Molecular deciphering of nitrate response in Pooideae species

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Molecular deciphering of nitrate response in *Pooideae* species

Dissection moléculaire de la réponse au nitrate chez les Pooideae

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SUMMARY

ABREVIATIONS LIST

5PTASE2	INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 2
5PTASE9	INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 9
5PTASEs	INOSITOL POLYPHOSPHATE 5-PHOSPHATASES
ABA	Abscisic acid
ABA-GE	ABA-Glucose Ester
ABIs	ABA-INSENSITIVEs
ACC	Aminocyclopropanecarboxylate
ACO1	ACC OXIDASE 1
ACT2	ACTIN 2
AFB3	AUXIN SIGNALING F-BOX3
AFBs	AUXIN SIGNALING F-BOXes
amiRNA	Artificial miRNA
AMTs	AMMONIUM TRANSPORTERs
AMT1	AMMONIUM TRANSPORTER 1
ANOVA	ANalysis Of VAriance
ANR1	ARABIDOPSIS NITRATE REGULATED 1
ANT1	AROMATIC AND NEUTRAL TRANSPORTER 1
ARFs	AUXIN RESPONSE FACTORs
At	*Arabidopsis thaliana*
ATP	Adenosine triphosphate
Bd	*Brachypodium distachyon*
BT1	BRIC-A-BRAC/TRAMTRACK/BROAD (BTB) AND TAZ DOMAIN PROTEIN1
BT2	BRIC-A-BRAC/TRAMTRACK/BROAD (BTB) AND TAZ DOMAIN PROTEIN2
bZIP	BASIC REGION/LEUCINE ZIPPER MOTIF
BZIP60	BASIC REGION/LEUCINE ZIPPER MOTIF 60
C	Carbon
C_2H_2	Ethylene
$Ca(NO_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
CBL9	CALCINEURIN B-LIKE

cDNA	Complementary DNA
CIPK23	CBL-INTERACTING PROTEIN KINASE 23
CKs	Cytokinins
CLC	CHLORIDE CHANNEL
CPKs	CALCIUM-DEPENDENT PROTEIN KINASEs
CPM	Count Per Million
CRFs	CYTOKININ RESPONSE FACTORs
Ct	Cycle threshold
CYP707A2	CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE 2/ ABA 8'-HYDROXYLASE
Cys	Cysteine
DEGs	Differentially Expressed Genes
DNA	Deoxyribonucleic Acid
DW	Dry Weight
EDTA	Ethylenediaminetetraacetic acid
EF1 α	EUKARYOTIC TRANSLATION ELONGATION FACTOR 1 α
eFP Browser	electronic Fluorescent Pictograph Browser
EGTA	Disodium ethylenediaminetetraacetate dehydrate
ES	Enrichment Scores
EXPA7	EXPANSIN A7
Fd-GOGAT	ferredoxin dependant GOGAT
FDR	False Discovery Rate
Fe	Iron
Fe^{2+}	Ferrous ion
FNR	FERREDOXIN-NADP(+) OXIDOREDUCTASE
G3P	GLYCERALDEHYDE 3-PHOSPHATE
G6PDH	GLUCOSE-6-PHOSPHATE DEHYDROGENASE
GA	Gibberellins
GA20OX-1	GIBBERELLIN 20-OXIDASE 1
GA20OX-4	GIBBERELLIN 20-OXIDASE 4
GAF-like	cGMP-specific phosphodiesterases, Adenylyl cyclases and FhlA like
GLN1	GLUTAMINE SYNTHETASE 1
GO	Gene Ontology
GOGAT	GLUTAMATE SYNTHASE
GS	GLUTAMINE SYNTHETASE

GTP	Guanosine-5'-triphosphate
H_2O	water
H_3BO_3	Boric acid
HATS	High Affinity Transport System
He	Helium
HHO1	HRS HOMOLOGUE 1
HRS1	HYPERSENSITIVE TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1
Hv	*Hordeum vulgare*
Ile	Isoleucine
IP3	Inositol trisphosphate
IPT4	ADENYLATE ISOPENTENYLTRANSFERASE 4
IPT5	ADENYLATE ISOPENTENYLTRANSFERASE 5
IPTs	ADENYLATE ISOPENTENYLTRANSFERASEs
IRMS	Isotope-Ratio Mass Spectrometry
K_2SO_4	Potassium sulfate
KCl	Potassium chloride
KH_2PO_4	Monopotassium phosphate
KNO_3	Potassium nitrate
LaCl3	Lanthanum (III) chloride
LATS	Low Affinity Transport System
LBD37	LOB DOMAIN-CONTAINING PROTEIN 37
LBD38	LOB DOMAIN-CONTAINING PROTEIN 38
LBD39	LOB DOMAIN-CONTAINING PROTEIN 39
LBDs	LOB DOMAIN-CONTAINING PROTEINs
Met	Methionine
$MgSO_4$	Magnesium sulfate
miR	micro-RNA
$Mo_7O_24(NH_4)_6$	Ammonium heptamolybdate
mRNA	messenger RNA
N	Nitrogen
N_2	dinitrogen
NACs	NAC DOMAIN CONTAINING PROTEINs
NADH	Nicotinamide adenine dinucleotide
NADH-GOGAT	GLUTAMATE SYNTHASE (NADH)

NAS	NICOTIANAMINE SYNTHASE
NAXT1	NITRATE EXCRETION TRANSPORTER1
NBIP1	NRT1.1B INTERACTING PROTEIN 1
NES	Normalize Enrichment Scores
NH_4^+	Ammonium
NIA1	NITRATE REDUCTASE 1
NIA2	NITRATE REDUCTASE 2
NIGT1	NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR1 / HRS HOMOLOGUE 1
NIGT1/HHO	NITRATE-INDUCIBLE, GARP-TYPE TRANSCRIPTIONAL REPRESSOR 1/HYPERSENSITIVE TO LOW PI-ELICITED PRIMARY ROOT SHORTENING
NIN	NODULE INCEPTION
NiR	NITRITE REDUCTASE
NITRs	NITRITE TRANSPORTERs
NLA	NITROGEN LIMITATION ADAPTION
NLPs	NIN-LIKE PROTEINs
NO_2^-	Nitrite
NO_3^-	Nitrate
NR	NITRATE REDUCTASE
NRE	Nitrogen Responsive Element
NRG2	NITRATE REGULATORY GENE 2
NRT1 / NPF	NITRATE TRANSPORTER 1 / NRT PTR FAMILY
NRT1.1 / NPF6.3	NITRATE TRANSPORTER 1.1 / NRT PTR FAMILY 6.3
NRT2	NITRATE TRANSPORTER 2
NRT3	NITRATE TRANSPORTER 3
NUE	Nitrogen Use Efficiency
NUpE	Nutrient Uptake efficiency
NUtE	Nitrogen Utilization efficiency
O_2	Dioxygen
OASTL	O-ACETYL (THIOL) LYASE
OGps	Orthogroups
Os	*Oryza sativa*
P	Phosphate
PB1	Phox and Bem1 protein domain
PCR	Polymerase Chain Reaction

PDX1	PYRIDOXINE BIOSYNTHESIS 1
PDX2	PYRIDOXINE BIOSYNTHESIS 2
PDX3	PYRIDOXINE BIOSYNTHESIS 3
PEPC1	PHOSPHOETHANOLAMINE/PHOSPHOCHOLINE PHOSPHATASE 1
PHL1	PHOSPHATE STARVATION RESPONSE PROTEIN LIKE 1
PHO2	PHOSPHATE 2
PHR1	PHOSPHATE STARVATION RESPONSE 1
PHR2	PHOSPHATE STARVATION RESPONSE 2
PHT1	PHOSPHATE TRANSPORTER 1
Pi	inorganic Phosphate
PLC	PHOSPHOLIPASE C
PLP	Pyridoxal phosphate
PMP	Pyridoxamine 5-phosphate
PNR	Primary Nitrate Response
PPsPase1	PYROPHOSPHATE-SPECIFIC PHOSPHATASE1 / PHOSPHATE STARVATION-INDUCED GENE 2
PSR	Phosphate Starvation Response
qPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Locus
R5P	Ribose-5-phosphate
RAB	Ras-associated binding proteins
RCF	Relative Centrifugal Force
RNA	ribonucleic acid
RNAseq	RNA sequencing
ROS	Reactive Oxygen Species
rRNA	ribosomal RNA
RT-qPCR	Real-time polymerase chain reaction
SAMDC	S-ADENOSYLMETHIONINE DECARBOXYLASE
SAT	SERINE ACETYL TRANSFERASE
SD1	SEMI DWARF 1
Ser	Serine
SO_4Cu	Copper (II) sulfate
SO_4Mn	Manganese (II) sulfate
SO_4Zn	Zinc sulfate
SOS4	SALT OVERLY SENSITIVE 4

SPL9	SQUAMOSA PROMOTER BINDING PROTEIN-LIKE9
SPX4	SPX DOMAIN GENE 4
SSU-rRNA	Small subunit ribosmal ribonucleic acid
Ta	*Triticum aestivum*
TAR	TRYPTOPHAN AMINOTRANSFERASE RELATED
TCA cycle	Tricarboxylic Acid cycle
TCP20	TEOSINTE BRANCED, CYCLOIDEA, PCF (TCP)-DOMAIN FAMILY PROTEIN 20
TGA1	TGACG SEQUENCE-SPECIFIC BINDING PROTEIN 1
TGA4	TGACG SEQUENCE-SPECIFIC BINDING PROTEIN 4
Thr	Threonine
TILLING	Target Induced Local Lession IN Genomes
TMM	Trimmed Mean of M-value
UBC18	PROBABLE UBIQUITIN-CONJUGATING ENZYME E2 18
UBI10	POLYUBIQUITIN 10
UPM1	UROPHORPHYRIN METHYLASE 1
VCl_3	Vanadium (III) chloride
WABI5	wheat putative ortholog of AtABI5
WAK4	WALL ASSOCIATED KINASE 4
WRK40	WRKY DNA BINDING PROTEIN 40
WT	Wild Type
YUCCA	Flavin monooxygenase-like gene
Zm	*Zea mays*
Zn^{2+}	Zinc (II) ion

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The supplementary data are available until 15/04/21 at:

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RESUME DE LA THESE EN FRANÇAIS

L'azote est un macro-élément essentiel pour les êtres vivants. Dans les terres agricoles, l'azote un nutriment très souvent limitant qu'il est nécessaire de complémenter à l'aide d'engrais. Cependant, l'utilisation massive d'engrais est très couteuse pour les agriculteurs et génère d'importante pollution des sols et de l'eau. Le nitrate étant la source d'azote préférée de la plupart des plantes, l'amélioration de son utilisation par les plantes cultivées pourraient limiter ses effets indésirables.

Le but de mon projet de thèse était de caractériser la réponse moléculaire au nitrate principale chez *Brachypodium distachyon*, une espèce modèle des monocotylédones mais aussi chez l'orge. Ces deux plantes appartiennent toutes les deux à la sous-famille des *Pooideae* qui rassemble d'importante céréale tempérée telle que le blé, le seigle et l'avoine. Pour ce faire, deux stratégies ont été adoptée.

La première avait pour but d'identifier des processus biologiques ou des gènes régulés par le nitrate spécifiquement chez les *Pooideae*. Une analyse transcriptomique comparative a donc été réalisée chez *Brachypodium distachyon*, l'orge et la plante modèle dicotylédone, *Arabidopsis thaliana* qui a été utilisée comme référence puisque la réponse moléculaire au nitrate chez cette espèce est bien caractérisée. Notre principale hypothèse était que même si une majeure partie de la réponse moléculaire au nitrate est commune entre les dicotylédones et les monocotylédones, des spécificités doivent aussi exister. Ainsi, la comparaison de la réponse au nitrate chez les trois espèces étudiées a permis d'identifier des processus biologiques et des gènes qui n'ont pas pu être mis en évidence dans les études uniquement réalisées sur Arabidopsis et qui présentent des régulations divergentes en réponse au nitrate. Par exemple, les gènes nécessaires à la biosynthèse des gibbérellines sont plus régulés chez Brachypodium que chez Arabidopsis et l'orge. De manière intéressante, plusieurs

processus biologiques régulés principalement chez Brachypodium et l'orge ont été identifiés dans cette étude tels que la biosynthèse de sérine à partir de cystéine ou encore la biosynthèse de pyridoxal phosphate. Des études expérimentales complémentaires seront nécessaire pour approfondir la compréhension de la régulation spécifique par le nitrate de ces processus chez les *Pooideae.* Ainsi, des candidats intéressants ont été mis en évidence pouvant servir l'amélioration de l'utilisation du nitrate chez les céréales.

Le second objectif, était de caractériser le rôle des protéines BdNLP6 et BdNLP7 dans la réponse au nitrate chez Brachypodium. Ces protéines sont phylogénétiquement les plus proches de la protéine d'*Arabidopsis thaliana*, AtNLP7. Cette dernière, a un rôle majeur dans la régulation de la réponse moléculaire au nitrate. Notre principale hypothèse, la plus simple, était que BdNLP6 et BdNLP7 soient impliqués dans la réponse moléculaire au nitrate de façon similaire à AtNLP7 chez *Brachypodium distachyon*. Par une caractérisation de deux lignées mutantes pour le gène *BdNLP6* et une lignée exprimant un micro-ARN artificiel ciblant l'expression des gènes *BdNLP6* et *BdNLP7*, nous avons finalement montré que BdNLP6 semble avoir un rôle complètement divergent de celui décrit pour AtNLP7. En effet, il semblerait que BdNLP6 ait un rôle négatif en présence de nitrate dans le développement et la croissance de la plante.

Ce manuscrit est rédigé en anglais et comporte quatre chapitres. Le premier chapitre est une introduction générale décrivant l'importance du nitrate en tant que nutriment et molécule signal, principalement chez Arabidopsis mais aussi chez les céréales. Les chapitres II et III sont rédigés sous forme d'articles en préparation et correspondent aux résultats de l'étude transcriptomique comparative en réponse à un traitement nitrate entre Arabidopsis, Brachypodium et l'orge (chapitre II) et aux résultats de l'étude des rôles de BdNLP6 et BdNLP7 chez Brachypodium en relation avec la disponibilité en azote (chapitre III). Pour finir, le quatrième chapitre est une discussion

générale dans laquelle l'ensemble des résultats sont discutés et des hypothèses quant à la réponse au nitrate chez les *Pooideae* sont faites.

CHAPTER I

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GENERAL INTRODUCTION

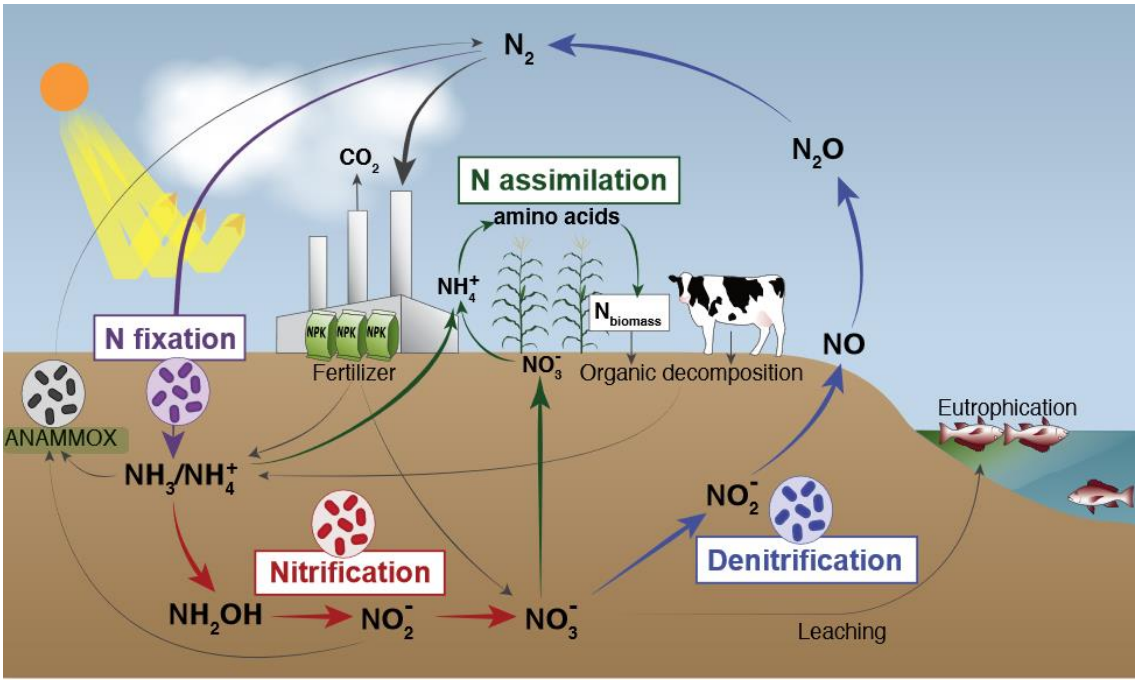

Figure 1: Nitrogen cycle main processes.
ANAMMOX stands for anaerobic ammonium oxidation. From Corruzzi et al., 2015

GENERAL INTRODUCTION

Nitrogen cycle, the use of fertilizers and the importance of enhancing NUE

Nitrogen cycle (Figure 1) is one of the most important biogeochemical cycles on Earth. Indeed, nitrogen (N) is an essential macro-element for all known life forms. In the air, nitrogen under its gaseous form (dinitrogen N2), represent 79% of the air we breathe. In *Arabidopsis thaliana* (Arabidopsis), nitrogen represents 5 to 6% of its dry matter while phosphorus and potassium only represent 0,4 and 2,5%, respectively (Castaings *et al.*, 2009). However, for its utilization by plants, nitrogen must be available thanks to fixation into mineral forms such as ammonium (NH_4^+) or nitrate (NO_3^-). Ammonium and/or nitrate found in soils come from different sources and processes (Figure 1). It can comes from processes such as the fixation of atmospheric nitrogen by specific bacteria, or from organic decomposition of animal dejections or dry plants biomass (Figure 1). In agricultural fields, nitrogen is often limiting, thus ammonium and nitrate are brought through industrial fertilizers (Figure 1). Afterward, nitrate and/or ammonium is assimilated into cultivated plants. However, it has been estimated that more than 170 million tons of nitrogenous fertilizers have been used in the word (FAO, 2017) while it has been shown that less than half of the applied fertilizer is utilized by crops and leftover are leached and lead to severe environmental pollutions (Peoples *et al.*, 1995).

For guaranteeing high yields to meet global food demand while reducing fertilizers inputs and thus reduce costs for farmers as well as pollutions, improving crops nitrogen use efficiency (NUE) appears as a solution. Indeed, estimations suggest that increasing NUE by only 1% could save around 1.1 billion dollars every year (Kant et al., 2011). NUE in cereals is defined as the grain yield per unit of nitrogen available in the soil (Moll *et al.*, 1982).

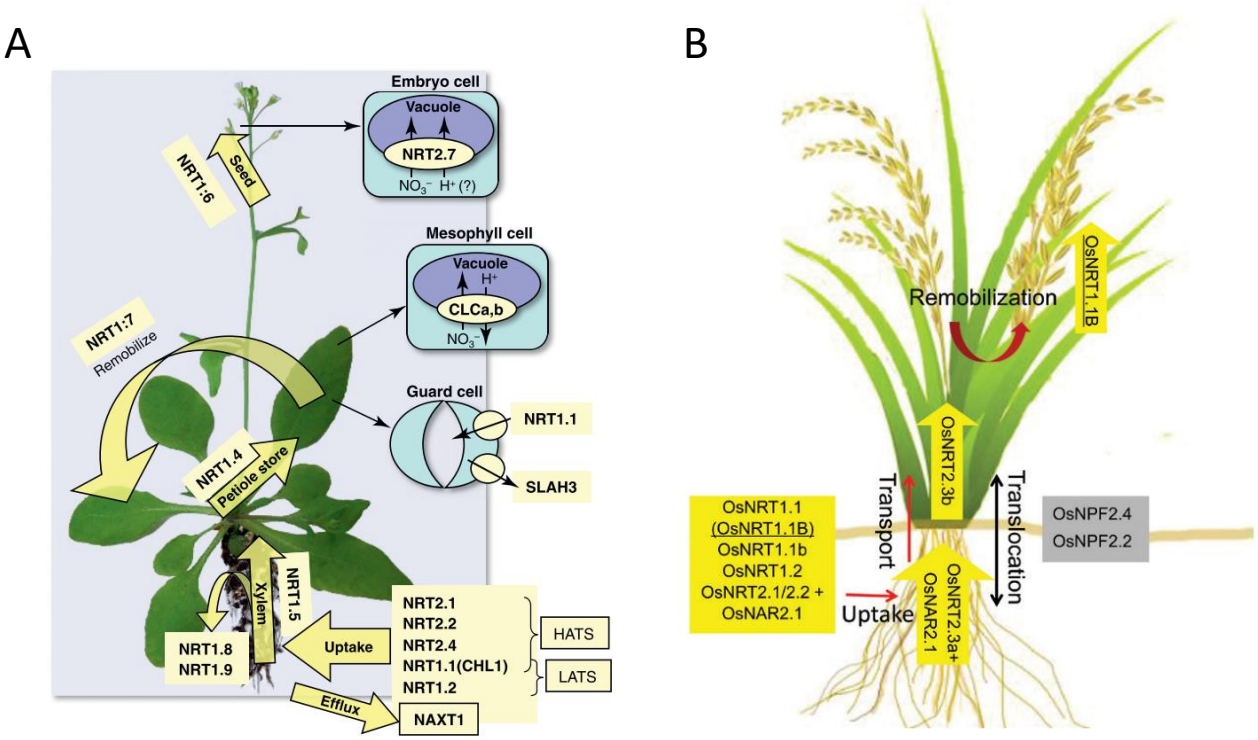

Figure 2: Summary of the contribution of nitrate transporters in (A) Arabidopsis and (B) rice. Arabidopsis figure comes from Wang et al., 2012 and Rice one from Fan et al., 2016.

It is the result of the plant uptake efficiency (NUpE) and its utilization efficiency (NUtE). In other words, it corresponds to the combination of how effectively plants capture N and how plants use the N that is taken up (Hansen *et al.*, 2018).

Nitrate is the preferential nitrogen source for most land plants. Even though, other sources can be managed by plants such as ammonium (especially appreciated by rice) as well as amino acids for example. My work focus on the molecular comprehension of nitrate as a signal molecule in Brachypodium (*Brachypodium distachyon*), barley (*Hordeum vulgare*) and to a lesser extent in Arabidopsis. This introduction will thus focus on nitrate metabolism and its known role as a signaling molecule.

Nitrate uptake from the soil and transport within the plant

When nitrate is present in soils, the first crucial step for its use by the plant is its uptake by the roots. Nitrate uptake is an active transport mediated by H^+ cotransport that occurs in the outer cell layers of the roots. Nitrate concentration in soils is not stable. In agricultural fields, nitrate concentration can vary from 1 mM to 10 mM. To deal with those variations of nitrate availability, plants have developed two uptake mechanisms: a high affinity transport system (HATS), whose Km for nitrate ranges from 10-100 µM and that is saturated under high nitrate concentrations; and a low affinity transport system (LATS) which activity depends on nitrate concentration in a linear manner and is dominating for nitrate concentration above 500 µM (Epstein, 1953). The contribution of HATS and LATS transporters in nitrate uptake depends on soil nitrate availability, but also on root developmental stage and N status of the plant (Wang et al., 2012). In Arabidopsis, LATS and HATS have been linked to two main nitrate transporter families: NRT1/NPF responsible for the LATS and partially for the HATS, and NRT2 responsible for most of the HATS. It is important to note that NRT1/NPF transporters family gathered also transporters that are able to transport other molecule than nitrate.

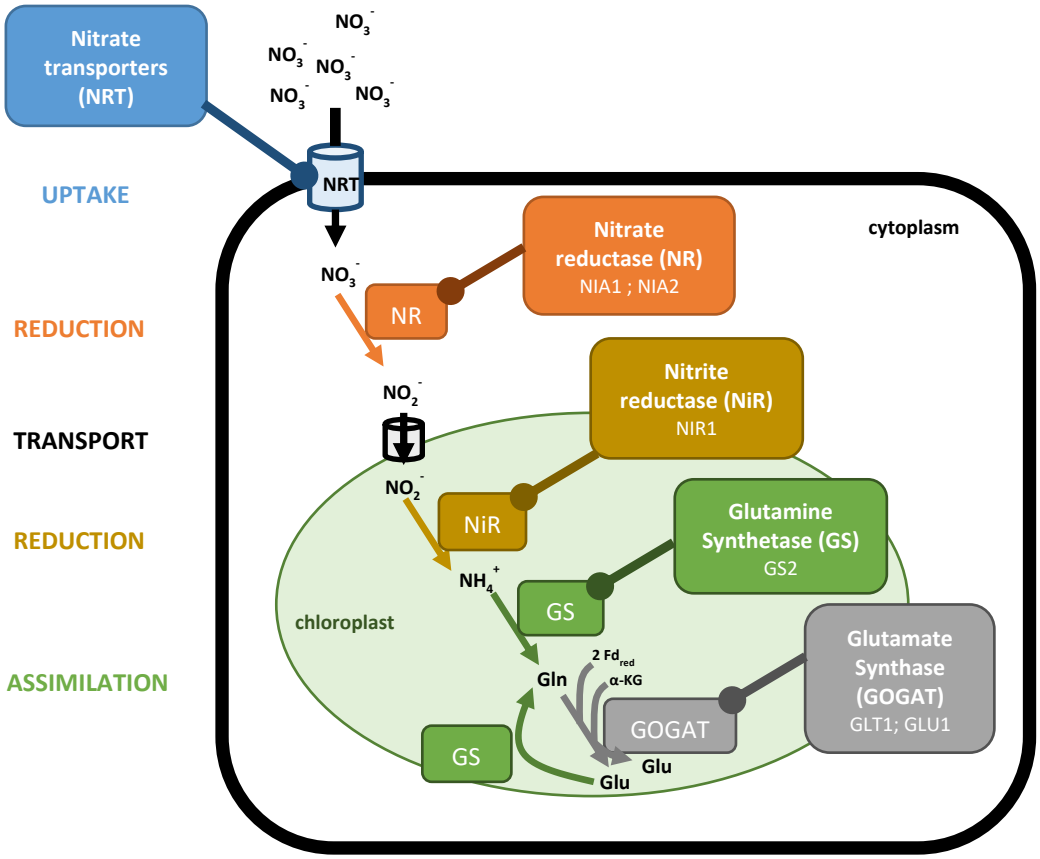

Figure 3: Main steps of nitrate assimilation.

Both in Arabidopsis and rice, nitrate uptake from the soil is carried by NRT1 and NRT2 nitrate transporters (Figure 2, for review see Fan et al., 2017; Wang et al., 2012). Then, nitrate is transported in shoots and seeds for further assimilation of storage (Figure 2). Other NRT/NPFs as well as NRT2s have been shown to be involved in root to shoot translocation or relocalization from source to sinks tissues (during growth or remobilization; Figure 2). In Arabidopsis, other types of transporters and channels have been characterized such as CLC and SLAC/SLAH protein members which facilitate nitrate transport inside the plant (Figure 2A). NAXT1 is a root nitrate excretion transporter (Figure 2A; (Segonzac *et al.*, 2007). It is expressed in seeds and controls nitrate content and dormancy (Chopin *et al.*, 2007).

Interestingly, it has been shown using reciprocal best hit approach that cereals and dicot present divergent number of genes and organization of NRT families (Plett *et al.*, 2010). Thus, deepen the characterization of cereal NRT transporters seems necessary.

Nitrate reduction and assimilation

Once inside the plant, nitrate is reduced and assimilated to produce the first amino acid, glutamine (Figure 3). First, nitrate is reduced into nitrite in the cytosol thanks to Nitrate Reductase (NR) enzyme which uses NADH as reduction power (Figure 3; Crawford, 1995). In Arabidopsis, this enzyme is encoded by *AtNIA1* and *AtNIA2* genes. Then, nitrite is transported into chloroplast through AtNITR2 transporters (Figure 3, Maeda et al., 2014) where it is reduced into ammonium by the Nitrite Reductase (NiR) which uses reduced ferredoxin as a reduction power (Wray, 1993). Ammonium is then assimilated thanks to the GS/GOGAT cycle. Glutamine synthase (GS) presents two forms: GS1 which is cytosolic, and GS2 which is plastidial. GS1 is found mainly in roots while GS2 is localized in shoots (Ishiyama *et al.*, 2004). Glutamine synthetase fixes

ammonium to a glutamate molecule using ATP to form glutamine. Together, glutamine and glutamate allow the synthesis of the other amino acids.

On the other hand, glutamate synthase (GOGAT) allow the formation of two glutamate molecules from one molecule of glutamine using 2-oxoglutarate coming from Krebs cycle (Foyer *et al.*, 2011). Two forms of GOGAT exist depending on their electron donor. One GOGAT is ferredoxin dependent (Fd-GOGAT) while the other is NADH dependent (NADH-GOGAT) (Lancien *et al.*, 2002). Thus, Fd-GOGAT is localized in shoots where ferredoxin is produced by photosynthesis while NADH-GOGAT is found in the roots using NADH produced by pentose-phosphate cycle (Bowsher *et al.*, 2007).

Contribution of nitrate-responsive transcriptomics in elucidating nitrate signaling

Since early 2000's, many transcriptomic studies have shown that nitrate controls the expression of thousands of genes (about 10% of Arabidopsis genome) (Krouk et al., 2010a; Marchive et al., 2013; Scheible et al., 2004). This part will describe some of the main biological processes that have been found to be nitrate-responsive in Arabidopsis and cereals species (maize, rice, wheat and sorghum) by transcriptomic analyses.

Nitrate uptake and assimilation related pathway

Not surprisingly, the first genes that have been discovered to be nitrate-responsive belong to nitrate assimilation pathway. First, mRNA accumulation of gene encoding Nitrate Reductase (*NIA*) and Nitrite Reductase (*NII*) was observed in response to nitrate in Arabidopsis (Castaings *et al.*, 2009), maize (Gowri *et al.*, 1992*a*) and barley (Sueyoshi *et al.*, 1999). With arrival of transcriptomic approaches, it appeared that the global nitrate uptake and assimilation pathway is regulated by nitrate. Nitrate transporters such as *AtNRT2.1*, *AtNRT2.2* (low-affinity transporters) and the dual-affinity transceptor *AtNRT1.1 (NPF6.4*) are shown to be induced by nitrate (Scheible *et al.*, 2004). For rice

(Obertello *et al.*, 2015), wheat (Dissanayake *et al.*, 2019), maize (Zamboni *et al.*, 2014; Du *et al.*, 2020) and sorghum (Du et al., 2020), the gene ontology term "Nitrate transport" is always induced by nitrate in the studies. Since nitrate transporter families are highly divergent between Arabidopsis and cereals (Plett *et al.*, 2010) it is however difficult to directly compare the response gene by gene between the species.

As mentioned before, genes encoding enzymes of the assimilation pathway are also always induced by nitrate such as *NIA1*, *NIA2*, *NII* and this is confirmed by transcriptomic in many species (Scheible et al., 2014; Du et al., 2020; Zamboni et al., 2014; Obertello et al., 2015; Dissanayake et al., 2019). Also, specific isoforms of GS (Gln synthetase), GOGAT (Glu synthase) are known to be induced. Interestingly, in Zamboni et al., (2014), the authors compared two maize inbred lines, differing in Nitrogen Use Efficiency (NUE), and show that the induction of nitrate transporters and enzymes for nitrate assimilation is much higher in the low NUE line. The high NUE line has a higher basal expression of these genes and thus a reduced induction.

Genes indirectly involved in nitrite reduction are also regulated by nitrate. Since NR and NiR require reductants to function, processes such as oxidative and non-oxidative pentose-phosphate pathway providing NADPH and genes involved in the production of Ferredoxin are also induced by nitrate (UPM1, FNR, G6PDH). These pathways are directly related to carbon metabolism. Related pathways such as glycolysis, TCA cycle and the synthesis of organic acids are also always induced by nitrate and provide energy for growth (Ortebello et al., rice, Dissanayake et al., 2019 wheat, Zamboni et al., 2014 maize; Du et al., 2020 Sorgho and maize). Altogether, these observations suggest a good conservation among species of the regulation of genes necessary for the nitrate metabolism in response to nitrate which was expected.

Phytohormones related genes

Apart from directly nitrate-related processes, other pathways are known to be regulated and seem very conserved between Arabidopsis and cereals. Phytohormones biosynthesis and degradation are pathways often described to be nitrate-responsive in transcriptomes, suggesting that they participate in nitrate signaling. Cytokinins (CKs) are known to be produced in response to nitrogen in roots (Ruffel *et al.*, 2011; Poitout *et al.*, 2018). In Arabidopsis, it occurs mainly via an up-regulation by nitrate of *AtIPT3*, a gene encoding a key enzyme in CKs biosynthesis. CKs are then translocated to the shoots *via* xylem sap to control shoot growth (Takei *et al.*, 2001, 2004; Sakakibara *et al.*, 2006; Osugi *et al.*, 2017). The elevation of CKs in rice is not directly nitrate-dependent, but more regulated by Glutamine that enhances at least *OsIPT4* and *OsIPT5* expression. This lack of nitrate-dependent regulation might be linked to the preference of rice for ammonium nutrition (Kamada-Nobusada *et al.*, 2013).

The auxin receptor *AtAFB3* expression is directly induced by nitrate, and not by a product of its assimilation. Indeed*, AtAFB3* is not regulated by nitrate anymore in NR-null mutants (Vidal et al., 2010). Auxin is responsible for the control of root growth in response to nitrate. Auxin transport by AtNRT1.1 is another evidence of the crosstalk between nitrate signaling and auxin in Arabidopsis (Krouk et al., 2010).

A cross-species analysis between Maize and Sorghum (Du *et al.*, 2020) identified orthologs of AtWRK40, a key regulator of ABA signaling, that are regulated by nitrate. In Arabidopsis, nitrate treatment could stimulate ABA level in root tips by deconjugating ABA-glucose ester (ABA-GE) instead of ABA biosynthesis (Ondzighi-Assoume *et al.*, 2016). More recently in wheat, this same mechanism of accumulation of ABA in roots in response to nitrate *via* deconjugation of ABA-GE has been demonstrated as conserved. The expression of *TaANR1* (homolog of *AtANR1*, a MADS-box transcription factor) is induced in response to nitrate and binds and activates

TaBG1 expression, which is responsible for ABA-GE deconjugation into ABA. The authors also show that this elevation of ABA level in response to nitrate can in turn activate *TaNRT2/TaNAR2* expression *via* an up-regulation of TaWabi5 transcription factor, a putative ortholog of *AtABI5* known to be involved in the response to ABA in Arabidopsis (Wang *et al.*, 2020).

Finally, phytohormones appear to have a key role in integrating N signals into plant growth and development.

Cell wall related genes

Plant growth and development trigger cell division and cell expansion. Not surprisingly, in one of the first Arabidopsis transcriptome in response to nitrate, cell wall related proteins were found to be nitrate-regulated, such as cell-wall modification enzymes like xyloglucan endotransglycosylases or expansins (Scheible et al., 2004). In a publication from Canales et al. (2014), a metanalysis of nitrate response in Arabidopsis led to the identification of a nitrate-regulated module enriched in genes associated with root hair development, such as *AtEXPA7*, an expansin specifically expressed in root hairs. Its reduced expression reduces root hair length (Lin et al., 2011a; Lin et al., 2011b). Interestingly, *AtEXPA7* can complement the rice *Osexpa17* mutant, suggesting a conservation of expansins function in these two species (ZhiMing *et al.*, 2011). This Arabidopsis module contains other proteins such as extensins or endotransglucosylases, suggesting that the root morphogenic changes in response to nitrate are mediated by cell wall modification. Moreover, ontology term related to cell-wall are also found to be regulated by nitrate in wheat (Dissanayake et al., 2019), maize and sorghum (Du et al., 2020), showing the importance and probably the conservation of cell wall modifications between Arabidopsis and *Poaceae*.

Protein synthesis related genes

Although nitrate activates the so-called Primary Nitrate Response (PNR) which, *inter alia*, do not requires de novo protein synthesis (Gowri et al., 1992), activation of all the machinery necessary for synthetizing new proteins is required for further nitrogen acquisition. In Arabidopsis, it is one of the first biological processes to be induced, after only 3 minutes, through induction of ribosomal proteins (Krouk, et al., 2010). Timing here is interesting because, in another study, the activation of genes required for protein synthesis was only slightly seen after 30 minutes and more effectively detected after 3 hours (Scheible et al., 2004), suggesting the machinery required for protein synthesis is gradually activated in response to nitrate. No such early point (3 minutes) is available in *Poaceae* transcriptomic studies. In wheat, a significant enrichment of gene ontology related to maturation of specific categories of rRNA has been observed as induced by 4 hours of nitrate treatment, suggesting that the response in *Poaceae* might be slower (Dissanayake et al., 2019).

Transcriptomes have been very useful for discovering new molecular actors of Arabidopsis nitrate signaling. Many other biological processes could have been detailed here, since the nitrate response seems globally conserved between Arabidopsis and *Poaceae* when considering the main biological pathways. However, cereals transcriptomes in response to nitrate have always been analyzed by comparing to already published knowledge on Arabidopsis. Thus, it is hard to highlight specificities of cereals. Also, considering the fact that a meta-analysis of 27 Arabidopsis transcriptomic sets led to the identification of new processes involved in Arabidopsis nitrate response (Canales et al., 2014), it can be expected that many analyses will be needed in cereals to identify process that might not be seen in Arabidopsis. Due to phylogenetic and physiological differences between Arabidopsis and cereals, nitrate-response at a molecular level might present differences even within conserved processes. This question must be addressed to identify new molecular actors of cereal

nitrate response, and this knowledge could subsequently be valuable to enhance their NUE.

Nitrate signaling: molecular players of nitrate signal transduction

In Arabidopsis, the dual-affinity nitrate transporter AtNRT1.1 (NPF6.4) has been characterized as a nitrate sensor (Ho et al., 2009; Figure 4). Indeed, a complete knock-out of this gene expression provokes a reduction of about 30% of the gene induction in response to nitrate. Moreover, this lack of transcriptional nitrate response is not due to a defect in nitrate uptake, since a single amino acid substitution in *Atnrt1.1* mutant impairs the nitrate uptake but not the transcriptional nitrate response. This confirms that AtNRT1.1 is able to transport and sense nitrate in a decoupled manner (Ho *et al.*, 2009). The fact that a low but significant induction of gene expression in response to nitrate is still occurring in *Atnrt1.1* mutant suggests that other nitrate receptors/sensors exist, which have still to be discovered. In Rice, OsNRT1.1B, the functional homolog of AtNRT1.1, have also been demonstrated to be a nitrate sensor: *Osnrt1.1b* mutant is impaired in *OsNIA1* and *OsNIA2* expression (Hu *et al.*, 2015). This suggests a conservation of NRT1.1 function in *Poaceae*, but no NRT1.1 ortholog in other *Poaceae* species have been functionally characterized yet in a nitrate signaling context to confirm this conservation.

Early studies on maize and barley showed that treating detached leaves with a calcium chelator (EGTA) or a calcium channel blocker ($LaCl_3$) can reduce the expression of nitrate-responsive genes (Sakakibara *et al.*, 1997; Sueyoshi *et al.*, 1999), identifying calcium as a good candidate to be a second messenger. Later, the involvement of calcium as a second messenger was confirmed in Arabidopsis: nitrate treatment increases cytosolic calcium concentration and the level of IP_3 (inositol triphosphate) (Figure 4), but this is abolished in *Atnrt1.1* mutants. Thus, sensing of nitrate by AtNRT1.1 provokes calcium waves through the action of a phospholipase C (PLC) (Riveras *et al.*, 2015).

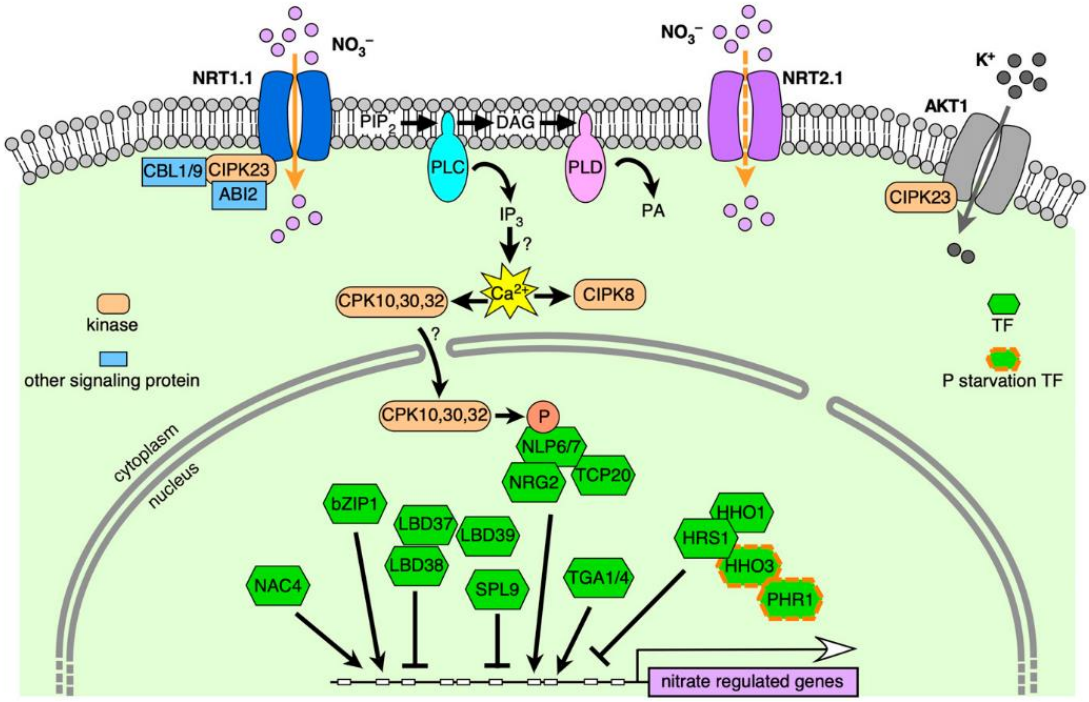

Figure 4: Summary of nitrate signaling pathways in Arabidopsis.
From Vidal et al., 2020.

