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Genetic Analysis of Nitrate Transporter 2 Family in Brachypodium distachyon, with an Essential Role of BdNRT2.1 to Nitrogen Use Efficiency

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Abstract

Plant agricultural productivity relies heavily on nitrogenous fertilizers. Excess N fertilizer application can often lead to lower nitrogen use efficiency (NUE) along with energy waste and environmental problems. Therefore, understanding and improving NUE in plants are of key importance. The small monocot plant *Brachypodium distachyon* (*Brachypodium*) is rapidly emerging as a powerful model system to study questions unique to monocot crops (wheat, maize, rice, etc.).

Here, through an intensive BLAST search, six putative orthologues of the *Arabidopsis* NRT2 gene family were identified in the fully sequenced *Brachypodium* genome (Phytozome v11.0), among which I isolated a T-DNA mutant (*bdnrt2.1*) lacking *BdNRT2.1*. Analysis of individual BdNRT2 gene expression, plant nitrogen uptake, assimilation, remobilization, metabolic change under different nitrogen sources, concentrations, and developmental stage in wild-type and the *bdnrt2.1* was performed.

Results demonstrate that *BdNRT2* gene expressions are governed by internal nitrogen status rather than external nitrate concentrations. Genes in the BdNRT2 family have diverse roles differing from *AtNRT2* in response to different nitrogen conditions. The BdNRT2.1 knock-out mutant showed an impaired inducible high-affinity transport system (iHATS), reduced nitrogen utilization efficiency (NUtE) and overall NUE (37% on average) under a N non-limited condition, whereas the constitutive high-affinity transport system (cHATS), low-affinity transport system (LATS) and nitrogen uptake efficiency (NUpE) were not affected. The mutant's reduced NUE and iHATS phenotype could be rescued by complementation. Furthermore, BdNRT2.1 had a contrasting impact on nitrogen metabolism at different developmental stages, suggesting it serves a more important role (signal transducer) after anthesis. Finally, *BdNRT2.1* overexpressing transgenic *Brachypodium* lines had significantly higher grain yield. This demonstrates that BdNRT2.1, serving as a key member of the family, is involved in nitrogen remobilization, and it has potential application for more efficient use of nitrogen fertilizer

in monocot crops. These results provide the possibility for future experiments to elucidate the specific roles of each NRT2 transporter in monocot plants.

Keywords

Nitrate transporter, NRT2, nitrogen response, nitrogen use efficiency, yield, remobilization, glutamine synthetase, metabolomics, senescence, monocots.

Co-Authorship Statement

The following thesis contains material from manuscripts in preparation which are co-authored by Jiang Wang (JW), Yajun Gao (YG), Shrikaar Kambhampati (SK), Frederic Marsolais (FM), Norman P.A. Hüner (NH), and Lining Tian (LT). My supervisors Lining Tian and Norman P.A. Hüner provided insight and strategic direction for the projects and also edited the final manuscripts (Chapters 2-4).

Chapter 2. Author's contributions

JW designed the research, performed the experiments and drafted the manuscript. NH, and LT conceived the study and participated in its design. JW, NH, and LT edited the manuscript.

Chapter 3. Author's contributions

JW designed the research, performed the experiments and drafted the manuscript. YG, NH, and LT conceived the study and participated in its design. JW, NH, and LT edited the manuscript.

Chapter 4. Author's contributions

JW designed the research, performed the experiments and drafted the manuscript. NH and LT conceived the study and participated in its design. SK conducted the metabolomics measurement, including data analysis, and wrote the corresponding methods. FM provided material and lab space. JW, NH, and LT edited the manuscript.

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List of Abbreviations

2OG	2-oxoglutarate
35S	Cauliflower mosaic virus 35S promoter
AMT	Ammonium transporter
ANOVA	Analysis of variance
Arg	Arginine
AS	Asparagine synthetase
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
att	Gateway cloning attachment site
BAR	BASTA resistant gene
cDNA	complementary DNA
cHATS	Constitutive high-affinity transport system
DAG	Day after germination
ddH_2O	Double distilled water
Dicot	Dicotyledonous
DNA	Deoxyribonucleic acid
Fd	ferredoxin
GDH	Glutamate dehydrogenase
Gln	Glutamine
Glu	Glutarate
GOGAT	2-oxoglutarate amino transferase or glutamate synthetase
GS	Glutamine synthetase
HA	Human influenza hemagglutinin
HATS	High-affinity transport system
HI	Harvest index
Hyg^r	Hygromycin resistant gene
iHATS	iducible high-affinity transport system
JGI	Joint genome institute
LATS	Low-affinity transport system
LB	Left boarder
Lys	Lysine
MFS	Major facilitator superfamily
mM	millimolar

