Nitrogen use efficiency:
Absorption, metabolism and regulation

http://www-ijpb.versailles.inra.fr/
Laboratory of Plant Nitrogen Nutrition
(National Institute of Agronomy)
Versailles, France
Laboratory of Plant Nitrogen Nutrition

10-12 mM nitrate

0.2-3 mM nitrate

Maize -yield

Arabidopsis plants (35DAS)
Laboratory of Plant Nitrogen Nutrition

Plant response to nitrogen availability: transport, signaling and natural variation
*F. Vedele, A. Krapp, S. Ferrario, F. Chardon, S. Chaillou*

Functional genomic of nitrogen recycling in plants
*C. Meyer, H.N. Truong, C. Masclaux, M. Cren*

Nitrogen management from vegetative stage to grain filling
*B. Hirel, A. Suzuki, T. Tercé, I. Quilleré*
Nitrogen use efficiency

Economic and environmental stakes
Nitrogen use efficiency (NUE = NupE + NutE)

Capacity of the plant to produce a supplement of yield (dY) for each added unit of N fertiliser (dNf)

N fertilization

Yield

\[ \text{NUE} = \frac{dY}{dN} \]

\[ Y_{\text{max}} \]

\[ Y_0 \]

\[ \text{NO}_3^- \text{ leaching} \]

\[ \text{NO release} \]

N mineralization

0

N fertilization

N total
World population and mineral fertilisation

- Current growth rate is about 1.3%, (doubling time of 54 years).
- Now 6 billion to become 12 billion by 2054.
Impact on environnement and health

- **Nitrate is mobile in soils:**
  - pollution of ground water
  - Eutrophisation
  - Human health problem
    (methemiglobinemia)

Limitation for nitrate content in water: <50 mg/l
Mineral N nutrition: major component of agronomic production

**Requirements:**
- High NUE
- Sustainable agriculture (surface limitation, preservation of environment, quality of products)

**Goals:**
- Adjust nutrition to plant demand
- Adaptation of the plant to nutrient limitation
Key functions of N nutrition – whole plant level

Seeds

Minerals

Roots

Absorption

Transport

Storage

Assimilation

Leaves

Growth

Demand of the plant
Absorption

Cytosol

Transporter

NADH (2e⁻)

NR

Reduction

NO₃⁻ → NO₂⁻

NIR

Chloroplast

NO₂⁻ → NH₄⁺

Photorespiration

Assimilation

Glu → 2 Glu

GOGAT

Protein synthesis

Export

Storage

Amino acids

Protein synthesis

Export

Transaminases

Storage

Amino acids

Export

N Assimilation pathway – cellular level
How to increase NUE?

**Targeted approaches:**

- Modify level of major player in N metabolism
  - Nitrate transport (NRT2)
  - N utilisation (GS)

- Modify regulatory circuits of N metabolism
  - DOF transcription factor
  - NLP7 transcription factor

More basic knowledge required

**Untargeted approaches:**

- Exploiting Natural variation
Physiological studies: Two Nitrate transport systems

Absorption of nitrate in the roots

HATS
Km 10-100µM,
linear kinetics

LATS
linear kinetics
Model species: Arabidopsis thaliana
Cloning of NRT1.1 (« LATS »):

Chlorate resistant mutant (chl1, Tsay, 1993)

- 12 TM domains, nitrate inducible, root specific expression, dual affinity transporter (HATS + LATS)
Fig. 1. Phylogenetic tree of the Arabidopsis and rice NRT1/PTR family. Multiple sequence alignments of 53 Arabidopsis NRT1/PTR transporters, 80 rice NRT1/PTR transporters, and BaNRT1-2, AgDCTR1, and HvPTR1 were performed using the BLOSUM protein weight matrix and the phylogenetic tree was constructed using the neighbor-joining method of the ClustaX program [87]. The tree was displayed and manipulated using the MEGA3 program [88].
Cloning of the Nrt2 genes in higher plants

CrnA mutant
(chlorate resistant, A Nidulans)

Deletion strain
(C.reinhardtii)

CrnA

Consensus sequences, PCR amplifications

CrNrt2.1

Plant Nrt2
N.plumbaginifolia, Arabidopsis, tomato, barley, rice, maize....
NRT2 protein structure

60 KDa
Major Facilitating Superfamily
12 transmembrane domain

Introduction
NRT 2 genes in *Arabidopsis thaliana*.