Downstream of this, it has been shown that CPKs of the subgroup III are specially activated by calcium waves and phosphorylate one of the major regulator of nitrate response: the transcription factor AtNLP7 (Liu et al., 2017; Figure 4). Phosphorylated AtNLP7 is retained in the nucleus, where it can regulate the transcription of many nitrate-responsive genes (see below).

In rice, OsCBL1 was found to regulate nitrate response. CBL proteins are calcium sensors that specifically interact and activate CIPKs to transduce calcium signals. *OsCBL1* expression is not induced by nitrate treatment but its mutation provokes a decrease of expression of some nitrate-responsive genes such as *OsNRT2.1* and *OsNRT2.2*. Taken together, these results suggest that OsCBL1 respond to calcium variation triggered by nitrate supply, and in turn, activate downstream OsCIPKs (Yang *et al.*, 2019). In Arabidopsis, AtCBL9 and/or AtCBL1 have been characterized as involved in the phosphorylation of AtNRT1.1 which confer its dual-affinity transport function. AtCBL9 and AtCBL1 interact with AtCIPK23, which in turn phosphorylates AtNRT1.1 at Thr-101 under low nitrate condition (Ho *et al.*, 2009). This suggest at least a partial conservation even if the link between OsCBL1 and OsNRT1.1B has not been demonstrated yet.

Many questions on nitrate signal transduction are still remaining since some of nitrate-responsive genes are still induced in response to nitrate treatment in *Atnrt1.1* mutants. For example, is there another nitrate transduction pathway, different from the one downstream of AtNRT1.1? Can other molecules act as second messengers, such as reactive oxygen species or G-protein complexes?

Principal transcription factors of the nitrate response

Downstream of signal transduction mediated via calcium and protein kinases, transcription factors have been identified as regulating, directly or indirectly, the expression of nitrate-responsive genes (Figure 4).

Among the identified transcription factors, AtNLP7 appears to be one of the most important as it controls the rapid induction of *NRT2.1*, *NRT2.2*, *NIA1* and *NIA2*, *inter alia* (Castaings *et al.*, 2009; Marchive *et al.*, 2013; Alvarez *et al.*, 2020). AtNLP7 and the global NLP family are discussed thereafter. The first identified transcription factor identified as involved in nitrate signaling pathway was ANR1. ANR1 encodes a MICK-type MADS-box transcription factor involved in lateral root development in response to nitrate. Now, thanks to bioinformatics tools and gene regulatory network a myriad of transcription factors have been confirmed or identified as involved in nitrate response such as TGA4, NAC4, HRS1, LBDs (Varala *et al.*, 2018). In wheat, TaNAC2-5A transcription has been identified as a regulator of nitrate response. Interestingly, TaNAC2-5A belongs to a cereal-specific clade of NAC transcription factors underlying the importance to study nitrate response in cereals species as well.

NIN-Like Proteins

Leguminous plants can fix atmospheric nitrogen via association with rhizobia bacteria. This symbiotic beneficial association happens in roots where a novel organ is formed, called nodule. The NIN (Nodule INception) transcription factor has been characterized as essential for nodule formation (Schauser et al., 1999). Homologs of this protein have been found in non-leguminous plants based on sequence homology and domain conservation, and have been called NLPs for NIN-Like Proteins. NIN and NLP proteins

both contain a very conserved 60 amino acids region called RWP-RK domain and known to be responsible for DNA binding (Konishi & Yanagisawa, 2013; Schauser et al., 2005). At the N-terminal part, NLP proteins also carry a GAF-like domain, closely related to GAF domains that are known to be able to fix low-molecular weight ligand such as cGMP, 2-oxoglutarate, nitric oxide and nitrate (Möglisch *et al.*, 2009; Niemann *et al.*, 2014; Shi *et al.*, 2014). However, based on sequence analysis, this N-term part seems actually quite divergent from the GAF sequence (Mu and Luo, 2019) and, to date, no study reveals the involvement of this putative GAF domain in binding low-molecular weight ligand. At the C-terminal part, NLP proteins have a conserved PB1 domain that have been shown to be responsible for protein-protein interaction (Guan *et al.*, 2017; Konishi and Yanagisawa, 2019). The fact that NLPs are homologs of NIN, which is involved in nodule formation in leguminous plants, led to the hypothesis that NLPs are also involved in regulating nitrogen metabolism in non-leguminous plants.

A screening of *nlp* mutants in Arabidopsis identified AtNLP7 as a putative actor of nitrate response: *nlp7-1* mutant exhibits a smaller rosette, especially on non-limiting nitrate condition, compared to wild-type plants (Castaings *et al.*, 2009). And among available *nlp* mutants, *nlp7-1* was the only one to show this growth reduction (Anne Krapp early observation). Now, AtNLP7 is one of the most studied transcription factor involved in nitrate signaling. *Atnlp7* mutants display phenotypes in non-limiting nitrate condition that are characteristic of nitrogen-starved plants, such as a reduced rosette growth and a longer primary roots (Castaings *et al.*, 2009). This suggest the mutant is no longer able to sense the nitrate availability in the soil. At molecular level, up-regulation of sentinel genes such as *AtNIA1*, *AtNIA2*, *AtNRT2.1* and *AtNRT2.2* in response to short nitrate treatment is reduced in *Atnpl7* mutants (Castaings *et al.*, 2009). AtNLP7 and other AtNLPs are able to fix the NRE *cis*-element (Nitrate Responsive Element) present in many promoters of nitrate-responsive genes, and to activate their transcription (Konishi and Yanagisawa, 2013).

Gene Name	Transcriptional regulation (induction experiments)	Shuttling	Binds NRE	Restoration of *Atnlp7* phenotype	Additionnal informations	References
AtNLP1	NC (in silico experiment)	ND	ND	ND	-	Chardin et al., 2014
AtNLP3 , *AtNLP4* , *AtNLP5* , *AtNLP9*	induced by nitrate starvation (in silico experiment)	ND	ND	ND	-	Chardin et al., 2014
AtNLP6	unregulated	ND	ND	ND	interacts with NLP7 and have a partial redundant role	Chardin et al., 2014 Guan et al. 2017
AtNLP7	unregulated	Yes	Yes		involved in primary nitrate response	Castaings et al., 2009 Marchive et al., 2013
AtNLP8	induced by nitrate starvation	No (constitutively localized in nucleus)	Yes (e.g *AtCYP707A2*)	No	regulation of seed germination by inducing ABA catabolism in response to nitrate	Chardin et al., 2014 Yan et al, 2016b
ZmNLP1 , *ZmNLP7* , *ZmNLP9*	unregulated	ND	ND	ND	-	Cao et al., 2017
ZmNLP2	induced by nitrate (10mM)	ND	ND	ND	-	Cao et al., 2017
ZmNLP3	induced by low nitrate (0,2 mM)	ND	ND	ND	-	Cao et al., 2017
ZmNLP4	induced by low nitrate (0,2 mM) (Cao et al., 2017) induced by nitrate supply (Ge et al. 2018)	ND	ND	ND	-	Cao et al., 2017; Ge et al., 2018
ZmNLP5	unregulated (Cao et al., 2017) induced by nitrate (Ge et al. 2018; Ge et al., 2020)	ND (mainly localized in nucleus but more concentrated under deficient N conditions)	Yes (e.g *ZmNIR1.1*)	ND	modulating N assimilation especially under N deficient conditions	Ge et al., 2020; Ge et al, 2018
ZmNLP6 , *ZmNLP8*	unregulated (Cao et al, 2017) induced by nitrate (Ge et al., 2018)	Yes	Yes (e.g *ZmNRT1.2* , *ZmNiR2* in vitro)	Yes	may improve NUE under low nitrate conditions	Cao et al., 2017
OsNLP1	induced by N starvation repressed by N resupply	No	Yes (e.g *OsNRT1.1A* , *OsNRT1.1B* , *OsNRT2.4* , *OsNIA1* , *OsNIA3* , *OsAMT1.1* , *OsGRF4*)	Yes	role in N utilization at the seedling stage; maintains basal N utilization; positively impacts NUE under low N conditions; response to both ammonium and nitrate	Hsieh et al., 2018 Alfatih et al., 2020
OsNLP3	induced by N starvation and repressed by N resupply in shoot induced by N resupply in root	Yes (in response to nitrate only)	Yes (*OsNIA1* , *OsNIA3* , *OsNRT1.1B* , *OsNRT2.4* , *OsGRF4*)	Yes	more important in nitrate utilization rather than ammonium utilization	Hu et al., 2019 Zhang et al. 2021
OsNLP4	Induced by N starvation Repressed by N resupply	Yes (in response to nitrate and ammonium)	Yes (e.g *OsNRT2.1* , *OsNRT2.2* , *OsNRT2.3* , *OsNRT2.4* , *OsNRT1.1B* , *OsNIA1* , *OsNIA3* , *OsAMT1.1* , *OsGRF4* , *OsNiR*)	Partially	positively impacts NUE under different N conditions; maximizes N utilization	Wu et al., 2020 Yu et al., 2021
OsNLP2 , *OsNLP6*	unregulated	ND	ND	ND	-	Jagadhesan et al., 2020
OsNLP5	induced by N starvation	ND	ND	ND	-	Jagadhesan et al., 2020
TaNLP1	induced by starvation (in silico experiment)	ND	ND	ND	-	Kumar et al., 2018
TaNLP2	induced by starvation	ND	ND	ND	higher expression in shoot ; putative involvement in N translocation/remobilzation	Kumar et al., 2018
TaNLP7	induced by starvation (in high NUE genotype) induced bu N resupply (in low NUE genotype)	ND	ND	ND	-	Kumar et al., 2018
TaNLP3 , *TaNLP4* , *TaNLP5*	unregulated (in silico experiment)	ND	ND	ND	-	Kumar et al., 2018

Table 1: Summary of characterized NLP in Arabidopsis, maize, rice and wheat.

AtNLP7 is also able to bind and induce genes related to nitrate that do not have an NRE in their promoters (Marchive *et al.*, 2013). AtNLP7 regulation have been recently uncovered: the transcription factor is localized in the cytoplasm in absence of nitrate, and is rapidly retained (within minutes) in the nucleus after nitrate treatment (Marchive et al. 2013). This nuclear retention is governed by nitrate perception *via* AtNRT1.1, which then transduced signal through calcium waves decrypted by CPK proteins that will, in turn, phosphorylate AtNLP7 at Ser-205 residue and promote its nuclear localization (Liu *et al.*, 2017). AtNLP7 interacts via its PB1 C-terminal domain with AtNLP6 and TCP20 and, together, they localize in the nucleus in both ample and starved nitrate conditions (Guan *et al.*, 2017). AtNLP6 is the closest paralog of AtNLP7, and the *Atnlp6/Atnlp7* double mutant shows a higher reduction of sentinel-gene response to nitrate compared to *Atnlp7* single mutant. *Atnlp6* single mutant does not exhibit obvious phenotype, thus suggesting a partial redundancy between AtNLP7 and AtNLP6. TCP20 is also able to fix NRE *cis*-element and is involved in cell cycle processes and in the control of root growth (Guan *et al.*, 2014). AtNLP7 is now considered as one of the key regulators of nitrate response. Also, it has been once shown that overexpression of *AtNLP7* can improve plant biomass under both ample and limiting nitrogen conditions, identifying AtNLP7 as a good candidate for improving plant performance (Yu *et al.*, 2016).

Nitrate is important in controlling seed dormancy and germination processes. It acts as a signal for the induction of germination (Alboresi *et al.*, 2005). ABA promotes seed dormancy and its levels is reduced after nitrate addition. This reduction is mediated by AtNLP8 which, in response to nitrate, binds the promoter of *CYP707A2*, a P450 cytochrome encoding the main ABA catabolic enzyme. AtNLP8 is also necessary for the expression of nitrate responsive genes such a *NIA2*, *NIA2* and *NIR* in seeds. Interestingly, AtNLP8 is constitutively localized in the nucleus, regardless of nitrate application, while AtNLP7 localizes is the nucleus only in the presence of nitrate. This

suggest that nitrate regulates AtNLP8 in a different manner than AtNLP7 (Yan *et al.*, 2016). Besides AtNLP7, AtNLP6 and AtNLP8, no other AtNLP has been well characterized yet. However, using expression data from transcriptomes in response to nitrate, it has been observed that *AtNLP4*, *AtNLP5*, *AtNLP8* and *AtNLP9* are possibly transcriptionally regulated by nitrate (Chardin *et al.*, 2014).

In *Poaceae* species, many studies have now been published on the identification of NLPs and on their potential regulations, notably in rice, maize and wheat. All the characteristics of cereals NLP have been gathered in Table 1. Interestingly, it appears that similarly to AtNLP7, ZmNLP6, ZmNLP8, OsNLP3, OsNLP4 are localized in the nucleus after nitrate treatment (shuttling) and that they are able to binds the Nitrate Responsive *cis* Element (Cao et al., 2017; Hu et al., 2019; Wu et al., 2020; Zhang et al., 2021). However, unlike AtNLP7, most of them are also transcriptionally regulated by nitrate availability. This suggest a partial conservation of functions and regulations between AtNLP7 and cereals NLPs. Further studies are necessary to finely characterized cereals NLP and uncover their molecular regulations.

Opening on N and P signaling crosstalks

The physiological link between N and P is now well established and observed in several species, at different level (physiology, root development, biomass, uptake...). But the identification and comprehension of the molecular nodes explaining the physiological observations is still at its early stage. The first clues came from transcriptomics analyses studying N starvation that showed deregulation of genes involved in Phosphate Starvation Response (PSR) in maize leaves (Schülter et al., 2012). When comparing transcriptome of N starved plant versus P starved plants, a large overlap of common genes was identified (Cai et al., 2013).

More recently, proteins known to be involved in N or P pathways individually were shown to also play a role in the other pathway: the nitrate transporter NPF7.3/ NRT1.5 modulates root architecture in response to –P (Cui et al., 2018). OsPHR3, a close homolog of OsPHR4 which mediates Pi homeostasis under Pi starvation, is also induced by phosphate starvation but has no influence on expression of downstream genes of the PSR. However, its expression is induced by different source of N and regulates N homeostasis in rice (*Osphr3* displays reduction of expression of nitrogen transport and assimilation genes) (Sun et al., 2018).

The established nodes: from up to downstream

Molecular actors of this crosstalk start to be identified (Figure 5). The current first node is the interaction between the rice transceptor of nitrate, OsNRT1.1B, and the Pi sensor, SPX4. This interaction takes place only in +N-P and facilitates the ubiquitination of SPX4 via the newly identified E3 ligase NBIP1 (Figure 5, Hu et al., 2019a; Medici et al., 2015). SPX proteins are known to interact and repress the action of the major transcription factor, OsPHR2 in rice and AtPHR1 in Arabidopsis, which are master regulators of phosphate starvation response (Rubio *et al.*, 2001). OsSPX4 also interacts with OsNLP3 (closest ortholog of AtNLP7), the major regulator of the primary nitrate response, which makes another molecular connection between the two pathways (Figure 5, Hu et al., 2019).

Downstream of that, another node is mediated by the AtNIGT1/HHO protein family, which integrates both the N and P signals (Medici et al., 2015). AtNIGT1/HHO are transcription factors that permit a feedback regulatory loop of the nitrate response: they are induced by nitrate via NLP7 and are negative transcriptional regulators of *AtNRT2.1* (Maeda et al., 2018). AtNIGT1/HHO are also induced by phosphate starvation *via* AtPHR1 and inhibit repressors of PSR (such as SPXs and PHO2) (Kiba *et al.*, 2018).

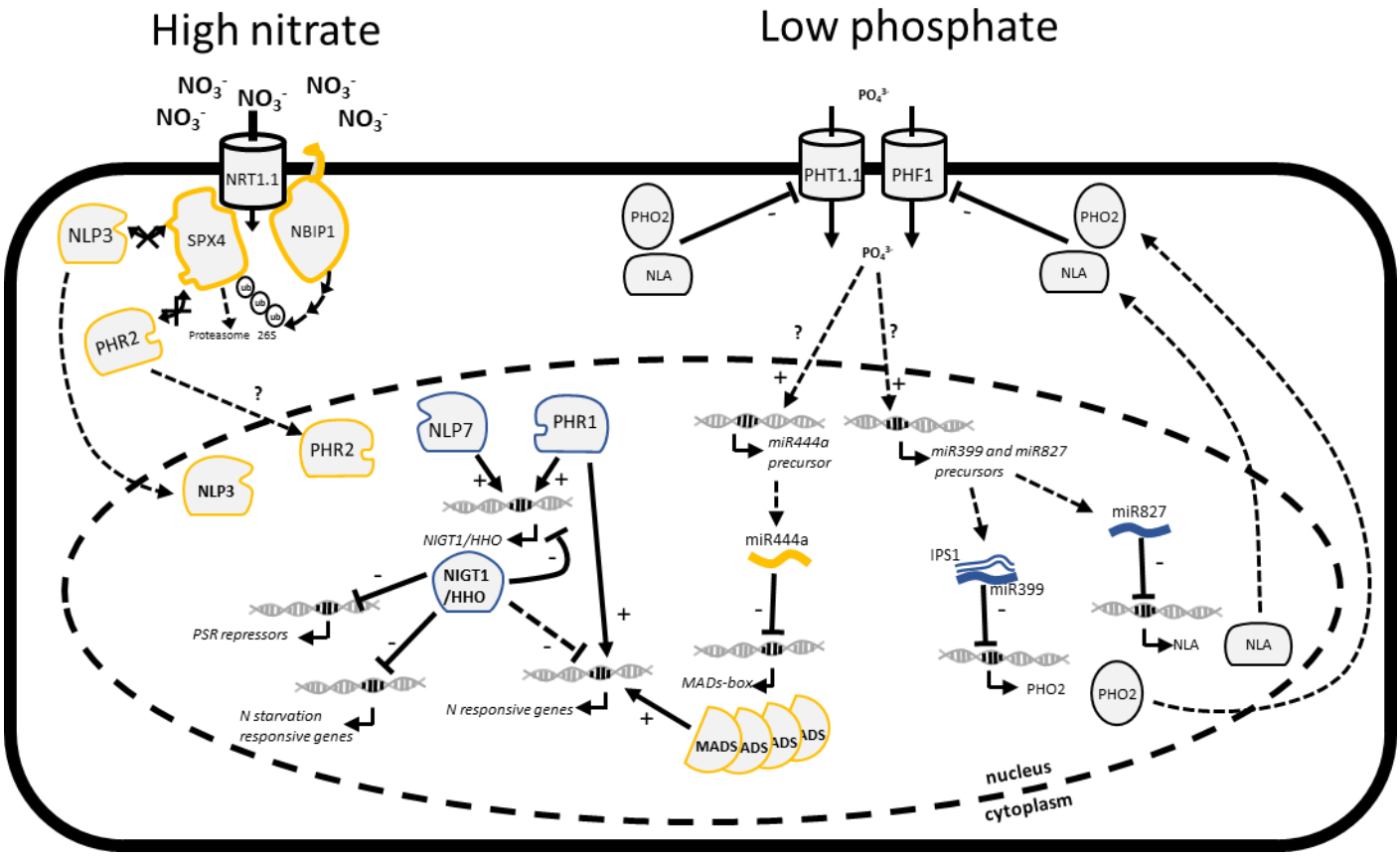

Figure 5: Current model for molecular N-P crosstalk under high nitrate and low phosphate conditions. In rice (yellow pathway), the nitrate perception by NRT1.1 lead to the ubiquitination of SPX4 by NBP1 and thus its degradation. OsNLP3 and OsPHR2 are consequently released to the nucleus where they will coordinately activate N and PSR responses. The downstream mechanism is then better described in Arabidopsis (blue pathway) : *NIGT1/HHO* expression is controlled by AtNLP7 and AtPHR1, NIGT1/HHO then act as a repressor first of the N starvation genes and the PSR genes but also in a second time of the N responsive genes to perform a feed back regulation loop.

NLA and PHO2, which encode two ubiquitin conjugases, work cooperatively to regulate Pi transporter trafficking (Kant et al., 2011; Lin et al., 2013). Regulations by microRNA are another important part of molecular N and P crosstalks: these two proteins are targeted by microRNA (miR827 and miR399) induced by phosphate starvation (Fujii *et al.*, 2005; Chiou *et al.*, 2006; Kant *et al.*, 2011*b*). Interestingly, miR399 is itself regulated by the non-protein coding gene *IPS1* which will paired but with a mismatch loop to the miR399 sequence and thus inhibit its activity without cleavage (target mimicry mechanism) (Puga *et al.*, 2007). *IPS1* expression is highly induced by phosphate starvation but only in the presence of nitrate (Medici *et al.*, 2019). Yan et al. (2014) identified miR44a, strongly induced by nitrate, which targets in rice four genes homologous of *AtANR1*, a MADS-box transcription factor triggering lateral root growth in response to nitrate.

A conserved process?

As shown before, studies of N and P crosstalks were carried out in diverse species, mainly in the dicot Arabidopsis but also in the monocot rice. It is known that the phenotype of root architecture in response to phosphate and nitrate differs between Arabidopsis and monocots (Smith and de Smet, 2012; Shahzad and Amtmann, 2017). Some studies, such as Medici et al. (2019), tend to show that pathways are conserved in Arabidopsis and wheat by showing that the expression of PSR genes such as *IPS1*, *SPX* and *PHT1* are induced in -P only in the presence of N. Also, Hu et al. (2019) and Medici et al. (2019) both showed that the accumulation of PHR1 is controlled by NRT1.1 (via the NBIP1 E3 ligase in rice). On the contrary, some studies point out specificities among monocots. For example, the miR444a identified in Yan et al. (2014) is specific of monocots. More recently, Dissanayake et al (2019), by studying the transcriptome of wheat in different N and P combination, shows that the closest homologue of *AtHRS1/NIGT1*, *TaHRS1*, is regulated after 24h of nitrate treatment while it happen within some minutes in Arabidopsis and in about 1 hour in rice (Krouk *et al.*, 2010*b*;

Sawaki *et al.*, 2013). Consequently, the downstream genetics targets of TaHRS1 may also be regulated in a different manner.

To date, the vast majority of molecular understanding of nitrate response is in Arabidopsis. The rice molecular response is also well described now, and new processes are now first discovered in rice such as the molecular link between nitrate and phosphate crosstalk (Hu et al., 2019). Even though this discovery directly in a crop are very interesting, rice is not representative of all cereals. Indeed, its growth conditions are very divergent compared to temperate cereals which grow in soils and which prefer nitrate.

Thesis objectives

In this introduction, the importance of nitrate as a signal molecule has been highlighted. Many molecular mechanisms have been deciphered in the dicot model species Arabidopsis. Recently, more and more studies are now performed on rice and, to a lesser extent, in maize or wheat. The identification of key molecular actors and processes of nitrate response that are specific to cereals are still missing and are necessary to further target NUE. Thus, the understanding of molecular response to nitrate in cereals has to be deepen. My main study models are member of *Pooideae* sub family which gather important temperate cereal such as wheat, barley, oat and rye. Arabidopsis is now a well-known model plant which has been extensively use for understanding plant biology. *Brachypodium distachyon* is a small *Pooideae* family member (a sub group of Poaceae) which gather all the characteristics of a good model: it has a small and well sequenced diploid genome (272 Mb), it is transformable, has a short time life cycle (around 3 months from grain to grain) and a small size which facilitate its growth under controlled conditions (for review, Girin et al., 2014). On the other hand, barley (*Hordeum vulgare*) is also a member of *Pooideae* and is an important crop for livestock feed, human food and malt production. Unlike wheat, which possesses a complex polyploid genome, barley has a sequenced 5.3 Gb diploid genome.

First, I used a comparative transcriptomic approach on Arabidopsis, Brachypodium and barley to describe and identify common and specific pathways responding to nitrate (Chapter II). Afterwards, I studied the role of two BdNLP (BdNLP6 and BdNLP7) in Brachypodium in relation to nitrogen availability (Chapter III). Finally, I discussed my results and formulate hypotheses about *Pooideae* nitrate response (Chapter IV).

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CHAPTER II

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Comparative cross-species transcriptomic analysis identifies new candidates of *Pooideae* nitrate response

----------Manuscript in preparation----------

Comparative cross-species transcriptomic analysis identifies new candidates of *Pooideae* nitrate response

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ABSTRACT

Using transcriptomic approaches allowed the comprehension of an important part of the molecular Arabidopsis nitrate response. On the other hand, there are still many missing points concerning nitrate response in other species, including crops. Here we performed a comparative transcriptomic analysis based on Arabidopsis, Brachypodium and barley nitrate responses. Combining Gene Ontology (GO) to an orthology analysis, we confirmed already suspected common nitrate-responsive genes and pathways, such as genes involved in nitrate metabolism or response to phytohormones in the three species. We also identified genes and pathways differentially or specifically regulated by nitrate in the two monocot species, such as a specific branch of the cysteine biosynthesis pathway. Finally, we established a list of good candidates for further studies to better understand cereal nitrate response.

INTRODUCTION

Pooideae is a sub-family of *Poaceae* which regroups most temperate C3 cereals, such as barley (*Hordeum vulgare*), wheat (*Triticum sp.*), rye (*Secale cereale*) and oat (*Avena sativa*). Together, they provide a major source of food for humans and cattle. Brachypodium (*Brachypodium distachyon*), as member of *Pooideae*, has been used as a model for this family (for review, Girin et al., 2014). As the global population increases, demand for food becomes critical. To obtain optimal growth and production, crops need a complex combination of multitude of environmental traits such as amount of light, specific temperature and, also, proper amount of nutrients. Nitrogen, is a major limiting macro-element in fields, taken up by herbaceous plants mainly as nitrate and ammonium. Thus, nitrogen fertilizers are largely applied to enhance growth and production. In addition to ecological and economical costs of nitrogen fertilizers production, nitrate rapidly leaches into soils causing severe environmental problems such as eutrophication and biodiversity depletion. The main challenge of today's agriculture is to maintain or enhance crop production while diminishing polluting fertilizers amounts. However, actual cultivars have been bred under high levels of nutrient availability. Thus, dissection of molecular mechanisms of bred and non-bred plants is necessary to engineer new cultivars able to provide high yield under limited amount of nutrients.

Nitrate is the main source of nitrogen for many plants, including Arabidopsis (*Arabidopsis thaliana*) and *Pooideae* crops. It is acquired from the soil by NRT nitrate transporters, and then mostly translocated to aerial organs to be successively assimilated into nitrite, ammonium and glutamine and glutamate, respectively through Nitrate Reductase (NR), Nitrite Reductase (NiR) enzymes and the GS/GOGAT (Glutamine Synthetase/Glutamate Synthase) cycle (Xu *et al.*, 2012). Nitrate is also a signal molecule governing gene expression, plant growth and development (Krouk *et al.*, 2010*a*).

Since early 2000's, many transcriptomes have been used to describe the transcriptional response of Arabidopsis to nitrate (known as "nitrate response", or "Primary Nitrate Response" (PNR)). Many genes and processes have thus been identified as nitrate responsive, such as the global nitrate transport and assimilation pathway, genes related to carbon assimilation, hormone pathways particularly involved in modulating root system architecture, amino acids biosynthesis pathway, and others (Scheible *et al.*, 2004; Canales *et al.*, 2014). Besides nitrate responsive processes, principal molecular actors of PNR have been identified in Arabidopsis. NRT1.1 is a dual-affinity nitrate transporter also known to be a nitrate sensor (Ho *et al.*, 2009). In response to sensing nitrate, PNR is rapidly induced thanks to calcium signals perceived by CPKs which in turn phosphorylate NLP7 transcription factor (Liu *et al.*, 2017). Phosphorylated NLP7 is retained to the nucleus and will physically interact with NLP6, TCP20 and NRG2 to govern expression of genes necessary for nitrate metabolism (Marchive *et al.*, 2013; Xu *et al.*, 2016; Guan *et al.*, 2017). A lot of other transcription factors have been characterized as important for nitrate response such as TGA1 and TGA4 (Alvarez *et al.*, 2014), HRS1 which is integrator of both nitrate and phosphate signals (Medici *et al.*, 2015; Maeda *et al.*, 2018) or LBD37, LBD38 and LBD39 (Rubin *et al.*, 2009; Brooks *et al.*, 2019).

Recently, a cross-species network analysis between Arabidopsis and Rice allowed to identify N-regulatory modules that are conserved between species, and thus good candidates for translational studies (Obertello *et al.*, 2015). Another cross-species analysis identified conserved regulated modules in nitrate response between Maize and Sorghum and led to pinpoint interesting targets that might be specific to monocot (Du *et al.*, 2020). They especially identified a module related to hormones, confirming the involvement of hormones signaling in response to nitrate in monocots. Since global root architecture are different between monocots and dicots (Smith and de Smet, 2012), a divergence of regulation by hormones in response to nitrate is expected. Thus,

going deeper into this module could identify monocot' specificities, but it is rather difficult without a reference specie.

In this study, we aimed to compare nitrate response in Arabidopsis (a wild dicot), Brachypodium (a wild *Pooideae*) and barley (a domesticated *Pooideae*). We performed an RNAseq analysis on roots of each species in response to 1.5 and 3 hours of 1mM nitrate treatment, preceded by 4 days of nitrogen starvation. We then compare their responses by using gene ontology enrichment, to estimate the conservation/divergence of the responses in terms of biological processes. To deepen the comparison to gene level, we produce a global orthology analysis using OrthoFinder, which allow us to define Orthogroups (OGps) gathering closest orthologs based on protein sequence similarities. We thus identified common regulated processes such as global nitrate transport and assimilation system as well as hormone related processes. However, by looking at gene level within most of studied processes, we identified differential regulations between species. To finish, combining gene ontology enrichments and OGps allowed us to identify key processes specifically regulated in one or two species only such as the rRNA pathway which is specifically up-regulated in Arabidopsis or the cysteine biosynthesis from serine pathway which is specifically up-regulated in Brachypodium and barley.

RESULTS AND DISCUSSION

Adjusting plants growth conditions for an optimal comparison between Arabidopsis, Brachypodium and barley

The goal of this study is to identify by transcriptomics common and specific genes, groups of genes and processes that are responding to nitrate in the three species. Growing all these species in the same growth chamber was not possible since the light requirement (both the amount and the duration) of Brachypodium and barley is much higher than for Arabidopsis culture. However, to ensure the best comparison as possible it was chosen to grow them in the same hydroponic medium, as described in Materials and Methods. In order to identify the maximum of responding genes, harvesting was done at two timepoints after nitrate treatment, thus avoiding biases due to possible differences in response kinetics between the species. Plant growth conditions have been studied and adjusted on Brachypodium to maximize the nitrate response and validated on the other species. Thus, the duration of starvation before induction (4 days), the nitrate concentration for induction (1mM) and the two different timepoints of induction (1h30 and 3h) were set. Plantlets were cultivated for 18 days on a basal medium containing 0.1mM NO_3^-. Then, they were transferred on nitrogen-deprived medium for 4 days. 22 after sowing plantlets were treated with 1mM NO_3^- (nitrate treatment) or mock-treated during 1h30 or 3h (Figure 1). These conditions were validated by measuring *NRT2A/NRT2.1*, *NIA1* and *NIA2* sentinel genes expression levels in the three species in response to 1h30 or 3h nitrate treatment (Figure 2). For all species, these three genes were significantly induced after 1h30 and/or 3h nitrate treatment, validating our conditions. Globally, the response appeared stronger in Arabidopsis than in the other species (induction ratios ranging from 3,7 to 30,55 in Arabidopsis, 1.7 to 3,9 in Brachypodium and 1.8 to 2,9 in barley). This was unexpected, as the experimental conditions have been set up to maximize the response in Brachypodium.

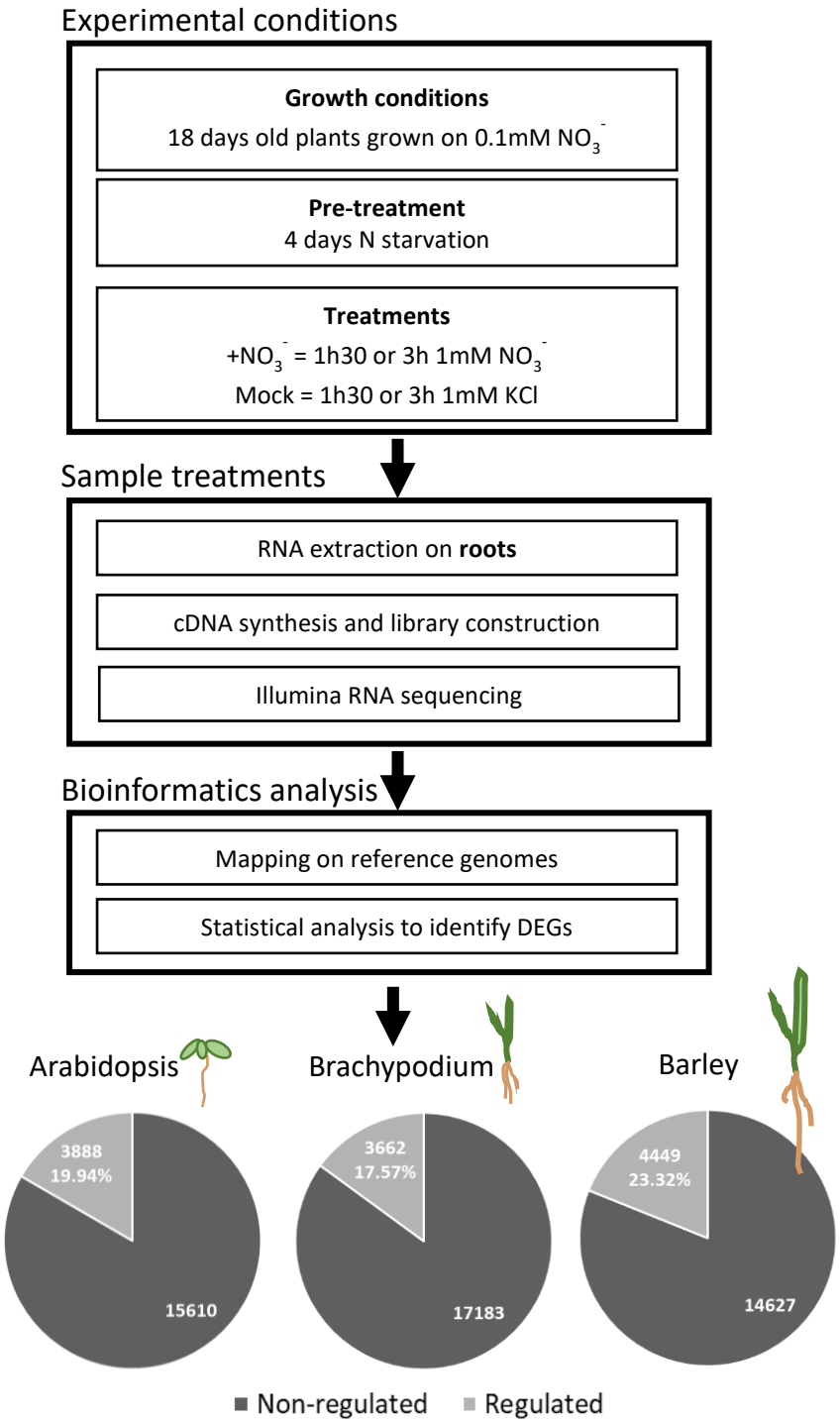

Figure 1: Schematic diagram of the experimental approach and RNAseq data extraction. Arabidopsis, Brachypodium and barley plantlets were grown hydroponically for 18 days on basal media. After 4 days of nitrogen starvation, plantlets were treated for 1h30 or 3h with 1mM NO_3^- (mix of KNO_3 and $CaNO_3$) or 1mM KCl (mock-treatment). Roots were collected and RNA were extracted and then treated for RNAseq. Mapping and differential analysis finally leads to the identification of Differentially Expressed Genes (DEGs) between nitrate- and mock-treated samples for each timepoint and each species. proportion of genes for each species regulated by nitrate after nitrogen starvation. Proportion among detected genes of regulated genes at 1h30 and/or 3h are represented by pie charts.

Effect of 1mM Nitrate treatment after 4 days of nitrogen starvation on Arabidopsis, Brachypodium and barley's transcriptomes

Using the set up conditions, 3 independent experiments were performed for each species, and roots were collected for RNA extraction and RNAseq analysis (Figure 1).

Mappings of the RNAseq reads on Arabidopsis, Brachypodium and barley led to the unambiguous detection of 19498, 20845 and 19076 genes, respectively (corresponding to 66%, 57% and 48% of annotated genes; lists available in Supplementary Table S1).

After differential analysis comparing nitrate-treated samples to mock, 3888 genes for Arabidopsis, 3662 genes for Brachypodium and 4449 genes for barley were found to be differentially expressed at 1h30 and/or 3h (pie charts, Figure 2; lists available in Supplementary Table S1). Differentially Expressed Genes will be called DEGs for the rest of the article. By comparing numbers of Differentially Expressed Genes (DEGs) to the total number of detected genes for each species, it appeared that 19.9%, 17.6% and 23.3% of Arabidopsis, Brachypodium and barley expressed genomes were impacted by nitrate treatment, respectively. Interestingly, the Brachypodium proportion seems lower than Arabidopsis and barley. This was especially true at 1h30 (11.4% *versus* 14.0%, and 14.7% for Brachypodium, Arabidopsis and barley, respectively) and the trend was similar at 3h (13.1% *vs* 15.5% and 17.6%). This could suggest that Brachypodium is globally less responsive to nitrate, as the experimental conditions have initially been set up to maximize the response in this species.

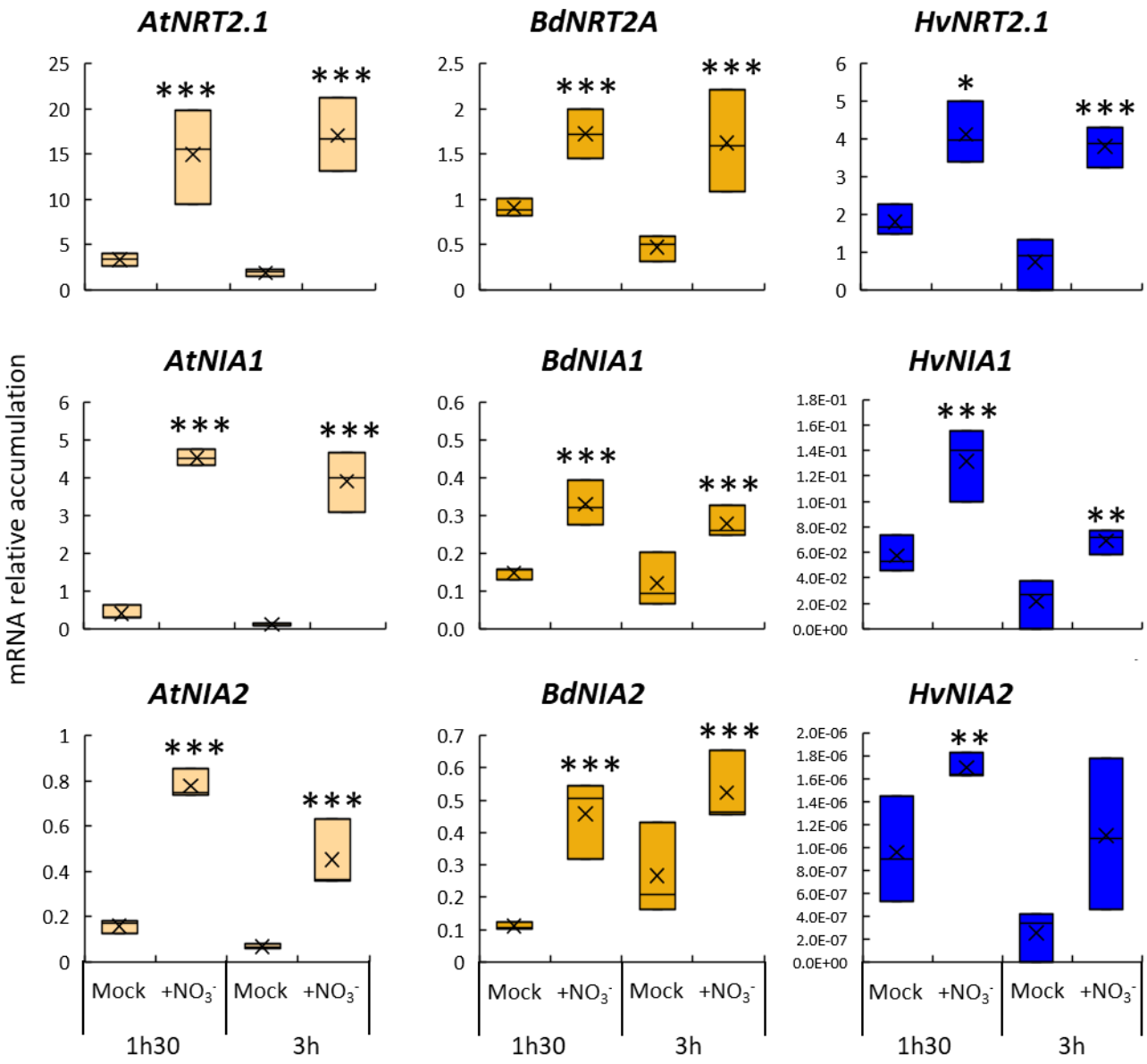

Figure 2: Sentinel gene expression in response to 1mM NO_3^- in Arabidopsis (At), Brachypodium (Bd) and barley (Hv). RT-qPCR have been performed on root tissues of plantlets grown in the exact same conditions as those set up for the transcriptome experiment. The boxplots represent minimum, median, mean (cross) and maximum values. Stars (*) represent the statistical significance between nitrate treated samples and their corresponding mock sample by a non-parametric ANOVA test (*pvalue<0,05 ; **pvalue<0,01 ; ***pvalue<0,001).