Monocot	Monocotyledonous
mRNA	messenger RNA
MU	*bdnrt2.1* mutant
N	Nitrogen
NAC	NAM, ATAF, and CUC transcription factor
NADH	Nicotinamide adenine dinucleotide H
NAE	Nitrogen assimilation efficiency
NiR	Nitrite reductase
NR	Nitrate reductase
NRE	Nitrgen remobilization efficiency
NRT1/PTR	Nitrate transporter 1/Peptide transporter
NRT2	Nitrate transporter 2
NUE	Nitrogen use efficiency
NUpE	Nitrogen uptake efficiency
NUtE	Nitrogen utilization efficiency
OCS	Octopine synthase terminator
PCD	Programmed cell death
PCR	Polymerase chain reaction
qPCT	quantative-polymerase chain reaction
RB	Right boarder
RNA	Ribonucleic acid
SAGs	Senescence-associated genes
SamDC	S-adenosylmethionine decarboxylase proenzyme
Ser	Serine
T-DNA	Transfer-DNA
WT	Wild type
YFP	Yellow fluorescent protein

1 General Introduction

1.1 Current problems resulting from excess agricultural nitrogen supply

In the last 45 years, the global annual amount of synthetic nitrogen (N) fertilizer applied to crops has risen dramatically, from merely 10.2 Tg in 1960 (Frink *et al*., 1999) to 109 Tg in 2014 (FAOSTAT), resulting in significant increased crop yield and decreased world hunger (Good *et al*., 2004). However, nitrogen fertilizer captured by crops is usually inefficient, with only 30% to 50% of applied nitrogen being utilized (McAllister *et al*., 2012). Thus, more than 50% of the applied nitrogen is lost through a combination of different processes, including ground run-off, leaching, denitrification and ammonia volatilization (Garnett *et al*., 2009). The excess nitrogen (especially nitrate) can contaminate drinking water and adversely affect human health, including reproductive problems, methemoglobinemia, and cancer (Townsend *et al*., 2003). Nitrate excess in fresh water can lead to algal blooms (dead zone) and result in substantial disruption of marine biodiversity (Vitousek *et al*., 2009). Additionally, the excess use and production of N fertilizer also plays a role in ozone depletion and global warming through excess emissions of nitrous oxide (Wuebbles , 2009). These, along with increasing N fertilizer costs resulting from the energy intensive Haber-Bosch process (Xu *et al*., 2012), have created an urgent need to enhance nitrogen use efficiency (NUE) in crops, that is, crops that are better able to uptake, utilize and remobilize the nitrogen available to them.

1.2 Understanding nitrogen use efficiency

For crops, NUE is defined as grain yield per unit of applied N in the soil; for *Arabidopsis*, NUE is expressed as fresh or dry biomass per nitrogen content in the plant (Good *et al*., 2004). In both cases, NUE is the combination of nitrogen uptake efficiency (NUpE, calculated as the ratio of plant acquired N to N supply) and nitrogen utilization efficiency (NUtE, calculated as the ratio of plant yield to plant acquired N), which is also the same expression as the optimal integration of nitrogen assimilation efficiency (NAE) and nitrogen remobilization efficiency (NRE) (Xu *et al*., 2012).

1.2.1 Nitrogen uptake

Nitrate is the major form of inorganic N that can be used by crop plants in aerobic soils (see review of Xu *et al*., 2012) due to rapid nitrification of applied fertilizer and organic nitrogen. Plants take up nitrate mainly through members of two gene families, namely NPF (the nitrate transporter 1/peptide transporter family) which can be activated when external nitrate is abundant (>1 mM, mainly responsible for low-affinity transport system, LATS) and NRT2 (the nitrate transporter 2 family) which can be activated at low external nitrate (<1 mM, mainly responsible for high-affinity transport system, HATS) (see review of Tsay *et al*., 2007; Masclaux-Daubresse *et al*., 2010). Additionally, a secondary protein, NAR2, needs to be co-expressed with NRT2 to yield nitrate uptake (Zhou *et al*., 2000; Orsel *et al*., 2006; Yan *et al*., 2011). In response to changes in nitrogen demands and availability, the activity of these two nitrate uptake systems can be further fine-tuned by transcriptional regulation and post-translational modifications. Transporters of both families belong to the major facilitator superfamily (MFS) of secondary active transporters and are dependent on protons for nitrate transport (Pao *et al*., 1998; Law *et al*., 2008).Other plants, especially ammonium-preferring rice, take up ammonium mainly through the membrane-located AMT/MEP/Rh transporter (Khademi *et al*., 2004).

