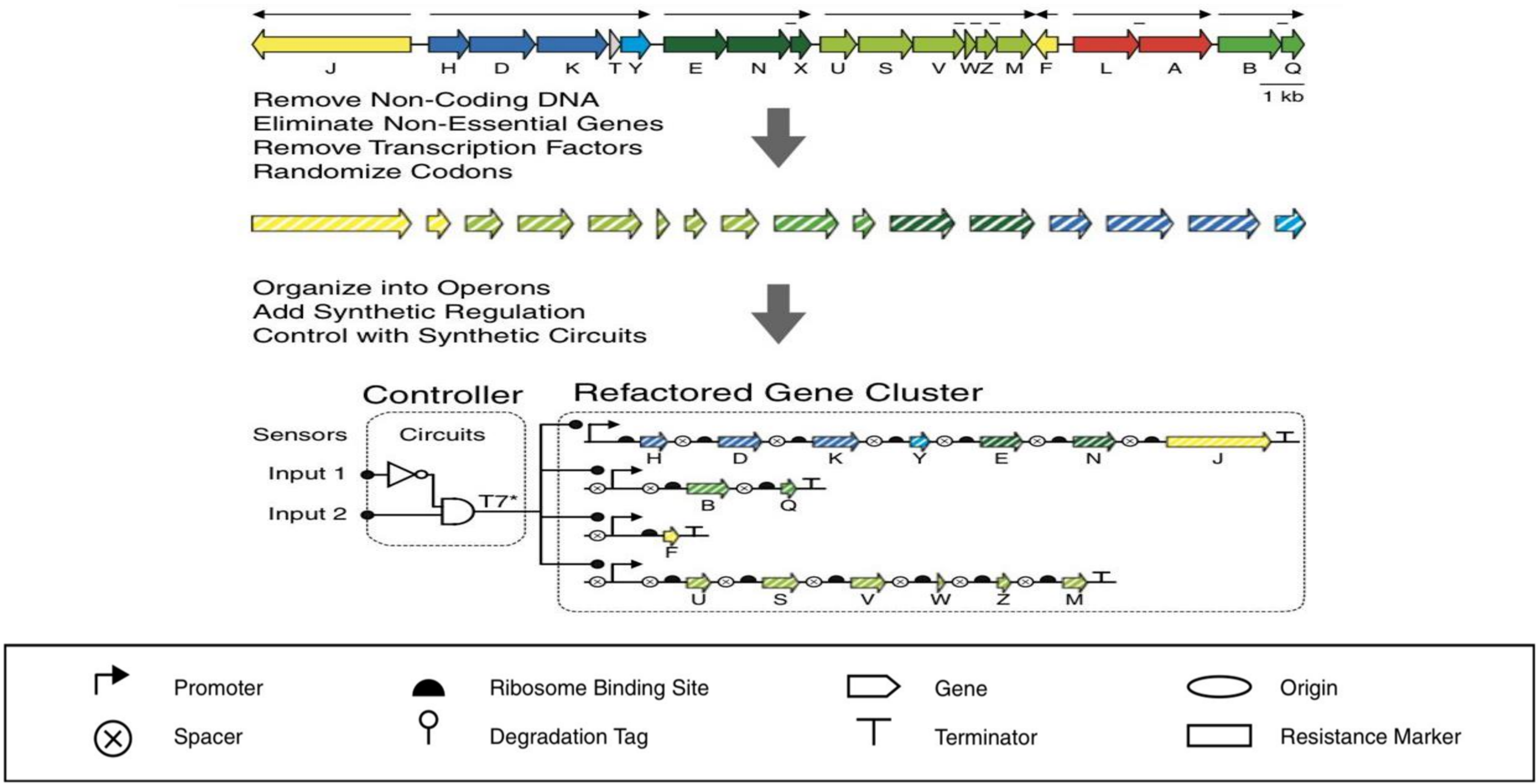
1. Removal of ncDNA + regulatory genes
2. Essential genes recoded (selecting DNA producing codons distant from Wt))
3. Recoded genes grouped into artificial operons + expression controlled
4. Refactored gene cluster containing "organized discrete well characterized parts"

Method Contd.