Description of biological processes associated with identified DEGs

The next step of the analysis was to identify biologic processes associated with identified DEGs for each species. To do so, we used Gene Ontology which provides uniform terms to describe biological processes for different species encoded by different genomes. Enrichment analyses and calculations have been performed using topGO R package, which takes the GO hierarchy into account thus enabling the identification of most relevant GO terms. Figure 3 describes the analysis process. First, we provided to topGO the gene ontology databases available on ensemblPlants, restricted to genes that have been detected in our RNAseq. Then we confronted this corrected database to our DEG lists for each species individually. To characterize the global response to nitrate in each species, the GO terms enriched in the full DEG set (*ie* combination of DEGs at 1h30 and 3h after nitrate treatment) were identified (Figure 4, panels A1-A9; Supplementary Tables S2, sheets A1 to A9). We first performed the enrichment analysis on total DEGs (Figure 4, panels A1, A4 and A7) and then separated the up-regulated DEGs (Figure 4, panels A2, A5 and A8) from the down-regulated DEGs (Figure 4, panels A3, A6 and A8). Similarly, analyses of gene ontology enrichments were performed on 1h30 DEGs and 3h DEGs separately, using all-DEG, up-DEG and down-DEG sets (Supplementary Figure S2 and Supplementary Tables S3 for 1h30 treatment; Supplementary Figure S3 and Supplementary Tables S4 for 3h treatment). No major difference was seen between 1h30, 3h and global analyses (except for GOs related to replication in barley, see below), suggesting the processes are steadily responding over the experimental time period.

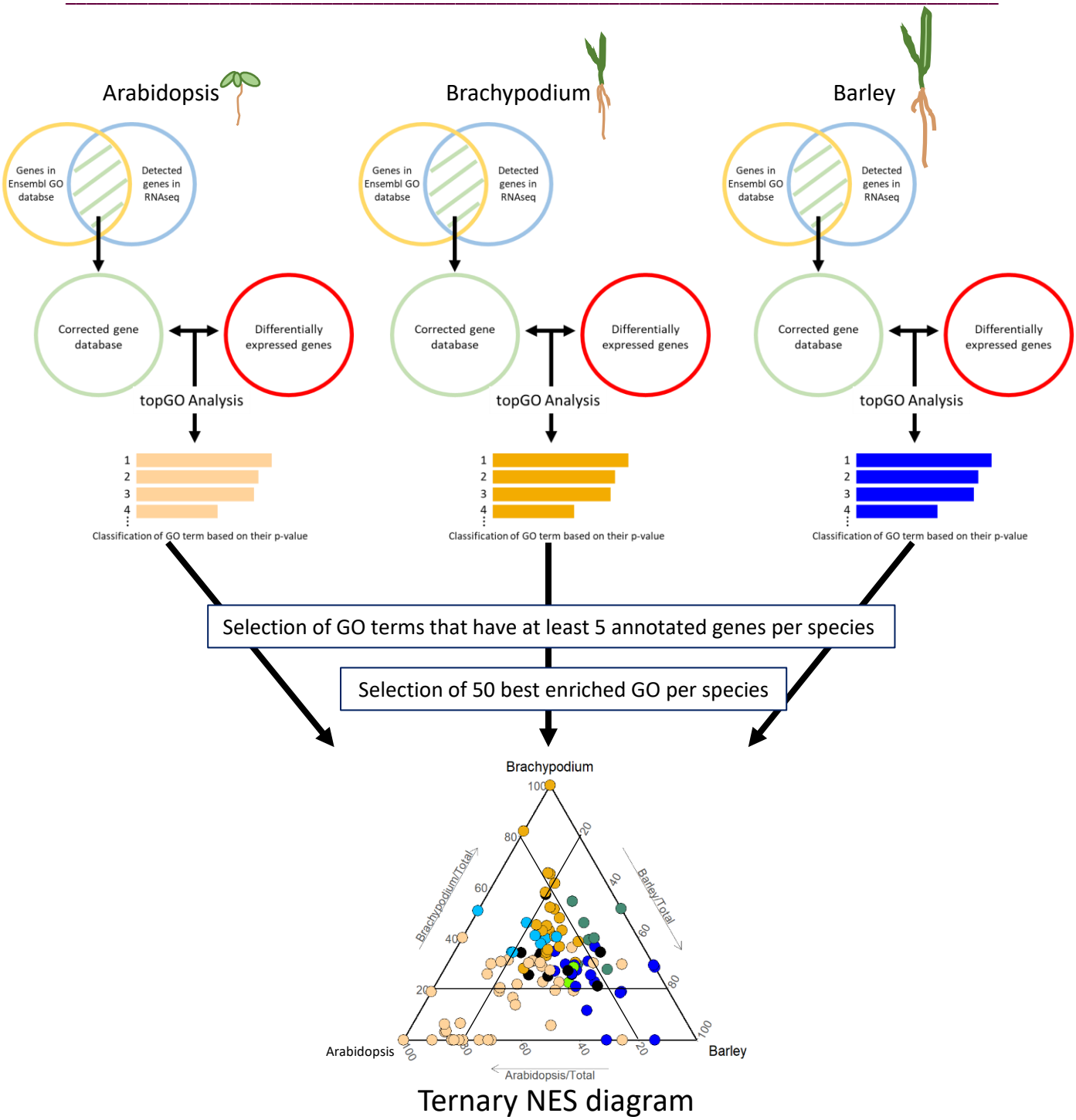

Figure 3: **Schematic diagram of the Gene ontology enrichment analysis using topGO R package.** First, Ensembl databases are restricted to genes that have been detected in our RNAseq. Then, lists of differentially expressed genes are loaded and topGO R function generates a classification from the most to the less enriched GO terms based on their p-value scores (Elim Fisher test). From these lists, GO terms that are not available for the 3 species are suppressed as well as those displaying less than 5 annotated genes. The 50 most-enriched GO per species are selected (giving a total of less than 150 GOs, due to overlaps between species), their Normalized Enrichment Scores (NES) are calculated and plotted on the ternary NES diagram. The NES correspond to the enrichment of a GO in a species relative to the enrichment in the three species (the sum of normalized scores for a GO is equal to 100).

Regulated processes in response to nitrate in Arabidopsis

Several of the main regulated biological processes in Arabidopsis are related to nitrate (Figure 4, panel A1): "nitrate assimilation" (GO:0042128, p-value=8E-11), "nitrate transport" (GO:0015706, pvalue=4.9E-9) and "response to nitrate" (GO:0010167, pvalue=1.40E-5) are among the 10 best enriched GO terms. More precisely, "response to nitrate" and "nitrate transport" terms are enriched in the up-regulated DEGs in response to our nitrate treatment (Figure 4, panel A1; Supplementary Table S2A2). This validates furthermore our experimental conditions. Interestingly, GO term "nitrate assimilation" is the best enriched term for down-regulated DEGs (1.7E-7) but is also found significatively enriched in up-regulated DEGs (Supplementary Figure S4A2, 0.004). Sentinel genes such as *AtNIA1*, *AtNIA2* and *AtNRT2.1* belong to this GO term and are up-regulated (Figure 2). Down-regulated genes of this GO term are mainly AtNPF nitrate transporters (*AtNPF3.1*, *AtNPF4.6*, *AtNPF2.13*, *AtNPF6.2*, *AtNPF2.3*…). NRT2 nitrate transporters that are known to be induced by nitrate starvation such as *AtNRT2.5* (Lezhneva *et al.*, 2014) are also found down-regulated. Three forms of glutamine synthetase are found down-regulated: *AtGLN1.1*, *AtGLN1.3* and *AtGLN1.4*, suggesting the start of the negative feedback of the assimilation pathway by products of nitrogen assimilation. Also, very interestingly, the master regulator of nitrate response, *AtNLP7*, which is also part of this GO term, is found down-regulated both after 1h30 and 3h nitrate treatment. As this gene has been described as non regulated by nitrate (Marchive *et al.*, 2013) or transiently induced by nitrate treatment (Varala *et al.*, 2018) it suggests our timepoints correspond to late stages of nitrate response.

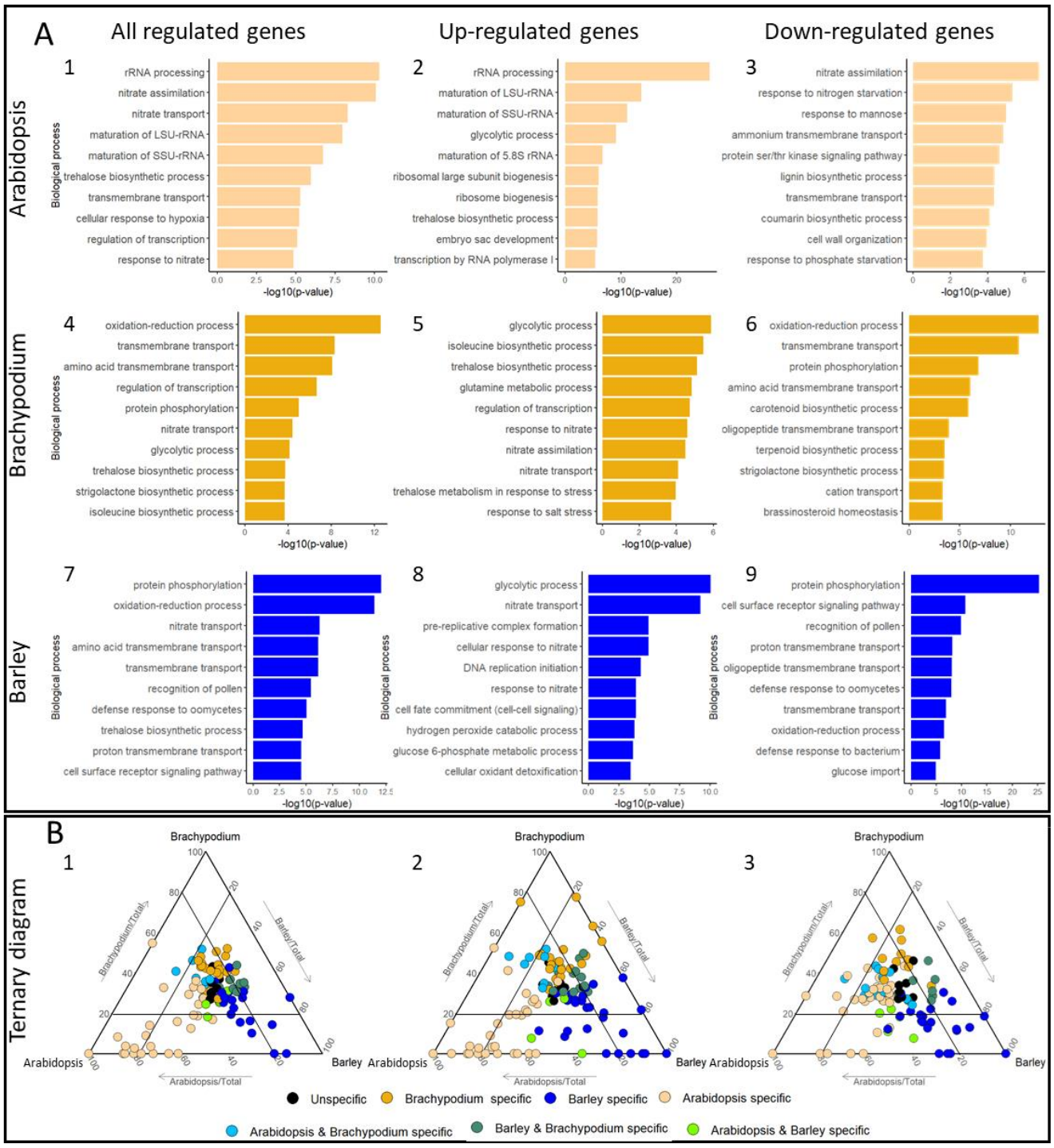

Figure 4: **Gene Ontology (GO) enrichment analysis for Arabidopsis, Brachypodium and Barley after 1mM NO_3^- treatment (combination of 1h30 and 3h).** (A) The 10 best enriched GO terms (based on their p-value score) were plotted for each species. The full lists of enriched GO terms are available in Supplementary Tables S2, sheets A1-A9. (B) Ternary diagrams represent the distribution of the 50 best enriched GO terms per species (giving totals of 109, 118 and 95 GO terms for B1, B2 and B3, respectively, due to overlaps between the species). Each axes represent the Normalized Enrichment Score (NES) for one species and each point represents a GO term. Colors indicate for which species the GO term is significantly enriched based on ElimFisher test (pval<0,05). Internal bars represent a 20% threshold which is used for comparing enrichment scores between species (see Results). The lists of selected GOs and their NES are available in Supplementary Tables S2, sheets B1-B3. For both (A) and (B), the first column represents biological process GO terms considering all the DEGs; the second column represents biological process GO terms considering only the up-regulated DEGs; the third column, considering only down-regulated DEGs. Gene ontology terms, their enrichment scores and their p-values were generated using topGO R package.

Other biological processes are found regulated in Arabidopsis such as carbon related pathways ("glycolytic process GO:0006096", p-value=0.00075; "pentose-phosphate shunt" GO:0009051, pvalue=0.018; "tricarboxylic acid cycle" GO:0006099, pvalue=0.039). This is expected and well described as nitrate response and carbon pathways are closely related and dependent on one another to sustain plant growth. "Trehalose biosynthesis process" (GO:0005992, pvalue=1.70E-6) is also induced in Arabidopsis and is indirectly linked to carbon metabolism since it improves growth and productivity of plant through regulation of carbon metabolism (Lunn *et al.*, 2014).

Hormone related pathways ("abscisic acid signaling pathway" GO:0009738, p-value=0.00023; "response to auxin" GO:0009733, pvalue=9.3E-5; "response to cytokinin" GO:0009735, pvalue=0.022; "response to gibberellin" GO:0009739, pvalue=0.014; "brassinosteroid biosynthetic process" GO:0016132, pvalue=0.037) are also found regulated in Arabidopsis in response to nitrate treatment. Similarly to carbon related processes, it is well known now that hormones have an essential role in nitrate response and in turn promote growth.

Also, processes related to transcription ("regulation of transcription", GO:0006355, p-value=8E-6) and translation ("rRNA processing" GO:0006364, pvalue=1.3E-26; "maturation of SSU-rRNA GO:0000462", pvalue=8.3E-12; "ribosome biogenesis" GO:0042254, pvalue=1.5E-6) took a big part of regulated processes in Arabidopsis and, more specifically, were induced. It can be explained by the fact that in response to nitrate, gene transcription is activated and followed by protein synthesis to produce tools to respond nitrate and promote growth in Arabidopsis.

Finally, two interesting GO terms are found down-regulated in response to nitrate treatment: "response to nitrogen starvation" (GO:0006995, p-value=4.6E-6) and "response to phosphate starvation" (GO:0016036, pvalue=0.00018). This perfectly illustrates the crosstalk of nitrogen and phosphate pathways(Kant *et al.*, 2011; Krapp *et al.*, 2011) that have been recently molecularly uncovered(Hu *et al.*, 2019; Medici *et al.*,

2019): phosphate response requires nitrate. In our data we can see that in response to nitrate, Arabidopsis down-regulates its response both to nitrogen and phosphate starvation, suggesting that nitrate addition allows phosphate response.

Regulated processes in response to nitrate in Brachypodium

Brachypodium response to nitrate is described on Figure 4, panels A4 to A6. As well as in Arabidopsis, GO terms related to nitrate are found regulated by our treatment, and more specifically up-regulated (Figure 4, panels A4 and A5): "nitrate assimilation" (GO:0042128, p-value=3.30E-5), "nitrate transport" (GO:0015706, 8.2E-5) and "response to nitrate" (GO:0010167, 2.50E-5). This again validates our experimental approach.

Similarly to Arabidopsis, pathways related to carbon are regulated: "glycolytic process" (GO:0006096, p-value =7.3E-6), "pentose-phosphate shunt" (GO:0006098, 0.013) tricarboxylic acid cycle (GO:0006099, 0.02) as well as trehalose biosynthetic process (GO:0005992, 0.00019) (Figure 4, panels A4 and A5, and Supplementary Tables S2A4 and S2A5).

Hormone related pathways are also represented in Brachypodium response: "response to auxin" (GO:0080167, p-value=0.045), "response to gibberellin" (GO:0009739, 0.041), "cytokinin metabolic process" (GO:0009690, 0.041) and "response to abscisic acid" (GO:0009737, 0.041).

Interestingly, genes involved in the biosynthesis of amino acid are induced in response to nitrate in Brachypodium (notably, isoleucine GO:0009097, p-value=1E-5; valine GO:0009099, pvalue=0.0006 and glutamine GO:0006542, pvalue=0.0006), while genes related to amino acid transport (GO:0003333, pvalue=9.3E-7) are repressed. It suggests a priority for recycling N sources instead of transporting the newly produced amino acids.

Finally, genes linked to "regulation of transcription" (GO:0006355, p-value=2.37) and to "protein phosphorylation" (GO:0006468, pvalue=1.1E-5) are responding to the treatment, suggesting a global regulation of signaling pathways *via* expression of both transcription factors and protein kinases that are known to be involved in signal transduction in response to nitrate.

Regulated processes in response to nitrate in barley

Barley response to nitrate is presented on Figure 4, panels A7 to A9. First, as for Arabidopsis and Brachypodium, nitrate related GO terms are found significantly enriched such as "nitrate transport" (GO:0015706, p-value=5.4E-7), "cellular response to nitrate" (GO:0071249, pvalue=0.00015) and "response to nitrate" (GO:0010167, pvalue=0.0073) and are all positively regulated, confirming again, the adequation of our experimental set up.

As expected, carbon-related terms are also found regulated in barley in response to nitrate: "glycolytic process" (GO:0006096, p-value=3.6E-5), "tricarboxylic acid cycle" (GO:0006099, pvalue=0.00036), "carbon fixation" (GO:0015977, pvalue=0.012), "pentose-phosphate shunt" (GO:0006098, pvalue=0.037) and also "trehalose biosynthetic process" (GO:0005992, pvalue=2.2E-5). This shows that barley clearly produces energy to sustain its growth in response nitrate nutrition.

On the other hand, some pathways related to defense are repressed in barley, such as "defense response to oomycetes" (GO:0002229, p-value=1.1E-8) and "defense response to bacterium" (GO:0009617, pvalue=1.60E-6). This illustrates well the balance that plants have to face between growth and defense. No contamination was noticed is our culture, but since barley is a domesticated crop, defense pathways have been surely selected and might be more responsive than in wild plants.

As in Arabidopsis and Brachypodium, hormones pathways are regulated in barley in response to nitrate. GO terms have been found enriched such as: "response to auxin"

(GO:0009733, p-value=0.0052), "cytokinin metabolic process" (GO:0009690, pvalue=0.0036) and "response to gibberellin" (GO:0009739, pvalue=0.02) (Supplementary Tables S2A7 to S2A9).

Finally, it seems that in response to nitrate, processes related to DNA replication are activated in barley: "DNA replication initiation" (GO:0006270, p-value=8.E-6) and "pre-replicative complex assembly" (GO:0006267, pvalue=3.2E-6). Interestingly, these GO terms are among the few that seem to be time-dependent. They are indeed over-represented among DEGs at 3h after nitrate treatment (Supplementary Figure S3 and Supplementary Tables S4), but not among DEGs at 1h30 of (Supplementary Figure S2 and Supplementary Tables S3), suggestion they belong to a late phase of response to nitrate.

Comparison of significantly enriched biological processes GO terms between Arabidopsis, Brachypodium and barley

By analyzing significantly enriched GO terms in response to nitrate in Arabidopsis, Brachypodium and barley independently, we were able to identify very expected common biological processes that are regulated by nitrate: nitrate related pathways such as nitrate assimilation and nitrate transport, carbon related pathways to generate energy for growth through activation of glycolytic pathway and the tricarboxylic acid pathway, and also pathways related to phytohormones. On the other hand, some biological processes seemed more enriched in only one species such as pathways related to rRNA processing, and thus translation, that were specifically regulated in Arabidopsis, or such as pathways related to DNA replication, that were specific of barley. To deepen this analysis, a quantitative method was needed to compare the levels of enrichment of the GOs between the species.

To ensure the best comparison between species, filters were added to lists of significant enriched GO terms per species (Figure 3): we selected GO terms that have at least 5 annotated genes per species. Indeed, GO databases are not equal for the three species, the Arabidopsis one being well more complete than Brachypodium and barley ones. We calculate that among all the Arabidopsis genes annotated in TAIR10, 85.2% of them were effectively associated to a GO term. On the other hand, only 47.7% and 48.4% of annotated Brachypodium and barley genes are associated with a GO term, respectively. We thus needed to counteract this annotation imbalance by suppressing GO terms that were inexistant in Brachypodium and/or barley. After these filters, we kept a maximum of 50 significantly enriched GO terms per species (giving a total of less than 150 GO terms, due to overlaps between species) and plotted them on a ternary diagram based on their Normalize Enrichment Scores (NES_specie1 = [ES_specie1/(ES_specie1+ES_specie2+ES_specie3)]x100, with ES_speciesX = (number_DEGs_in_GOterm_specieX/number_annotated_genes_in_GOterm_specieX)) (Figure 3). Each point represents a GO term. Each corner of the triangle represents the maximum NES for one species (*ie*: GO terms that include no regulated gene in the 2 other species). Colors give indication on the significance of enrichment scores per species (pvalue<0,05) which creates 7 clusters. For example, "Arabidopsis and barley specific" category (yellow points) means that the GO term is significatively enriched in Arabidopsis and barley but not in Brachypodium, based on ElimFisher statistical tests. This information is important but cannot be the only tool of decision, as ElimFisher statistical tests have been calculated independently for each species, and thus do not test whether the enrichment is different between the three species. Thus, we decided to add a classification of the GOs based on their relative enrichment between the species. A threshold (represented by internal bars on the ternary diagram, Figure 3) is arbitrarily defined: a GO term is considered as under-represented in a species relative to the other species if its NES is below 20%. In Supplementary Tables S2B1 to S2B3 describing the ternary diagrams, GO have been clustered following this 20%-threshold

rule. In our analysis below, we will consider a GO-term as specific or common between species based mainly on its localization on the diagram. A GO will be considered as non-specific when localized at the center of the diagram, regardless of the results of ElimFisher tests. On the other hand, a GO term will be considered as more enriched in a species if its NES is below 20%, regardless of the results of ElimFisher tests

Similarly to species-independent enrichment analysis, we performed the analysis on the combination of 1h30 and 3h DEGs (Figure 4, panel B1; Supplementary Table S2B1), on only up-regulated DEGs (Figure 4, panel B2; Supplementary Table S2B2) and on only down-regulated DEGs (Figure 4, panel B3; Supplementary Table S2B3). We also performed the analyses specifically after 1h30 nitrate treatment and 3h nitrate treatment (Supplementary Figures S2 and S3 and Supplementary Tables S3 and S4).

Most GO terms gathered in the center of the ternary diagrams (Figure 4, panels B1-B3; Supplementary Tables S2, sheets B1-B3) suggesting that most of nitrate-responsive biological processes are common to Arabidopsis, Brachypodium and barley. Interestingly, we can also identify GO terms located more on borders and even on corners of the diagram, showing that some processes are more regulated in one or two species.

Unspecific processes that are globally regulated in response to nitrate (central points in Figure 4, panel B1; Supplementary Tables S2, sheets B1) regroup GO terms already discussed before such as nitrate related pathways: “nitrate transport” (GO:0015706), “response to nitrate” (GO:0010167) and “cellular response to nitrate” (GO:0071249). Carbon related pathways are also largely represented, such as “glycolytic process” (GO:0006096), “pentose-phosphate shunt” (GO:0006098), “tricarboxylic acid cycle” which is specifically up-regulated (GO:0006099) and “trehalose biosynthetic process” (GO:0005992), confirming that the core response to nitrate is conserved among species. Responses to phytohormones such as cytokinin, auxin, gibberellin, jasmonic acid, karrikin and brassinosteroid are also considered as common regulated processes

in response to nitrate treatment. Even if two cytokinin related GO terms appear in the center of triangle ("cytokinin metabolic process" (GO:0009690) and "cytokinin biosynthetic process" (GO:0009691)), another one found enriched only in Arabidopsis and Brachypodium "response to cytokinin" (GO:0009735)). Some specificities can thus be pointed out, despite a global conservation of the effect of nitrate treatment on phytohormones pathways. Characteristics common to Arabidopsis and Brachypodium could be related to the undomesticated nature of these species, contrary to barley.

Processes described above in Arabidopsis, related to rRNA processing (GO:0006364) and ribosome biogenesis (GO:0042254), appear here to be extremely specific to this species. It suggests that Arabidopsis produces all the necessary machinery to increase translation, but not the other two species. A hypothesis could be that Arabidopsis is already at an advanced stage of nitrate response, while Brachypodium and barley are at an early stage. This timing difference in cereals has already been evoked in Dissanayake et al. (2019) where wheat induces the expression of *TaHRS1* after 4h of nitrate treatment while its ortholog gene in Arabidopsis is induced within minutes.

On barley and Brachypodium side, pathway related to cell wall seems slightly more regulated than in Arabidopsis (glucoroxylan biosynthetic process, GO:0010417).

Comparison of up-regulated processes (Figure 4, panel B2) gives us some precisions. Again, the majority of nitrate-related GO terms are localized in the center of the diagram, except "nitrate transport" (GO:0015706) which is clearly more enriched in Brachypodium and barley. It can be related to the highly divergence of NRT transporters between Arabidopsis and cereals (Plett *et al.*, 2010). "Photosynthesis" (GO:0009768) has been found enriched especially in barley. This can be an effect of domestication, as an efficient increase of photosynthesis following the release of a stress will have a beneficial effect on crop production.

Interestingly, by looking at down-regulated processes (Figure 4, panel B3; Supplementary Tables S2, sheet B3), "response to starvation" (GO:0042594), "cellular

response to nitrogen starvation" (GO:0006995) and "cellular response to phosphate starvation" (GO:0016036) appear more enriched in Arabidopsis and barley than in Brachypodium. It suggests that before nitrate addition, the processes related to starvations are more activated in Arabidopsis and barley. Indeed, before nitrate treatment, plants were nitrate-starved for 4 days. The fact that several nutrient starvation pathways, at least phosphate and nitrate, are responding illustrates once more the importance of nitrate for assimilation of other nutrients. Exploring more precisely the genes involved in these pathways could give supplemental indications on the role of this repression. This kind of characteristics common to Arabidopsis and barley could be linked to the adaptation of these two species to rich soils, while Brachypodium is adapted to dry and poor soils. Brachypodium might thus have been less affected than the other species by the nitrogen starvation preceding the nitrate treatment. Comparing nitrogen deficiency tolerance of Arabidopsis, Brachypodium and barley could further validate this hypothesis.

Generation of orthogroups for a comparison between Arabidopsis, Brachypodium and barley responses at the gene level

To further investigate the conservation and divergence of the response between the species at the gene level, it was necessary to compare the regulations between orthologs of the three species. Very few orthologous genes were identified between Arabidopsis, Brachypodium and barley. Thus, we performed a whole-genome analysis using Orthofinder, to identify orthologs at a large scale based on protein sequence similarity between the three species. Orthofinder is based on selecting reciprocal best hits after protein sequence comparison. Orthologs are then clustered and regrouped into orthogroups (OGps). A total of 36754 OGps were identified, including 9974 OGps composed of genes from the three species (27% of OGps, regrouping 43% of genes),

6509 OGps genes of only two species (bi-species OGps; 18% of OGps, regrouping 21% of genes) and 20271 OGps composed of genes from a single species (55% of OGps, regrouping 36% of genes). For one-species OGps, 4741 OGps are composed of paralogous genes (13% of total OGps, regrouping 21% of total genes) or 15530 OGps composed of only one gene each (42% of total OGps, regrouping 15% of total genes). Figure 5 represents genes' repartition into OGp types, for each species. In Arabidopsis, 40% of annotated genes are in OGps containing only Arabidopsis genes and 55% are in tri-species OGps. Only few genes are in bi-species OGps. For Brachypodium and barley about 34% of their genes are in mono-species OGps and about 39% are in tri-species OGps. Interestingly, they have about 25% of their genes that are in Brachypodium-barley bi-species OGps, contrary to Arabidopsis which has less than 5% of its genes in bi-species OGps. This can be explained by the phylogenic proximity between Brachypodium and barley which both are *Pooideae*. The high proportion of genes for which no orthologs in other species have been identified (mono-species OGps) can partially be explained by the analysis method, with a threshold effect. It cannot be excluded that within these three species, some protein sequences are divergent despite being involved in common biological processes. Some mono-species OGps may also be linked to species-specific biological processes.

Distribution of DEGs within the types of OGps where investigated (Figure 5, striated bars). Interestingly, the proportion of genes belonging to tri-species OGps is increased in DEGs as compared to the whole genomes. And, on the contrary, genes belonging to mono-species OGps are under-represented in DEG sets. This means that genes that respond to nitrate treatment mainly possess close orthologs in other species.

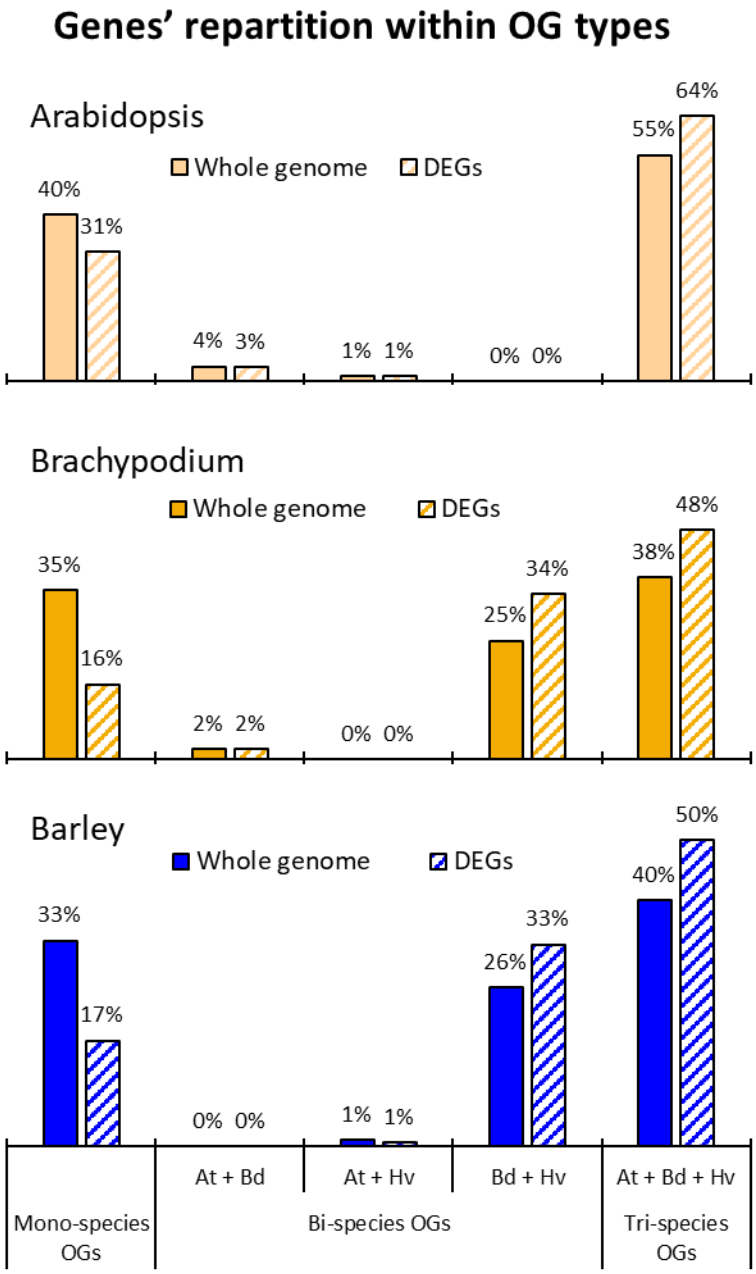

Figure 5: **Description of orthogroups composition**. 8111 orthogroups were generated using OrthoFinder bioinformatic tool. Gene distrubtions within OGs types are represented taking into account the whole genome (left full bars) or only DEGs of the three species (rigth striated bars).

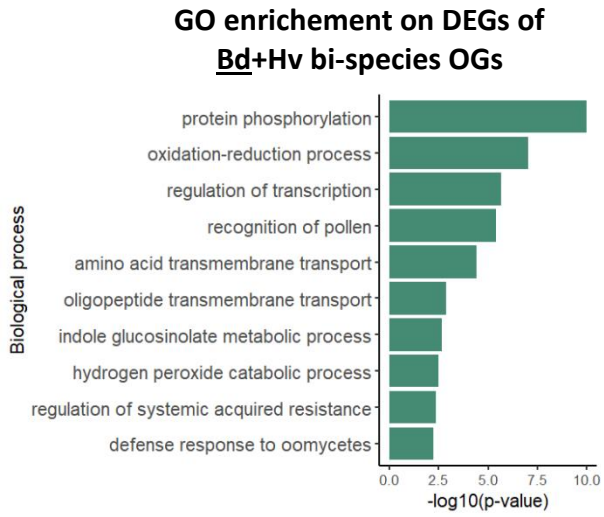

Figure 6: **Brachypodium and Barley specific best 10 enriched biological process GO terms in response to 1mM nitrate treatment.** Using topGO R package, significant enriched GO terms were identify on Brachypodium and Barley DEG list belonging to Bd+Hv bi-species OG. All significant enriched GO terms are available in supplementary table Fig8. We arbitrarily chose to show enrichment based on Brachypodium GO database but enrichment based on Barley GO database is available in supplementary figure and table S4 .

Brachypodium and barley DEGs are more distributed in Brachypodium-barley bi-species OGps comparing to their whole-genome genes (Figure 5), suggesting that there is a part of nitrate response in these species that is carried by specific genes, that are divergent from Arabidopsis ones. TopGO R package was used to analyze the biological functions of the DEGs belonging to Brachypodium-barley bi-specific OGps (Figure 6 and Supplementary Table S5). Interestingly, the best enriched GO terms are related to regulation of transcription and protein phosphorylation. This suggest that a non negligeable number of proteins such as transcription factors and kinases present highly divergent protein sequence comparing to Arabidopsis. These genes constitute good candidates that might have a role in nitrate response in *Pooideae*.

The biological functions of the different types of OGps was characterized, to assess a possible bias in the gene distribution. We extracted gene list associated with tri-species OGps, bi-species and mono-species OGps and input them into topGO R package to calculate enrichments in biological process GO terms (Supplementary Figure S3 and Supplementary Table S6). Tri-species OGps were enriched in general biological functions, such as "mRNA processing" (GO:0006397, 5.5E-13), "DNA repair" (GO:0006281, 1.1E-12) and "cell division" (GO:0051301, 1.2E-11). This was expected and validates our OGps, since genes involved in these functions might have very conserved sequences. However, some general biological functions were also enriched in mono- and bi-species OGps. For instance, "translation" (GO:0006412) was both enriched in tri-species OGps (p-value=1.1E-22) and in barley-specific OGps (p-value=1E-9); "regulation of transcription (GO:0006355) was enriched in Arabidopsis-specific OGps (1.3E-2), in Brachypodium-specific OGps (p-value=3.2E-19) and in Brachypodium-barley bi-species OGps (pvalue=7E-12). This suggest that among very general processes, specificities exist between species and genes that may have similar function are divergent at protein sequence level.

	Canales *et al*., 2014	At								Bd								Hv							
OGp name	**Arabidopsis gene and/or family names representative of the orthogroup**																								
OGp0000971	*LATE EMBRYOGENESIS ABUNDANT 3 (LEA3); SENESCENCE-ASSOCIATED GENE 21 (SAG21); DROUGHT-INDUCED 21 (DI21)*	4,281	3,624								3,073								2,069						
OGp0002372	*NITRATE REDUCTASE (NIA1) (NIA2)*	1,765	3,936							2,647	3,211							1,851	2,594						
OGp0002924	***BTB AND TAZ DOMAIN PROTEIN (BT1) (BT2)***	4,804	4,418							3,998								3,831	3,575	3,298					
OGp0003174	*GRAS family transcription factor family protein*	3,532								0,96								1,914							
OGp0004350	*LOB DOMAIN-CONTAINING PROTEIN (LBD37) (LBD38) (LBD39)*	2,824	3,838	3,602						1,421								1,233							
OGp0004275	*sulfate/thiosulfate import ATP-binding protein, putative (DUF506)*	4,219								5,498								4,798							
OGp0005686	*Glycosyl hydrolase superfamily protein*		2,693							1,847								1,707							
OGp0005850	*6-PHOSPHOGLUCONATE DEHYDROGENASE (PGD1) (PGD3)*	2,535	3,213							1,933								2,749							
OGp0006122	*ROOT FNR (RFNR1) (RFNR2)*	3,315	4,278							2,712								2,448							
OGp0006704	*Integral membrane HPP family protein (ATNITR2.2) (ATNITR2.1)*	1,665	3,762							5,17								3,825							
OGp0008657	*GLYCERATE KINASE (GLYK)*	3,918								1,776								1,022							
OGp0010222	***NITRITE REDUCTASE 1 (NIR1)***	3,074								2,89								3,387							
OGp0010601	*CBL-INTERACTING PROTEIN KINASE 3 (CIPK3)*	3,174								0,931								2,128							
OGp0010999	*Protein phosphatase 2C family protein*	3,264								1,381								1,239							
OGp0012661	*UROPHORPHYRIN METHYLASE 1 (UPM1)*	2,893								2,575								2,758							
OGp0013156	*L-ASPARTATE OXIDASE (AO)*	1,737								2,829								1,372							
OGp0000527	***NITRATE TRANSPORTER 2 (NRT2.1) (NRT2.2) (NRT2.4)***	2,492	2,303	-1,569							1,581	2,228						2,992	2,608	2,752	1,11				
OGp0001900	*TRANSKETOLASE (TKL1) (TKL2)*	-0,523	2,971							1,191	-1,293									1,844					
OGp0004680	*CYTOCHROME P450, FAMILY 707, SUBFAMILY A, POLYPEPTIDE (CYP707A1) (CYP707A3)*	-0,999	1,316							0,922									1,183						
OGp0002366	*HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1 (HRS1) ; HRS1 HOMOLOGUE 1 (HHO1) (HHO2) (HHO3)*	4,011	3,123	2,242	6,503					2,076															
OGp0006384	*HEMOGLOBIN 1 (HB1)*	3,478								4,67															
OGp0007415	*Protein phosphatase 2C family protein*		2,088							0,782															
OGp0004614	*P-loop containing nucleoside triphosphate hydrolases superfamily protein*	4,936																1,726							
OGp0001967	*STELAR K+ OUTWARD RECTIFIER (SKOR) ; GATED OUTWARDLY-RECTIFYING K+ CHANNEL (GORK)*	2,385																							
OGp0000199	***NICOTIANAMINE SYNTHASE (NAS1) (NAS2) (NAS3) (NAS4)***		1,492	2,959	2,081																				
OGp0002923	*PLANT U-BOX (PUB29) (PUB28) ; CYS, MET, PRO, AND GLY PROTEIN 2 (CMPG2)*		1,781																						
OGp0005499	*Ephrin-A3 protein (AT1G16170) (AT1G79660)*	2,194	0,616																						
OGp0002919	*SENESCENCE ASSOCIATED GENE 20 (SAG20) (AT5G01740)*	-0,779	5,883																						
OGp0009452	*GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD3) (G6PD2)*	4,029	2,622							3,289															
OGp0001679	*BASIC LEUCINE-ZIPPER (bZIP58) (bZIP42) (bZIP3)*	1,007	2,887			2,546																			
OGp0005583	*MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 13 (MAPKKK13) (MAPKKK14)*	3,892	3,031																						
OGp0001272	*GLUTAREDOXIN (GRXS3) (GRXS1) (GRXS6) (GRXS8) (GRXS7) (GRXS4) (GRXS5)*		3,927	4,463		6,172	5,153																		
OGp0015402	*Avr9/Cf-9 rapidly elicited protein (AT1G32920) (AT1G32928)*	2,327	2,113																						
OGp0016273	*Nitric oxide synthase-interacting protein (AT5G10210) (AT5G65030)*	4,459	2,697																						
OGp0015052	*Transcription initiation factor TFIID subunit 1b-like protein (AT1G49500) (AT3G19030)*	5,09	2,791																						
OGp0016023	*BTB AND TAZ DOMAIN PROTEIN (BT4) (BT5)*	1,689	0,617																						
OGp0015229	*TREHALOSE-6-PHOSPHATE PHOSPHATASE C (TPPC) ; TREHALOSE-6-PHOSPHATE PHOSPHATASE B (TPPB)*		2,585																						
OGp0022230	*ATALKBH10A (ALKBH10A)*	3,363																							
OGp0021531	*PHOSPHOGLYCERATE/BISPHOSPHOGLYCERATE MUTASE (PGM)*	3,182																							
OGp0021715	*GLUTATHIONE S-TRANSFERASE (CLASS PHI) 14 (GSTF14)*	1,567																							
OGp0024813	*Eukaryotic aspartyl protease family protein (AT5G19120)*																								

	At	Bd	Hv
UP/tot	74%	58%	46%
DOWN/tot	4%	2%	2%
DEGs/tot	79%	60%	46%

Table 1: **Nitrate-response of top 50 most consistent genes induced by nitrate in Arabidopsis (list from Canales et al., 2014) and of their orthologs in Brachypodium and barley.** Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue up-regulated; purple, up-regulated at 1h30 and down-regulated at 3h). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Table below the OGp list shows the proportions of regulated genes among the considered genes for each species. OGps in bold font are discussed in the text.