1.2.2 Nitrogen assimilation

Schematic presentation of the enzymes involved in the primary nitrogen assimilation pathway is illustrated in Fig. 1.1. Once nitrate is taken up by the cell, it is first reduced to nitrite by the enzyme nitrate reductase (NR) in the cytosol (Meyer and Stitt, 2001). Subsequently, nitrite is relocated to the plastid, where it is further reduced to ammonium by the enzyme nitrite reductase (NiR) (Meyer and Stitt, 2001). Ammonium, whether it originated from nitrate reduction, ammonium uptake or from photorespiration or amino acid recycling, is mainly assimilated through the GS/GOGAT cycle (Lea and Miflin, 1974). The enzyme glutamine synthetase (GS) incorporates an ammonium onto a glutamate molecule to form glutamine. This glutamine subsequently reacts with 2-oxoglutarate (from Krebs cycle) to form two molecules of glutamate, which is catalyzed by the enzyme 2-oxoglutarate amino transferase (or glutamate synthetase, GOGAT).

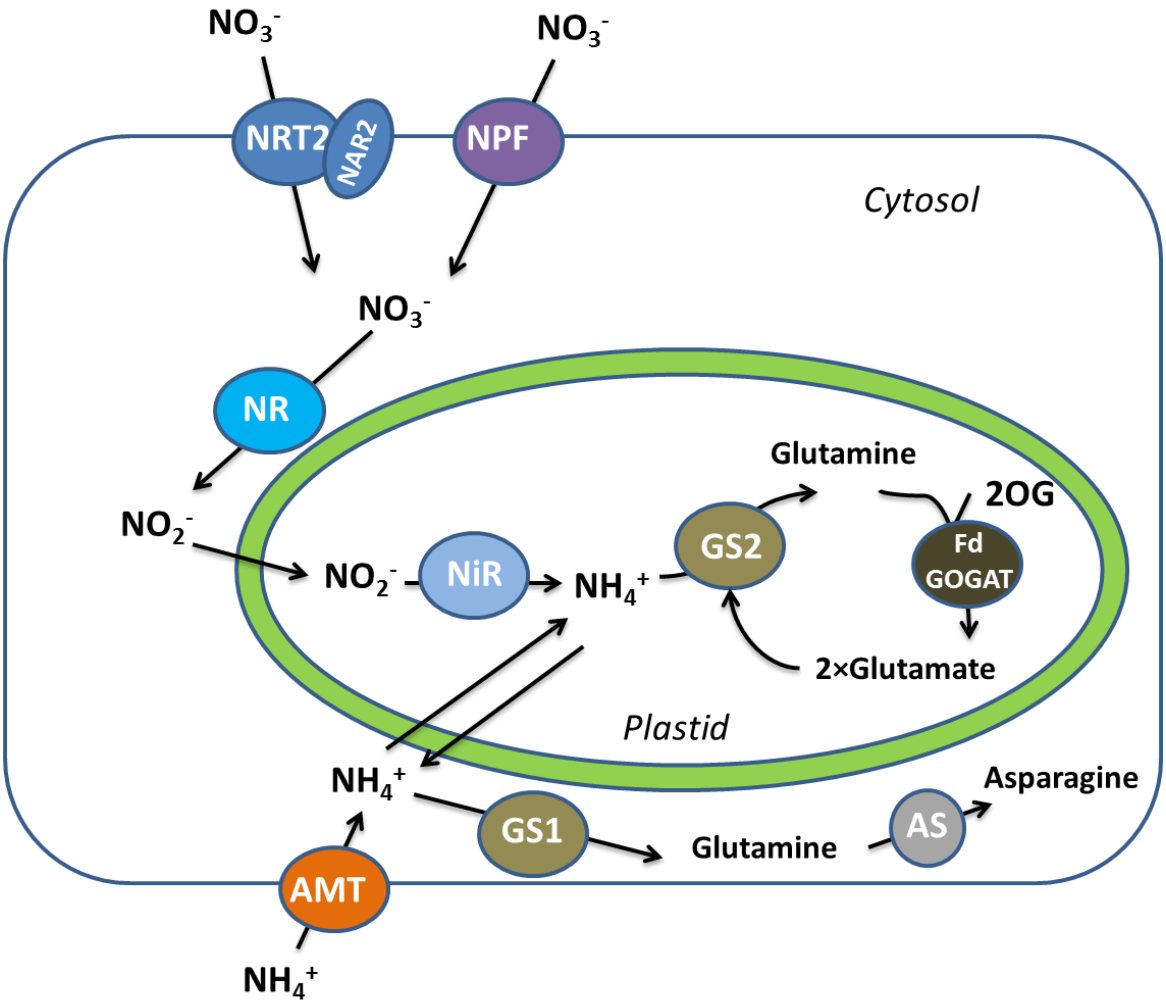

Figure 1.1 Schematic presentation of enzymes involved in the primary nitrogen assimilation pathway.

Nitrate reductase (NR), glutamine synthetase isoenzyme 1 (GS1), and asparagine synthetase (AS) are localized in the cytosol; nitrite reductase (NiR), glutamine synthetase 2 isoenzyme (GS2), glutamate synthase (GOGAT) within the plastids of mesophyll cells.

Thus, the net outcome from the GS/GOGAT cycle is the production of glutamate, which can be further converted into other amino acids through aminotransferases or transaminases (Forde and Lea, 2007). Two types of decameric GS isoforms are described in higher plants, including cytosolic GS1 and chloroplastic GS2 (Unno *et al*. 2006). Chloroplatic GS2 is mainly present in green leaf tissue and is responsible for the primary assimilation of ammonium reduced from nitrate in both C_3 and C_4 plants; it is also involved in re-assimilation of ammonium originating from photorespiration in C_3 plants (Masclaux-Daubresse *et al*., 2010). In contrast, cytosolic GS1 present in various organs such as roots and stems is mainly responsible for ammonium recycling during particular developmental stages like leaf senescence; it is also involved in glutamine synthesis for relocation into the phloem