Which are the roles of each gene for nitrate transport?
Expression levels of the AtNRT2 genes

- Quantitative analysis of expression by Real time PCR
- Plants grown in hydroponics, 35 days, SD, 10 mM NO₃⁻

AtNRT2.1 is the main component in roots, whereas in leaves AtNRT2.4, 2.6 and 2.7 are predominantly expressed.
Analysis of the k.o.mutant *atnrt2.1-1*

A 25 kb deletion occurs in the *atnrt2.1-1* mutant...

...and affects the nitrate influx

Filleur et al 1999
Phenotype of the *atnrt2.1-1* mutant

...under different nitrogen regimes (100µE)

*atnrt2.1-1* shows nitrogen limitation in conditions in which the wildtype is not yet limited.

Orsel et al 2002
Localisation of GFP-NRT2.1

Fig. 2. Localization of AtNRT2.1::GFP by confocal microscopy. Root meristem of GFP expressing transformants using the TCSNT confocal microscope (Leica, Heidelberg, Germany). Green color indicates GFP fluorescence. Red color indicates FM4–64 staining. A: Fluorescent image from clone 3. Scale bar is 75 μm. B: Fluorescent image from clone 8. Scale bar is 65 μm. C: Detailed image from clone 8. Arrows indicate the yellow color obtained with co-localisation of GFP and FM4–64 staining. Scale bar is 56 μm.
Model of the different roles of NRT2 family

- **Loading of nitrate into seeds**
  - AtNRT2.7: Tonoplast; storage vacuoles?
  - AtNRT2.6?
    - Transporter: nitrite/nitrate
  - Chloroplast

- **AtNRT2.5? Leaf vascular tissue**

- **AtNRT2.4? Root vascular tissue**
- **Xylem loading**

- **HATS**
- **AtNRT2.1/NAR2.1**

- **HATS+LATS AtNRT1.1**

- **Meristeme**

- **AtNRT1.4**

- **Transport via xylem**

- **• Storage of nitrate**
- **• Metabolism of nitrate**
- **• Distribution of nitrate**
- **• nitrate uptake**
How to increase NUE?

• Modification of Nitrate transport was not successful for increasing NUE

• but the function of more transporters needs to be investigated
**N Assimilation pathway**

**Absorption**
- Nitrate ($\text{NO}_3^-$) enters the cytosol via transporters.

**Transporter**
- $\text{NO}_3^-$ is transported into the cytosol.
  - Reduced by NADH (2e⁻).

**Reduction**
- Nitrite ($\text{NO}_2^-$) is formed from nitrate.
  - Reduced by NR to ammonium ($\text{NH}_4^+$).

**Chloroplast**
- Nitrite is further reduced to ammonium by NiR.
  - Requirements: ATP, α-Ketoglutarate (αKG), and Ferredoxin (Fd).

**Assimilation**
- Ammonium enters the chloroplast and assimilates into amino acids.
  - Transaminases convert α-Ketoacids to amino acids.
  - GOGAT converts Glu to 2 Glu.
  - ATP is used for Glutamine (Gln) synthesis.

**Protein synthesis**
- Amino acids are used for protein synthesis.

**Storage**
- Ammonium is stored in the vacuole as amino acids.

**Export**
- Amino acids can be exported for further use.
- Nitrite can be exported or used for assimilation.
Studies of insertion mutants for cytosolic GS (GS1) :
function of GS1.3 and GS1.4 in maize