Identification of conserved and divergent gene regulations of genes involved in nitrate response using OGps

To go further into comparison of gene regulation of genes belonging to nitrate response pathway, we decided to analyze OGps that contains genes known to be molecular players of nitrate response in Arabidopsis. We based our analysis on 2 available lists, which are partially overlapping. The first one regroups the 50 more consistently regulated genes in response to nitrate, identified from a meta-analysis of 27 transcriptomic datasets (Canales et al., 2014). The second list is an inventory of the main proteins/genes involved in nitrate transport, assimilation and signaling pathways, which was published in a recent review (Vidal et al., 2020). From those Arabidopsis lists, we identified orthologs in Brachypodium and barley based on our OGps, and compared their regulations in response to nitrate treatment using heatmaps (Tables 1 and 2).

The 50 main nitrate-responsive genes identified by Canales et al. (2014) belong to 41 different OGps, grouping a total of 89 Arabidopsis genes (Table 1). Seventy of these genes were regulated in our experiment. In particular, 49 out of the initial list of 50 genes were induced, which is a good indicator of the quality of our Arabidopsis transcriptomic analysis of nitrate response. By analyzing gene regulations through OGps, we observe that when orthologs of these genes could be identified in Brachypodium and barley, they are also regulated in response to nitrate. Indeed, 68% of tri-species OGps (19 out of 28) are responding in the three species, and 18% more are responding in two species (3 OGps for Arabidopsis-Brachypodium and 2 for Arabidopsis-barley). This suggests a high conservation of core genes responding to nitrate within plants.

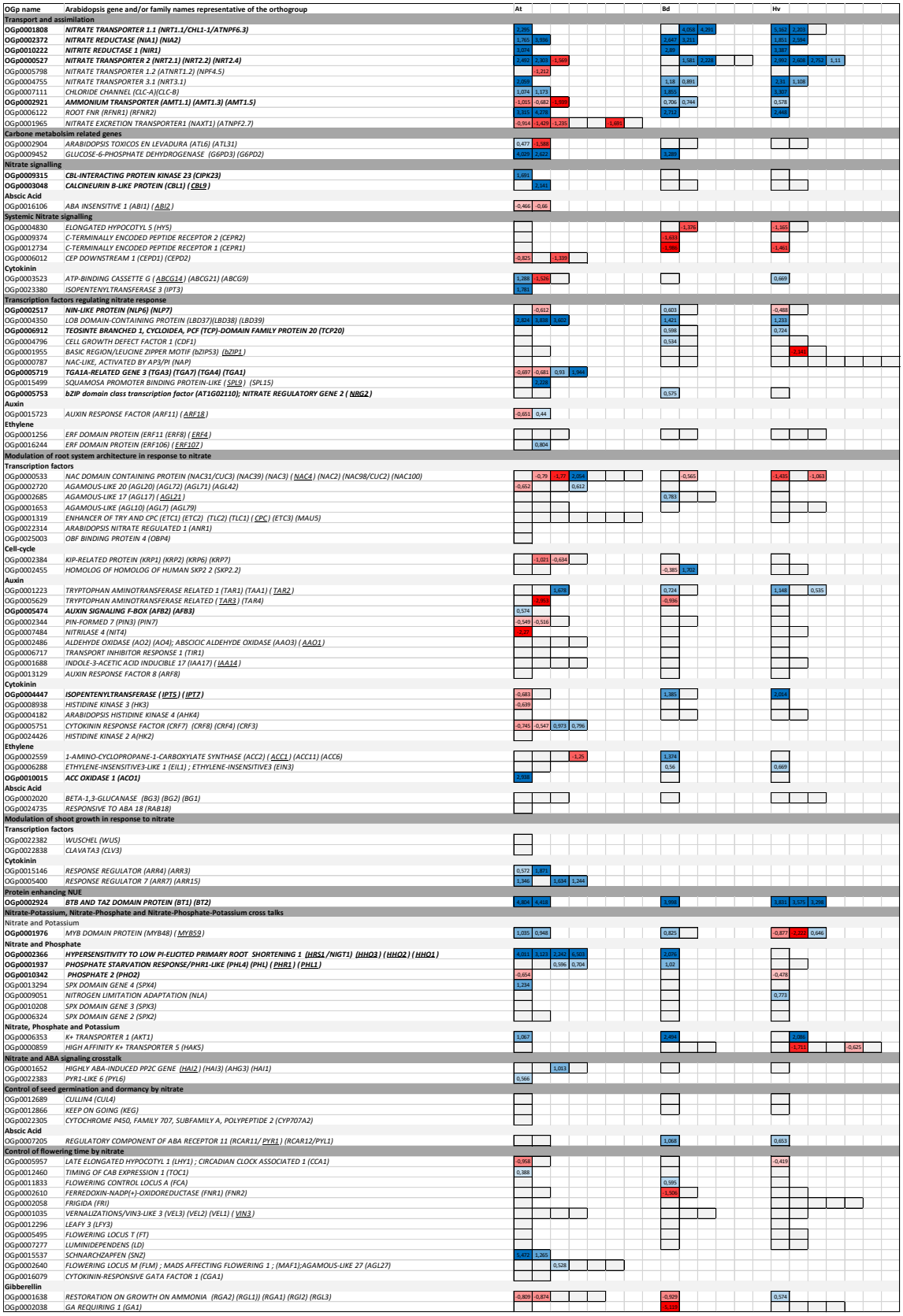

Table 2: **Nitrate-response of genes involved in nitrate transport, assimilation and signaling pathway (list from Vidal et al., 2020).** Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Tables on the right show the proportions of regulated genes among the considered genes for each species. OGps in bold font are discussed in the text and underlined genes have been discussed in Vidal et al., 2020 review.

The case of *NAS* genes retains our attention. *NAS* genes, encoding key enzyme of nicotianamine biosynthesis (Table 1). Nicotianamine is a chelator of various divalent cations such as Fe^{2+} and Zn^{2+} and is necessary for plants internal transport of Fe^{2+} and other cations to maintain metal homeostasis. *NAS* expression is known to be induced by Fe deficiency. Here, we show that nitrate treatment induced *NAS* expression specifically in Arabidopsis. Indeed, while 3 of the 4 paralogs are stimulated in Arabidopsis, none of the 3 Brachypodium and 12 barley ortholog is seen regulated (Table 1). Since Arabidopsis and grasses plants have different strategies to acquire Fe from the soil (Marschner and Romheld, 1994), our result suggest that they might also have a different strategy for maintaining the homeostasis of such cations in response to nitrate treatment.

The list from Vidal et al. (2020) has been organized by processes in which each gene has been described (12 categories; Table 2). For that second list, genes are not necessarily transcriptionally regulated by nitrate but have been shown to be part of nitrate response in Arabidopsis. In agreement, nearly half of the OGps (44 out of 98) did not contained responsive genes in Arabidopsis.

Among the "**transport and assimilation**" category, most of the OGps are similarly regulated between the three species, and mostly up-regulated (Table 2). As expected, genes encoding enzymes necessary for nitrate assimilation such as *AtNIA1*, *AtNIA2* and *AtNIR* are up-regulated in our three species (Table 2). Our method identifies 3 *NRT1.1* orthologs for Brachypodium and barley, which is consistent with the fact that cereals present a divergent dichotomy of nitrate transporters (Plett *et al.*, 2010). Two out of the 3 orthologs are up-regulated in response to nitrate in each *Pooideae* species, suggesting a high capacity of response for nitrate uptake. Genes of the *AtNRT2.1* orthogroup, known to be responsible for a large part of nitrate uptake from the soil, are also regulated in our three species in response to nitrate. As for *AtNRT1.1* OGp, there is more orthologs in Brachypodium and barley than in Arabidopsis. In barley, all the genes of the *AtNRT2.1* OGp are up-regulated, suggesting again a high response of

nitrate uptake. Interestingly, it has already been suggested a higher response of high affinity nitrate uptake in barley than in Brachypodium (David *et al.*, 2019). This could be related to a very low level of barley uptake under nitrate limitation and/or to a high level in non-limiting conditions. It could also mean that barley's nitrate transporters are less efficient and thus that it requires more protein to ensure the same level of transport as in Arabidopsis and Brachypodium. To confirm those hypotheses, nitrate influx could be compared in our experimental conditions: it will test whether barley is able to uptake more nitrate than Brachypodium and Arabidopsis, and thus if its higher number of regulated nitrate transporters ensure it a better uptake or not. In complement, the efficiency of transporters from the three species could be tested by heterologous expression, for instance in Xenopus oocytes. The *AtNRT2.1* OGp also contains *AtNRT2.4*, known to be involved in nitrate uptake specifically under severe nitrate limitation (Kiba *et al.*, 2012). As expected, this gene is down-regulated in our conditions. The fact that no Brachypodium or barley gene of this OGp is down-regulated suggest a divergence of physiological function between orthologs.

Interestingly, genes coding for ammonium transporters (*AtAMT1.1*, *AtAMT1.3* and *AtAMT1.5*) are found down-regulated in response to nitrate in Arabidopsis (Table 2), while closest identified orthologs in Brachypodium and barley are up-regulated. In Arabidopsis, ammonium transporters are known to be induced by N starvation (Loqué and von Wirén, 2004). Since our plants faced 4 days of N starvation, it is possible that *AtAMT* transporters were up-regulated before our nitrate treatment. Application of nitrate then down regulate *AtAMT* since a preferential N source is now available for Arabidopsis. Indeed, Arabidopsis is described as an ammonium-sensitive species, compared to ammonium-unsensitive species such as rice (Britto *et al.*, 2001; Loqué *et al.*, 2006). Nonetheless, Brachypodium and barley showed and induction of their *AMTs* expression in response to nitrate, while they are also ammonium sensitive plants (Girin, personal communication, Britto et al., 2001). This might highlight a physiology difference, where *Pooideae* are less sensitive to ammonium than Arabidopsis or are

more efficient in their ammonium utilization. A comparison of GS activity between these 3 species could help us confirmed this last hypothesis.

Nitrate signaling requires several actors, such as NRT1.1, calcium signals, and kinases. Under low nitrate conditions AtCIPK23 and AtCBL9 interact and phosphorylate AtNRT1.1 to switch to its high affinity nitrate transport activity. *AtCIPK23* is known to be induced in response to nitrate (Ho *et al.*, 2009), while nothing is described concerning the transcriptional regulation of *AtCBL9* by nitrate. Here we show that these genes are up-regulated in response to nitrate, but that their closest orthologs in Brachypodium and barley are not (Table2). This suggest that the mechanism of regulation in response to nitrate is at least partially divergent between our three species, but does not exclude a role of AtCIPK23 and AtCBL9 orthologs in Brachypodium and barley in this response. To further study this point, it would be interesting to test their phosphorylation activity in response to nitrate and to perform mutant analyses in the three species.

Nitrate response mobilize a huge amount of **transcription factors**. Among them, AtNLP6 and even more AtNLP7 are described as major regulators of nitrate response by governing expression of a lot of nitrate-responsive genes (Marchive *et al.*, 2013). AtNLP7 is usually described either as transcriptionally unsensitive to nitrogen availability (Castaings *et al.*, 2009) or as transiently up-regulated in response to nitrate, with a maximum at 30 minutes after nitrate addition (Varala *et al.*, 2018). In our conditions, *AtNLP7* and *HvNLP7* are found down-regulated, while *BdNLP7* is up-regulated (Table 2). This diversity of regulation suggests specificities of *NLP* regulations in response to nitrate in the different species. Down regulation of *AtNLP7* can be explained by a temporal difference, with Arabidopsis being at a later stage of its nitrate response and thus starts to decrease its level of response. But the fact that a close ortholog in barley is also down regulated can suggest that the two species are at the same stage of response, despite a possible delayed response described in cereals (Dissanayake et al., 2019).

AtTCP20 is a transcription factor that has been shown in Arabidopsis to binds *AtNRT2.1* and *AtNIA* promoters. AtTCP20 interacts with AtNLP6 and AtNLP7, and they control together the nitrate response of cell cycle and root growth (Guan *et al.*, 2014, 2017).To our knowledge, no study have shown a transcriptional regulation of this gene by nitrate neither in Arabidopsis nor in *Pooideae*. In our conditions *AtTCP20* is not regulated in Arabidopsis (Table 2). Very interestingly, one of its two orthologs in Brachypodium and its unique ortholog in barley are up-regulated. This suggests an additional level of regulation (transcriptional) in our *Pooideae* species as compared to Arabidopsis. It also suggests that TCP20 acts downstream of another transcriptional regulators in *Pooideae*, while it might be a primary regulator in Arabidopsis.

AtNRG2 encodes a transcription factor that interacts with AtNLP7 and which mediates nitrate signaling. Its expression in not regulated by nitrate, ammonium or nitrogen deprivation (Xu *et al.*, 2016). Two paralogs exist in Arabidopsis (*AtNRG2* and *AT1G02110*), while only one ortholog protein has been identified for Brachypodium and barley (Table 2). Interestingly, only the ortholog from Brachypodium is regulated in our experiment. This suggests as new level of regulation of the NRG2-dependent nitrate signaling pathway in Brachypodium, as compared to Arabidospsis.

TGA transcription factors, and especially AtTGA1 and AtTGA4, have an important role in Arabidopsis nitrate response: they can bind At*NRT2.1* and At*NRT2.2* promoters and are involved in the regulation of lateral root elongation (Alvarez *et al.*, 2014). Surprisingly, no TGA orthologs have been identified by our method in Brachypodium or barley (Table 2). However, we were able to manually identify regulated *TGA* genes in Brachypodium and barley that were in mono-specie or bi-species Bd+Hv OGps using simple Blasts. This illustrates the strength, which can also be a weakness, of our OGps. Indeed, they gathered the closest orthologs in term of protein sequence similarity, which is useful to identify candidate genes and to understand the evolution of gene function. On the other hand, this strictness can exclude genes that are fairly divergent in the family. In the case of TGAs, the protein sequences are likely very divergent

between the closest orthologs. But the function of TGAs in the nitrate response is probably conserved, as regulated genes can be found in the three species. Yet, this huge protein sequence divergence between Arabidopsis and *Pooideae* suggest different type of post translational regulation that might be species-specific and would be very interesting to analyze. This reasoning of divergent orthologs having similar function could be applied to all the mono specie OGps, such as those containing *AtSPL9*, *AtANR1*, *AtCRF* and other regulators involved in hormone pathways such as ARFs and ABI (Table 2). Alternatively, it is also possible that these genes are truly specific of Arabidopsis, meaning they have no close ortholog in the other species and they have no furthest orthologs sharing the same function in nitrate response. Detailed and exhaustive analyses of candidate gene families would be needed to distinguish between the two situations.

Within several categories of known genes linked to nitrate response in Arabidopsis (Table 2), phytohormones are sub-divided separately, such as auxin, cytokinin, abscisic acid (ABA) and ethylene. They especially have roles in the nitrate regulation of **root system architecture** (Table 2). Root system architecture is a complex trait that can be very different between monocot and dicot (Hochholdinger *et al.*, 2004; Osmont *et al.*, 2007) and is governed, *inter alia*, by nutrient availability. This roots plasticity confers to the plants a range of responses to adapt to variation of their environment.

AtAFB3 encodes an auxin receptor which is transcriptionally regulated by nitrate and controls root system architecture in Arabidopsis (Vidal *et al.*, 2010). In our conditions, *AtAFB3* is also up-regulated, but its orthologs in Brachypodium and barley are not regulated by our nitrate treatment (Table 2). Several hypotheses can be made. It is possible that Brachypodium and barley orthologs identified in this OGp are not those which are regulated in these species. In other words, the closest ortholog based on protein sequence similarity might not be the one carrying the function. Or, it is also possible that in Brachypodium and barley, root system architecture in response to nitrate do not follow the same auxin regulation pathway than the one described for

Arabidopsis. Further analysis on auxin pathway in response to nitrate in *Pooideae* would be interesting to clarify the role of AtAFB3 orthologs. In rice, OsAFBs have been characterized and shown to be involved in rice height, tillering, grain yield and even root architecture (Guo *et al.*, 2020). To our knowledge, no study of the relation between nitrate pathways and OsAFBs has been conducted so far.

Cytokinins, besides their involvement in systemic nitrate signaling, have been shown to be involved in primary root development in response to nitrate (Naulin *et al.*, 2020) through the regulation of *IPT* genes necessary for cytokinins biosynthesis. In particular, *AtIPT5* is regulated by nitrate addition (and also by cytokinin and auxin) (Miyawaki *et al.*, 2004). In our transcriptomic analysis, *AtIPT5* appears down-regulated after 3hours (Table 2). It suggests that *AtIPT5* expression might be regulated by products of nitrate assimilation after a certain amount of time to regulate cytokinins levels. On the other hand, orthologs found in Brachypodium and barley are both up-regulated in response to nitrate treatment. Since a difference of roots system architecture exists between monocots and dicots, it is possible that *Pooideae* requires more cytokinin to develop their primary root. Or, it can also be timing of response that is different in Arabidopsis comparing to Brachypodium and barley: repression by nitrate assimilation product will occur later in the *Pooideae* species. Taking a closer look into the regulation of *IPT5* in response to nitrate or in response to Glutamine could help us better understand this regulation differences.

Ethylene, which is synthesized from methionine, is a phytohormone involved in different plant development processes such as fruit maturation. In 2009, a role of ethylene in root development in response to nitrate have been identify in Arabidopsis: nitrate induces *AtACO1*, encoding an enzyme of ethylene biosynthesis pathway (Tian *et al.*, 2009). This will lead to a rapid increase of ethylene in lateral roots primordia coordinated with a repression of lateral roots in response to high nitrate. In our analysis, *AtACO1* expression is induced by 1mM of nitrate treatment, but neither Brachypodium

nor barley orthologs are regulated (Table 2), suggesting again the existence different mechanisms between Arabidopsis and *Pooideae*.

Nitrate Phosphate cross talk is now well established. In Arabidopsis, AtHRS1 and AtHHO1 are transcription factors highly induced in response to nitrate. They repress primary root growth in response to P deficiency only in presence of nitrate (Medici *et al.*, 2015). AtHRS1, also known as AtNIGT1, acts as a repressor of *AtNRT2.1* expression through AtNLP7 (Maeda *et al.*, 2018). By our method, we identified only 1 ortholog for Brachypodium and 1 for barley, against 4 paralogs in Arabidopsis, suggesting a divergence between Arabidopsis and *Pooideae* species. It cannot be excluded that other members of the family exist in Brachypodium and barley, but their protein sequences would be fairly divergence. Whereas all 4 Arabidopsis genes and the Brachypodium ortholog are highly induced, the barley gene is unresponsive (Tables 1 and 2). This could suggest either 1/ a regulation divergence possibly linked to domestication, 2/ that it is not the closest ortholog that is regulated and that carries the function in barley or 3/ that its regulation appears later than 3 hours after nitrate treatment. A combined situation has been observed in wheat, where the closest ortholog of *AtHRS1* was regulated but at a later stage than in Arabidopsis, while a more distant ortholog was rapidly regulated (Dissanayake et al., 2019). Such a case could indicate a molecular divergence linked to evolution and/or domestication, as a common function might be carried by relatively distant orthologs rather than by close orthologs. In Arabidopsis, *AtHRS1* regulation in response to nitrate is known to depend on AtPHR1 transcription factor in a phosphate deficiency context (Maeda *et al.*, 2018). *AtPHR1* expression is not governed by Pi status (Rubio *et al.*, 2001). *AtPHR1* and one of its paralogs (*AtPHL1*) are slightly up-regulated in Arabidopsis as well as its unique ortholog in Brachypodium, but the barley ortholog is insensitive (Table 2). This difference between barley and the 2 other species recalls and is in agreement with the case of HSR1/HHO OGp. It should be noted that *AtPHL1* expression was not seen as regulated by nitrate in a published study (Medici *et al.*, 2019). This regulation has thus

to be confirmed by RT-qPCR in our conditions, but the fact that Brachypodium ortholog is also regulated suggest 1/ that genes of this OGp are indeed responsive to nitrate and 2/ a putative role of PHR1 in regulating genes involved in nitrate response. However, *Atphr1 Atphl1* double mutant is still able to induce *AtNRT1.1* and *AtNiR* in response to nitrate (Medici et al., 2019) which suggest that AtPHR1 does not have a role in nitrate response. Identifying PHR1 targets through a transcriptome in this mutant genetic background could help us identify potential target of the transcription factors among nitrate-responsive genes. This could add another layer on N-P crosstalk regulation, which would be conserved between Arabidopsis and Brachypodium, but not in barley. In Arabidopsis, PHR1 is also known to induce expression of miR399 which represses PHO2, an E2 ubiquitin conjugase responsible for phosphate transporters degradation (Bari *et al.*, 2006; Liu *et al.*, 2012; Park *et al.*, 2014). Moreover, *AtPHO2* has been described as induced by nitrogen starvation even in presence of phosphate (Medici et al., 2019). In our transcriptome, *AtPHO2* and its ortholog in barley are down-regulated which seems logical since it has been probably induced by our nitrogen starvation treatment and then, nitrate treatment repressed its expression since there is no phosphate starvation. In Brachypodium, *AtPHO2* ortholog is not regulated, suggesting either a different mechanism or a low level of regulation, which would need to be confirmed by RT-qPCR.

To finish, BT2 is a member of the Bric-a-Brac/Tramtrack/Broad family of scaffold proteins known to play an essential role in female and male gametophyte (Gingerich *et al.*, 2007; Robert *et al.*, 2009) and also to be a major **actor of NUE** (Araus *et al.*, 2016). Our results show that both *AtBT1* and *AtBT2* are highly induced in response to nitrate treatment, as well as their orthologs in *Pooideae* (Table 2). Interestingly, barley displays 3 paralogs that are all induced, suggesting an impact of domestication on this gene family. On the other hand, Brachypodium, which grows on poor soil, has only one member.

Finally, those Arabidopsis based lists give a general vision of best characterized actors of nitrate response. Globally, nitrate response seems well conserved between Arabidopsis and *Pooideae* based on core genes, such as enzymes required for nitrate assimilation and several regulators like LBD. However, some differences in regulation can be pointed out suggesting a partial conservation of the gene regulation between Arabidopsis, Brachypodium and barley. This supports the importance of studies on cultivated species to identify the best candidate that could enhance cereals nitrate response.

Analysis of biological processes regulated by nitrate and belonging to tri-species OGps

The aim of this part is to combine DEG results with OGps by analyzing processes responding to nitrate within OGps tri-species subset of genes. This way, we will be able to identify specificities and generalities of nitrate response among genes that are conserved between the three species. In other words, divergence between species will be due to regulation differences and not due to orthologs absence. To do so, we performed a combined analysis using gene ontology and orthology datas. This combination allows us to identify enriched Gene Ontology biological processes belonging to tri-species OGps and to compare gene regulations within our three species of interest. Moreover, it will allow to verify the enrichment specificities since all the genes belonging to the OGp will appear and, especially for Brachypodium and barley, thus potential orthologs or paralogs that are not annotated in the GO term. Finally, it will enable us to identify conserved and divergent regulated actors of nitrate response. This will give us clues for understanding evolution of nitrate response and also, it can lead to identification of actors more specific of *Pooideae* that will be good candidates for improving NUE.

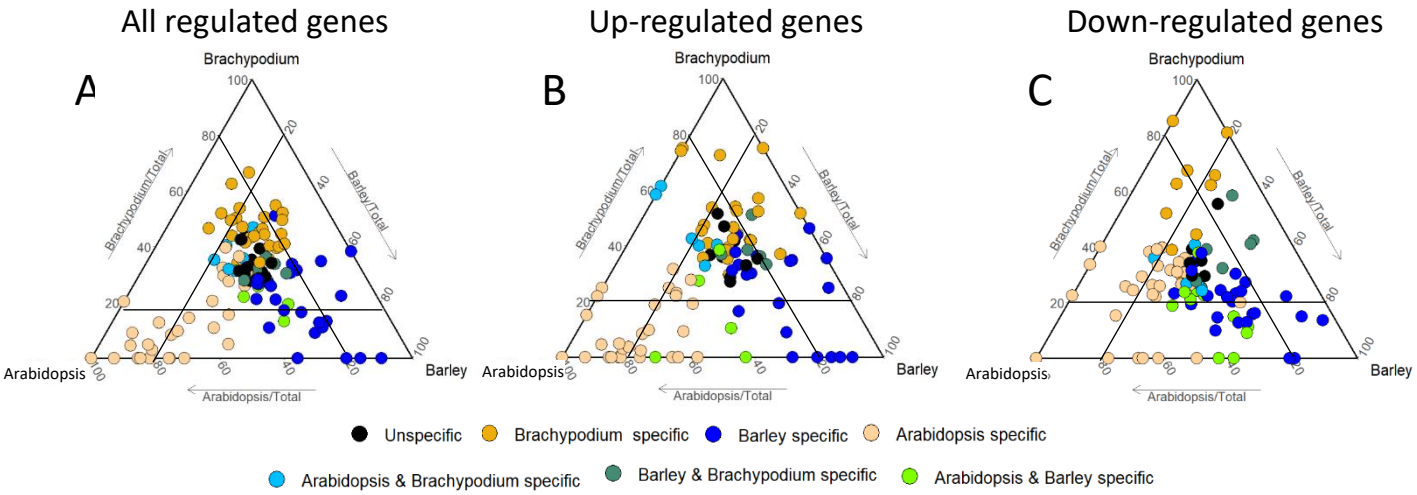

Figure 7: Gene Ontology (GO) comparative enrichment analysis for Arabidopsis, Brachypodium and barley after 1mM nitrate treatment (combination of 1h30 and 3h) for DEGs belonging to tri-species OGps. Ternary diagrams represent the distribution of the 50 best enriched GO terms per species (giving totals of 109, 93, 84 GO terms for A, B and C, respectively, due to overlap between the species). Each axes represent the Normalized Enrichment Score for one species and each point represents a GO term. Colors indicate for which species the GO term is significantly enriched based on ElimFisher test (pval<0,05). Internal bars represent a 20% threshold which is used for comparing enrichment scores between species. (A) biological process GO terms considering all the DEGs from tri-species OGps; (B) biological process GO terms considering only the up-regulated DEGs; (C) biological process GO terms considering only down-regulated DEGs. Gene ontology terms, their enrichment scores and their p-values were generated using topGO R package. Complete data lists are available in Supplementary Tables S7.

For this analysis, we used a very similar pipeline as for the analysis of whole DEG sets (Figure 3). However, the input DEG lists and the Ensembl databases were restricted to genes belonging to tri-species OGps. This way, we will be able to compare gene regulations within common pathways (at least one ortholog exists in other species). Figure 7 represents ternary diagrams of Normalized Enrichment Scores while considering all DEGs (Fig 7A), only up-regulated DEGs (Fig 7B) or down-regulated genes (Fig 7C). Similarly as in Figure 4, the majority of GO terms are found equally enriched in our three species since they clustered in the center of the diagram. However, a non-negligible number of GO terms are found more enriched in one or two species, in particular, many GO terms specific to Arabidopsis, especially when analyzing the whole DEG set or restricting to the up-regulated DEGs. Interestingly, all combinations of two species are associated with an enrichment in some biological processes. Based on these diagrams, we selected a subset of GO terms for detailed analysis at the gene level using our orthology data.

Among common enriched GO terms between Arabidopsis, Brachypodium and barley, 3 GO terms related to nitrate are found: "response to nitrate" (GO:0010167), "nitrate transport" (GO:0015706) and "cellular response to nitrate" (GO:0071249). First, it means that genes belonging to the global nitrate transport, assimilation and signaling pathway are conserved within our three species, since they belong to common OGps. Then, the fact that those genes are found regulated in response to nitrate in our three species shows that core part of nitrate response seems conserved between Arabidopsis and *Pooideae*. To verify this, we extracted genes annotated in those GO terms that belong to commons OGps (Supplementary Table S8). Majority of genes present in those GO terms are also in Vidal et al. 2020 list.

A

At&Hv spe	GO:0006995 cellular response to nitrogen starvation																			
OGp name	Arabidopsis gene and/or family names representative of the orthogroup	At						Bd						Hv						
OGp0012489	*DEGRADATION OF UREA 3 (DUR3)*	-2,25						-2,88						-2,69						
OGp0012092	*ALLANTOINASE (ALN)*	-1,22						-1,56						-0,65						
OGp0011214	*AUTOPHAGY 8H (APG8H/ATG8I)*	-0,5						-0,53						-0,5						
OGp0001713	*CATALASE (CAT3/SEN2) (CAT1) (CAT2)*	-0,6		1,124				0,679	2,871					1,114						
OGp0006194	*AUTOPHAGY 8C (ATG8C)*	-0,58												-0,49						
OGp0007681	*GENERAL REGULATORY FACTOR 3 (GRF3)*								0,657					0,536						
OGp0004315	*AUTOPHAGY 10 (ATG10)*							-0,56												
OGp0006772	*SUGAR WILL EVENTUALLY BE EXPORTED (SWEET16) (SWEET17)*													-0,77						
OGp0007236	*ABC1-LIKE KINASE RELATED TO CHLOROPHYLL DEGRADATION AND OXIDATIVE STRESS 1 (ACDO1)*													-0,45						
OGp0011120	*AUTOPHAGY 6 (ATG6)*													-0,4						
OGp0012333	*AUTOPHAGY 5 (ATG5)*																			
OGp0012948	*AUTOPHAGY 7 (ATG7)*																			
OGp0001006	*COPPER AMINE OXIDASE ALPHA (CUAO α) (CUAOα2) (CUAOα3) (CUAOα)*																			

	At	Bd	Hv
UP/tot	5%	19%	10%
DOWN/tot	26%	25%	35%
DEGs/tot	32%	44%	45%

B

At&Bd spe	GO:0003333 amino acid transmembrane transport																			
OGp name	Arabidopsis gene and/or family names representative of the orthogroup	At						Bd						Hv						
OGp0000529	*LYSINE HISTIDINE TRANSPORTER 2 (LHT2); AMINO ACID TRANSPORTER-LIKE PROTEIN 2 (AATL2); (LHT1)*			-1,2					-0,94								-1,05			
OGp0000758	*AMINO ACID PERMEASE 3 (AAP3) (AAP2) (AAP4)*		-1,09	-1,15					-1,63								-1,45			
OGp0003040	*AMINO ACID TRANSPORTER 1 (AAT1)*	-2,18						-1,3								-0,96				
OGp0004800	*Transmembrane amino acid transporter family protein (AT5G41800)*	-1,05						-1,01	-2,42					-0,63	-2,28					
OGp0012722	*AMINO ACID PERMEASE 6 (AAP6)*	-1,24						-1,75						0,56						
OGp0010057	*Transmembrane amino acid transporter family protein (AT2G41190)*	0,621						0,898												
OGp0012770	*GLUTAMINE DUMPER 3 (GDU3)*	-0,87						1,314												
OGp0001664	*CATIONIC AMINO ACID TRANSPORTER 8 (CAT8) (CAT5)*	-0,41												-0,73	-1,97					
OGp0000764	*LIKE AUXIN RESISTANT 2 (LAX3) (LAX2); AUXIN RESISTANT 1 (AUX1); (LAX1)*		-0,56		-0,54										0,374					
OGp0002730	*Transmembrane amino acid transporter family protein (AVT3B) (AT4G38250) (AVT3)*							0,794						1,177						
OGp0009451	*Transmembrane amino acid transporter family protein (AT1G25530)*							-0,9						-0,8						
OGp0002892	*AROMATIC AND NEUTRAL TRANSPORTER 1 (ANT1)*							2,033								-1,22				
OGp0002690	*Transmembrane amino acid transporter family protein (AT2G39130) (AT3G54830)*	0,815	8,502																	
OGp0004060	*PROLINE TRANSPORTER 3 (PROT3) (PROT1) (PROT2)*		-0,97																	
OGp0009517	*Transmembrane amino acid transporter family protein (AT1G80510)*	-0,53																		
OGp0003590	*USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 19 (UMAMIT19) (UMAMIT18) (UMAMIT17)*	-3,57	1,322	-0,83																
OGp0004006	*Transmembrane amino acid transporter family protein (AT2G40420) (AT3G56200)*							1,152												
OGp0009061	*MITOCHONDRIAL BASIC AMINO ACID CARRIER 2 (MBAC2)*							1,442												
OGp0011133	*PLANT UNCOUPLING MITOCHONDRIAL PROTEIN 1 (PUMP1)*							0,89												
OGp0002889	*Transmembrane amino acid transporter family protein (AT3G30390) (AT5G38820)*							-0,53												
OGp0007694	*A BOUT DE SOUFFLE (BOU)*							-0,67												
OGp0009812	*Transmembrane amino acid transporter family protein (AT1G08230)*							-0,65												
OGp0012188	*Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein (ATOEP16-S)*							-1,22												
OGp0006617	*BIDIRECTIONAL AMINO ACID TRANSPORTER 1 (BAT1)*														-0,85					
OGp0013238	*AMINO ACID PERMEASE 7 (AAP7)*													-0,35						
OGp0006795	*CATIONIC AMINO ACID TRANSPORTER 7 (CAT7)(CAT6)*																			
OGp0009410	*Transmembrane amino acid transporter family protein (AT1G47670)*																			
OGp0006505	*OUTER PLASTID ENVELOPE PROTEIN 16-L (ATOEP16-L)*																			
OGp0010256	*Tryptophan/tyrosine permease (AT2G33260)*																			
OGp0010448	*Mitochondrial substrate carrier family protein (MBAC1)*																			
OGp0012453	*Tryptophan/tyrosine permease (AT5G19500)*																			
OGp0013170	*DICARBOXYLATE TRANSPORT 2.1 (DIT2.1)*																			

	At	Bd	Hv
UP/tot	8%	15%	6%
DOWN/tot	28%	24%	21%
DEGs/tot	36%	39%	26%

Table 3.1: Gene nitrate-response in tri-species OGps belonging to significantly enriched nitrogen-related GO terms (part 1). GO terms "cellular response to nitrate starvation" (A), "amino acid transmembrane transport" (B), "cystein biosynthetic process from serine" (C, next page) and "leaf senescence" (D, next page) were identified as specific of Arabidopsis-barley, Arabidopsis-Brachypodium, Brachypodium-barley and Arabidopsis subgroups, respectively (indicated at the top of each table). Genes associated to those GO terms and belonging to tri-species OGps were extracted. Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Tables below the OGp lists show the proportions of regulated genes among the considered genes for each species.

Analysis of Nitrogen metabolism enriched GO terms

Besides GO terms directly linked to nitrate, we identified four other GO terms that are related to nitrogen metabolism, based on the ternary diagrams (Figure 7 and Supplementary Table S7; Table 4): "cellular response to nitrogen starvation" (GO:0006995) which is more enriched in Arabidopsis and barley, "amino acid transport" (GO:0003333) which is more enriched in Arabidopsis and Brachypodium, "cysteine biosynthetic process from serine" (GO:0006535) which is more enriched in Brachypodium and barley and "leaf senescence" (GO:0010150) which appears more enriched in Arabidopsis. Annotated genes for these GO terms were extracted and associated with OGps.

"Cellular response to nitrogen starvation" biological process was expected to be highly down-regulated in response to nitrate, which was confirmed by heatmap representing this GO term (Table 3.1A). According to ternary diagram, this GO term was more enriched in Arabidopsis and barley. However, when considering the list of annotated genes in this category plus their orthologs, this GO term appears regulated in all three species (Table 3.1A). The discrepancy between the two analyses is likely due to an inequal annotation of the species in GO databases and illustrates the importance of crossing GO analysis to OGps. Response to nitrogen starvation seems globally conserved, even if more genes are repressed in barley. This could suggest differences in the strategy to respond to nitrogen starvation in barley.