sap (Bernard and Habash, 2009). Two types of GOGAT forms exist in higher plants, including Fd-GOGAT and NADH-GOGAT, which use ferredoxin and NADH as electron donors, respectively (Vanoni *et al*., 2005). Fd-GOGAT is predominantly present in leaf chloroplasts, while NADH-GOGAT mainly locates in non-photosynthetic tissues such as roots, etiolated leaf tissues and companion cells (Masclaux-Daubresse *et al*., 2010). In addition to the GS/GOGAT cycle, other enzymes probably play important roles in ammonium assimilation. Cytosolic asparagine synthetase (AS) facilitates the amino group transfer from glutamine to aspartate to form glutamate and asparagine in an ATP dependent way. Asparagine has a higher N/C ratio than glutamine and can be used for long-distance transport and storage (Lam *et al*., 2003). Mitochondrial NADH-glutamate dehydrogenase (GDH) catalyzes the equilibrium between ammonium and glutamate. However, the reverse reaction whereby GDH acts as a glutamate deaminase is proposed as its main function (Masclaux-Daubresse *et al*., 2006).

1.2.3 Nitrogen remobilization

During the vegetative growth stage, the leaves are the major sink for N; during the reproductive stage (senescence), this N is remobilized and makes it way to the developing seeds, mainly as amino acids (Okumoto *et al*., 2011). However, the mechanisms of amino acid transporters which are coded by multiple gene families have been poorly understood for their roles in N remobilization during leaf senescence (Okumoto *et al*., 2011).

Increased asparagine (Asn) and glutamine (Gln) have been proposed to play a key role during N remobilization from senescent leaves (Masclaux-Daubresse *et al*., 2010). Additionally, leaf senescence involves age-dependent PCD (Programed cell death). PCD in leaf senescence is responsible for remobilization of nutrients from the leaf to other organs, and especially developing seeds (Lim *et al*., 2007). Many senescence-associated genes (SAGs) or enzymes, including some isoforms of GS1, GDH, AS, and certain transcriptional factors, such as NAC, WRKY, are strongly activated during leaf senescence and play key role in N remobilization and seed yield (Lim *et al*., 2007, Masclaux-Daubresse *et al*., 2010, Xu *et al*., 2012).