A. Martin, B. Hirel
### The multigenic Zm GS1 family

<table>
<thead>
<tr>
<th>Annotation (Li et al., 1993)</th>
<th>Localisation cellulaire</th>
<th>Position sur le génome</th>
<th>Expression tissulaire</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GS1.1</strong></td>
<td>Cytosolique (GS1)</td>
<td>Chromosome 1</td>
<td>Racines (1)</td>
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<td></td>
<td></td>
<td></td>
<td>Grains (2)</td>
</tr>
<tr>
<td><strong>GS1.2</strong></td>
<td>Cytosolique (GS1)</td>
<td>Chromosome 1</td>
<td>Tissu vasculaire racinaire (1)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Grains (2)</td>
</tr>
<tr>
<td><strong>GS1.3</strong></td>
<td><strong>cytosolic</strong></td>
<td>Chromosome 5</td>
<td>Constitutive</td>
</tr>
<tr>
<td><strong>GS1.4</strong></td>
<td><strong>cytosolic</strong></td>
<td>Chromosome 4</td>
<td>Senescent leaves roots kernels</td>
</tr>
<tr>
<td><strong>GS1.5</strong></td>
<td>Cytosolique (GS1)</td>
<td>Chromosome 9 (non publié)</td>
<td>Racines (1)</td>
</tr>
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<td>Tige (1)</td>
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<td></td>
<td></td>
<td></td>
<td>Grains (2)</td>
</tr>
<tr>
<td><strong>GS2</strong></td>
<td>Chloroplastique (GS2)</td>
<td>Chromosome 10</td>
<td>Feuilles jeunes (1)</td>
</tr>
</tbody>
</table>
Three insertion mutants (transposon Mu):
Keith Edwards, University of Bristol

gs1.4
gs1.3
gs1.3/gs1.4

Shoot transcript

Enzyme activity GS

Martin et al., The Plant Cell 2006 18: 3252-3274.
Phenotyping of *gs1-3* and *gs1-4* mutants

(kernel weight + number)

*Martin et al., The Plant Cell 2006 18: 3252-3274.*
Arabidopsis plants overexpression a ZmDOF transcription factor grow better on limiting N compared with the wildtype.

Yanagisawa et al 2004, PNAS 101, 20, 7833-7838
Assimilation of N requires C skeletons

Absortion

Plasmalemme

Transporter

Vacuole

Storage

Chloroplast

Protein synthesis

Amino acids

Cytosol

Reduction

Vacuole

Export

Absortion

Amino acids

Protein synthesis

Export

Absortion

Absortion

Absortion

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Absoration
Little is known about the regulation of the N metabolism in plants. Find new regulatory genes by a comparative approach.
Symbiosis, a highly regulated process

Carbohydrates

Nod factors (LCO)

Rhizobia

Flavonoids

Legume

- N

+ N
The NIN gene is necessary for Rhizobium-legume symbiosis.
NIN genes and N metabolism

**In legumes**

- N

WT

Schauser _et al._ 1999

Ljin

**In Chlamydomonas**

NIT2 (a NIN homologue) regulates nitrate assimilation

Camargo _et al._ 2007

+NO₃⁻

MID (a NIN homologue) regulates gametogenesis

Ferris _et al._ 1997

- N

The NIN genes are well conserved

**In rice**

**In wheat**

NIN=NLP (Nin Like Protein)

**In Arabidopsis**

Schauser _et al._ 2005
NLP: a conserved protein structure

Hydrophobic domain

The most conserved domain

DNA-binding and dimerization domain

PB1 domain
protein-protein interaction/heterodimerization

Leucine-zipper

Transcription factors?

NIN genes are good candidates to be regulators of the N metabolism

From Schauser et al. 2005
A reverse genetics strategy

- Isolation of nlp mutants
- Preliminary characterisation of the nlp mutants

NLP6 and NLP7 have been chosen for a more complete study

Delphine Pocholle

Delphine Pocholle

Schauser et al., 2005
Two allelic mutants: smaller plants with a delayed flowering time

NLP7 expression

Bolting

Castaings et al
Three allelic mutants: smaller plants with a delayed flowering time

Impaired nitrate assimilation in the mutants

<table>
<thead>
<tr>
<th></th>
<th>col8</th>
<th>nlp7-1</th>
<th>nlp7-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol / g FW)</td>
<td>113,3</td>
<td>146,5</td>
<td>137</td>
</tr>
<tr>
<td>± 4,9</td>
<td>± 2,8</td>
<td>± 13,6</td>
<td></td>
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<tr>
<td>Amino acid content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(µmol / g FW)</td>
<td>10,3</td>
<td>8,1</td>
<td>7,6</td>
</tr>
<tr>
<td>± 0,7</td>
<td>± 0,2</td>
<td>± 1,3</td>
<td></td>
</tr>
</tbody>
</table>
Three allelic mutants: smaller plants with a delayed flowering time.
Nitrate assimilation is modified in the mutants.