C

Bd&Hv spe	GO:0006535 cysteine biosynthetic process from serine									
OGp name	Arabidopsis gene and/or family names representative of the orthogroup	At					Bd		Hv	
OG0001079	*CYSTEINE SYNTHASE D1 (CYSD1); O-ACETYLSERINE (THIOL) LYASE (OAS-TL) ISOFORM A2 (OASA2) (OASA1); (CYSD2); L-CYSTEINE DESULFHYDRASE 1 (DES1)*						1,502			0,552
OG0006408	*SERINE ACETYLTRANSFERASE (SAT3.1) (SAT3.2)*						1,057		0,644	
OG0011030	*CYSTEINE SYNTHASE 26 (CS26)*	0,815								
OG0006961	*CYSTEINE SYNTHASE C1 (CYSC1)*									1,81
OG0004781	*SERINE ACETYLTRANSFERASE 1.1 (SERAT1.1)*									
OG0008788	*Pyridoxal-5'-phosphate-dependent enzyme family protein (AT1G55880)*									

	At	Bd	Hv
UP/tot	3%	44%	44%
DOWN/tot	0%	0%	0%
DEGs/tot	3%	44%	44%

D

At spe	GO:0010150 leaf senescence													
OGp name	Arabidopsis gene and/or family names representative of the orthogroup	At						Bd			Hv			
OGp0000971	*LATE EMBRYOGENESIS ABUNDANT 3 (LEA3) (LEA38/SAG21); DROUGHT-INDUCED 21 (DI21)*	4,281	3,624						3,073			2,069		
OGp0002411	*MILDEW RESISTANCE LOCUS O 2 (MLO2) (MLO6) (MLO12)*			-1,36				-0,67			-0,35			
OGp0004211	*ASPARAGINYL ENDOPEPTIDASE 1 (AEP1)(AEP3)*	-0,99	-1,9					-1,59			-1,68	-1		
OGp0000282	*NAC DOMAIN CONTAINING PROTEIN 2 (ANAC002) (ANAC018) (ANAC025) (NAC32) (NAC47) (NAC56) (NAC81) (NAC102)*	-0,69	-2,31		-2,21		-0,83	-0,92		0,913		-1,88		-1,7
OGp0000533	*NAC DOMAIN CONTAINING PROTEIN (NAC31/CUC3) (NAC39) (NAC3) (NAC4) (NAC2) (NAC98/CUC2) (NAC100)*		-0,79	-1,77	2,054				-0,56		-1,43		-1,06	
OGp0002657	*ABSCISIC ACID RESPONSIVE ELEMENT-BINDING FACTOR 1 (ABF1) (ABF4) (ABF3)*			1,163					-1,57		-0,84			
OGp0001696	*Matrixin family protein (AT3-MMP) (AT5-MMP) (AT2-MMP)*		0,594					1,132						
OGp0012840	*LA RELATED PROTEIN 1B (LARP1B)*	1,198						0,397						
OGp0005517	*AUXIN RESPONSE FACTOR 1 (ARF1)*	-0,46						-0,37	-0,41					
OGp0000815	*GLUTAMINE SYNTHETASE (GLN1.5) (GLN1.2) (GLN1.3) (GLN1.4) (GLN1.1)*		1,292	-1,22	-3,16	-1,22		-1,12	0,888	0,532				
OGp0001776	*2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (SRG1)*					-1,13					-0,47			
OGp0001656	*ORGANIC CATION/CARNITINE TRANSPORTER 6 (OCT6) (OCT3) (OCT1) (OCT2) (OCT5)*			-4,81							-0,77			
OGp0002001	*SENESCENCE-ASSOCIATED SUBTILISIN PROTEASE (SASP)*	-0,85											-1,14	
OGp0004682	*PLASMA-MEMBRANE ASSOCIATED CATION-BINDING PROTEIN (PCAP1/MDP25) (PCAP2/MAP18)*	-0,84									-0,35			
OGp0004710	*AUXIN RESPONSE FACTOR 2 (ARF2)*	-0,74										-0,45	-0,51	
OGp0007711	*DOWNY MILDEW RESISTANT 6 (DMR6)*	-1,7									-1,11			
OGp0004620	*TRICHOME BIREFRINGENCE-LIKE 18 (TBL18) (TBL17)*	-0,36									0,602			
OGp0004545	*WRKY DNA-BINDING PROTEIN (WRKY22) (WRKY29)*								1,62		1,204			
OGp0006912	*TEOSINTE BRANCHED 1, CYCLOIDEA, PCF (TCP)-DOMAIN FAMILY PROTEIN 20 (TCP20)*							0,598			0,724			
OGp0007531	*MYB HYPOCOTYL ELONGATION-RELATED (MYBH/KUODA1)*							1,046			-0,6			
OGp0001652	*HIGHLY ABA-INDUCED PP2C GENE (HAI2) (HAI3) (AHG3/SAG113) (HAI1)*			1,013										
OGp0003646	*TEOSINTE BRANCHED 1, CYCLOIDEA AND PCF TRANSCRIPTION FACTOR (TCP3) (TCP10) (TCP4/MEE35)*			0,519										
OGp0003943	*ASPARTATE AMINOTRANSFERASE (ASP4) (ASP3/YLS4) (ASP2)*			0,435										
OGp0006371	*RNA-binding (RRM/RBD/RNP motif) family protein*		0,528											
OGp0007093	*OUTER MEMBRANE 47 (OM47)*	0,588												
OGp0007323	*ARABIDOPSIS ABNORMAL SHOOT3 (ABS3); EARLY LEAF SENESCENCE 1 (ELS1)*	1,182	2,109											
OGp0000290	*MAP KINASE (MPK11)(MPK13)(MPK6)(MPK12)(MPK3) (MPK10) (MPK4)(MPK5)*					-0,9								
OGp0000818	*EPITHIOSPECIFIER PROTEIN (ESP/TASTY); NITRILE SPECIFIER PROTEIN 2 (NSP2) (AT3G07720) (NSP3) (NSP1) (NSP4) (AT3G59620) (NSP5)*			-0,78										
OGp0000761	*MAP KINASE KINASE 7 (MKK7) (MKK9) (MKK8)*		-0,92											
OGp0004187	*CDPK-RELATED KINASE 3 (CRK3)*	-0,62												
OGp0004117	*AUTOPHAGY 18A (AtATG18a); PEROXISOME UNUSUAL POSITIONING 2 (PEUP2)*		-0,4											
OGp0004167	*LEUCYL AMINOPEPTIDASE (LAP1) (AT4G30910) (LAP2)*		-0,67											
OGp0006864	*NAC DOMAIN CONTAINING PROTEIN 46 (NAC046) (NAC087)*	-1,45	-1,02											
OGp0009566	*PHOSPHOLIPID STEROL ACYL TRANSFERASE 1 (PSAT1)*	-1,2												
OGp0008938	*HISTIDINE KINASE 3 (HK3)*	-0,64												
OGp0002919	*SENESCENCE ASSOCIATED GENE 20 (SAG20) (AT5G01740)*	-0,78	5,883											
OGp0002720	*AGAMOUS-LIKE 20 (AGL20) (AGL72) (AGL71) (AGL42)*	-0,65			0,612									
OGp0005537	*NITRATE TRANSPORTER (NRT1.5/NPF7.3) (NRT1.8/NPF7.2)*	0,699	-1,21											
OGp0005657	*RELA/SPOT HOMOLOG 3 (RSH3)(RSH2)*	-0,73	0,598											
OGp0006375	*TCP DOMAIN PROTEIN (TCP9) (TCP19)*	-1,71	0,804											
OGp0002599	*DONGLE (DGL); DAD1-LIKE LIPASE 5 (DALL5)*								1,976					
OGp0009712	*TRIPHOSPHATE TUNNEL METALLOENZYME 1 (TTM1)*							0,579						
OGp0002073	*ENHANCED DISEASE RESISTANCE 2 (EDR2)*							-0,46						
OGp0002581	*OROSOMUCOID-LIKE 1 (ORM1) (ORM2)*							-0,99						
OGp0012756	*SENESCENCE-ASSOCIATED GENE 29 (SAG29/SWEET15)*							-0,85						
OGp0001066	*POWERDRESS (PWR)*										0,365			
OGp0010353	*CURLY LEAF (CLF)*										0,432			
OGp0010515	*RUBISCO ACTIVASE (RCA)*										1,139			
OGp0001052	*SIGNAL RESPONSIVE 1 (SR1/CAMTA3)*											-0,49		
OGp0003704	*ORESARA15 (ORE15)*										-0,7			
OGp0004185	*NAC DOMAIN CONTAINING PROTEIN 42 (NAC042/JUB1)*											-1,01		
OGp0006707	*AUTOPHAGY 2 (ATG2)*										-0,59			
OGp0006749	*PHYTOALEXIN DEFICIENT 4 (PAD4); SENESCENCE-ASSOCIATED GENE 101 (SAG101)*										-0,73			
OGp0007405	*SET DOMAIN-CONTAINING PROTEIN 10 (SDG10/SWN)*											-0,55		
OGp0011299	*BIG BROTHER (BB)*										-0,39			
OGp0000122	*SENESCENCE-ASSOCIATED GENE 12 (SAG12)*													
OGp0000681	*WRKY DNA-BINDING PROTEIN (WRKY41)(WRKY53)(ATWRKY30)*													
OGp0000787	*NAC DOMAIN CONTAINING PROTEIN 29 (NAC029)*													
OGp0001688	*INDOLE-3-ACETIC ACID INDUCIBLE 17 (IAA17/AXR3) (IAA16) (IAA7) (IAA14)*													
OGp0001972	*KH domain-containing protein / zinc finger (CCCH type) family protein (KHZ1) (KHZ2)*													
OGp0002044	*GLUCAN SYNTHASE-LIKE 5 (GSL05)*													
OGp0002529	*MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE (MAPKKK18) (MAPKKK17) (MAPKKK16) (MAPKKK15)*													
OGp0002874	*HISTONE DEACETYLASE (HDA17)(HDA10)(HDA9)*													
OGp0003023	*UBIQUITIN PROTEIN LIGASE 5 (UPL5)*													
OGp0002397	*Senescence regulator (AT1G29640) (AT2G34340) (AtS40-3) (AT5G45630)*													
OGp0003099	*TCP DOMAIN PROTEIN (TCP17) (TCP5)*													
OGp0003006	*PSEUDO-RESPONSE REGULATOR 2 (PRR2); GOLDEN2-LIKE 2 (GLK2)*													
OGp0001648	*AGP GALACTOSYLTRANSFERASES5 (GALT5) (GALT6)*													
OGp0003680	*SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE 1 (SAUL1/PUB44); PLANT U-BOX 43 (PUB43)*													
OGp0004829	*HYPERSENESCENCE 1 (HYS1)*													
OGp0006644	*GBF'S PRO-RICH REGION-INTERACTING FACTOR 1 (GPRI1)*													
OGp0006233	*UBP1-ASSOCIATED PROTEIN 2A (UBA2A)*													
OGp0006770	*METHYLENE BLUE SENSITIVITY (MBS1)(MBS2)*													
OGp0007386	*DMR6-LIKE OXYGENASE 2 (DLO2) (DLO1)*													
OGp0007503	*ETHYLENE INSENSITIVE 2 (EIN2)*													
OGp0006777	*ARGININE-TRNA PROTEIN TRANSFERASE (ATE2) (ATE1/DLS1)*													
OGp0007515	*MECHANOSENSITIVE CHANNEL OF SMALL CONDUCTANCE-LIKE 10 (MSL10) (MSL9)*													
OGp0008915	*NUCLEOSIDE HYDROLASE 2 (NSH2/URH2)*													
OGp0009582	*MEDIATOR 2 (MED2)*													
OGp0009657	*senescence-associated family protein (AT1G66330)*													
OGp0009865	*SWEETIE (SWEETIE)*													
OGp0011522	*SNF1 KINASE HOMOLOG 10 (KIN10)*													
OGp0011421	*MEDIATOR28 (MED28)*													
OGp0010964	*PROTEIN ACYLTRANSFERASE 14 (PAT14)*													
OGp0011534	*CHARGED MULTI-VESICULAR BODY PROTEIN 7 (CHMP7)*													
OGp0012201	*RELA/SPOT HOMOLOG 1 (RSH1)*													
OGp0011632	*CERAMIDASE (ATACER)*													
OGp0011794	*AUTOPHAGY-RELATED 11 (ATG11)*													
OGp0012074	*GENETIC VARIANTS IN LEAF SENESCENCE (GVS1)*													
OGp0011882	*MULTIUBIQUITIN CHAIN BINDING PROTEIN 1 (MBP1)*													
OGp0012333	*AUTOPHAGY 5 (ATG5)*													
OGp0012948	*AUTOPHAGY-RELATED 7 (ATG7)*													
OGp0012869	*NEET GROUP PROTEIN (NEET)*													
OGp0013137	*ABC2 HOMOLOG 13 (ATH13)*													

	At	Bd	Hv
UP/tot	10%	7%	4%
DOWN/tot	18%	7%	13%
DEGs/tot	28%	15%	17%

Table 3.2: Gene nitrate-response in tri-species OGps belonging to significantly enriched nitrogen-related GO terms (part 2). See legend in Table 3.1.

Amino acids are the first molecules formed after nitrate assimilation. They are the currency of nitrogen exchange. In our transcriptome, **amino acids transporters** are mainly down-regulated in our species (Table 3.1B). According to ternary diagram (Figure 7 and Supplementary Table S7), this GO term is enriched in all the species. By considering OGps, the proportion of regulated genes is higher in Arabidopsis and Brachypodium, but is still non-negligeable in barley. Interestingly, roots amino acids transporters are mostly down-regulated in response to nitrate. This suggest that in response to nitrate, plants use their newly made amino acid for protein biosynthesis instead of transporting them throughout the plant. Once again, by analyzing the regulation at individual OGps level, we can point out some specificities: only 20% of regulated OGps (5/25) are regulated in the three species, whereas 52% and 28% are responsive in only one and two species, respectively. These specifically-responsive OGps contain some strongly regulated genes: the closest ortholog of AtANT1, known in Arabidopsis to transport aromatic amino acids, neutral amino acids, arginine and auxin (Chen *et al.*, 2001) is highly induced only in Brachypodium, and a non-characterized amino acid transporter (AT3G54830) is very highly and specifically up-regulated in Arabidopsis, suggesting that they might be key actors of nitrate response but only in specific species.

According to ternary diagram (Figure 7) GO term "**cysteine biosynthetic process from serine**" is specifically enriched in Brachypodium and barley, which is confirmed by the heatmap of common OGps (Table 3.2C). Cysteine is an amino acid containing reduced sulfur synthetized by the plant through sulfur assimilation. In our transcriptome, two enzymes involved in its biosynthesis from serine are up-regulated in Brachypodium and barley but not in Arabidopsis: serine acetyl transferase (SAT) and O-acetyl (thiol) lyase (OASTL), suggesting a higher production of cysteine in response to nitrate in

Pooideae species. In Arabidopsis, this pathway occurs in mitochondria (Hesse *et al.*, 1999). OASTL requires pyridoxal phosphate (PLP), also known as the active form of Vitamine B6, as a cofactor to ensure its function. Very interestingly, genes necessary for PLP biosynthetic process are also induced only in Brachypodium and barley (Table 9A; see below), confirming the regulation specificity to *Pooideae* of this pathway. Since cysteine is the precursor of many molecules such as Glucosinolates, Camalexin, Ethylene and Glutathione, dosage of those metabolites in response to nitrate could help us to determine the effect of this putative over production of cysteine in *Pooideae*. As mentioned before, cysteine is a product of sulfur assimilation. Thus, the question arises concerning the sulfur response in our transcriptome. No direct GO terms related to sulfur assimilation is found enriched but "sulfur compound metabolic process" (GO:0006790) appears evenly enriched in our three species (Figure 7A and Supplementary Table S7A), suggesting that there is no imbalance of sulfur availability in our three species. However, here, specifically regulated enzymes are only those necessary for the biosynthesis of cysteine from serine, suggesting again that the upper stream of sulfur assimilation is not the cause of the putative imbalanced cysteine production in *Pooideae* versus Arabidopsis. To confirm an imbalance level of cysteine, again a dosage could help answering this question.

To finish on nitrogen related processes, senescence is the process that enables the recycling of molecules from source organs to sink organs following ageing. Senescence have been extensively described in leaves and only few studies are available in roots. In our transcriptome, exclusively performed on roots tissues, "**leaf senescence"** GO term is found enriched and more specifically in Arabidopsis according to ternary diagram (Figure 7A and Supplementary Table S7A). Table 3.2D shows that this GO term is effectively slightly more enriched in Arabidopsis and that most of the genes are down-regulated. This suggests that after N starvation, the root senescence pathway is activated, before being suppressed after the nitrate treatment since a nitrogen source

becomes available again. Once again, there is about 20 OGps (38% of regulated OGps) that are exclusively regulated in Arabidopsis, highlighting a potential divergence in the response of root senescence to nitrate availability between Arabidopsis and *Pooideae*.

Analyzing nitrogen related pathways allows us to confirm that before nitrate treatment, plants activated their nitrogen starvation response as well as senescence-related genes. It suggests that the nitrate response studied here correspond at least partially to a recovery from nitrogen starvation. This observation is consistent with a repression of genes involved in amino acid transport, that might have been induced by starvation as well. Nitrate treatment thus appears to reduce recycling processes and stimulate the use of newly synthetized amino acid for the biosynthesis of protein and other amine molecules. Interestingly, a *Pooideae*-specific activation of cysteine biosynthesis from serine is seen in response to nitrate. This is supported by a specific induction in these two species of PLP biosynthesis, which is required as a cofactor for this cysteine biosynthesis. This last pathway deserves extended experiments to be confirmed and explained.

Bd spe	GO:0009686 gibberellin biosynthetic process	At	Bd	Hv
OGp name	Arabidopsis gene and/or family names representative of the orthogroup			
OG0007247	***GIBBERELLIN 20 OXIDASE (GA20OX1) (GA20OX2)***	-4	-2	1
OG0003860	*GIBBERELLIN 2-OXIDASE 1 (GA2OX1)*	2		-1
OG0006284	*2-oxoglutarate and Fe(II)-dependent oxygenase superfamily (AT2G06960); GERMINATION INSENSITIVE TO ABA MUTANT 2 (GIM2)*	-3		2
OG0000991	*GA REQUIRING 2 (GA2)*		-1	-1 -1 -1
OG0001482	*BETA HLH PROTEIN 93 (bHLH093)*		-1	-1
OG0000321	*HOMEOBOX PROTEIN (HB31) (HB21) (HB24) (HB28) (HB22) (HB25)*		-2	
OG0002038	*GA REQUIRING 1 (GA1)*		-5	
OG0003150	*GA REQUIRING 3 (GA3)*		-1 -2 -2	
OG0001749	*CYTOCHROME P450, FAMILY 88, SUBFAMILY A, POLYPEPTIDE 3 (CYP88A3); ENT-KAURENOIC ACID HYDROXYLASE 2 (KAO2)*		-4 -1 1	
OG0004702	*TUDOR-SN PROTEIN (TUDOR1) (TUDOR2)*			0
OG0002069	*HOMEOBOX GENE 1 (ATH1)*			
OG0002358	*GIBBERELLIN 2-OXIDASE (GA2OX6) (GA2OX4)*			
OG0002940	*FUSCA3 (FUS3)*			
OG0007316	*GIBBERELLIN 2-OXIDASE 8 (GA2OX8)*			
OG0013085	*CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1)*			

	At	Bd	Hv
UP/tot	3%	5%	12%
DOWN/tot	9%	31%	12%
DEGs/tot	12%	36%	24%

Table 4: Gene nitrate-response in tri-species OGps belonging to "gibberellin biosynthetic process" GO term. Genes associated to this GO term and belonging to tri-species OGps were extracted. Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Table below the OGp list show the proportion of regulated genes among the considered genes for each species.

Gibberellin biosynthesis pathway is more regulated in Brachypodium and barley

The "response to gibberellin (GA)" GO term were found enriched in our three species (Figure 7A and Supplementary Table S7A) and confirmed by heatmap on OGps (data not shown). However, "**gibberellin biosynthetic process**" GO term is found specifically regulated in Brachypodium according to ternary diagram (Figure 7A and Supplementary Table S7A) but appeared also regulated in Barley according to heatmap (Table 4). Indeed, 36% and 24% of OGps are respectively specifically regulated in Brachypodium and Barley against only 12% for Arabidopsis. These observations suggest that GAs are part of nitrate response in our three species since the GO term "response to gibberellin" is regulated in our three species but on the other hand, the GA biosynthesis is differentially regulated by nitrate in *Pooideae* compared to Arabidopsis. All the genes will not be fully detailed here, but the case of *GA20OX-1* expression is interesting. *GA20OX-1* encodes an enzyme involved in the last steps of gibberellin biosynthesis. GAs are involved in shoots growth and more specifically in stem elongation (Rieu *et al.*, 2008). In rice and other domesticated species, this gene is also known as *SD1* for *SEMI-DWARF1* which have been one of the key gene of Green Revolution (Jia *et al.*, 2009). Indeed, domesticated plants such as rice, barley and wheat, carry a mutation in this gene leading to a semi-dwarf phenotype that have been selected during domestication to avoid lodging. Interestingly, in our root transcriptomes, *AtGA20OX1* and its closest ortholog in Brachypodium are highly repressed in response to nitrate, while it is up-regulated in barley. *GA20OX* are also involved in root development, at least for *GA20OX-4* (Rieu *et al.*, 2008). To our knowledge, there is no defined role of *GA20OX-1* in root development in a nitrate response context. Our findings reveal a potential negative role of *GA20OX-1* in root development in response to nitrate in Arabidopsis and Brachypodium, since its expression is highly repressed by nitrate addition. Interestingly, this high repression in Arabidopsis cannot be due to a high induction during N starvation since its expression is not regulated by N starvation (manually check in Chardon et al., 2020; Krapp et al., 2011).

At&Hv spe	GO:0016036 cellular response to phosphate starvation	At						Bd						Hv						
OGp name	**Arabidopsis gene and/or family names representative of the orthogroup**																			
OGp0000518	*PEP(PHOSPHOENOLPYRUVATE) CARBOXYLASE (PPC1) (PPC2) (PPC3)*		1,348	1,363				3,65			0,627			4,509		1,807				
OGp0003124	*SULFOQUINOVOSYLDIACYLGLYCEROL 2 (SQD2)*	-0,93								-0,75				-0,41						
OGp0000226	*PHOSPHATE TRANSPORTER 5 (PHT5) (PHT1.4) (PHT1.7) (PHT1.1) (PHT1.3) (PHT1.2)*		-0,75		-0,98		-1,6	-1,44	-1,39			0,845			-2,56					-0,93
OGp0001713	*CATALASE (CAT3)(CAT1) (CAT2)*	-0,6		1,124				0,679	2,871					1,114						
OGp0001937	*PHR1-LIKE/PHOSPHATE STARVATION RESPONSE (PHL4) (PHR1) (PHL1)*			0,596	0,704			1,02												
OGp0002366	*HYPERSENSITIVITY TO LOW PI-ELICITED PRIMARY ROOT SHORTENING 1 (HRS1 /NIGT1) (HHO3) (HHO2) (HHO1)*	4,011	3,123	2,242	6,503			2,076												
OGp0007363	*PHOSPHOENOLPYRUVATE CARBOXYKINASE (PCK1)(PCK2)*		2,223					2,724												
OGp0003815	***PHOSPHOETHANOLAMINE/PHOSPHOCHOLINE PHOSPHATASE 1 (PEPC1); PHOSPHATE STARVATION-INDUCED GENE 2 (PS2/PPsPase1)***	-0,96	-1,12					-1,25												
OGp0002585	*WRKY DNA-BINDING PROTEIN (WRKY6) (WRKY42) (WRKY31)*		2,314						-0,8											
OGp0003773	*ALUMINUM-ACTIVATED MALATE TRANSPORTER 3 (ALMT3) (ALMT9)*		0,68											1,668						
OGp0002970	*PHR1-LIKE (PHL2) (PHL3); UNFERTILIZED EMBRYO SAC 16 (UNE16)*	-0,55	-0,91											-0,38						
OGp0010342	*PHOSPHATE 2 (PHO2)*	-0,65												-0,48						
OGp0006205	*SABRE (SAB); HYPERSENSITIVE TO PI STARVATION 4 (HPS4)*	0,455												-0,46						
OGp0007681	*GENERAL REGULATORY FACTOR 3 (GRF3)*								0,657					0,536						
OGp0002015	*PHOSPHOENOLPYRUVATE CARBOXYLASE KINASE 2 (PPCK2)*							-0,96						-1	-1,31		-0,7	0,851		
OGp0004460	*PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 (PHF1)*	0,558																		
OGp0008796	*EXS (ERD1/XPR1/SYG1) family protein (PHO1.H1)*	1,489																		
OGp0013294	*SPX DOMAIN GENE 4 (SPX4)*	1,234																		
OGp0000980	*RIBONUCLEASE (AT1G14210) (AT1G14220) (RNS3) (RNS1)*				-3,21															
OGp0001412	*NON-SPECIFIC PHOSPHOLIPASE C (NPC3) (NPC4) (NPC5)*		-0,6																	
OGp0004644	*SULFOQUINOVOSYLDIACYLGLYCEROL 1 (SQD1)*	-0,73																		
OGp0006360	*MONOGALACTOSYL DIACYLGLYCEROL SYNTHASE (MGD3) (MGD2)*	-1,55	-1,28																	
OGp0005473	*TMO5-LIKE1 (T5L1); TARGET OF MONOPTEROS 5 (TMO5)*		-0,38																	
OGp0007140	*ETHYLENE OVERPRODUCER 1 (ETO1); ETO1-LIKE 2 (EOL2)*	-0,47																		
OGp0008938	*HISTIDINE KINASE 3 (HK3)*	-0,64																		
OGp0002735	*MYO-INOSITOL-1-PHOSPHATE SYNTHASE (MIPS1) (MIPS2) (MIPS3)*	-0,97	-1,36	0,731																
OGp0003567	*ALFIN-LIKE (AL7) (AL6)*								0,788											
OGp0009024	*VENOSA 3 (VEN3)*							0,406												
OGp0010594	*PHOSPHATASE UNDERPRODUCER 1 (PUP1)*													0,737						
OGp0004499	*uridine 5'-monophosphate synthase / UMP synthase (PYRE-F) (UMPS)*													-1,01						
OGp0007065	*PHOSPHATE 1 (PHO1)*														-0,49					
OGp0011951	*DIGALACTOSYL DIACYLGLYCEROL DEFICIENT 2 (DGD2)*													-0,99						
OGp0003102	*SAP AND MIZ1 DOMAIN- CONTAINING LIGASE1 (SIZ1)*																			
OGp0004182	*CYTOKININ RESPONSE 1 (CRE1)*																			
OGp0004279	*UDP-GLUCOSE PYROPHOSPHORYLASE (UGP1) (UGP2)*																			
OGp0005741	*LOW PHOSPHATE ROOT1 (LPR1) (LPR2)*																			
OGp0006324	*SPX DOMAIN GENE (SPX2) (SPX1)*																			
OGp0006876	*PHOSPHATIDIC ACID PHOSPHOHYDROLASE (PAH1) (PAH2)*																			
OGp0006998	*VENOSA 6 (VEN6)*																			
OGp0006717	*TRANSPORT INHIBITOR RESPONSE 1 (TIR1); AUXIN SIGNALING F BOX PROTEIN 1 (AFB1)*																			
OGp0010208	*SPX DOMAIN GENE 3 (SPX3)*																			
OGp0010914	*POLYNUCLEOTIDE PHOSPHORYLASE (PNP)*																			
OGp0011493	*PYRIMIDINE B (PYRB)*																			
OGp0011501	*PYRIMIDINE R (PYRR)*																			
OGp0013171	*putative galactose-1-phosphate uridyltransferase*																			
OGp0012490	*F-BOX PROTEIN 2 (FBX2)*																			
OGp0013174	*PHOSPHATE DEFICIENCY RESPONSE 2 (PDR2)*																			

	At	Bd	Hv
UP/tot	20%	16%	9%
DOWN/tot	23%	9%	15%
DEGs/tot	43%	24%	24%

Table 5: Gene nitrate-response in tri-species OGps belonging to "cellular response to phosphate starvation" GO term. Genes associated to this GO term and belonging to tri-species OGps were extracted. Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Table below the OGp list show the proportion of regulated genes among the considered genes for each species.

No such data are available in Brachypodium and a measurement of *BdGA20OX1* expression in response to N starvation by RT-qPCR could help to understand this regulation. And on the contrary, the mutated version of *GA20OX-1* in barley is induced by nitrate suggesting that this mutated allele might carry another function in roots.

Closer analysis on nitrate and phosphate crosstalk

As mentioned above, nitrate and phosphate responses are closely related. Recent studies on Arabidopsis (Medici *et al.*, 2019) and Rice (Hu *et al.*, 2019) show that phosphate starvation response is possible only if nitrogen is present. This seems conserved also in wheat (Dissanayake *et al.*, 2019; Medici *et al.*, 2019). GO analysis shows that the "**cellular response to phosphate starvation"** GO term was more enriched in response to nitrate in Arabidopsis and barley than in Brachypodium (Table 5 and Supplementary Table S7). By looking at gene expression considering the OGps belonging to this GO term (Table 5), we observe that this process seems actually more enriched in Arabidopsis, while it is evenly regulated between Brachypodium and barley. Besides HRS1, PHR1 and PHO2, which have already been discussed above, AtPEPC1 and AtPPsPase1, two phosphatases induced by phosphate starvation retained our attention. AtPEPC1 and AtPPsPase1 have a role in recycling lipid membrane polar head triggered by phosphate starvation (Hanchi *et al.*, 2020). In our analysis, they belong to the same OGp. Both of these genes are down-regulated in response to nitrate in Arabidopsis, as well as the closest ortholog in Brachypodium but not the two barley ones. This suggests that in Arabidopsis and Brachypodium, these enzymes might have been induced by nitrogen starvation and subsequently downregulated by nitrate application. PEPC1 and PPsPase1 are thus two interesting candidates for a new molecular player of nitrogen and phosphate response in Arabidopsis and Brachypodium as soon as this hypothesis of a regulation by nitrogen starvation is confirmed by RT-qPCR.

Table 6: Gene nitrate-response in tri-species OGps belonging to "rRNA processing GO term". Genes associated to this GO term and belonging to tri-species OGps were extracted. Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Table on the right of the OGp list show the proportion of regulated genes among the considered genes for each species.

The fact that genes involved in phosphate starvation response are regulated in response to nitrate in our three species further confirm the established link between nitrate and phosphate signal. This crosstalk appears at least partially conserved among plant species. However, the combination of transcriptomic and OGps analysis suggests a partial divergence of the mechanism between Arabidopsis and *Pooideae* species. The lower proportion of genes responding to nitrate in our considered set in Brachypodium and barley could be due to a lower quality of GO annotations in these species as compared to Arabidopsis.

rRNA processing is activated in response to nitrate almost exclusively in Arabidopsis

In several analysis of our study, "**rRNA processing**" GO term appears highly regulated in Arabidopsis. To ensure it, we made the heatmap regrouping genes belonging to this GO term and to tri-species OGps (Table 6). Results are unequivocal: genes related to rRNA processing are massively induced in Arabidopsis only. The induction is quite low, probably because these genes must have a high basal level of expression to maintain a level of protein synthesis even in difficult conditions such as nitrogen starvation. Since rRNA are necessary for ribosome activity and thus for mRNA translation, we hypothesized that the apparent Arabidopsis-specificity was due to a different timing of response between the species, Arabidopsis being at a later stage of response in our experiment. To test the hypothesis of rRNA genes being late markers of the response, we used topGO tool on a list of early-responsive Arabidopsis genes (genes induced by nitrate for maximum 20 minutes; Krouk et al., 2010). No GO term related to rRNA processing or ribosome biogenesis was enriched (data not shown), in agreement with our hypothesis. Even though experiment conditions was different as ours (before induction, plants were nitrate-starved but not nitrogen-starved, the basal medium containing 0.5mM of ammonium), it gives an indication that rRNA processing process might be induced later. Moreover, in one of the first transcriptomic analysis of nitrate response in Arabidopsis (Scheible *et al.*, 2004), genes related to protein biosynthesis were found more induced after 3h treatment than after 30 minutes treatment.

Bd spe	GO:0032482 Rab protein signal transduction																		
OGp name	**Arabidopsis gene and/or family names representative of the orthogroup**	**At**										**Bd**					**Hv**		
OGp0008929	*RAB GTPASE HOMOLOG G3B (RABG3B)*	-0,88										0,681							
OGp0005976	*RAB GTPASE HOMOLOG A3 (RABA3)*	-0,72																-0,81	
OGp0005445	*RAB GTPASE HOMOLOG A2 (RABA2B) (RABA2C)*											0,685					0,551		
OGp0002639	*RAB GTPASE HOMOLOG 5 (RABA5E) (RABA5D) (RABA5C) (RABA5B)*	-0,62																	
OGp0004769	*Ras-related small GTP-binding family protein (SGP1)*	-0,81																	
OGp0006818	*RAB GTPASE HOMOLOG C (RABC2B) (RABC2A)*	-1,09	-0,51																
OGp0000249	*ARABIDOPSIS THALIANA RAB GTPASE HOMOLOG A1A (RABA1A) (RABA1B) (RABA1I) (RABA1H) (RABA1G) (RABA1E) (RABA1D) (RABA1C) (RABA1F)*					0,706		0,352		-0,94									
OGp0001966	*RAB GTPASE HOMOLOG A4 (RABA4D) (RABA4B) (RABA4C) (RABA4A)*												0,757						
OGp0004539	*RAB GTPASE HOMOLOG B1B (RAB-B1B)*												0,493						
OGp0005410	*RAB GTPASE HOMOLOG B18 (RAB18)*											0,442							
OGp0006756	*RAB GTPASE HOMOLOG (RAB5C)*												0,449						
OGp0013090	*RAB GTPASE HOMOLOG A2D (RABA2D)*											0,928							
OGp0000976	*RAB GTPASE HOMOLOG G (RABG3E) (RABG3D) (RABG3C) (RABG1F)*																		
OGp0001062	*RAB GTPASE HOMOLOG 8 (RAB8A) (RAB8) (RAB8B) (RAB8C)*																		
OGp0001734	*RAB GTPASE HOMOLOG (RAB1C) (RABD2C) (RABD2B)*																		
OGp0004042	*RAB GTPASE HOMOLOG (RABH1D) (RABH1B) (RABH1E)*																		
OGp0004602	*RAB GTPASE HOMOLOG (ATRAB5B) (RAB5A)*																		
OGp0008677	*RAB GTPASE 11C (ATRAB11C)*																		
OGp0012485	*RAB GTPASE HOMOLOG A5A (RABA5A)*																		
OGp0007687	*LIGHT INSENSITIVE PERIOD1 (LIP1)*																		

	At	Bd	Hv
UP/tot	4%	21%	3%
DOWN/tot	15%	0%	3%
DEGs/tot	19%	21%	7%

Table 7: Gene nitrate-response in tri-species OGps belonging to "Rab protein signal transduction" GO term. Genes associated to this GO term and belonging to tri-species OGps were extracted. Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Table below the OGp list show the proportion of regulated genes among the considered genes for each species.

Taken together, these observations in other studies were consistent with our hypothesis that Arabidopsis response to nitrate occurs earlier than in *Pooideae*. It would thus be interesting to uncover the molecular explanation for this possible shift in the response between the species. To confirm these observations, it would be useful to perform an induction kinetics in *Pooideae* species, especially in longer times that 3 hours of treatment. A recent study on Maize and Sorgho analyzed nitrate induction upon 8 hours (Du *et al.*, 2020). Using topGO and their lists of DEGs, we did not detect any enrichment in rRNA processing pathway of ribosome biogenesis GO terms (data not shown). Thus, as an alternative explanation to a response-shift, it might be that the basal expression level of genes related to rRNA processing or their intensity of response to nitrate is different between Arabidopsis and cereals, making the detection of response harder in Brachypodium and barley. To test whether the apparent difference in rRNA response to nitrate has a functional impact, a comparison between the three species of the translation level before and after nitrate treatment could be performed.

Genes related to membrane trafficking and cell wall biogenesis are differentially regulated in response to nitrate

The GO term "**Rab protein signal transduction**" retains our attention due to its divergence of regulation between our three species. According to ternary diagram, this GO term is specifically enriched among Brachypodium up-regulated genes (Figure 7B and Supplementary Table S7B), which was confirmed by heatmap on corresponding OGps (Table 7). Surprisingly, these genes are mainly repressed in Arabidopsis and unresponsive in barley. Rab proteins, and more specifically Rab GTPase proteins, regulate membrane trafficking. In our transcriptome, several Rab GTPase are regulated and most of them belonging to RabA, B and C subfamily involved in transport of vesicles at precise membrane-bound compartments (see Woollard & Moore, 2008 for review).

A

At&Bd spe	GO:0016998 cell wall macromolecule catabolic process	At	Bd	Hv
OGp name	Arabidopsis gene and/or family names representative of the orthogroup			
OGp0009715	*ECTOPIC ROOT HAIR 1 (ERH1)*	-0,51	-0,63	
OGp0001388	*HOMOLOG OF CARROT EP3-3 CHITINASE (AT2G43580) (AT2G43590) (AT3G47540) (EP3)*	-0,98		
OGp0001442	*PATHOGENESIS-RELATED 3 (PR3)*	-0,9		
OGp0012918	*ENDO-BETA-MANNASE 7 (MAN7)*	-0,97		
OGp0010938	*XYLOGLUCAN ENDO-TRANSGLYCOSYLASE-RELATED 8 (XTR8/XTH31)*			
OGp0003839	*Chitinase family protein (AT1G02360) (AT4G01700)*		-1,57	
OGp0010553	*XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 32 (XTH32)*			-0,57
OGp0006223	*ENDO-BETA-MANNASE 2 (MAN2) (MAN5)*			
OGp0007482	*ENDO-BETA-MANNASE 6 (MAN6)*			

	At	Bd	Hv
UP/tot	7%	0%	0%
DOWN/tot	36%	14%	8%
DEGs/tot	36%	14%	8%

B

At spe	GO:0009969 xyloglucan biosynthetic process	At	Bd	Hv
OGp name	Arabidopsis gene and/or family names representative of the orthogroup			
OGp0002576	*UDP-GLUCOSE 4-EPIMERASE (UGE4/REB1) (AT3G28530) (UGE5) (UGE2)*	-0,69 -0,63	0,601	-1,17
OGp0001768	*XYLOGLUCAN XYLOSYLTRANSFERASE (XXT4) (XXT5) (XXT3)*	-0,47		
OGp0006824	*XYG XYLOSYLTRANSFERASE 1 (XXT1) (XXT2)*	-0,7 -0,51		
OGp0007614	*XYLOGLUCAN L-SIDE CHAIN GALACTOSYLTRANSFERASE POSITION 2 (XLT2/GT18)*	-1,21		
OGp0010488	*SHORT ROOT IN SALT MEDIUM 3 (RSA3/MUR3)*			1,188
OGp0001493	*Glycosyl hydrolase family protein (BGLC1)*			
OGp0010030	*FUCOSYLTRANSFERASE 1 (FUT1/MUR2)*			

	At	Bd	Hv
UP/tot	0%	10%	8%
DOWN/tot	46%	0%	8%
DEGs/tot	46%	10%	17%

Table 8: Gene nitrate-response in tri-species OGps belonging to significantly enriched cell wall -related GO terms. GO terms "cell wall macromolecule catabolic precess" (A) and "xyloglucan biosynthetic process" (B) were identified as specific of Arabidopsis-Brachypodium and Arabidopsis subgroups, respectively (indicated at the top of each table). Genes associated to those GO terms and belonging to tri-species OGps were extracted. Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Tables below the OGp lists show the proportions of regulated genes among the considered genes for each species.

Trafficking is important for the proper localization of hormone receptors and ion channels, and for the recycling of components, since it relocalizes the component to the vacuole for example. It is also important for cell wall biogenesis and thus for growth (see Lycett, 2008 for review). Indeed, enzymes necessary for synthetizing polysaccharides need to be trafficked to the cell wall, as well as cell wall components. Interestingly, cell wall related processes are also mostly down-regulated in Arabidopsis while only few orthologs are regulated in Brachypodium and barley (Table 8A and 8B).

These observations of different regulations between species in response to nitrate seem interesting and deserves much more investigations. But finally, these pathways seem related to global growth response that happens in response to nitrate nutrition.

Analysis of pathways enriched exclusively in Brachypodium and barley

One of our main goal is to better characterize nitrate response in *Pooideae* species. Thus, processes that appeared only regulated in Brachypodium and barley particularly retains our attention. Two GO terms of this kind have already been discussed above: "cysteine biosynthetic process from serine" (GO:0006535, Table 3.2C) and "**biosynthesis of pyridoxal phosphate"** (PLP) (GO:0042823, Table 9A). PLP is the co-factor of OASTL enzyme required for cysteine biosynthesis from serine (see above). PLP is necessary for about 200 enzymes reactions, mostly involved in amino acid, sugar, and fatty acid metabolism. There is two ways for producing PLP. The de novo biosynthetic pathway generates PLP from ribose-5-phosphate (R5P), glyceraldehyde 3-phosphate (G3P) or from glutamine through PDX1 and PDX2 enzymes. The expression of these enzymes is induced in response to nitrate specifically in Brachypodium (both of them) and barley (only *PDX1*). The other pathway, known as the salvage pathway, recycles other existing Vitamine B6 forms, such as pyridoxamine 5'-phosphate (PMP) into PLP through the action of PDX3 or SOS4.

A

Bd&Hv spe	GO:0042823 pyridoxal phosphate biosynthetic process			
OGp name	Arabidopsis gene and/or family names representative of the orthogroup	At	Bd	Hv
OGp0001858	***PUTATIVE PDX1-LIKE PROTEIN 4 (PDX1L4) ; PYRIDOXINE BIOSYNTHESIS 1.1 (PDX1.1) (PDX1.3/RSR4)***		1,191	1,147
OGp0004727	***PYRIDOXINE BIOSYNTHESIS 2 (PDX2)***		1,217	
OGp0013068	*SALT OVERLY SENSITIVE 4 (SOS4)*		0,767	
OGp0010356	*Pyridoxamine 5'-phosphate oxidase family protein (AT2G46580)*			
OGp0013368	*PYRIDOXIN (PYRODOXAMINE) 5'-PHOSPHATE OXIDASE (PPOX)*			
OGp0012567	*PYRIDOXAL REDUCTASE 1 (PLR1)*			

	At	Bd	Hv
UP/tot	0%	38%	13%
DOWN/tot	0%	0%	0%
DEGs/tot	0%	38%	13%

B

Bd&Hv spe	GO:0046856 phosphatidylinositol dephosphorylation			
OGp name	Arabidopsis gene and/or family names representative of the orthogroup	At	Bd	Hv
OG0003909	*DNAse I-like superfamily protein (AT1G71710)*		0,634	0,652
OG0004208	***INOSITOL POLYPHOSPHATE PHOSPHATIDYLINOSITOL 5-PHOSPHATASE9 (5PTASE9)***		-1,36 -1,42	-1,97
OG0001499	*DNAse I-like superfamily protein (AT5G04980)*		-0,84	0,49
OG0001689	*INOSITOL-POLYPHOSPHATE 5-PHOSPHATASE (5PTASE13) (5PTASE14) (5PTASE12)*			-1,12
OG0002856	*ROOT HAIR DEFECTIVE4 (RHD4) ; SUPPRESSOR OF ACTIN 1B (SAC1B)*			
OG0007100	*DNAse I-like superfamily protein (AT3G63240)*			
OG0009299	*INOSITOL POLYPHOSPHATE 5-PHOSPHATASE 11 (5PTASE11)*			
OG0010757	*SAC DOMAIN-CONTAINING PROTEIN 8 (SAC8)*			

	At	Bd	Hv
UP/tot	0%	6%	14%
DOWN/tot	0%	19%	14%
DEGs/tot	0%	25%	29%

Table 9: Gene nitrate-response in tri-species OGps belonging to GO terms specifically regulated in Brachypodium and Barley. GO terms "pyridoxal phosphate biosynthetic process" (A) and "phosphatidylinositol dephosphorilation" (B) were identified as specificaly enriched the two *Pooideae* species. Genes associated to those GO terms and belonging to tri-species OGps were extracted. Each line represents an OGp and each colored rectangle represents a gene belonging to this OGp. Color scale and numbers indicate their maximum value among 1h30 and 3h timepoints of LogFC between nitrate- and mock-treated conditions (red, down-regulated; blue, up-regulated; no gene had opposite response between the timepoints). Grey rectangles represent genes that are not regulated or not detected in our RNAseq. Tables below the OGp lists show the proportions of regulated genes among the considered genes for each species.

The *SOS4* OGp is also specifically induced in Brachypodium. A role of PDX3 in nitrogen metabolism has been proposed in Arabidopsis by studying *pdx3* mutants: an increased ammonium level due to nitrate assimilation promotes the accumulation of PMP, which in turn inhibit nitrate reductase to avoid ammonium accumulation and thus toxicity. In *pdx3* mutants, PMP accumulates (since it is no longer converted into PLP by PDX3) and thus constitutively represses nitrate reductase activity (Colinas *et al.*, 2016).

PDX1 and PDX2 have been shown to be involved in root development in Arabidopsis, but no link with nitrate have been made yet. Both *pdx1* and *pdx2* single mutants are impaired in their hormone homeostasis, since PLP is required for *TAR* genes necessary for auxin biosynthesis and *ACC* required for ethylene biosynthesis (Boycheva *et al.*, 2015). Very interestingly, a study of root development in Brachypodium shows that the crosstalk of auxin and ethylene for root growth differs from the Arabidopsis model. Indeed different mechanisms were highlighted: in Brachypodium ethylene represses *YUCCA* expression (a P450 cytochrome responsible for the conversion of idole-3-pyruvic acid into auxin), while it induces its expression in Arabidopsis (Pacheco-villalobos *et al.*, 2013). It would be interesting to test these in a nitrate context, but this difference of mechanism can partially explain the specific regulation of Brachypodium and barley for producing PLP.