1.3 Nitrate transporter 2 family

The high-affinity nitrate transporter (NRT2) family plays a critical role as the gate keeper of nitrate uptake and is heavily involved in internal nitrate translocation (Tsay *et al*., 2007). For the time being, NRT2 has been studied thoroughly in *Arabidopsis thaliana*, which is a model plant for dicots. There are seven members in the NRT2 family that have been identified in *Arabidopsis* as high-affinity nitrate transporters (Orsel *et al*., 2002). The high-level of redundancy suggests that members in the NRT2 family should function in various tissues, growth stages, and different environmental conditions. AtNRT2.1, AtNRT2.2, and AtNRT2.4 are involved in high-affinity nitrate transport in roots, and AtNRT2.1 is the main component, while AtNRT2.2 makes a small contribution; all three members can be induced by nitrate addition (Li *et al*., 2007; Kiba *et al*., 2012). AtNRT2.4 and AtNRT2.5 are expressed in both root and shoot, and are strongly induced by N starvation and may be involved in phloem loading of nitrate (Kiba *et al*., 2012; Lezhneva *et al*., 2014). AtNRT2.7 is expressed in mature seeds and is responsible for nitrate accumulation in the seeds (Chopin *et al*., 2007).

1.4 *Brachypodium distachyon* as a genetic model system

Angiosperms (flowering plants) can be categorized into two groups; monocotyledonous plants (monocots, one cotyledon per embryo) and dicotyledonous plants (dicots, two cotyledons per embryo). It is estimated that monocots branched off from dicots 140-150 million years ago (Chaw et al., 2004), and since then many differences have evolved. For

example, the structure and physiology of plant cell walls, the development and chemical composition of endosperm, and the genetic basis for cold tolerance (Kellogg, 2015). The vast majority of plant molecular biology research has been conducted in dicots, with *Arabidopsis thaliana* as a model system. However, the major cereal crops, such as rice, wheat and maize, are all monocots, and fundamental differences had been shown between *Arabidopsis* and monocot grass species in the gene number, family structure and the phylogenetic tree of NRT2 (Plett *et al*., 2010), which does not make it possible to determine the function of NRT2 in cereals based on sequence homology to NRT2 that has been characterized in *Arabidopsis.*

Rice (*Oryza sativa* L.) has often been used as a model monocot plant because of its relatively small genome (441MB), and it is also an agriculturally important crop. However, in many aspects, rice is not an ideal model organism for monocots NUE research, because it does not share some important agricultural traits with other cereal members. For instance, the root anatomy of rice is not a good model for Pooideae grasses, because rice roots normally grow submerged in water and have adapted to anaerobic conditions (Chochois et al., 2012). Ammonium is the main form of nitrogen (N) in a paddy soil, and consequently rice uses ammonium more than nitrate (Wang et al. 1993) unlike other agriculturally important monocots (wheat, maize, barley, etc.) which use nitrate predominantly. Moreover, the large size of adult rice plants makes it inconvenient to monitor plant growth across a full generation under controlled experimental conditions. The small monocot plant *Brachypodium distachyon* (*Brachypodium*), which diverged from wheat 32-39 million years ago, and from rice 40-53 million years ago (The International Brachypodium Initiative, 2010), is rapidly emerging as a powerful model system (reviewed in Girin *et al*., 2014; Kellogg, 2015) to study questions unique to the monocot crops, due to its small genome (270 Mbp, Vogel *et al*., 2010), plant size (~20 cm), short life cycle (~2 month), efficient transformation, and ease of cultivation.

1.5 Research objectives

Better understanding of nitrogen uptake, assimilation, and nitrogen remobilization is needed to deal with the challenge of trying to increase NUE in agricultural crops. With

the aid of *Brachypodium*, a cereal model plant, I can pursue the questions that are unique to monocot crops, such as how plants take up nitrate efficiently from the soil, how nitrogen can be efficiently transported from N source to sink, and eventually lead to the question of how to increase crop yield. Because drastic divergence of the NRT2 gene family between *Arabidopsis* and *Brachypodium* has been found, their functional differences need to be addressed. The *Brachypodium* NRT2 gene family is comprised of a set of gene members. Whether the individual NRT2 members have redundant function or each member serves a different role needs to be investigated. Furthermore, NRT2.1 has been proposed as the key member in the NRT2 family in *Arabidopsis* and rice. Whether this can be extended to *Brachypodium* needs to be confirmed. Finally, functions of BdNRT2.1 other than nitrate uptake need to be explored. Therefore, the objectives of my research were:

1) Characterizing genes of the BdNRT2 family and their nitrogen responses;

2) Functional analysis of BdNRT2.1 and testing its potential to increase NUE when over-expressed;

3) Exploring potential functions of BdNRT2.1 other than root nitrate uptake.