How do the *nlp7* mutants sense NO$_3^-$ as a signal?

![Diagram showing the interaction of nitrate with the plant's cellular processes.]

- Reduction in the plastid
- Export from the cell
- Storage in the vacuole
- Signaling pathway leading to changes in gene expression and root morphology

- NO$_3^-$ as a signal:
  - Modification of gene expression
  - Modification of root morphology
  - etc..

- NO$_3^-$ as a nutrient:

Castaings et al
The fusion proteins accumulates in the nucleus and the cytoplasm.
Regulation of N metabolism and perception and NUE??

Increased NLP activity may allow higher NUE??

Untargeted approaches to increase NUE:

Natural variation in Arabidopsis, a tool to identify genetic bases of nitrogen use efficiency
Accessions of Arabidopsis thaliana
Searching for QTL of nitrogen use efficiency in Arabidopsis

- Creation of an Arabidopsis recombinant inbred lines population from two genetically distant ecotypes (Bay-0 and Shahdara)

- Obtaining a genetic map of the population with markers (genotypical data)

- Growing plants on two types of nitrogen nutrition, normal (10 mM) or limiting (3mM) and measuring several parameters (phenotypical data)

- Correlation between genotypical and phenotypical data for QTL detection
Recombinant Inbred Lines - RILs

Bay-0 from Germany

Shahdara from Central Asia

Single Seed Descent

420 RILs
Bay-O × Shahdara RIL population

Genetic map of the population of 420 F6 Recombinant Inbred Lines

I
- T1G11
- F21M12
- MSAT1-10
- NGA248
- T27K12
- NGA128
- F5I14
- MSAT1-13
- MSAT1-5

II
- MSAT2-5
- MSAT2-38
- MSAT2-36
- MSAT2-41
- MSAT2-7
- MSAT2-10
- MSAT2-22

III
- NGA172
- ATHCHIB2
- MSAT3-19
- MSAT3-32
- MSAT3-21
- MSAT3-18

IV
- MSAT4-39
- MSAT4-8
- MSAT4-15
- MSAT4-18
- MSAT4-9
- MSAT4-37

V
- NGA225
- NGA249
- MSAT5-14
- NGA139
- MSAT5-22
- MSAT5-9
- MSAT5-12
- MSAT5-19

38 microsatellites markers

Loudet et al, TAG 2002
Phenotyping of the population

10 mM N

3 mM N
View of a set of 24 Arabidopsis lines after 34 days of culture

(Loudet et al., 2001)

N+ (10 mM)  N- (3 mM)
Isolation of several QTL linked to biomass, total N and amino-acid content

Loudet et al, 2002
After isolation of the QTL, we must validate the QTL. The use of Heterozygous Inbred Families (HIF) allows the validation of QTL.
Heterogeneous Inbred Family - HIF

RILXXX (F6)

- HIF - Sha
- HIF - Het
- HIF - Bay

Legend:
- Green: Shahdara
- Light Purple: Bay-0
- Gray: Heterozygous
Example of QTL validation using HIFs

**Amino acid content**

- **Mean of ILs 421 B = 173 nmole aa /mg of DM**
- **Mean of ILs 421 S = 155 nmole aa /mg of DM**
Then, you may be lucky if a candidate gene is present in the chromosome area where the QTL is located.

If no remarkable gene is mapped in the region of interest, we have to go through a fine mapping approach which allows to restrict the length of the interval on the chromosome.
This fine mapping approach has to be repeated until the QTL interval is reduced to a portion containing about 20 genes in order to be able to find a candidate gene.
The fine mapping approach is rather rapid in Arabidopsis, but QTL analysis is also very performant in Maize
Conclusion:

- GS is a key player for NUE in Maize
- It is still difficult to make plants « better » in terms of NUE
- Fundamental knowledge on N assimilation and regulation are still required.

Thank you very much for your attention
The fine mapping approach is rather rapid in Arabidopsis, but QTL analysis is also very performant in Maize