The GO term "**phosphatidylinositol dephosphorylation**" (GO:0046856) was also specifically regulated in Brachypodium and barley (Table 9B). No gene of the corresponding OGps was regulated in Arabidopsis, while 25% and 29% were responsive in the other species. When phosphorylated, phosphatidylinositols play an important role in lipid signaling, cell signaling and membrane trafficking, notably through the IP3 pathway. 5PTASE genes encode phosphatases of a large protein family. They hydrolyze 5-phosphates from a variety of phosphatidyl inositol phosphates. In

our conditions, closest orthologs of *At5PTASE9* are down-regulated in response to nitrate in Brachypodium and barley. In Arabidopsis, At5PTASE9 has been shown to be involved in plant salt tolerance: *At5ptase9* mutants present a decreased of calcium influx which coincide with a diminution of ROS production while these two molecules are necessary for induction of abiotic stress tolerance (Golani *et al.*, 2013). Interestingly, nitrate signaling also occurs through calcium signals (see Liu et al., 2020 for review). *At5PTASE2* expression is described as induced by nitrate (Berdy *et al.*, 2001; Canales *et al.*, 2014). This gene is indeed induced in Arabidopsis in our experiment, but it belongs to a mono-specie OGp so does not appear in Table 9B. Manually identification of the closest ortholog in Brachypodium and barley permit to identify a bi-species OGp in which they are grouped. Both Brachypodium and barley *5PTASE2* are also induced in response to nitrate. Taken together, these observations suggest that besides a putative common role of 5PTASE2 in nitrate signaling, there are 5TPASEs that are only regulated in Brachypodium and barley, suggesting some differences in signaling pathways.

CONCLUSIONS

The aim of this study was to precisely describe transcriptional nitrate response of Brachypodium and barley as member of *Pooideae*. To do so, we performed RNAseq on root tissues of plantlets grown on hydroponic basal medium before 4 days of nitrogen starvation followed by 1mM nitrate treatment during 1h30 and 3h to ensure the most exhaustive transcriptional response. Since little is known on nitrate response in *Pooideae* and since their genomes, besides being entirely sequenced, remain poorly annotated, we decided to add Arabidopsis as control and reference to our analysis. Even though a multitude of transcriptomes in response to nitrate exist in Arabidopsis, it would have been very difficult to perform comparison as deep as we did due to huge variability of the conditions. The main message of this study is that among biological processes that are commonly regulated by nitrate in Arabidopsis, Brachypodium and

barley, molecular players of these processes are not always similarly regulated between species. These divergences highlight importance to identify and study molecular players within cultivated plants. Studying model plants is very useful to acquire a global view of involved processes and to serve as a basis for the translation of knowledge to crops, but is not sufficient *per se* to understand the response in every single species.

Our first analysis consisted in factually describing DEGs by using Gene Ontology Enrichment. Analysis per species (Figure 4, panels A1 to A9) allow us to confirm RNAseq set ups since GO terms related to nitrate response were found enriched in our three species. We also highlight expected nitrate-regulate GO terms related to carbon metabolism or hormone signaling. These observations were further confirmed by the ternary analysis (Figure 4, panels B1 to B3) which place all those GO terms as commonly regulated in the three species. Ternary analysis also provides arguments to identify GO terms that appeared enriched in only one species (*ie*, "rRNA processing" GO term which is specifically enriched in Arabidopsis). This first step of analysis identifies many very expected common regulated process.

To deepen the analysis, we decided to have a closer look into processes by focussing at gene regulations. To do so, it was necessary to identify genes orthologs in Arabidopsis, Brachypodium and barley which were then gathered into OGps. Interestingly, DEGs gathered more into bi-species and tri-species OGps (Figure 5). DEGs present in Brachypodium-barley bi-specific OGps are, *inter alia*, involved in protein phosphorylation and regulation of transcription processes (Figure 6). It suggests that potential nitrate signaling actors such as kinases and transcription factors are specific to *Pooideae* due to high protein sequence divergence that are good candidates to further investigate. Nitrate pathway is well documented in Arabidopsis and is our main interest. Thus, we first decided to use OGps onto known genes in Arabidopsis that are up-regulated in response to nitrate (Table 1) or known to be part of nitrate response (Table 2). Among these two lists of OGps, respectively 57% and 30% of them present the same regulation (or non-regulation) pattern between the three species. These

numbers illustrate the existence of a non negligeable response divergence between Brachypodium, Arabidopsis and barley. Some divergences are supported by known physiological differences between Arabidopsis and *Pooideae* such as differentially regulated *NAS* genes involved in Fe homeostasis in response to nitrate which support a different strategy of Fe acquirement between Arabidopsis and grasses (Marschner & Romeld, 1994). Similarly, difference of gene regulation in hormone pathways necessary for root system architecture support different root phenotype in response to nitrate between Arabidopsis and cereal (Smith and de Smet, 2012; Shahzad and Amtmann, 2017). Thus, we identified here candidate genes explaining physiological differences between Arabidopsis and *Pooideae*.

The last part of the study consisted in setting up a method that have less biases as possible to identify specific pathways and then genes that are regulated by nitrate in one or two species only and more specifically, regulated in Brachypodium and Barley only. We thus decided to combine Gene Ontlogy analysis with OGps by calculating DEG enrichments on genes belonging to tri-species OGps only. This way, conservations and divergences between species will be due to regulations and not inexistence of orthologs of different gene dichotomy. We particularly highlight the divergence in cysteine biosynthesis from serine which seems more activated in Brachypodium and barley (Figure 3.2C). This activation is further supported by a specific induction of genes necessary for PLP biosynthesis which is required for the functioning of OASTL (one enzyme required for cysteine biosynthesis) in Brachypodium and barley. We thus identify an intriguing *Pooideae*-nitrate-specific process which will probably lead to a better understanding of nitrate response in cereals.

MATERIAL AND METHODS

Plant growth conditions

Arabidopsis (Col0) seeds were sowed on 0.1mM agar basal media put into cut tubes (0.35 mM K_2SO_4, 0.25 mM $CaCl_2$, 0.25 mM KH_2PO_4, 0.25 mM $MgSO_4$, 10 mg.L-1 Fer-EDTA, 243 µM $Mo_7O_24(NH_4)_6$, 0.4 µM H_3BO_3, 118 µM SO_4Mn, 10 µM de SO_4Cu and 34.8 µM SO_4Zn, 0.65% Plant Agar, pH5.8) supplemented with 0,05mM KNO_3 and 0,025mM $Ca(NO_3)_2$. Cut tubes are placed above hydroponics culture containing the basal medium without agar in long days growth chamber (21°C, 16h Day, 18°C, 8h Night) with 180 µE/m^2/s. Medium was renewed once a week for the first week and then twice a week. After 18 days, plantlets were transferred on basal medium without N source (5.5 mM KCl) for 4 days. Medium was renewed after 3 days. 22 days after sowing, plantlets were transferred on new medium containing either 0.5 mM KNO_3 and 0.25 mM $Ca(NO3)_2$ (treated) or 5.5 mM KCl (mock). Roots were harvested after 1.5 or 3 hours of treatment and frozen in liquid nitrogen.

Brachypodium (Bd21-3) and Barley (Golden promise), seeds were sowed in water and stratified for 3 days at 4°C. Seeds are then put at room temperature for germination for 7 days. Plantlets are transferred in extra-long days growth chambers (22°C, 18h Day; 19°C 6h Night) with 250 µE/m^2/s light on hydroponics on basal medium (0.35 mM K_2SO_4, 0.25 mM $CaCl_2$, 0.25 mM KH_2PO_4, 0.25 mM $MgSO_4$, 10 mg.L^{-1} Fer-EDTA, 243 µM $Mo_7O_24(NH4)_6$, 0.4 µM H_3BO_3, 118 µM SO_4Mn, 10 µM SO_4Cu and 34.8 µM SO_4Zn) supplemented with 0,05 mM KNO_3 and 0,025mM $Ca(NO_3)_2$. After 7 days, plants were transferred on basal medium without N source (5.5 mM KCl) for 4 days. Medium was renewed after 3 days. 22 days after sowing, plants were transferred on new medium containing either 0.5 mM KNO_3 and 0.25 mM $Ca(NO3)_2$ (treated) or no N source (5.5mM KCl) (mock). Roots were harvested after 1.5 or 3 hours of treatment and frozen in liquid nitrogen.

RNA extraction

For each species, 12 samples were produced including 3 independent biological replicates. 50 mg of fresh root tissue are frozen-ground, resuspended in 500µL NucleoZol and 200µL RNAse-free H_2O and incubated for 30minutes at room temperature. Tubes are then vortexed and incubated for 30minutes at room temperature. Afterwards, tubes are centrifuged for 15 minutes à 4°C (12000 RCF). The supernatants are collected and 2.5µL of 4-bromoanisole are added before vortex. Samples are centrifuged 10 minutes at 4°C (12000 RCF) and 500µL of supernatant are collected in new tubes. 500µL of Propane-2-ol are added before 40 minutes incubation at room temperature. After 25 minutes centrifugation (12000 RCF) at room temperature, pellets are washed twice with 75% ethanol. Dry pellets are resuspended with 50µL RNAse-free water. Samples are then purified using Clean and concentrator (Zymo research) following manufacturer instructions.

RT-qPCR on sentinels' genes

After RNA dosage, the same amount of RNA is used to synthetized cDNA. RevertAid H Minus Reverse Transcriptase (Thermo Scientific) kit is used following manufacturer instruction, using oligo$(dT)_{18}$. For qPCR, cDNA are diluted (1:20), and 2.5µL of this dilution is used with 2.5 SyBr mix (SsoAdvanced™ Universal SYBR® Green Supermix) which contains 0.03µM of primer forward and reverse. Relative quantification and statistics have been made using Rieu & Powers, 2009 methods. Normalization is made by dividing Ct of genes of interest by Ct of synthetic gene, which is the geometrical mean of 2 (for Barley) or 3 (for Arabidopsis and Brachypodium) Ct of housekeeping genes. List of primers used: *AtNIA1* (At1g77760) Forward (5'-ACGGAGCATGGATGAGTT-3'); *AtNIA1* Reverse (5'- ATCGTCAAAGAAACCGAAGTC-3'); *AtNIA2 (At1g37130)* Forward (5'- CATGCACGAACAGCAATC-3'); *AtNIA2* Reverse (5'-GGTTACGCATATTCCGGAG-3'); *AtNRT2.1* (At1g08090) Forward (5'-

AGTCGCTTGCACGTTACCTG-3'); *AtNRT2.1* Reverse (5'- ACCCTCTGACTTGGCGTTCTC-3'); *AtEF1α* (At5g60390) Forward (5'- CTGGAGGTTTTGAGGCTGGTAT-3'); *AtEF1α* Reverse (5'- CCAAGGGTGAAAGCAAGAAGA-3'); *AtUBI10* (AT4G05320) Forward (5'-GGCCTTGTATAATCCCTGATGAATAAG-3'); *AtUBI10* Reverse (5'-AAAGAGATAACAGGAACGGAAACATAGT-3'); *AtACT2* (AT3G18780) Forward (5'-GCCATCCAAGCTGTTCTCTC-3'); *AtACT2* Reverse (5'- CCCTCGTAGATTGGCACAGT-3'); BdNIA1 (Bradi3g37940) Forward (5'- CGAGTCCGACAACTACTACC-3'); *BdNIA1* Reverse (5'- CGTCGTGAATGCGTTGAT-3'); *BdNIA2* (Bradi3g57680) Forward (5'-GCTGGTGTTTCTGGTCAGTA-3'); *BdNIA2* Reverse (5'- GGAACCAGCAGTTGTTCATC-3'); *BdNRT2A* (Bradi3g01270) Forward (5'-GTGGAGACCGGCGACATG-3'); Reverse (5'-CGAAAGTGGAGACGAAGCAG-3'); *BdUbc18* (DV481689) (5'-GGAGGCACCTCAGGTCATTT-3'); *BdUBc18* Reverse (5'- ATAGCGGTCATTGTCTTGCG-3'); *BdEF1α* (DV482887) Forward (5'- CCATCGATATTGCCTTGTGG-3'); *BdEF1α* Reverse (5'-GTCTGGCCATCCTTGGAGAT-3'); *BdSamDC* (DV482676) Forward (5'-TGCTAATCTGCTCCAATGGC-3'); *BdSamDC* Reverse (5'- GACGCAGCTGACCACCTAGA-3'); *HvNIA1* (HORVU6Hr1G003300) Forward (5'- ACTACCCATCAACGCCTTCA-3'); *HvNIA1* Reverse (5'- TCTCTACCGACCAGAAGCAC-3'); *HvNIA2* (HORVU6Hr1G079700) Forward (5'- CTCATGTCCCACGGCTTCAT-3'); *HvNIA2* Reverse (5'-CGTGACGAGCTCCTCCATG); *HvNRT2.1* (HORVU6Hr1G005590) Forward (5'-TGCGTGTGGTGCTATCTTTG-3'); *HvNRT2.1* Reverse (5'- TGTACTCGAGACCCCTACCA-3'); *HvSamDC* primer used as in Francia et al., 2016; *HvUBI* Forward (5'-TCTCGTCCCTGAGATTGCCCACAT-3'); *HvUBI* (HORVU3Hr1G080790) Reverse (5'-TTTCTCGGGACAGCAACACAATCTTCT-3').

RNA-seq technology

Quality of total RNA was controlled on Agilent 2100 Bioanalyzer, according to manufacturer's recommendations. Library construction was generated by the IPS2-

POPS platform (Gif-sur-Yvette, France). Briefly, mRNAs were polyA selected, fragmented to 260 bases and libraries were built using TruSeq stranded mRNA kit (Illumina®, California, U.S.A.), with an Applied BioSystem 2720 Thermal Cycler and barcoded adaptors. Barcoded libraries were sequenced on Illumina NexSeq500 sequencer (IPS2 POPS platform). The RNA-seq samples were sequenced in Single-ends, stranded with a sizing of 260bp and a read length of 75 bases, lane repartition and barcoding giving approximately 20 to 40 millions of reads by sample.

RNA-seq bioinformatics treatments and analyses

To facilitate comparisons, each sample followed the same steps from trimming to count. RNA-Seq preprocessing included trimming library adapters and performing quality controls. The raw data (fastq) were trimmed with Trimmomatic (Bolger *et al.*, 2014) tool for Phred Quality Score Qscore >20, read length >30 bases, and ribosome sequences were removed with tool sortMeRNA (Kopylova *et al.*, 2012).

The genomic mapper STAR v2.7 (Dobin *et al.*, 2013) was used to align reads against the 3 genomes with options --outSAMprimaryFlag AllBestScore --outFilterMultimapScoreRange 0 to keep the bests results. The 3 genomes and annotation files used, coming from international consortium or repository, are *Hordeum vulgare* genome v1 from IBSC, *Brachypodium distachyon Bd21.3* v1 from Phytozome, *Arabidopsis thaliana* from TAIR v10. The abundance of each gene was calculated with STAR and counts only reads mapping unambiguously one gene, removing multi-hits. According to these rules, 94% of reads were associated to a unique gene for Arabidopsis and Brachypodium, and 88% for barley.

Differential analyses were performed independently for each species. They followed the procedure described in (Rigaill *et al.*, 2018). Briefly, genes with less than 1 read after a count per million (CPM) normalization in at least one half of the samples were

discarded. Library size was normalized using the trimmed mean of M-value (TMM) method and count distribution was modeled with a negative binomial generalized linear model. The logarithm of the mean expression was defined as a function of the time effect plus a treatment effect and the interaction between time and treatment. Dispersion was estimated by the edgeR method (Version 3.28.0, McCarthy et al., 2012) in the statistical software 'R' (Version 3.6.1 R Development Core Team (2005)). Expression differences of interest were tested using a likelihood ratio test and p-values were adjusted by the Benjamini-Hochberg procedure to control False Discovery Rate (FDR). In all tests, a gene was declared differentially expressed if its adjusted p-value < 0.05.

Data Deposition

All steps of the experiment, from growth conditions to bioinformatics analyses, were managed in CATdb database (Gagnot et al., 2008, http://tools.ips2.u-psud.fr/CATdb/) with ProjectID NGS2019_07_NiCe. This project is submitted from CATdb into the international repository GEO (Gene Expression Omnibus*, Edgard R. et al. 2002*, http://www.ncbi.nlm.nih.gov/geo) (submission to GEO in progress).

Orthology analysis

For the three species, the amino-acid sequences of proteomes were collected as fasta files, based on annotations TAIR v10 for *Arabidopsis thaliana*, Bd21-3 v1 for *Brachypodium distachyon* and High Confidence v2 for *Hordeum vulgare*. Only representative forms of proteins were used, excluding alternative forms and multiple variants. This represents a total of 103,797 protein sequences.

Orthologous genes were investigated using OrthoFinder software (v2.3.7, Emms and Kelly 2015). The first step of OrthoFinder*, i.e.* all-versus-all sequence comparisons, was performed with Diamond (v0.9.24, Buchfink *et al.* 2015). All subsequent steps were managed by OrthoFinder to produce orthogroups and identify orthologs.

Gene ontology analysis

Gene ontology analysis have been performed using topGO 'R' package (Alexa *et al.*, 2006; Alexa and Rahnenführer, 2007) using ElimFisher statiscal tests with 'R' version 3.6.3. GO databases from EnsemblPlants were used for *Arabidopsis thaliana*, *Brachypodium distachyon* and *Hordeum vulgare*.

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AUTHOR CONTRIBUTIONS

MG, AK, MLMM and TG conceived the experiments. MG and SP did the experiments. MG, VB and MLMM did bioinformatic and statistical analyses. J.PT and LB generated OGps. MG wrote the manuscript.

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SUPPLEMENTARY DATA

The supplementary data are available until 15/04/21 at:

https://filesender.renater.fr/?s=download&token=e00f394b-a381-4f8b-abab-696ad5e5eee9

Supplementary figures

Figure S1: Gene Ontology (GO) enrichment analysis for Arabidopsis, Brachypodium and barley after 1h30 of 1mM NO_3^- treatment.

Figure S2: Gene Ontology (GO) enrichment analysis for Arabidopsis, Brachypodium and barley after 3h of 1mM NO_3^- treatment.

Figure S3: Best 10 biological processes significantly enriched in different OGps types

Supplementary tables

Supp Table S1: RNAseq raw data

Supp Table S2: Enriched Gene Ontology terms per species

Supp Table S3: Significant enriched biological process GO after 1h30 nitrate treatment

Supp Table S4: Significant enriched biological process GO after 3h nitrate treatment

Supp Table S5: Significant enriched GO terms DEG OGp BdHv.

Supp Table S6: Significant enriched GO terms describing OGs functions

Supp Table S7: Enriched GO terms in Ternary diagrams in tri-species OGps

Supp Table S8: Gene nitrate-response in tri-species OGps belonging to significantly enriched nitrate-related GO terms.

CHAPTER III

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BdNLP6 represses plant growth under ample nitrate condition

----------Manuscript in preparation----------

BdNLP6 represses plant growth under ample nitrate condition

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ABSTRACT

NLP transcription factors have first been characterized in Arabidopsis, in which AtNLP7 have been described as a major regulator of Primary Nitrate Response (PNR). Later, many crops NLP (mainly rice, maize and some wheat NLPs) have been characterized as having globally the same function as AtNLP7 or at least a positive role in nitrate response. In our study, we aimed at characterizing Brachypodium NLPs, and more specifically BdNLP6 and BdNLP7 which are the closest orthologs of AtNLP7, to evaluate conservation or divergence between NLPs in Arabidopsis and cereals. We show that *BdNLPs* are transcriptionally dependent on nitrate source and availability, and that *BdNLP6*, which is repressed by nitrate is a growth repressor in a nitrate dependent way. *BdNLP6* and *BdNLP7* are not involved in the control of genes related to nitrate uptake and assimilation pathway at vegetative stage, neither in steady-state conditions nor after nitrate treatment. In Arabidopsis, *AtNLP8* and *AtNLP9* are also down-regulated by nitrate similarly to *BdNLP6*, and do not seem to be involved in regulating the response to nitrate. Finally, BdNLP6 might have a negative role on nitrate assimilation rate and on translocation from root to shoot. Further investigations are needed to complete and understand the role of BdNLP6 and BdNLP7 but still, they display a total divergent putative role compared to characterized Arabidopsis, rice, and maize NLPs.

INTRODUCTION

Nitrogen (N) is a macro-element essential for plants since it is a major component of nucleic acids, amino acids and hormones (Xu *et al.*, 2012). Absence or limitation of nitrogen have dramatic consequences on plant development and yields (Giehl and Wirén, 2014). In fields, Nitrogen is provided by addition of large amount of fertilizers which cause pollution and high costs for farmers. Nitrogen Use Efficiency (NUE) is defined as the ratio between grain yield and available N in the soil per unit of field-surface. Identifying new molecular players for improving crops NUE has become one of major interests in worldwide plant research. During the last decades, several strategies have been employed such as targeting genes directly involved in nitrate transport and assimilation or identifying novel regulators using quantitative trait locus (QTL) or transcriptomic approaches. Here, we aim at deciphering the role in *Brachypodium distachyon* (Brachypodium), a model plant for cereals (Girin *et al.*, 2014), of orthologs of AtNLP7, a major regulator of nitrate response characterized in the dicot model *Arabidopsis thaliana* (Arabidopsis).

Nitrate is the preferential nitrogen source for most land plants. Nitrate is uptaken from the soil *via* NRT nitrate transporters and then reduced to nitrite and ammonium through Nitrate Reductase (NR, encoded by *NIA* genes) and Nitrite Reductase (NiR, encoded by *NiR* gene). Afterwards, ammonium is assimilated into glutamate and glutamine through the GS/GOGAT cycle. Since the 90's, nitrate is known to be a signal molecule in addition to its nutrient function: it induces the *NIA* and *NiR* mRNA accumulation in maize and barley (Gowri *et al.*, 1992; Sueyoshi *et al.*, 1999). Now, nitrate have been shown to govern expression of thousands of genes in Arabidopsis (Krouk *et al.*, 2010). This rapid gene induction in response to nitrate which does not required *de novo* protein synthesis is called Primary Nitrate Response (PNR) (Gowri *et al.*, 1992). Molecular players of the nitrate-signaling mechanism are now well described in Arabidopsis. Nitrate is perceived by NRT1.1, a dual-affinity nitrate transporter also

called transceptor (Ho *et al.*, 2009). Then, nitrate causes a rapid increase of cytosolic calcium levels which triggers changes in gene expression in Arabidopsis roots (Riveras *et al.*, 2015). CPKs decode calcium waves which in turn phosphorylates AtNLP7 transcription factor, thus promoting its nuclear localization and the activation of its target genes (Liu *et al.*, 2017). Indeed, AtNLP7 is now considered as a master regulator of PNR (Castaings *et al.*, 2009; Konishi and Yanagisawa, 2013; Marchive *et al.*, 2013; Alvarez *et al.*, 2020)

AtNLP7 belong to the NLP (NIN-LIKE PROTEINS) family. They were identified due to their homology with NIN (NODULE INCEPTION) proteins that are necessary for legume nodule formation (Schauser et al., 1999). NIN are transcriptions factors with a DNA-binding bZIP domain which contain a highly conserved amino acid sequence displaying a RWPxRK signature (Borisov *et al.*, 2003). Thanks to phylogenetic analyses, NIN homologs were identified in non-legume plants, and named NLPs (Chardin et al., 2014; Schauser et al., 2005). NLP proteins share 3 conserved domains. In N terminal part, the precise function of GAF-like domain has not been characterized yet. The RWP-RK domain is necessary for DNA binding: it has been shown that it is through the RWP-RK domain that AtNLP is able to binds the Nitrate Responsive Element (NRE), a *cis* element present in the promoter of most nitrate responsive genes (Konishi and Yanagisawa, 2013). Finally, NLPs possess a PB1 domain at their C terminal part, which is necessary for protein-protein interactions (Guan *et al.*, 2017).

NLPs proteins have been characterized mainly in Arabidopsis, rice and maize. The first important NLP that has been characterized is AtNLP7. As mentioned before, AtNLP7 is considered as a master regulator of PNR. It is able to bind about 800 genes after only 10 minutes of nitrate treatment (Marchive *et al.*, 2013). AtNLP7 interacts with AtNLP6 through their PB1 domains, and together they regulate nitrate responsive genes (Guan *et al.*, 2017). While its transcriptional level seems globally not affected by nitrate, its subcellular localization is nitrate-dependent: the protein localizes in the nucleus only in the presence of nitrate (Marchive *et al.*, 2013). Interestingly, AtNLP7 overexpressing

lines present an enhanced growth both under limiting and sufficient nitrate conditions (Yu et al., 2016). On the other hand, *AtNLP8* is specially expressed in dry seeds and is necessary for nitrate-induction of germination (Yan et al., 2016). In maize, ZmNLP6 and ZmNLP8 seem to have the same function as AtNLP7, since they modulate nitrate signaling and assimilation and they are able to restore *Atnlp7* mutant (Cao *et al.*, 2017). Recently, OsNLP1, OsNLP3 and OsNLP4 have been found to also be involved in nitrate signaling and metabolism in rice (Alfatih et al., 2020; Hu et al., 2019; Yu et al., 2021; Zhang et al., 2021). Interestingly, unlike AtNLP7, they have been shown to be transcriptionally regulated by nitrate availability. However, they display the same post-translational regulation of subcellular localization which is dependent of nitrate. Together, they constitute interesting candidate for improving NUE.

To date, none of the 7 members of Brachypodium BdNLPs have been studied. Here, we characterized the transcriptional regulation by nitrogen availability of *BdNLPs* and subsequently studied the functions of BdNLP6 and BdNLP7, which are the closest orthologs of AtNLP7. We hypothesized that they are functional homologs, and thus tested their involvement in PNR. Unexpectedly, we show that BdNLP6 and possibly BdNLP7 seem to be growth repressors, specifically in sufficient nitrate conditions, by studying two *Bdnlp6* mutants and an *amiRNA* line expressing an artificial microRNA targeting both *BdNLP6* and *BdNLP7* genes. To finish, we identified two *AtNLPs* that display the same transcriptional regulation pattern as *BdNLP6* and showed that, similarly as *Bdnlp6* mutants, *Atnlp8* and *Atnlp9* are not affected in nitrate response. These results open the way for discovering new functions among NLP family.

RESULTS

The aim of the study is to characterize the roles of BdNLPs, and more specifically the ones of BdNLP6 and BdNLP7 since they are the closests orthologues of the well-characterized AtNLP7. AtNLP7 is known to be involved in nitrate response in Arabidopsis and more specifically in Primary Nitrate Response (PNR). More recently, several NLP in other species have be shown to be involved in nitrogen use efficiency. Thus, we decided to test whether these roles are conserved in Brachypodium. To do so, we studied 2 types of mutants: *Bdnlp6-1* and *Bdnlp6-2* mutants (later called *nlp6-1* and *nlp6-2*), displaying nonsense mutations leading to truncated forms of BdNLP6 proteins and *Bdnlp6/nlp7* transgenic line (later called *nlp6/nlp7*) which express an artificial microRNA targeting both *BdNLP6* and *BdNLP7*. The expression levels of the 2 genes are reduced in this line (supplementary Figure S1).

Expression of *BdNLPs* vary under contrasted nitrogen conditions

We characterized roots expression patterns of each *BdNLP* in various nitrogen conditions (Figure 1). Brachypodium plantlets were grown for 22 days in contrasted nitrate conditions (from 0 to 6mM) and 2 conditions of ammonium (0.002 and 0.2mM). To confirm these conditions, we monitored *BdNIA1* and *BdNRT2.5* expression (Figures 1A, B). As expected, *BdNIA1* expression is higher following increasing nitrate concentrations and stay low under ammonium nutrition, while *BdNRT2.5* expression is higher under nitrogen deprivation and extremely low nitrate concentrations (0.002 and 0.02mM), as well as under low ammonium conditions (0.002mM and 0.2mM) compared to higher nitrate concentrations (from 0.2 to 6mM) where its expression very low.

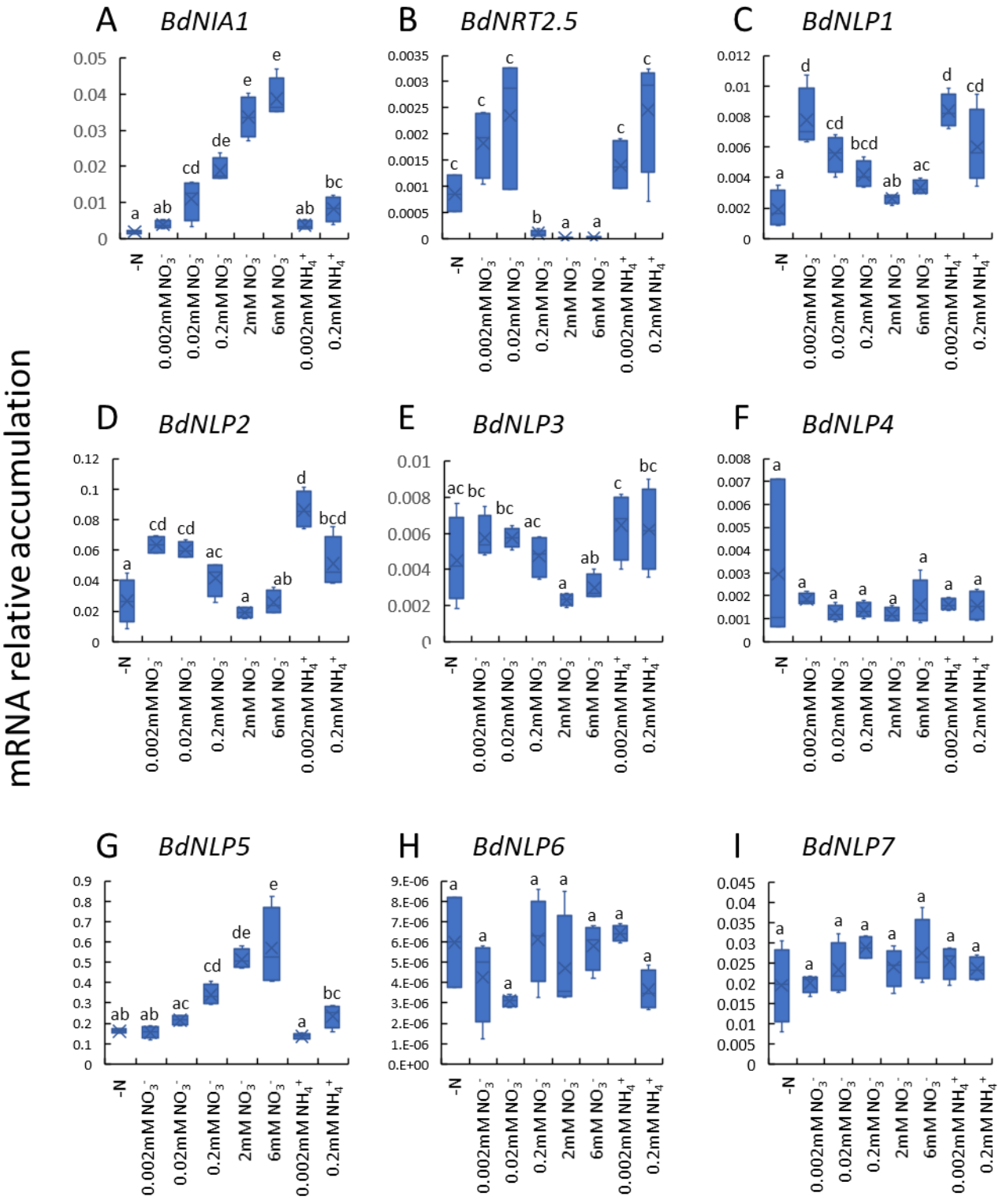
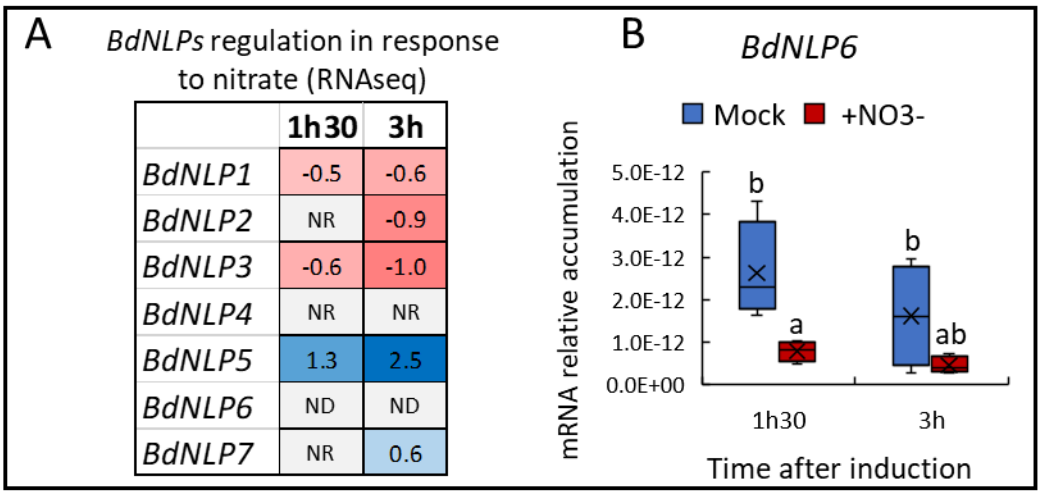


Figure 1: BdNLPs roots expressions patterns in various nitrogen conditions.
Wild-type (Bd21-3) plants, were grown for 22 days in different hydroponic media containing no nitrogen or a range of nitrate (0.002mM to 6mM) or ammonium (0.002mM and 0.2mM) concentrations Roots were collected and used for gene expression analysis. Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n=4 plants. Letters represent significant differences evaluated by ANOVA on log2-transformed data (pval<0.05).

Very interestingly, root expression levels of *BdNLPs* appear different from one another, and some of them are depending on nitrogen source and availability. *BdNLP1*, *BdNLP2* and *BdNLP3* expressions (Figures 1C, D, E) display almost the same expression pattern: their expression is higher under low nitrogen level (nitrate or ammonium) than on 2mM and 6mM nitrate conditions, suggesting that these BdNLPs have a role under low nitrogen conditions. Their expression is however uninduced in absence of nitrogen, especially concerning *BdNLP1* and *BdNLP2*. On the contrary, *BdNLP5* expression (Figure 1G) increased following nitrate increase and is low under ammonium conditions, similarly to *BdNIA1*. Expression under higher ammonium level would have been interesting to test, to clarify whether its regulation by nitrate is a more general regulation by nitrogen. In any case, these results suggest a different role for BdNLP5 compared to BdNLP1, 2 and 3. To finish, *BdNLP4*, *6* and *7* (Figures 1F, H, I) present no significant variation of expression in the tested nitrogen conditions, suggesting again different mechanisms of regulation and different roles from the others.

To better understand transcriptional regulation of *BdNLPs* by nitrate, we evaluated their transcriptional level in response to a nitrate treatment (Figures 2 A-C) or a nitrogen starvation treatment (Figures 2 D-K). From a transcriptomic analysis (RNA-seq, see Chapter II) in response to 1mM nitrate after 4 days of nitrogen starvation, we extracted *BdNLPs* expressions after 1h30 and 3h treatment (Figure 2A). *BdNLP1*, *2* and *3* appear to be downregulated in response to nitrate treatment, as compared to mock-treatment. Interestingly, these 3 genes are transiently induced by nitrogen starvation, but return to their basal level after 3 days (*BdNLP1* and *BdNLP3*) or 6 days (*BdNLP2*) (Figures 2 E, F, G). Thus, the repression in response to 1mM nitrate after starvation cannot be totally explained by their higher level of expression due to the starvation.

Response to nitrate induction after starvation



	1h30	3h
BdNLP1	-0.5	-0.6
BdNLP2	NR	-0.9
BdNLP3	-0.6	-1.0
BdNLP4	NR	NR
BdNLP5	1.3	2.5
BdNLP6	ND	ND
BdNLP7	NR	0.6

Response to nitrogen starvation

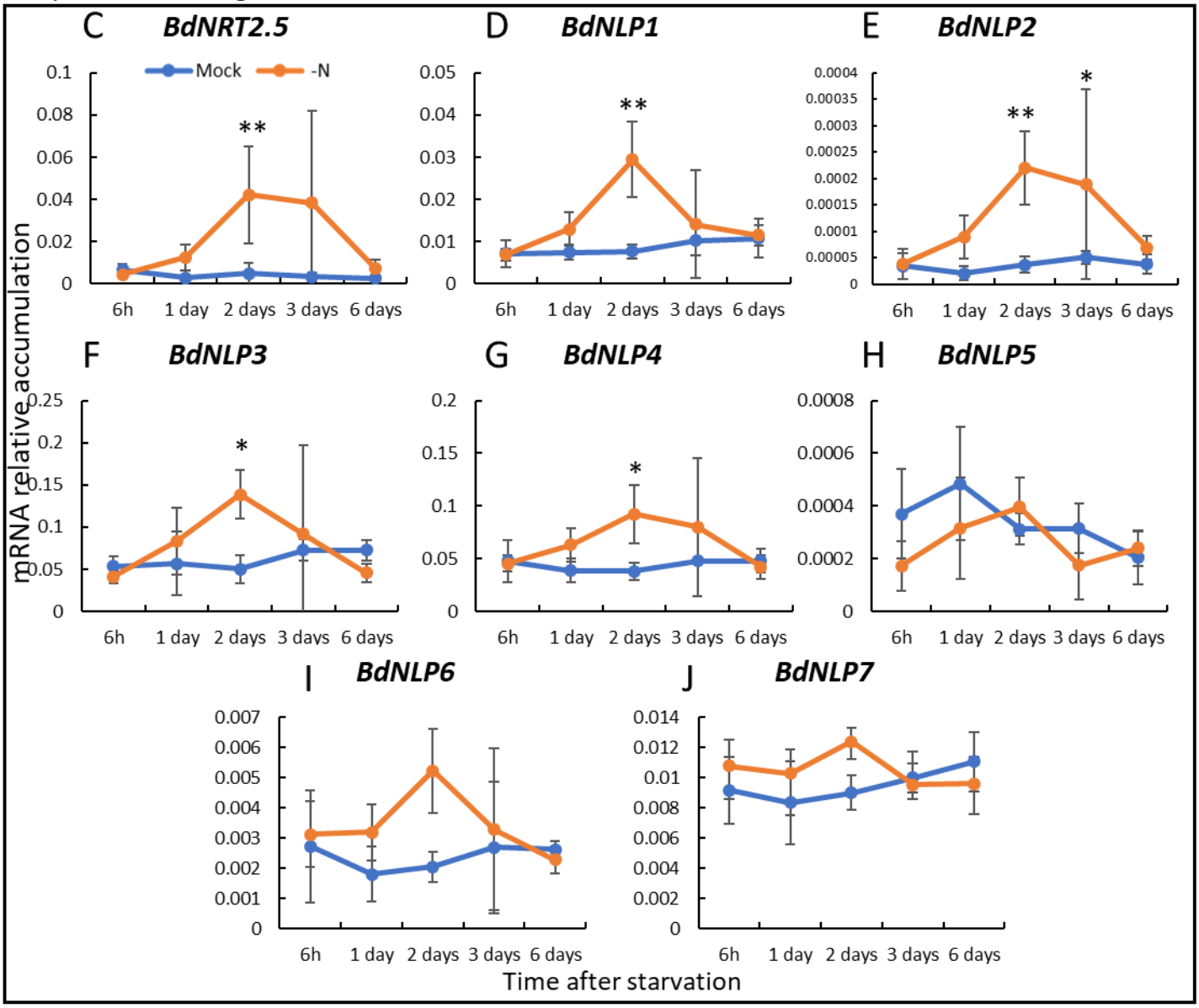


Figure 2: *BdNLPs* root expression patterns in response to nitrate induction and nitrogen starvation treatments.
22 days old Brachypodium plantlets were treated with 1mM nitrate for (A) 1h30 and 3h (RNAseq ; values correspond to Log2FC compared to mock condition) or (B) by RT-qPCR. Letters represent significant differences evaluated by ANOVA on log2-transformed data (pval<0.05).
(C-J) 15 days old plantlets were grown on basal hydroponic medium containing 0.1mM Nitrate and were then N starved from 6h to 6 days (-N) or not (Mock). Asteriscs (*) represent significant differences between Mock and treated value at each time point evaluated by ANOVA on log2-transformed data (pvalue < 0,05 * ; pvalue < 0,01 **).

However, their induction by starvation suggests a role of BdNLP1, 2, 3 in nitrogen starvation response. *BdNLP4* is not regulated by 1mM nitrate treatment (Figure 2A). However, its expression is induced after 2 days of nitrogen starvation. *BdNLP5* is up regulated in response to nitrate treatment (Figure 2A) while it is not regulated by nitrogen starvation. This is consistent with its higher expression in high nitrate concentration (Figure 1G). *BdNLP6* was not detected in our RNA seq. Indeed, its expression is extremely low comparing to others. We monitored its expression by RT-qPCR in response to 1mM nitrate (Figure 2B) and observed that its expression is repressed as compared to mock-treated plants. On the other hand, its expression is not significantly regulated by nitrogen starvation according to an ANOVA statistical test, even if it might have a transient peak of expression after 2 days of starvation (Figure 2I). Finally, *BdNLP7* is slightly induced after 3h of nitrate treatment (Figure 2A) and not regulated in response to nitrogen starvation (Figure 2J).

Taken together, these observations reveal different transcriptional responses for *BdNLPs* under various nitrogen conditions, suggesting non-redundant roles of the transcription factors. *BdNLP1*, *2* and *3* present similar expression patterns, being highly expressed under low nitrogen conditions and induced by nitrogen starvation. *BdNLP4* expression pattern seems globally to be independent on nitrogen level, similarly to *BdNLP6* and *7*, but is induced by nitrogen starvation. Finally, *BdNLP6* is repressed by nitrate treatment while *BdNLP7* is slightly induced.

Since *BdNLP6* and *BdNLP7* are the closest orthologs of *AtNLP7* which is known to be a master regulator of nitrate response, we decided to perform further investigations on these two proteins and test whether they display the same function.