- Bottom-up approach
- Gene clusters crucial for nitrogen fixation
- Nitrogenase subunits, metallocluster biosynthetic enzymes, chaperones, e-transport, and regulators
- Transfer to E. Coli
- Refactoring: reorganizes, simplifies regulation and assigns function

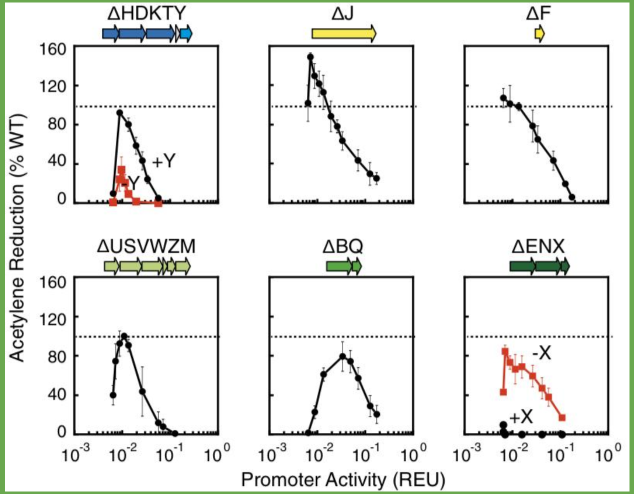


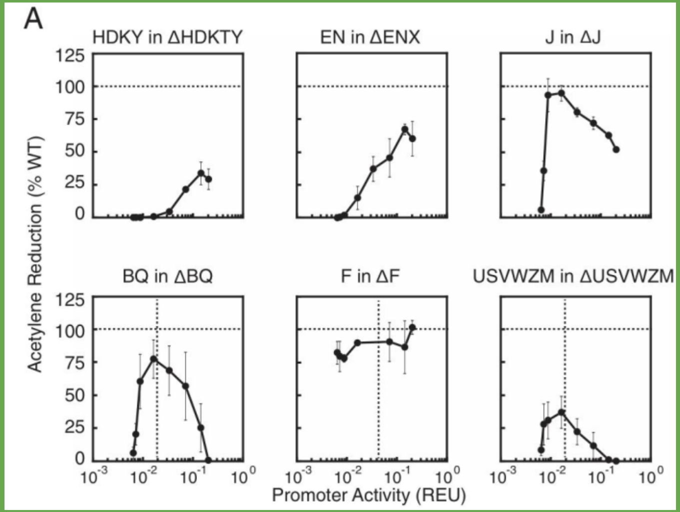
Native gene cluster



Results, achievements, success and problems

- NifY required for full activity and broadens tolerance
- NifT, no affect on activity
- NifJ & NifF need to be expressed at low levels
- NifX reduces activity
- NifT, NifX & NifLA not included in refactored cluster
- Ptac promoters replaced with T7
- Specialized promoters for NifHDK, NifJ & NifEN
- Synthetic cluster activity ~7.4% of WT nitrogen are activity, but uses ambient N2 as nitrogen source (3.5 X WT)





Achievements & success

- Synthetic cluster activity ~7.4% of WT nitrogen are activity, but uses ambient N2 as nitrogen source (3.5 X WT)
- Separation of controller and cluster simplifies regulation (New controller construction)
- Native regulation eliminated
- Nitrogen activity independent of environmental signals
- Cluster maintains activity in presence of ammonia
- Clean reference system, used for testing & creating improved functions

Problems

- Process of simplification and modularization reduces activity
- Expected outcome of refactoring a highly evolved system such as this
- Unknown genetics -> sequence errors (need for simple debugging methods)
- Context-independent parts needed for expressional control
- ‘Advanced computational methods equipped for scanning genetic designs for interfering functions’

What was achieved

- Rhizobium sp. IRBG74 can be modified to increase nitrogenase activity by the transfer of nif cluster.
- Nif cluster transferred from Rhodobacter sphaeroides or Klebsiella oxytoca.
- For P. protegens Pf-5, transfer of inducible cluster (from Pseudomonas stutzeri or Azotobacter vinelandii) causes ammonium tolerance and higher oxygen tolerance of nitrogenase activity than K. oxytoca.

Importance

- Identify how to deliver a high nitrogen flux to cereal crops.
- As known, nitrogen is used as fertilizer for crops -> higher nitrogen flux means better fertilizing effect. Nitrogen a limiting nutrient.
- Reduces need for nitrogenous fertilizer.
- Environmental, energy and economic gains.

In the future

- Ways to maximize how the microorganism catabolizes the carbon resources from plant.
- Redirect metabolism to increase the flux of nitrogen delivery.
- One possibility could be to genetically modify the plant for producing orthogonal carbon sources -> Place corresponding catabolic pathways to bacterium.

Pictures

Ryu et al. **Control of nitrogen fixation in bacteria that associate with cereals** Nature Microbiology volume 5, pages 314–330 (2020).

(Temme, K., Zhao, D., & C.A. Voigt (2012). Refactoring the nitrogen fixation gene cluster from *Klebsiella oxytoca*. *Proc. Natl. Acad. Sci.*, 109(18): 7085-7090.)

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