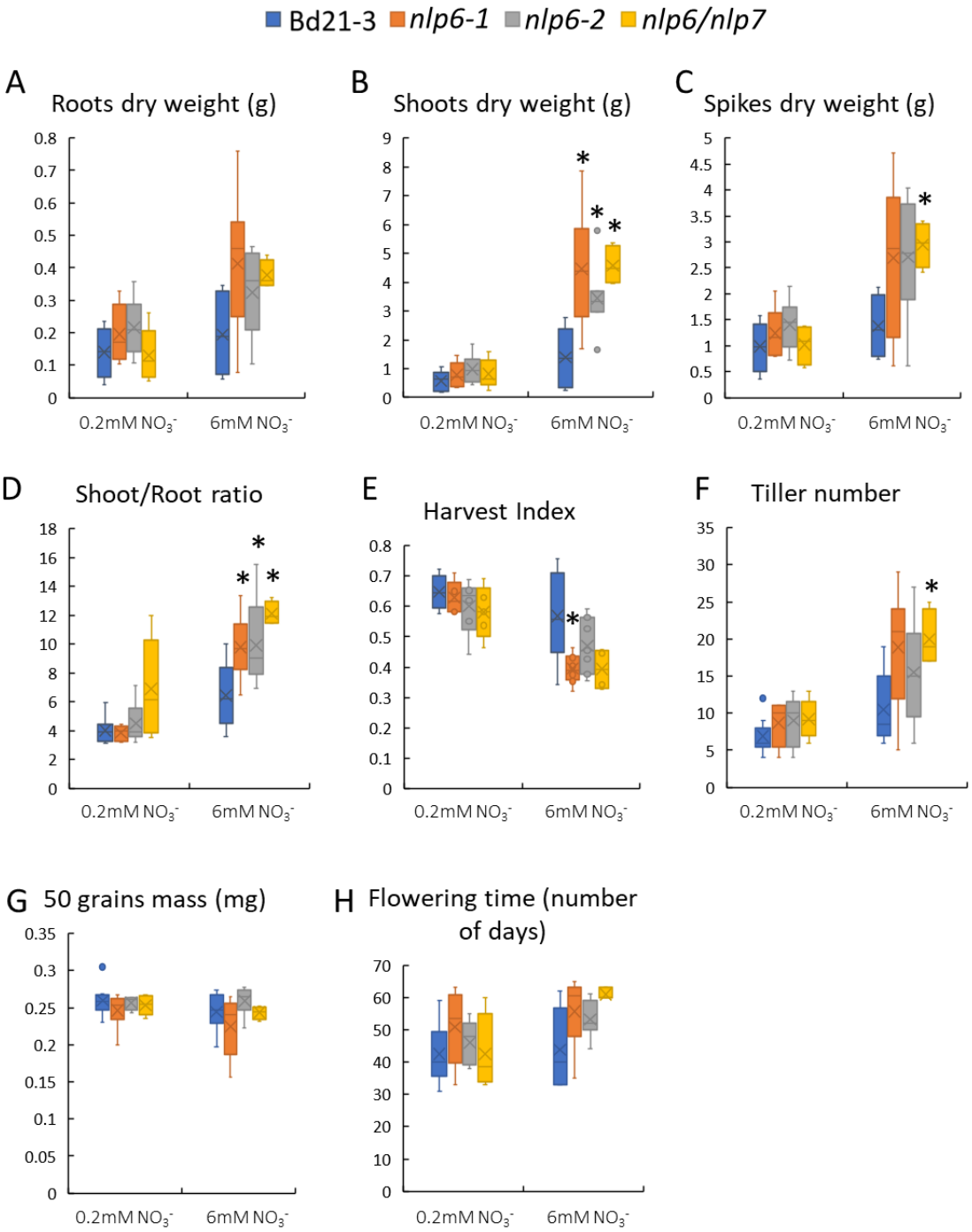


Figure 3: *Bdnlp* mutants are affected in shoots and roots growth in high nitrate steady-state condition.
Wild-type (Bd21-3) and mutants (*nlp6-1*, *nlp6-2*, *nlp6/nlp7*) plants were grown in hydroponics until the end of their cycle in medium containing 0.2mM or 6mM nitrate. Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n≥6 plants.
Asterixs (*) indicate statistical differences between wild-type (Bd21-3) and mutants within nitrate conditions according to a nonparametric ANOVA test.

BdNLP6 and BdNLP7 are involved in shoot development in high nitrate conditions

To test the involvement of Brachypodium BdNLP6 and BdNLP7 in nitrate/nitrogen metabolism we grew in hydroponics wild type (Bd21-3) and mutants (*nlp6-1*, *nlp6-2* and *nlp6/nlp7*) plants during the whole cycle on two nitrate conditions: a low nitrate condition (0.2mM) and a high nitrate condition (6mM). General growth and development parameters were measured (Figure 3). Surprisingly, *nlp* mutants display higher shoot dry mass compared to wild type, under high nitrate availability only (Figure 3B). This is consistent with a tendency to have a higher tiller number, despite not being always statistically significant (Figure 3F). Mutants also present a higher Shoot/Root ratio than WT plants (Figure 3D), which is consistent with a stronger enhancement of shoot dry weight than root dry weight (Figures 3A, B). Mutants display slightly higher spikes dry weight under 6mM conditions but interestingly the mass of 50 grains is not affected (Figure3C, G), suggesting that they produce more grains that has the same quality as those from Bd21-3. Finally, mutants show a slight increase of flowering time in 6mM conditions only, which is not statistically confirmed (Figure 3H).

These results allow us to identify a role of BdNLP6 and BdNLP7 only in ample nitrate conditions. Mutants present a better growth capacity compared to wild type, suggesting that the transcription factors are growth repressors acting in a nitrate dependent way.

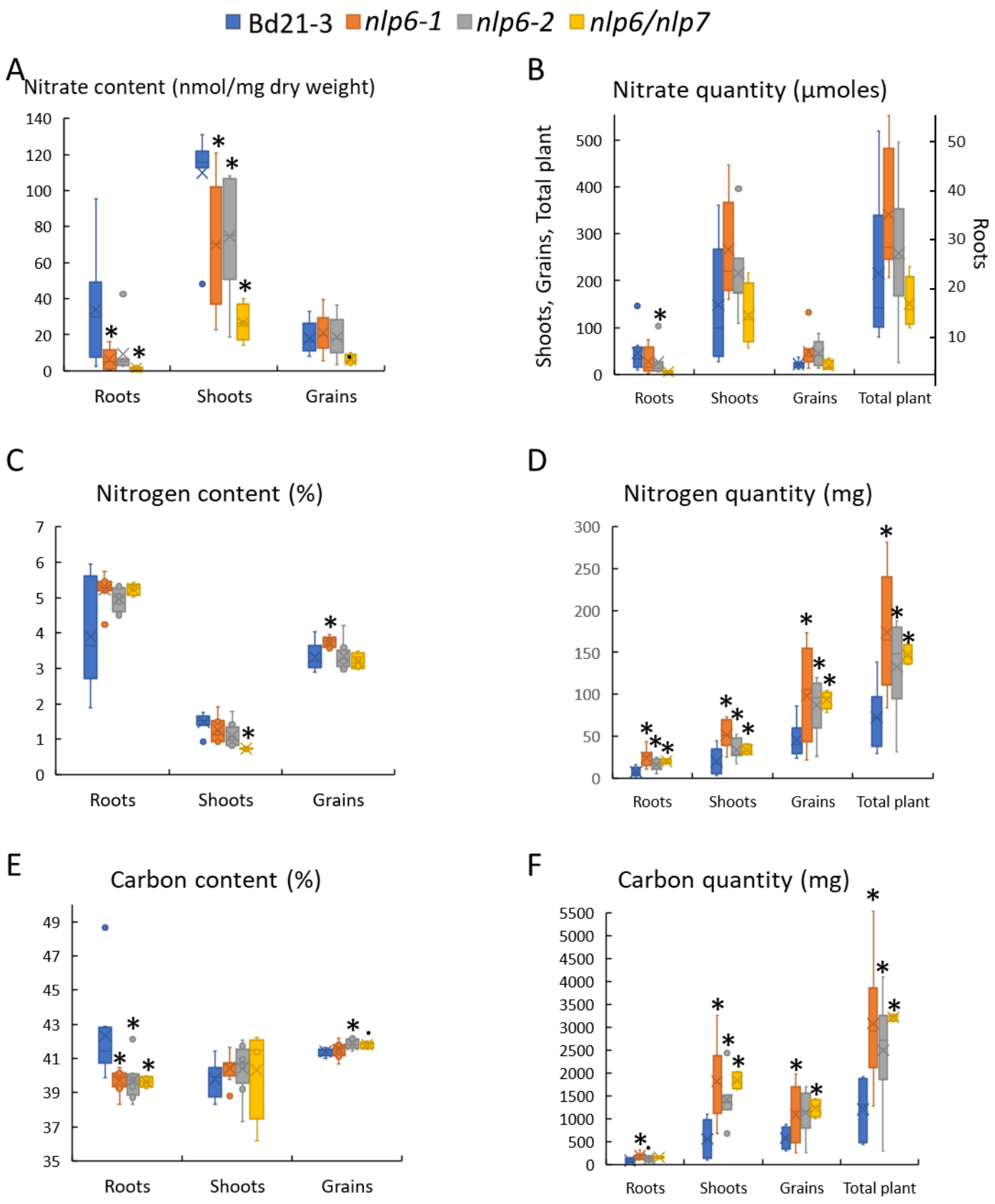


Figure 4: Bdnlp mutants are affected in plant nitrate, nitrogen and carbon contents under 6mM nitrate steady-state condition.

Wild-type (Bd21-3) and mutants (nlp6-1, nlp6-2, nlp6/nlp7) plants were grown in hydroponics until the end of their cycle in medium containing 6mM nitrate. (A-B) Nitrate content and total quantity, (C-D) nitrogen content and total quantity and (E-F) carbon content and total quantity have been quantified from all plant's parts on dry tissus. Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n≥6 plants. Asterixs (*) and dot (.) indicate statistical differences between wild-type (Bd21-3) and mutants according to a non parametric ANOVA test (pvalue < 0,1 . ; pvalue < 0,05 *)

Bdnlp6 and *Bdnlp6/nlp7* mutants are impaired in their nitrate, nitrogen and carbon contents and quantity specifically in 6mM nitrate condition

Since *Bdnlp6* mutants and *Bdnlp6/7* amiRNA line present a better growth under ample nitrate condition, the hypothesis arises that they may better use, uptake, assimilate and/or translocate nitrate. To confirm this, we quantified nitrate, nitrogen and carbon contents in roots, shoots and grains of plants grown on 0.2mM (supplementary Figure S2) or 6mM nitrate (Figure 4). On 0.2mM, similarly as seen before there is no effect of mutations or amiRNA construct neither on nitrate, nitrogen nor carbon contents and quantities (supplementary Figure S2), which confirm that BdNLP6 and BdNLP7 are not involved in controlling the plant development in this low-nitrate condition.

On 6mM nitrate condition, nitrate content in roots and shoots but not in grains is lower in the mutants than in Bd21-3 (WT) (Figure 4A). Nitrogen content (Figure 4C) is globally not affected in the lines, except a slight increase in shoots of *nlp6/nlp7* and a slight decrease in grains of *nlp6-1*. On the other hand, nitrate total quantity in different parts of the plant is not significantly affected, despite a possible decrease in mutant roots and a possible increase in mutant shoots and grains (Figure 4B). Nitrogen quantity is higher in all organs, and thus in total plants for all mutants (Figure 4D). Nitrogen and Carbon metabolisms are interdependent. Carbon content have been measured (Figure 4E) and appear to be affected in mutants only in roots. We can also denote a slight increase of carbon content in grains only for *nlp6-2* and *nlp6/nlp7* mutants. Similarly as for nitrogen quantity, mutants present a higher carbon total quantity compared to wild type in all the plant parts (Figure 4F).

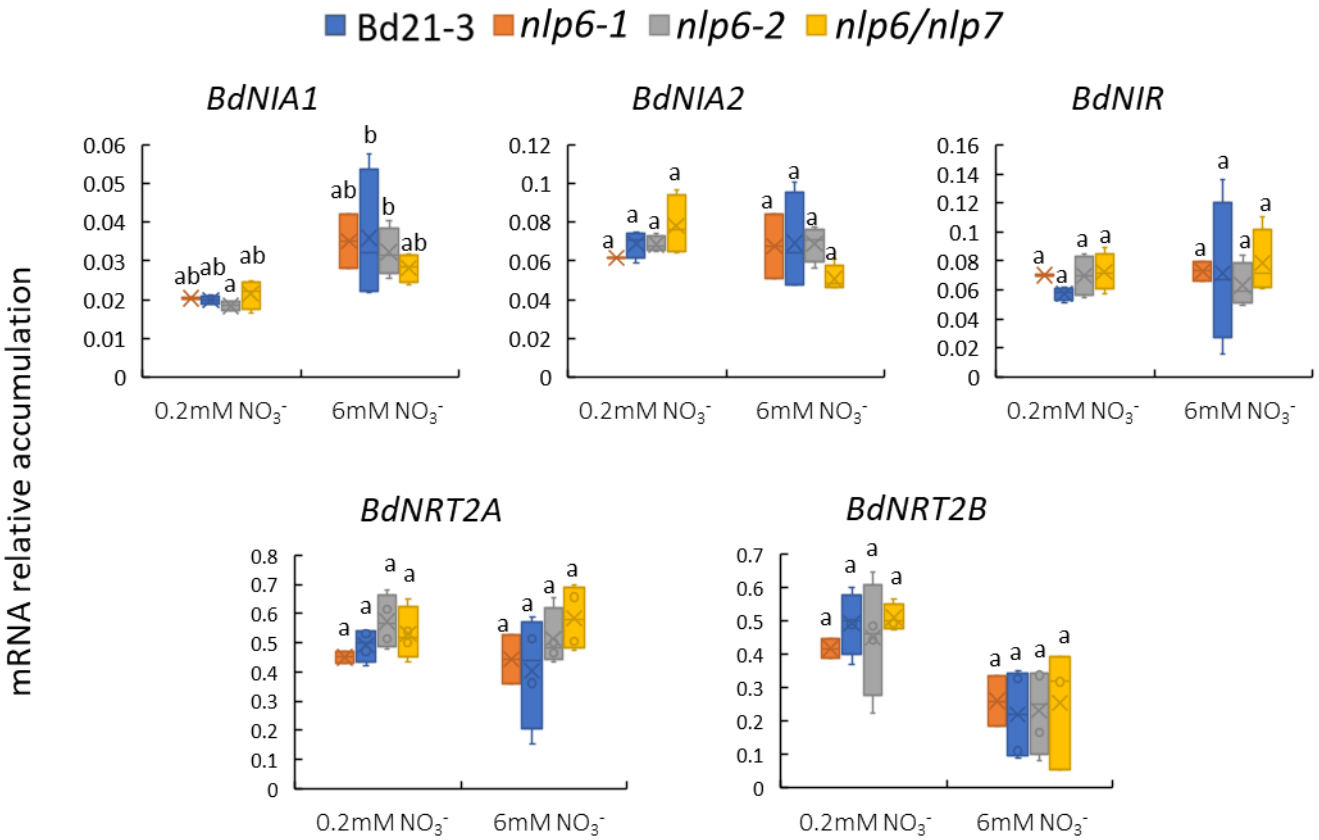


Figure 5: *Bdnlp* mutants are not affected in the expression of nitrate uptake and assimilation genes in roots in steady-state conditions.

Wild-type (Bd21-3) and mutants (*nlp6-1*, *nlp6-2*, *nlp6/nlp7*) plants were grown in hydroponics for 22 days in medium containing 0,2mM or 6mM nitrate. Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n=4 plants. Letters represent significant differences evaluated by ANOVA on log2-transformed data (pval<0.05).

These observations, combined with the general better growth of mutants, suggest that mutant plants present a general higher nitrate uptake, enabling a higher total nitrogen quantity and a better growth. Also, since the root nitrate quantity tends to be equal or diminished in mutants compared to wild-type, while there is a tendency of higher nitrate quantity in shoots for mutants, together with a general increase of nitrogen quantities, it suggests a high nitrate translocation from the roots to the shoots of mutants plants. Finally, mutants might also have an increased nitrate assimilation rate, enabling their better growth as compared to the wild-type. These hypotheses should be tested by monitoring nitrate uptake and translocation in the mutants, as well as by measuring the activities of nitrate-assimilation enzymes and the expression of genes necessary for nitrate metabolism. Still, these results confirm a negative role of BdNLP6 and BdNLP7 in the regulation of nitrate and nitrogen metabolism.

BdNLP6 and BdNLP7 do not control genes involved in nitrate uptake and assimilation in roots

Mutants display a deficit of nitrate content in roots and shoots, that could be due to a variation of expression of genes necessary for nitrate uptake and assimilation. To verify this, we measured expression level of genes encoding nitrate reductase (*BdNIA1* and *BdNIA2*), nitrite reductase (*BdNIR*) and transporters required for nitrate uptake (*BdNRT2A* and *BdNRT2B*) in roots of 22 days old plantlets grown in the exact same conditions of those used previously for end of cycle measurements (Figure 5). No difference of expression was observed in mutants compared to wild-type, neither on 0.2mM nor on 6mM nitrate media. It is to be stated that no clear phenotype has been seen in this stage (data not shown), thus it might be that BdNLP6 and BdNLP7 govern their expression at a later stage, which must be experimentally verified. Gene expression in the shoots and expression of genes involved in nitrate root-to-shoot translocation should also be characterized to further test our hypotheses.

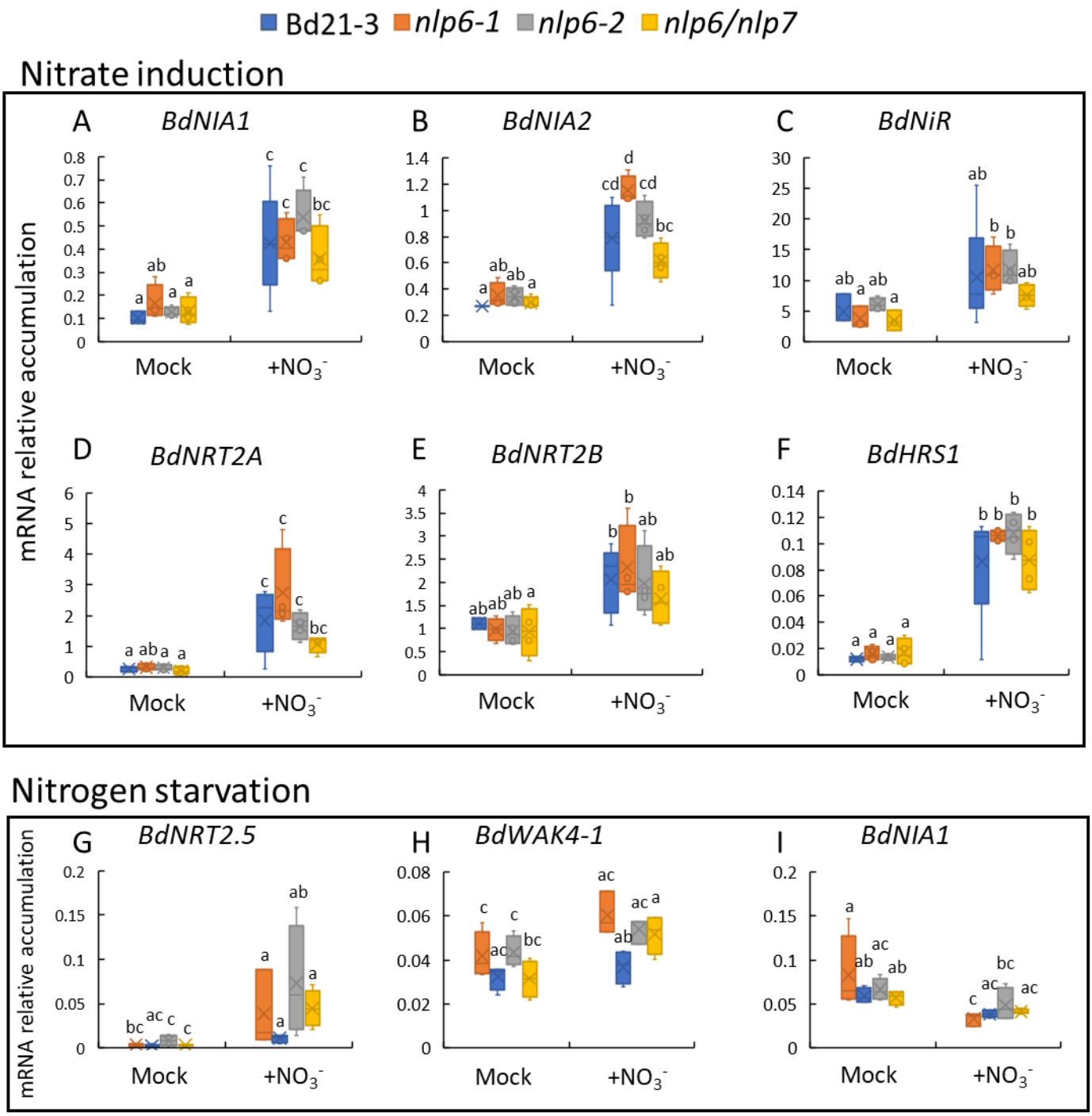


Figure 6: *Bdnlp* mutants are not affected in the regulation in roots of candidate genes by nitrate or starvation treatments.

(A-F) Wild-type (Bd21-3) and mutants (*nlp6-1*, *nlp6-2*, *nlp6/nlp7*) plants were grown in hydroponics for 18 days in normal medium containing 0,1mM CaNO3 + KNO3 and then transfered in a nitrogen starvation medium for 4 days and then treated for 3h with 1mM nitrate ($+NO_3^-$) or KCl (Mock).

(G-I) Wild-type (Bd21-3) and mutants (*nlp6-1*, *nlp6-2*, *nlp6/nlp7*), were grown in hydroponic basal media supplemented with 0.1mM nitrate for 15 days, and were then N starved (-N) or not (control, N) for 3 days. Roots were collected and used for gene expression analysis

Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n=4 plants. Letters represent significant differences evaluated by ANOVA on log2-transformed data (pval<0.05).

Interestingly, according to Brachypodium eFP Browser (Winter *et al.*, 2007; Sibout *et al.*, 2017), BdNLP6 is more expressed in young tissues: young leaf and young spikelet which further confirm its putative role in shoots (Supplementary Figure S3). On the other hand, *BdNLP7* expression seems more generalized the whole plant, similarly as *AtNLP7*.

AtNLP7 is known to be involved in primary nitrate response, notably by governing expression of genes necessary for nitrate uptake and assimilation. Since BdNLP6 and BdNLP7 are its closest orthologs, we tested whether BdNLP6 and BdNLP7 are involved in primary nitrate response. To do so, wild-type and mutant plants were grown on a basal medium supplemented with 0.1mM of nitrate before being nitrogen-starved for 4 days. Then, the 22 days old plantlets were treated with 1mM of nitrate for 3h and the roots expression of genes necessary for nitrate uptake and assimilation were monitored. The expression of *BdHRS1* was also quantified, as is it the closest ortholog of *AtHRS1,* known, *inter alia*, to be highly induced in response to nitrate treatment. Figure 6 A-F demonstrates that the Brachypodium mutants are not impacted in their nitrate response: the expression of all the tested genes is increased in response to nitrate-treatment in the different lines. Thus we checked if the BdNLPs are involved in the response to nitrogen starvation, by monitoring the root expression level of genes known to be regulated in this condition, such as *BdNRT2.5*, *BdWAK4* (which its closest ortholog in Arabidopsis is induced by N limitation in roots; Giehl et al., 2014) and *BdNIA1*. For this, plants were grown on basal medium supplemented with nitrate for 15 days, before being subjected to nitrogen starvation for 3 days. Despite a lower gene response to starvation than expected in the wild type, the response pattern seems conserved in the mutant lines, suggesting that BdNLP6 and BdNLP7 are not involved in response to nitrogen starvation.

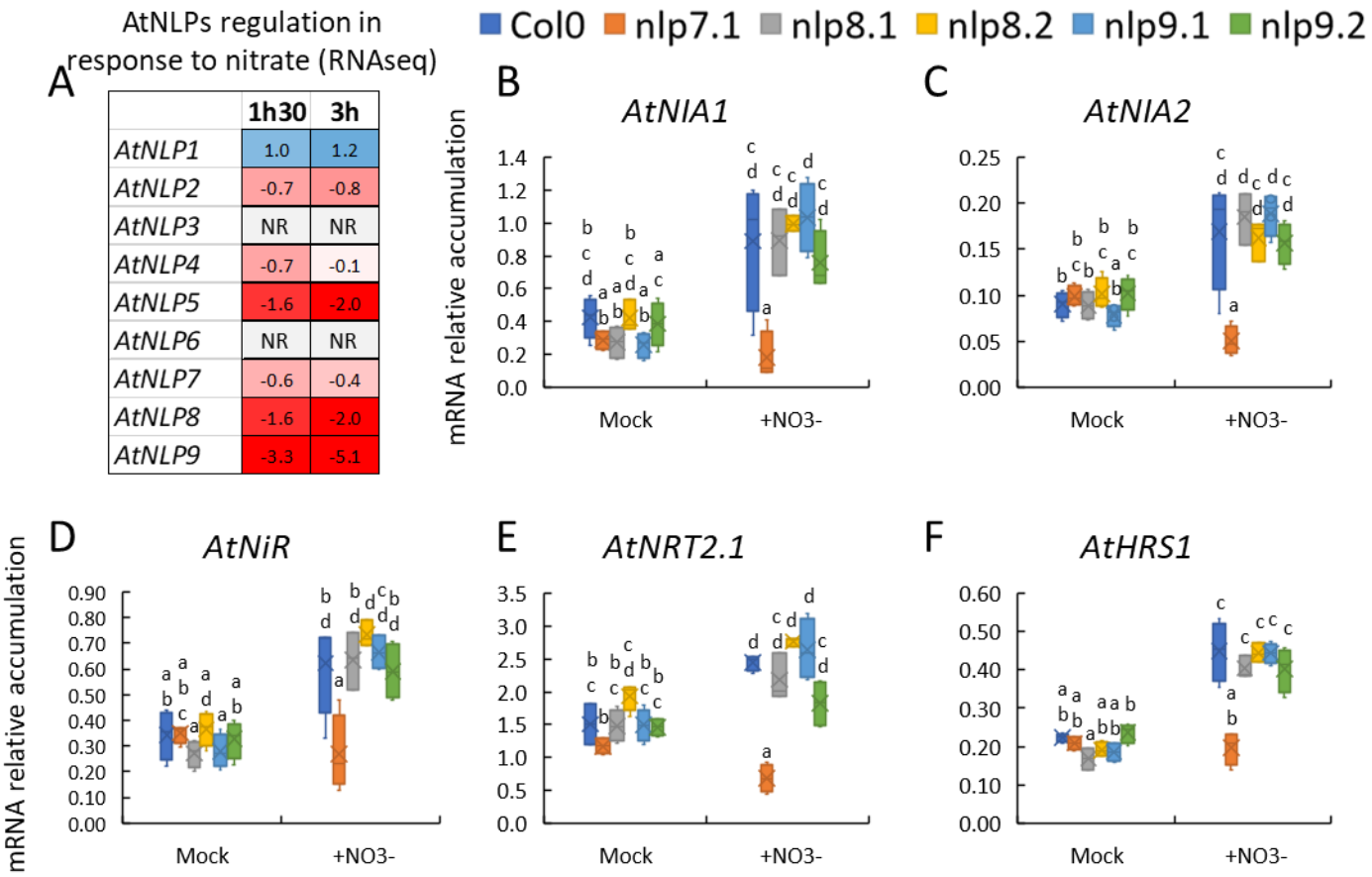


Figure 7: AtNLPs expressions are regulated by nitrate treatement, and *Atnlp8* and *Atnlp9* mutants are not affected in their nitrate response.

(A) 22 days old Arabidopsis (Col0) plantlets were treated with 1mM nitrate or mock-treated (1mM KCl) for 1h30 and 3h after 4 days starvation and roots were sampled for RNAseq analysis. Values correspond to Log2FC compared to mock condition.

(B-F) Wild-type (Col0) and mutants (*nlp8.1*, *nlp8.2*, *nlp9.1 and nlp9.2*) plants were grown in hydroponics for 18 days in basal medium supplemented with 0,1mM nitrate and then transfered on a nitrogen starvation medium for 4 days and then treated for 3h with 1mM nitrate ($+NO_3^-$) or KCl (Mock). Roots were collected and used for gene expression analysis. Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n=4 plants. Letters represent significant differences evaluated by ANOVA on log2-transformed data (pval<0.05).

Taking together, these results highlight that, unlike AtNLP6 and AtNLP7, BdNLP6 and BdNLP7 do not seem to be involved in regulating genes necessary for nitrate uptake and assimilation, neither in a primary nitrate response context nor in response to nitrogen starvation. As mentioned before, It would however be interesting to monitor these responses of gene expression at later stages of plant development, as the growth phenotypes of the mutants are stronger at the end of their cycle.

AtNLP8 and *AtNLP9* genes are also downregulated in response to nitrate treatment, and are not involved in primary nitrate response

Based on the expression and phenotype data acquired on *BdNLP6/7* in Brachypodium, we tested the hypothesis that their function in shared by Arabidopsis genes. Although *BdNLP6* and *BdNLP7* are phylogenetically the closest orthologs of *AtNLP7*, the fact that *BdNLP6* is downregulated by nitrate treatment, contrary to *AtNLP7*, suggested they have a different role. We thus searched for *AtNLPs* that were regulated the same way as *BdNLP6*, on Arabidopsis plants grown in similar conditions as for our Brachypodium experiments. By RT-qPCR (data not shown) and RNAseq (Figure 7A) we identified *AtNLP5*, *AtNLP8* and *AtNLP9* as strongly downregulated in response to 1mM nitrate. In parallel, *AtNLP1* was slightly induced, *AtNLP2*, *AtNLP4* and *AtNLP7* were slightly downregulated and *AtNLP3* and *AtNLP6* did not appear regulated in our conditions. *Atnlp8* and *Atnlp9* knock-out mutants were available in the lab, so we tested whether their nitrate response was not affected by the mutations, as observed for *Bdnlp6/7* mutants. Expression levels of sentinel genes of nitrate response were measured after 3h of nitrate treatment (Figure 7B-F). *Atnlp7.1* mutant, known to be affected in nitrate response, was used as a control and, as expected, was impaired in the induction of *AtNIA1*, *AtNIA2*, *AtNiR*, *AtNRT2.1* and *AtHRS1* expressions. On the other hand, induction of nitrate-responsive genes was still occurring in *Atnlp8* and *Atnlp9* mutants. These results illustrate that NLPs that are downregulated in response to nitrate

treatment might have other functions than those described for AtNLP7. To go further, characterization of *Atnlp8* and *Atnlp9* growth until the end of cycle would be interesting to compare phenotypes with those observed for *Bdnlp6* and *Bdnlp7* mutants. It also shows that unlike AtNLP7 and AtNLP6 that have been described to have redundant roles, other *AtNLP* may have different role that needs to be characterized.

DISCUSSION

In this study we show that besides BdNLP7, all BdNLPs are transcriptionally dependent on nitrate or nitrogen availability. *BdNLP1*, *BdNLP2*, *BdNLP3* are more expressed under low level of nitrate (Figure 1C, D, E), they are repressed in response to nitrate after nitrogen starvation (Figure 2A) and they are induced by starvation (Figure 2D, E, F). *BdNLP4* seems only regulated by nitrogen starvation (Figure 2G) contrary to *BdNLP5* which expression increases as the nitrate concentration increases (Figure 1G) and is induced by nitrate treatment (Figures 2A). *BdNLP6* is repressed by nitrate treatment (Figure 2B) while *BdNLP7* expression is slightly induced by nitrate treatment (Figure 2A). These differential nitrate-dependent transcriptional regulations between *BdNLPs* are consistent with transcriptional regulation of NLPs by nitrate in Arabidopsis (Chardin *et al.*, 2014), which have been confirmed by our study (Figure 7A), in maize (Cao *et al.*, 2017; Wang *et al.*, 2018*b*; Ge *et al.*, 2020), in rice (Alfatih et al., 2020; Wang et al., 2020; Wu et al., 2020), in wheat (Kumar *et al.*, 2018) and very recently in buckwheat (Liu *et al.*, 2021). Also, some NLPs shuttle between nucleus and cytoplasm, such as AtNLP7 (Marchive *et al.*, 2013), ZmNLP6 and ZmNLP8 (Cao *et al.*, 2017), OsNLP3 and OsNLP4 (Wang et al., 2018) while AtNLP8 is constrictively localizes in the nucleus (Yan *et al.*, 2016*a*). Variations of transcriptional responses of *NLP* genes as well as different post-translational regulations suggest specific non-redundant roles within the NLP family.

Further characterizations using *Bdnlp6-1*, *Bdnlp6-2* mutants and *Bdnlp6/Bdnlp7* transgenics show an unexpected phenotype: mutants present a higher shoot growth compared to wild-type. Thus, BdNLP6 and possibly BdNLP7 seem to be growth repressors at late stage of plant development only in presence of ample nitrate condition. Furthermore, mutants have greater nitrogen and carbon total quantities, and expression of gene related to nitrate uptake and assimilation are not impaired in mutants. These results are the total opposite of observed phenotype in other characterized *nlp* mutants. Indeed, all characterized *nlp* mutants display growth deficiency explained by lower expression of genes related to nitrate transport and assimilation, as well as a reduced activity of nitrate assimilation enzymes. Thus, BdNLP6 (and possibly BdNLP7) appears to have very divergent functions as compared to described NLPs. The phenotypes described here are similar to the ones of *Tabzip60* wheat mutant, affected in a transcription factor involved in nitrate response as a growth repressor (Yang *et al.*, 2019). Similarly as for *BdNLP6*, *TabZIP60* expression decreases in response to nitrate treatment. Moreover, reducing *TabZIP60* expression seems to increase shoot growth and increases N accumulation in shoots but do not impact grain weight, similarly as in *Bdnlp6* mutants and *Bdnlp6/Bdnlp7* amiRNA line, while overexpression *TabZIP60* has the opposite effect. These observations were further explained by an inhibitory effect of TabZIP60 on the expression of *TaNADH-GOGAT-3B*. Thus, testing the expression of the ortholog of *TaNADH-GOGAT-3B* in *Bdnlp* mutants could help us to understand our results. Also, TabZIP60 was firstly characterized as involved in abiotic stresses. Arabidopsis plants overexpressing TabZIP60 are more tolerant to drought and cold and have an increased sensitivity to ABA (Zhang *et al.*, 2015). In agreement, *TabZIP60* expression is induced by ABA application (Yang *et al.*, 2019). Investigating the impact of ABA on *BdNLP6* and *BdNLP7* expression and on *Bdnlp* phenotypes could also test whether this growth repressor role is linked to a role in plant response to stresses.

To further investigate the non-implication of BdNLP6 in transcriptional Primary Nitrate Response under our conditions, we look for Arabidopsis *AtNLP* that were down-regulated in response to nitrate, similarly to *BdNLP6*. It appears that *AtNLP2*, *AtNLP4*, *AtNLP7*, *AtNLP8* and *AtNLP9* expressions decrease after nitrate treatment (Figure 7A). Similarly to the observations in Brachypodium *nlp* mutants, nitrate-responsive genes were still induced in the *Atnlp8* and *Atnlp9* mutants (Figure 7 B-F), suggesting that AtNLP8 and AtNLP9 are not involved in the transcriptional control of root nitrate response. Unlike AtNLP7 which subcellular localization is nitrate-dependent, AtNLP8 is constitutively present in the nucleus (Yan *et al.*, 2016). AtNLP8 is involved in nitrate signaling during germination by activating expression of *CYP707A2*, encoding an ABA catabolic enzyme (Yan *et al.*, 2016). As ABA is a germination repressor, its catabolism led to activation of germination. BdNLP6 could have role more similar to AtNLP8 and AtNLP9 rather than to its closest ortholog, AtNLP7. Precisely evaluating the germination rate of *Bdnlp6-1* and *Bdnlp6-2* mutants might confirm a similar function between BdNLP6 and AtNLP8, even though we did not observe germination delays compared to wild-type (result not shown). Conversely, characterization of *Atnlp8* and *Atnlp9* growth until the end of their cycle could be done, to compare to the higher final shoot growth observed for *Bdnlp6-1*, *Bdnlp6-2* and *Bdnlp6/nlp7*.

Another parameter to consider for the interpretation of *nlp6-1* and *nlp6-2* phenotypes is that BdNLP6 protein sequence display a degenerated RWP-RK signature: HWPxRE. The RWPxRK domain is conserved in NLPs and is known to be necessary for binding *cis*-elements of target genes (Konishi and Yanagisawa, 2019). Thus, this sequence divergence could lead to different targets and thus a different role. Also, since the phenotypes of *Bdnlp6/Bdnlp7* amiRNA line are similar to the ones of *Bdnlp6* mutants and since the amiRNA line retains non-negligeable remaining expression of both *BdNLP6* and *BdNLP7* (Figure S1), the observed phenotypes might be solely due to BdNLP6, and not to BdNLP7. Thus, it would be interesting to study a *Bdnlp7* simple mutant to investigate the role of *BdNLP7*. Different roles for the two paralogs would

be in agreement with the fact that they are responding in opposite manners to nitrate treatment at the transcriptional level.

This present work lays the bases for an in-depth characterization of the functions of *BdNLPs* and suggests a negative control of nitrate assimilation and/or translocation plant growth by BdNLP6 in condition of ample nitrate availability.

MATERIAL AND METHODS

Plant material

Brachypodium distachyon Bd21-3 accession and *Arabidopsis thaliana* Col0 accession were used in this study. The mutants *Bdnlp6-1* and *Bdnlp6-2* carry respectively the non-sens mutations W513* and E298* identified by TILLING (Dalmais *et al.*, 2013). The amiRNA line contains the GAGGCTGTATATCTTAAAAG sequence, targeting a region at the beginning of the 3rd exon conserved between BdNLP6 and BdNLP7 but not with other BdNLPs. Arabidopsis mutants correspond to SALK_26134 (*Atnlp7.1*), SALK_140298 (*Atnlp8.1*), SALK_031064 (*Atnlp8.2*), SALK_025839 (*Atnlp9.1*), SALK_098057 (*Atnlp9.2*).

Growth conditions

For Brachypodium experiments, seeds were sowed in water and stratified for 3 days at 4°C. Seeds are then placed at room temperature for germination for 7 days. Plantlets are transferred in extra-long days growth chambers during 22 days (22°C, 18h Day; 19°C 6h Night) with 250 µE/m^2/s light on hydroponics on basal medium (0.35 mM K_2SO_4, 0.25 mM $CaCl_2$, 0.25 mM KH_2PO_4, 0.25 mM $MgSO_4$, 10 mg.L^{-1} Fer-EDTA, 243 µM $Mo_7O_24(NH4)_6$, 0.4 µM H_3BO_3, 118 µM SO_4Mn, 10 µM SO_4Cu and 34.8 µM SO_4Zn)

supplemented with different sources of nitrogen. For characterizing Brachypodium *BdNLP* expression upon various nitrate and ammonium conditions, the basal medium was supplemented either with no nitrogen or 0.002 mM, 0.02 mM, 0.2 mM, 2 mM or 6 mM of NO3- (mix of KNO_3 and $Ca(NO_3)_2$ at 1/0.5 molar ratio), or 0.002 mM or 0.2 mM of NH_4Cl. Medium was renewed twice a week. Roots were harvested 22 days after transfer on hydroponics and frozen in liquid nitrogen. For nitrate starvation characterization, the basal medium was supplemented with 0.05mM of KNO_3 and 0.025mM $Ca(NO_3)_2$. After 15 days, plantlets were transferred on basal medium without N source (5.5 mM KCl) and roots were sequentially harvested after 6h hours or 1, 2, 3 or 6 days for wild-type, and after 1 and 3 days for mutants, and frozen in liquid nitrogen. For end of cycle measurements, the basal medium was supplemented with either with 0,2 mM or 6 mM of NO_3^- (mix of KNO_3 and $Ca(NO_3)_2$ at 1/0.5 molar ratio) until end of cycle. Medium was renewed two to three times per weeks. Nitrate content of the medium was tested using Nitrachek (STEP Systems GmbH) to ensure its non-depletion between renewals. Plants were dried in a 70°C oven for 2 days. For nitrate induction and RNAseq experiments in Brachypodium and Arabidopsis, same conditions were used as previously described in Gregoire *et al.* 2021; Chapter II of this thesis manuscript).

Measurement of nitrate content

Nitrate content was measured by a spectrophotometric method adapted from Miranda and colleagues (Miranda *et al.*, 2001) after extraction in water of 1–2 mg dry weight ground tissues. The principle of this method is a reduction of nitrate by vanadium (III) through the acidic Griess reaction. The Griess reagent is a mixed solution of VCl_3 (2.5% w/v), N-1-naphtylethylenediamine (0.05% w/v) and sulfanilamide (1% w/v) in 0.5M HCl. It was added to plant extracts in equal proportion. After 2 hours of incubation at 60 °C, the diazoniium product was measured spectrophotometrically at 540 nm.

Measurement of N and C contents

Total N and C contents were quantified on 2–5mg DW aliquots of ground tissues, dried at 70°C for 48 h. The method is based on the Dumas combustion analysis which consist in a flash combustion of the samples in presence of He and O_2 at 950°C. The C and N elements are detected by gas chromatography on a FLASH 2000 Organic Elemental Analyzer (Thermo Fisher Scientific, Villebon, France). The $^{15}N/^{14}N$ isotopic ratio is subsequently quantified by a coupled mass spectroscope (Delta V advantage IRMS; Thermo Fisher Scientific, Villebon, France).

RNA extraction and RT-qPCR

Total RNAs were isolated using Trizol® reagent (Ambion, Life Technologies), treated by DNase I (Thermo Scientific) and reverse-transcribed using oligo(dT)18 primer and RevertAid H Minus Reverse Transcriptase (Thermo Scientific), according to the manufacturer's protocols. For qPCR, cDNA are diluted (1:20), and 2.5µL of this dilution is used with 2.5µL SyBr mix (SsoAdvanced™ Universal SYBR® Green Supermix) which contains 0.03µM of primer forward and reverse. Relative quantification and statistics have been made using Rieu & Powers, 2009 methods. Normalization is made by dividing Ct of genes of interest by Ct of a synthetic gene, which is the geometrical mean of 2 (for Barley) or 3 (for Arabidopsis and Brachypodium) Ct of housekeeping genes. Primer list is available in Supplementary Table S1. Primer efficiency (eff) was determined at each run using a standard curve on a pool of cDNA.

Statistical analyses

All statistical tests have been performed with the R software, using the multcomp, coin and RVAideMemoire packages. One-way ANOVA tests were used in conjunction with Tukey tests for the analysis of qRT-PCR data. Non-parametric one-way ANOVAs were performed using an approximate Fisher-Pitman permutation test, followed by a pairwise comparison test with the calculation of an adjusted P-value (FDR method).

ACKNOWLEDGMENTS

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AUTHOR'S CONTRIBUTIONS

MG, AK and TG conceived the experiments. MG, PB, LJ, QM, GD did the experiments. MG wrote the manuscript.

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SUPPLEMENTARY DATA

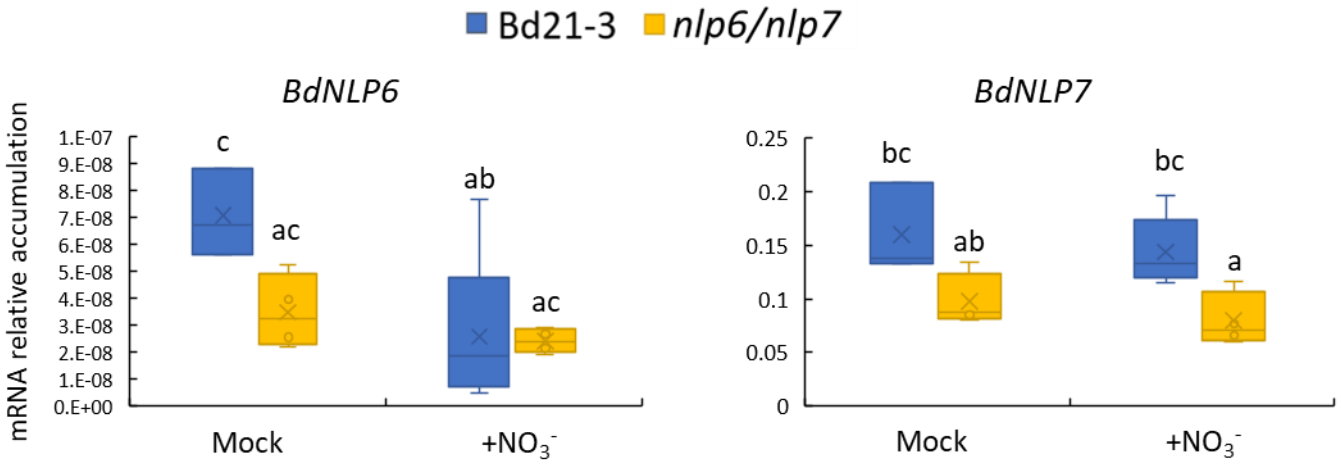

Figure S1: *BdNLP6* and *BdNLP7* root expression levels in amiRNA *nlp6/nlp7* line.
Wild-type (Bd21-3) and *nlp6/nlp7* plants were grown in hydroponics for 18 days in a medium containing 0,1mM NO_3^-, transfered in a nitrogen starvation medium for 4 days and then treated for 3h with 1mM NO_3^- (+NO_3^-) or KCl (Mock).
Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n=4 plants. Letters represent significant differences evaluated by ANOVA on log2-transformed data (pval<0.05).

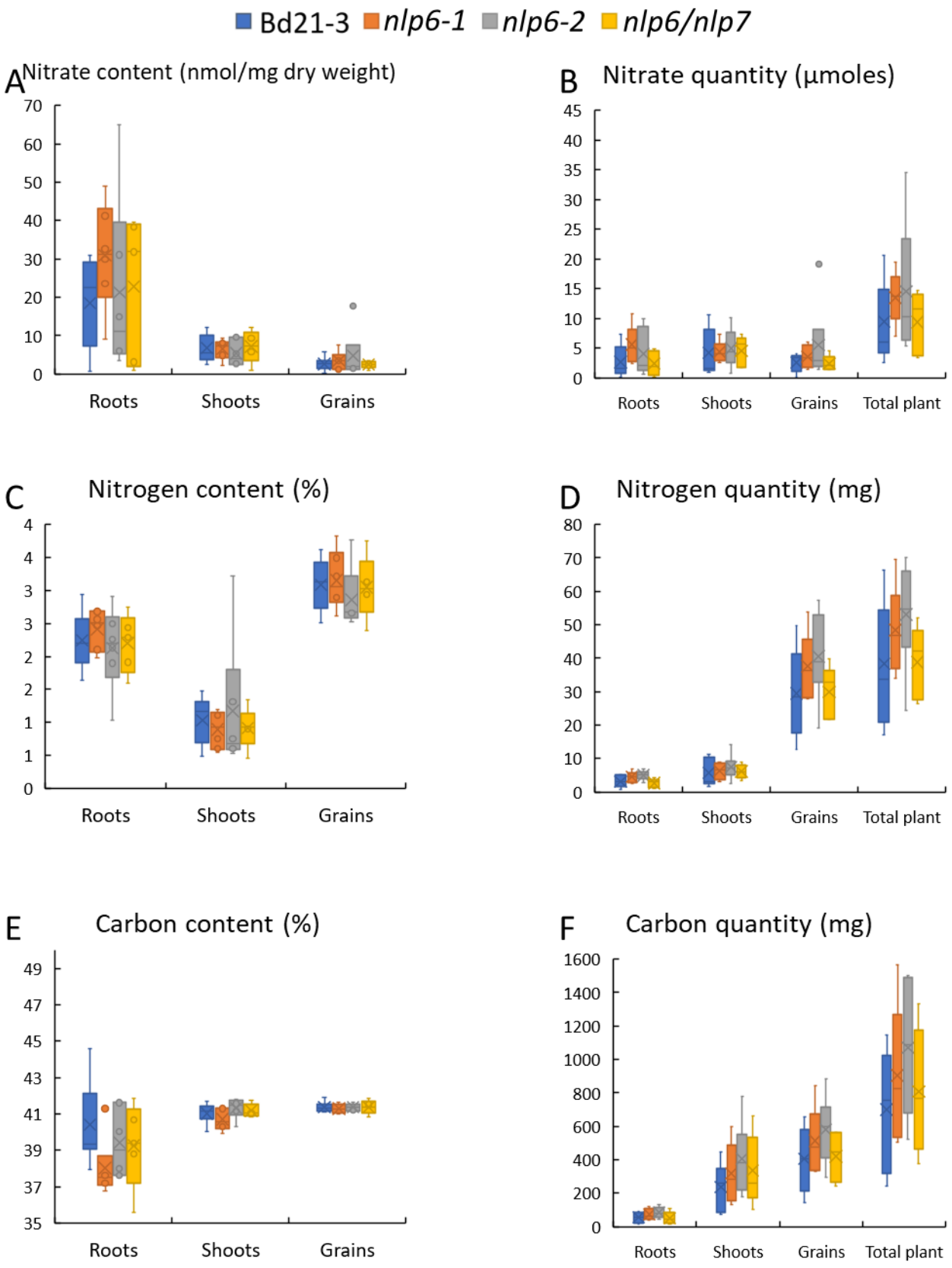

Figure S2: *Bdnlp* mutants are not affected in plant nitrate, nitrogen and carbon contents under 0.2mM nitrate steady-state condition.

Wild-type (Bd21-3) and mutants (*nlp6-1*, *nlp6-2*, *nlp6/nlp7*) plants were grown in hydroponics until the end of their cycle in medium containing 0.2mM nitrate. (A-B) Nitrate content and total quantity, (C-D) nitrogen content and total quantity and (E-F) carbon content and total quantity have been quantified from all plant's parts on dry tissus. Box-plots represent mean value (cross), median value (intern line) and minimum and maximum data points (extremities) of n≥6 plants. Asterixs (*) and dot (.) indicate statistical differences between wild-type (Bd21-3) and mutants according to a non parametric ANOVA test (pvalue < 0,1 . ; pvalue < 0,05 *)

BdNLP6 *BdNLP7*

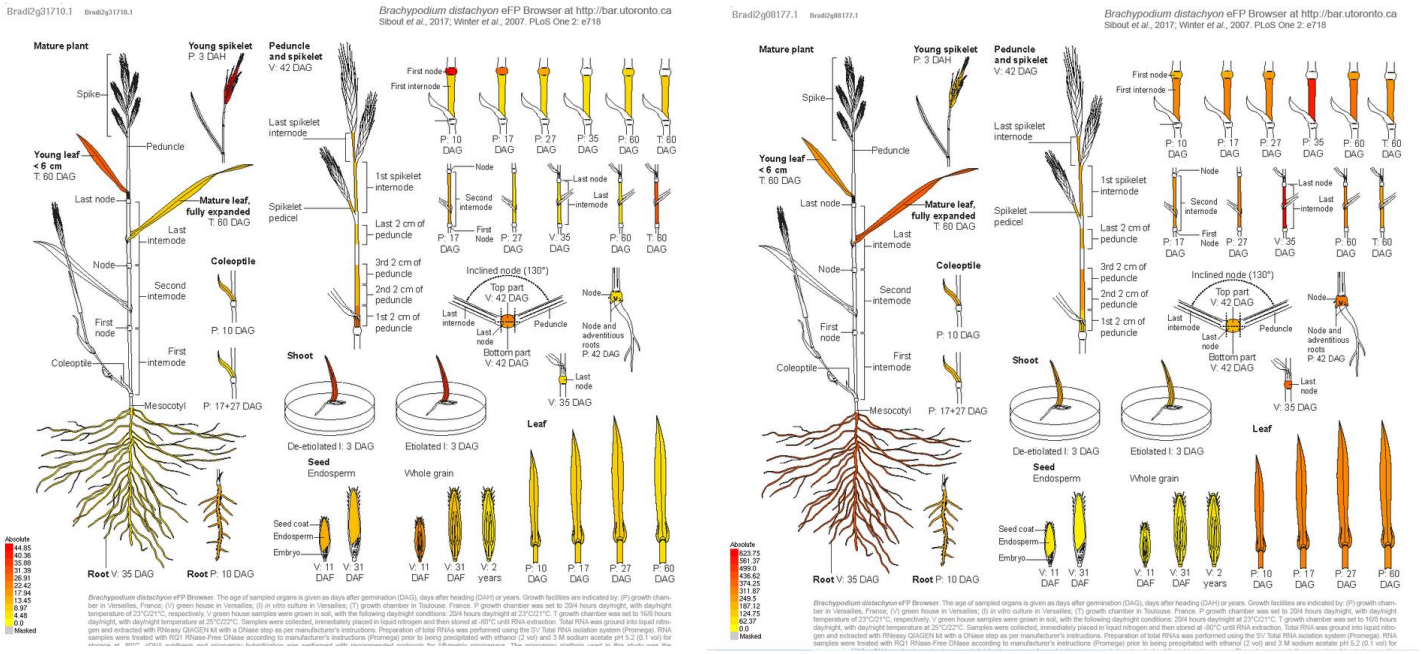

Figure S3: Expression localization of BdNLP6 and BdNLP7 according to Brachypodium eFP Browser (Winter et al., 2007; Sibout et al., 2017)

Gene		Primer sequence (5'->3')
UBC18 (DV481689)	Forward	GGAGGCACCTCAGGTCATTT
	Reverse	ATAGCGGTCATTGTCTTGCG
BdSAMDC (DV482676)	Forward	TGCTAATCTGCTCCAATGGC
	Reverse	GACGCAGCTGACCACCTAGA
BdNIA1 (Bradi3g37940)	Forward	CGAGTCCGACAACTACTACC
	Reverse	CGTCGTGAATGCGTTGAT
BdNIA2 (Bradi3g57680)	Forward	GCTGGTGTTTCTGGTCAGTA
	Reverse	GGAACCAGCAGTTGTTCATC
NRT2A (Bradi3g01270)	Forward	GTCGGGTTCCATCTTCTCG
	Reverse	CCGATGATCTTGCTGTTGAA
EF1α (DV482887)	Forward	CCATCGATATTGCCTTGTGG
	Reverse	GTCTGGCCATCCTTGGAGAT
BdNLP6 (Bradi2g31710)	Forward	TGGTCCTGCAAGCTCATCGT
	Reverse	TGGCTAAAAGGGTTTGTGGGGA
BdNLP7 (Bradi2g08177)	Forward	AGCCTTTCCAAGAACCAGAGCT
	Reverse	ATTGGCCCGAAATTGCTGGG
BdNLP1 (Bradi1g76340)	Forward	TGCAAGAACTTCGGAAGCAT
	Reverse	CCTTCAGCTCCATGTACCGA
BdNLP2 (Bradi4g37147)	Forward	AAAGGCGCACAAAGACAGAG
	Reverse	GATCTTTCGTGATGGCCAGC
BdNLP3 (Bradi4g20720)	Forward	CAGAAAATACGCAGCTCCACA
	Reverse	TTACGAGATGGCCACCTTGA
BdNLP4 (Bradi5g23300)	Forward	AGAGGCCAGCTTCTTCAACT
	Reverse	ACCACCTTCTCCTTTCTCCG
BdNLP5 (Bradi3g03170)	Forward	TTCCGGAAGATCAGCAAGGT
	Reverse	GTTCTGGTCAGTGGAGGAGG
BdNIR (Bradi3g57990)	Forward	CAGAAGACCCGCATGATGTG
	Reverse	TGCCTTGTCGATCATGTCCT
BdHRS1 (Bradi1g07630)	Forward	GGTGATGAAGGTTGATGGGC
	Reverse	GAGCTGCTTTGTTCCTGAGG
BdWAK4-1 (Bradi3g01157)	Forward	CTATGGGTGGGGATGCTGAT
	Reverse	CCCAAATGGCACCCTATTCG
BdNRT2A (Bradi3g01270)	Forward	GTGGAGACCGGCGACATG
	Reverse	ATGATAGGCACCAGGGGC
BdNRT2B (Bradi3g01250)	Forward	TAAGCTAGCTCGGACATGGA
	Reverse	ATGATAGGCACCAGGGGC
BdNRT2.5 (Bradi2g47640)	Forward	CAACTACCACAAGCTGCACA
	Reverse	TAGAAGTACTGCGCCACGAT
AtNIA1 (At1g77760)	Forward	ACGGAGCATGGATGAGTT
	Reverse	ATCGTCAAAGAAACCGAAGTC
AtNIA2 (At1g37130)	Forward	CATGCACGAACAGCAATC
	Reverse	GGTTACGCATATTCCGGAG
AtNRT2.1 (At1g08090)	Forward	AGTCGCTTGCACGTTACCTG
	Reverse	ACCCTCTGACTTGGCGTTCTC
AtEF1α (At5g60390)	Forward	CTGGAGGTTTTGAGGCTGGTAT
	Reverse	CCAAGGGTGAAAGCAAGAAGA
AtUBI10 (At4g05320)	Forward	GGCCTTGTATAATCCCTGATGAATAAG
	Reverse	AAAGAGATAACAGGAACGGAAACATAGT
AtACT2 (At3g18780)	Forward	GCCATCCAAGCTGTTCTCTC
	Reverse	CCCTCGTAGATTGGCACAGT
AtNiR (At2g15620)	Forward	TGCTGATGACGTTCTTCCACTCTGC
	Reverse	CTGAGGGTTGACTCCGAAATAGTCTC
AtHRS1 (At1g13300)	Forward	CCTAACAACGGAAACTCTCAA
	Reverse	TCCACAATAATCGACGTTCTA

Supp Table S1: Primers used for qRT-PCR analysis.

CHAPTER IV

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GENERAL DISCUSSION AND PROSPECTS

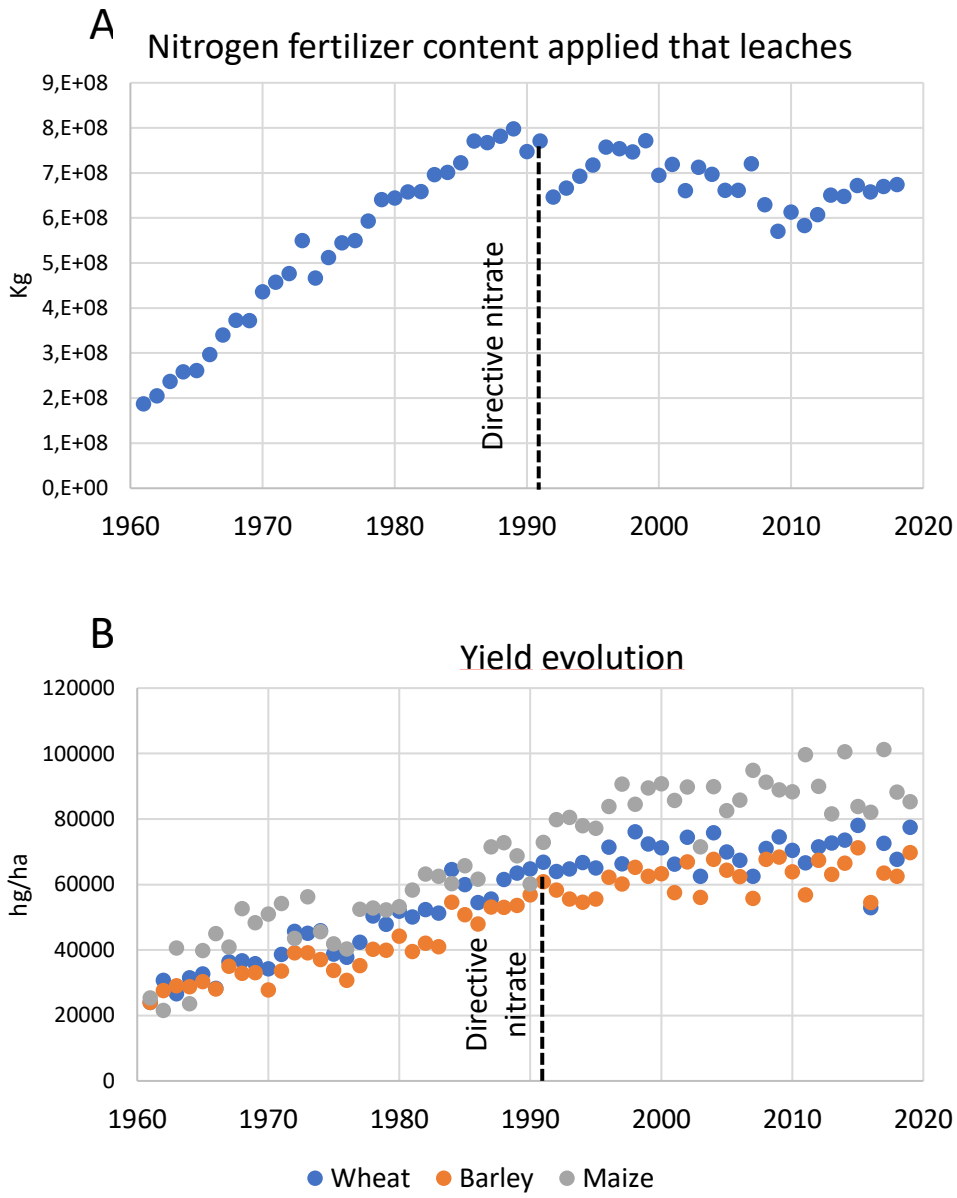

Figure 1: « Directive nitrate » impact on nitrogen fertilizer that leaches (A) and yield (B) in France (FAO STAT 2020).

GENERAL DISCUSSION AND PROSPECTS

The actual social and economic context highlights new issues on agricultural practices. Farmers are now more and more challenged on their agricultural practices, some of which being known to cause deleterious effect both on human health and on environment (phytosanitary products, fertilizers). Nitrogen (N) is a macro-element essential for plant growth and production and is often limiting in fields. Fertilizers are thus universally used by farmers to ensure yields. In fertilizers, nitrogen can be brought through nitrate and/or ammonium forms. Nitrate is the preferential source of nitrogen for most cereals, except rice which prefer ammonium. However, nitrate leaching due to overuse of fertilizers as well as nitrate from farm animal dejections cause dramatically water pollutions. To limit the impact of nitrate coming from agriculture on water pollution in Europe, European Union set the "nitrate directive" law since 1991. In response, states members had to identify vulnerable zones and to better control nitrate incomes in these zones. After around 30 years, this law allows the diminution of nitrogen fertilizers applied that leaches without decreasing yields in France (Figure 1, FAOSTAT). However, since 2010, a slight increase is denoted of nitrogen fertilizer that leaches. Reinforcement of regulations is not particularly welcome by farmers since they induce high economical costs and yield decrease. Finally, efforts that have been made seem not sufficient. A solution would be the development of new cultivars which require less nitrogen fertilizers while maintaining high yields. To achieve such a program, the molecular comprehension of nitrate response and metabolism can lead to identification of target genes or processes to include into selection programs.

The aim of my thesis project was to characterize molecular nitrate response mainly in Brachypodium, as a model for *Pooideae*, and also in barley. For this purpose, two strategies were adopted. The first one aimed at identifying nitrate responsive processes and/or genes that are specifically regulated in *Pooideae* using a cross-species

transcriptomic approach on Brachypodium, barley and Arabidopsis as a reference. Our main hypothesis was that even though monocots and dicots might share common nitrate responsive pathways, divergence of response must exist. Such a comparison will lead to identification of processes or at least genes that cannot be highlighted using Arabidopsis, which present divergent regulations in response to nitrate, and thus interesting candidate for improving NUE for example.

The second objective was to characterize BdNLP6 and BdNLP7 as they are closest orthologs of AtNLP7, a major regulator of molecular nitrate response. Our main hypothesis, the simplest one, was that BdNLP6 and BdNLP7 mediates nitrate response in Brachypodium in a similar way as AtNLP7. Finally, we show that BdNLP6 seem to carry a totaly divergent function than AtNLP7.

All along my manuscript (particularly in the transcriptome analysis), I choose to use "nitrate response" rather than "Primary Nitrate Response". Indeed, Primary Nitrate Response was defined by Gowri et al., 1992 as a rapid (within minutes in Arabidopsis) induction of nitrate responsive-genes in response to nitrate which does not require *de novo* protein synthesis. Since I have never checked Arabidopsis, Brachypodium and barley responses in presence of an inhibitor of protein translation (for instance, cycloheximide), I cannot be certain that the entire response I examine does fit to this definition, and I prefer not to use this term. However, as 1h30 and 3h after induction are still quite short times, I think that the observed response is a mix of Primary Nitrate Response and "early/late nitrate response". A transcriptome in response to nitrate in presence of cycloheximide thus seem an interesting experiment to properly discriminate primary response from early/late response.

Conserved elements of nitrate response between Arabidopsis and *Pooideae* within the global nitrate metabolism

The global mechanism of nitrate uptake, assimilation and remobilization of nitrate is well described and seems very conserved among species (Meyer and Stitt, 2001; Masclaux-Daubresse *et al.*, 2010). Briefly, nitrate is taken up from the soil through NRT nitrate transporters. Once nitrate is incorporated into plant cells, it is reduced to nitrite through nitrate reductase (NR) in the cytoplasm and then nitrite is in turn reduced into ammonium by nitrite reductase (NiR) in plastids and chloroplasts. Ammonium is then assimilated in the plastids by the GS/GOGAT cycle. Glutamine synthetase (GS) catalyzes the fixation of ammonium on a glutamate molecule to produce glutamine. Thereafter, glutamine reacts with 2-oxoglutarate to produce two molecules of glutamate in a reaction catalyzed by the glutamate synthase (GOGAT). Genes encoding Nitrate Reductase (*NIA*) and Nitrite Reductase (*NiR*) are now used as molecular markers of nitrate induction. Indeed, their expressions are governed by nitrate in maize (Gowri *et al.*, 1992), in barley (Sueyoshi *et al.*, 1999) and Arabidopsis (Castaings *et al.*, 2009), *inter alia*, and thus it is extrapolated to the majority of plants. I further confirm this conservation of regulation for Brachypodium and barley. Moreover, our orthogroup (OGp) analysis indicate very conservative protein sequences between Arabidopsis, Brachypodium and barley since they all gathered in the same OGps: 2 isoforms for *NIA* (OGp0002372) and 1 one for *NiR* (OGp0010222).

On a more global point of view, common nitrate-responsive modules have been identified in Arabidopsis and rice (Obertello *et al.*, 2015). This suggest also that the major part of nitrate response is conserved among species, including crosstalks between nitrate and phytohormones as well as the crosstalk between nitrate and phosphate. And this is further confirmed by our transcriptomic analysis. Indeed, the vast majority of identified processes through GO analysis were equally regulated in

Arabidopsis, Brachypodium and barley (see ternary diagrams in Article 1). This validates the study of model plants such as Arabidopsis to deepen molecular understandings.

Moreover, concerning NLPs, it has been shown that the shuttling mechanism that triggers AtNLP7 nuclear localization in response to nitrate is also conserved for ZmNLP6, ZmNLP8, OsNLP3 and OsNLP4 (Cao *et al.*, 2017; Hu *et al.*, 2019; Wu *et al.*, 2020). Also, ZmNLP6 and ZmNLP8 are able to rescue *Atnlp7.1* phenotypes (Cao *et al.*, 2017). Interestingly, it has been shown in rice that OsNRT1.1B also mediates nitrate signal transduction (Hu *et al.*, 2015), as it is known for the Arabidopsis AtNRT1.1 (Ho *et al.*, 2009). Together, these observations suggest at least a partial conservation of nitrate signaling pathway between Arabidopsis and cereals.

Working on finding conservations between species is necessary and quite usually done. In my thesis, I decided to focus more on identifying divergences, since data on specificities of cereals are lacking while it seems particularly important to understand them to better orient amelioration programs.

Divergent elements of nitrate response between Arabidopsis and *Pooideae* within the global nitrate metabolism

Nitrate is actively taken up from the soil *via* members of NRT transporters families. Using a reciprocal best hit (RBH) approach, it has been shown that the NRT family structures and gene numbers are very different between Arabidopsis and cereals (maize, rice, sorghum, Brachypodium ; Plett et al., 2010). This is further confirmed in barley by OGp analysis (see Article 1, Table 2, OGps 0000527 and 0005798). Cereals have more genes related to nitrate transport than Arabidopsis. To me, it could be explained by the divergence of root architecture and organization between Arabidopsis and cereals. Although cereals form primary and lateral roots almost similarly as dicots, the overall root architecture is more complex in monocots (Figure 2; Watt *et al.*, 2009;

Smith and de Smet, 2012; Atkinson *et al.*, 2014). Interestingly, Brachypodium have been shown to be a good model for cereal root development, as it shows a high degree of developmental and anatomical similarities to wheat root systems (Figure 2, Watt et al., 2009). Such anatomical divergences lead to the identification of genes specifically involved in root formation of cereals that are absent in Arabidopsis. For example ZmGRP3 and ZmGRP4 are glycine-rich proteins produced in maize root tips involved in cell wall formation (Goddemeier, 1998; Matsuyama *et al.*, 1999). It would be interesting to look up for orthologs of these genes in Brachypodium and barley to evaluate their regulation in our RNAseq as well as their OGps constitution to validate their cereal specificity.

Another example of cereal-specific genes concerns the nitrate-inducible *TaNAC2-5A* gene from wheat, encoding a transcription factor which does not have direct ortholog in Arabidopsis (He *et al.*, 2015). TaNAC2-5A is involved in nitrate signaling, as it was shown to regulate genes of nitrate uptake and assimilation pathway. Interestingly, orthologs of TaNAC2-5A were identified in Brachypodium and barley in our study, but not in Arabidopsis, confirming its cereal specificity. However, it was found up-regulated by nitrate only in barley, suggesting that the regulation is not conserved among all *Pooideae*. This regulation divergence can be an effect of domestication, that would have act similarly in wheat and barley, but not in Brachypodium. Identifying process or regulation divergences due to domestication was also one of my objectives. And the example of *GA20OX*, which is known as a Green Revolution gene, well illustrate this aspect: it was found hardly down-regulated in Arabidopsis and Brachypodium and up-regulated in barley roots (see Article 1). However, domestication effect was generally not as clear as we hoped, and we didn't angle our research too much in this sense but more in identifying divergences between monocots and dicots. Moreover, including an undomesticated barley ancestor in our study would have been useful to really evaluate a potential domestication effect on nitrate response.

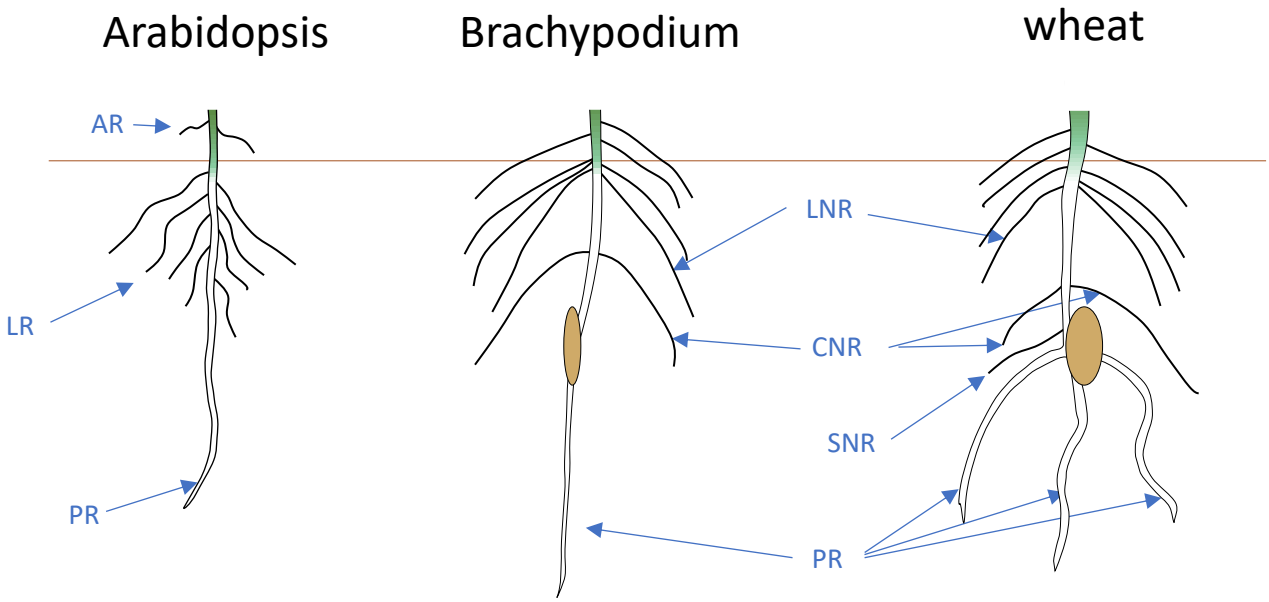

Figure 2: Comparision of root architecture of Arabidopsis, Brachypodium and wheat. Inspired from Atkinson et al., 2014, Watt et al., 2009 and Smith and de Smet, 2012. AR=Adventius root; LR=lateral root; PR=Primary root; LNR= Leaf node axile root; CNR=coleoptile node axile root; SNR=scutellar node root.

Thanks to OGps, we identified *Pooideae*-specifics nitrate-responsive genes, and specifically transcription factors and kinases (see Article 1, Figure 6). Characterization of such genes is very exciting. However, we decided not to focus too much on them for now since we think that a complementary OGps analysis is needed. OGps were designed to identify orthologs the more accurately as possible. On the other hand, we identified problematic OGps, such as OGps containing *AtTGA* gene family which do not carry any Brachypodium or barley gene, as well as *AtHRS1/HHO*-containing OGp which do not contain barley genes. We manually identified sequences that might be orthologs of *AtTGA* in Brachypodium and barley and close sequences of *AtHRS1* in barley. Thus, the generation of another set of less strict OGps that englobes actual OGps would be interesting for the identification of true nitrate-responsive *Pooideae*-specifics genes. On the other hand, working with too large OGps might have the opposite issue: regrouping together homologs that are finally quite distant in evolution and in function. Thus, working with two levels of OGp analysis in parallel seems like a good strategy. The other strategy to overcome these OGp issues is to focus on OGps that contain genes from the 3 species, and that is what we did in the final part of our analysis.

The comparative transcriptomic analysis also allowed the identification of other divergent responses: rRNA processing, which is only regulated in Arabidopsis; genes necessary for Gibberellins biosynthesis, that are more regulated in Brachypodium and barley; the pathway of cysteine biosynthesis from serine, together with the biosynthesis of pyridoxal phosphate, which are connected and exclusively regulated in Brachypodium and barley; many other precise regulations at the gene level all along the analysis, like the non-regulation of *CIPK23* and *CBL9* in Brachypodium and barley while they are known to be involved in nitrate signaling in Arabidopsis. This global approach and description were necessary and led to the identification of processes or genes which constitute good candidates that will pave the way for future studies.

Unexpectedly, the second part of my work also highlighted a divergence between monocots and dicots, with the discovery of an unexpected role of BdNLP6 (and to a lesser extent of BdNLP7). BdNLP6 seems to be a growth repressor in a nitrate dependent manner. To date, no similar phenotype has been observed neither in Arabidopsis nor maize and rice *nlp* mutants, as far as we know. Since all Arabidopsis and cereal NLPs are not characterized yet, it is difficult to conclude on a Brachypodium specificity or not. A hypothesis on BdNLP6 role as a repressor could be an involvement in a pathway of stress-resistance. Indeed, plants have to manage their balance between growth and defense against biotic and abiotic stresses. Interestingly, *Tabzip60* mutant displays a similar increased of growth in presence of nitrate. Also, *TabZIP60* expression is induced by abscisic acid (ABA), which is known as the stress phytohormone (Yang *et al.*, 2019) and its expression in Arabidopsis promotes stress tolerance (Zhang *et al.*, 2015). Thus, it is possible that BdNLP6 (as well as TabZIP60?) manages plant defense to biotic and/or abiotic stresses by maintaining energy and nutrient reserve by keeping growth to a lower level than its maximum potential. In other words, BdNLP6 could work as a guardian of reasonable nitrate metabolism level to save energy in case of biotic or abiotic stresses. This is an hypothesis that must be verified by testing *Bdnlp6* mutants in diverse stress conditions, as well testing the involvement of ABA in its transcriptional regulation. Another hypothesis could be that *BdNLP6* does actually not confer any advantage to the plant. It would simply be a future pseudogene that is currently under counter-selection, which expression is being more and more suppressed in Brachypodium (indeed, expression level of BdNLP6 is very low, in all tested tissues). In agreement, it has been shown that wheat does not possess a BdNLP6 ortholog (Kumar *et al.*, 2018). The authors suggest that its function diverges or that it has been lost during evolution/domestication. Also, barley displays only one ortholog for BdNLP6/7, HvNLP1 (Girin *et al.*, 2014), supporting again the hypothesis that BdNLP6 orthologs in domesticated cereals such as wheat and barley have been lost. This hypothesis is also consistent with the divergent RWP-RK signature motif of DNA binding that is found in

BdNLP6 (HWP-RQ) which suggest a divergence compared to other NLPs. However, the phenotypes observed in our *Bdnlp6* mutants show that the gene does have an effect on plant development, whether *BdNLP6* is under counter-selection or not.

Some work remains to decipher the roles of BdNLP6 and BdNLP7. For example, the lack of *Bdnlp7* simple mutants complexifies the interpretations of our mutant characterizations. Mutants' availability in Brachypodium is not as high as in some other model species, and even if the generation of CRISPR lines were initiated in the team, the success was not as expected. And even though I had the chance to work with two allelic *Bdnlp6* mutants coming from a TILLING project in Brachypodium, the difficulty of Brachypodium backcrosses impairs the cleaning of potential other mutations in the genomes. However, since similar phenotypes were observed for the amiRNA line and the two non-sens mutants, we are confident the described phenotypes are related to BdNLP6. To complete this work, subcellular localization was engaged as well as complementation of Arabidopsis *Atnlp7* mutant, but the cloning of *BdNLP6* and *BdNLP7* appeared very difficult (near impossible). To counteract the impossibility of localizing BdNLP6 protein, and also because we hypothesized that the low expression level of *BdNLP6* could be due to a very specific expression zone, I tried to localize *BdNLP6* (and *BdNLP7*) mRNA by *in situ* hybridization in different tissues and conditions of nitrate availability. Although many repeats were done, results were unfortunately hardly interpretable due to high variability between independent experiments, partially due to the difficulty to obtain perfect tissue sections.

Finally, this thesis draw many new perspectives of fundamental research which could lead to applied field concerning the molecular nitrate response of cereals.

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université PARIS-SACLAY
ÉCOLE DOCTORALE
Sciences du végétal : du gène à l'écosystème (SEVE)

Titre : Dissection moléculaire de la réponse au nitrate chez les *Pooideae*

Mots clés : Nitrate, *Pooideae*, transcriptomique, NIN-Like-Protéines

Résumé : L'azote est un macro-élément essentiel pour les êtres vivants. Dans les terres agricoles, l'azote est souvent limitant et il est généralement nécessaire de le complémenter à l'aide d'engrais. Cependant, l'utilisation massive d'engrais est très couteuse pour les agriculteurs et génère d'importante pollution des sols et de l'eau. Le nitrate étant la source d'azote préférée de la plupart des plantes, l'amélioration de son utilisation par les plantes cultivées pourraient limiter ses effets indésirables. Le but de mon projet de thèse était de caractériser la réponse moléculaire au nitrate principalement chez *Brachypodium distachyon*, une espèce modèle des monocotylédones, et aussi chez l'orge. Ces deux plantes appartiennent à la sous-famille des *Pooideae* qui rassemble d'importante céréale tempérées telles que le blé, le seigle et l'avoine. Pour ce faire, deux stratégies ont été adoptées. La première avait pour but d'identifier des processus biologiques et/ou des gènes régulés par le nitrate spécifiquement chez les *Pooideae*. Une analyse transcriptomique a donc été réalisée chez *Brachypodium distachyon*, l'orge et la plante modèle dicotylédon, *Arabidopsis thaliana*, qui a été utilisée comme référence puisque la réponse au nitrate est bien caractérisée au niveau moléculaire chez cette espèce.

Notre principale hypothèse était que même si une majeure partie de la réponse moléculaire au nitrate est commune entre les dicotylédones et les monocotylédones, des spécificités doivent aussi exister. Ainsi, la comparaison de la réponse au nitrate chez les trois espèces étudiées permettra d'identifier des processus biologiques ou au moins des gènes qui n'ont pas pu être mis en évidence dans les études uniquement réalisées sur Arabidopsis et qui présentent des régulations divergentes en réponse au nitrate. Ainsi, des candidats intéressants ont été mis en évidence pouvant servir l'amélioration de l'utilisation du nitrate chez les céréales. Le second objectif était de caractériser le rôle des protéines BdNLP6 et BdNLP7 chez *Brachypodium distachyon*. Ces protéines sont phylogénétiquement les plus proches de la protéine AtNLP7 d'*Arabidopsis thaliana*, qui a un rôle majeur dans la régulation de la réponse moléculaire au nitrate. Notre première hypothèse, la plus simple, était que BdNLP6 et BdNLP7 soit impliqués dans la réponse au nitrate de façon similaire à AtNLP7. De manière surprenante, nous avons montré que BdNLP6 semble avoir un rôle complètement divergent de celui décrit pour AtNLP7, puisqu'il pourrait avoir un rôle négatif sur le développement et la croissance de la plante en présence de nitrate.

Title : Molecular deciphering of nitrate response in *Pooideae* species

Keywords : Nitrate, *Pooideae*, transcriptomic, NIN-Like-Proteins

Abstract : Nitrogen is a macro-element essential for all living organisms. In agricultural fields, nitrogen is often limiting leading to the massive utilization of fertilizers. However, it represents high costs for farmers and generates important soils and water pollutions. As nitrate is the favorite nitrogen form for most plants, the enhancement of its utilization by crops plants could counteract these deleterious effects. The aim of my thesis project was to characterize molecular nitrate response mainly in the model monocot Brachypodium ditachyon and also in barley. They are both member of the *Pooideae* sub-family which contains important temperate cereal species such as wheat, rye and oat. For this purpose, two strategies were adopted. The first one aimed at identifying nitrate responsive processes and/or genes that are specifically regulated in Pooideae using a cross-species transcriptomic approach on Brachypodium distachyon, barley and the dicot model Arabidopsis thaliana, as a reference since molecular nitrate response is better characterized in this species.

Our main hypothesis was that even though monocots and dicots might share common nitrate responsive pathways, divergence of response must exist. Such a comparison will lead to the identification of processes or at least genes that cannot be highlighted by studying solely Arabidopsis thaliana, which present divergent regulations in response to nitrate, and thus constitute interesting candidates for improving cereal nitrate utilization. The second objective was to characterize BdNLP6 and BdNLP7 roles in Brachypodium distachyon, as they are closest orthologs of AtNLP7, a major regulator of molecular nitrate response in Arabidopsis thaliana. Our first hypothesis, the simplest one, was that BdNLP6 and BdNLP7 mediates nitrate response in a similar manner as AtNLP7. Unexpectedly, we show that BdNLP6 seem to carry a total divergent function than AtNLP7 as it might act as a growth repressor in presence of nitrate.