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2 Identification and Molecular Characterization of the *Brachypodium distachyon* NRT2 Family, with a Putative Major Role of *BdNRT2.1*

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2.1 Introduction

Nitrate is the major form of inorganic N that can be used by crop in aerobic soils (see the review of Xu *et al*., 2012) due to rapid nitrification of applied fertilizer and organic nitrogen. Plants take up nitrate mainly through members of two gene families. The high-affinity transport system (HATS), which consists of members of the NRT2 family, is mainly responsible for nitrate uptake with Michaelis constant (Km) values in the micromolar range, whereas the low-affinity transport system (LATS), comprising the PTR (the nitrate transporter 1/peptide transporter) family, transports nitrate at millimolar concentrations. (Tsay *et al*., 2007; Masclaux-Daubresse *et al*., 2010; Wang *et al*., 2012; Krapp *et al*., 2014; Krapp, 2015). Unlike NPF, which has diverse substrate specificity, all NRT2 isolated from land plants only transport nitrate (Tsay *et al*., 2007; Wang *et al*., 2012). NRT2 has been studied thoroughly in *Arabidopsis thaliana* which is a model plant for dicots. As described in Chapter 1.3, seven members of the AtNRT2 family have diverse functions in various tissues, growth stages, and under different nitrogen conditions (Li *et al*., 2007; Kiba *et al*., 2012; Lezhneva *et al*., 2014; Chopin *et al*., 2007). However, the major cereal crops, such as rice, wheat, and maize are all monocots, and fundamental differences have been shown between *Arabidopsis* and monocot species in gene number, family structure and phylogenetic tree of NRT2 (Plett *et al*., 2010), which does not make it possible to determine the function of NRT2 in cereals based on sequence homology to NRT2 characterized in *Arabidopsis*. The small monocot plant *Brachypodium distachyon* (*Brachypodium*) is rapidly emerging as a powerful model system (reviewed in Girin *et al*., 2014; Kellogg, 2015) to study questions unique to the monocot crops due to its small genome (270 Mbp, Vogel *et al*., 2010), plant size (~20 cm), short life cycle (~2 month), efficient transformation, and cultivation.

In this study, I analyzed individual *BdNRT2* gene expression under different nitrogen sources and concentrations in both the wildtype *Brachypodium* and the *BdNRT2.1* knock-out mutant. I discovered that *BdNRT2* gene expressions are governed by internal nitrogen status rather than external nitrate concentrations. *BdNRT2.1* is a key member of the family which exhibits strong regulatory ties with *BdNRT2.2* and *BdNRT2.6*. I hypothesized that genes of the BdNRT2 family have different roles from members of AtNRT2 family with respect to tissue specificity and nitrogen responses.

2.2 Materials and Methods

2.2.1 Plant material and growth conditions

The *BdNRT2.1* T-DNA insertion mutant line was obtained from the JGI *Brachypodium* collection (JJ12084). Homozygous T3 lines for the mutants were selected by genomic PCR using specific primers. Wild type (Bd21-3), and T3 homozygous mutant (based on the same ecotype Bd21-3) seeds were germinated and grown under aseptic conditions. Seeds were soaked in ddH_2O for 2 hours at room temperature before the lemma were removed. For sterilization, seeds were first soaked in 70% ethanol for 30 seconds with gentle shaking, and then transferred into 1.3% hypoclorite solution for 4 minutes with gentle shaking, followed by three washes with sterilized ddH_2O. Sterilized seeds were then placed on wet filter paper in a petri dish sealed with 3M Micropore tape and incubated for 4 d in the dark at 4 ^oC to synchronize germination. To initiate germination, seeds were transferred to a growth room with a 20 h photoperiod, a temperature of 23 ^oC, and a light intensity of 75 μmol/m^2/s, for 5 d. Successfully germinated seedlings of similar size were transferred to sterilized Magenta^{TM} GA-7 Plant Tissue Culture Boxes (Sigma-Aldrich) containing 125 ml of modified Hoagland nutrient solution with the desired nitrate/ammonium contents (49 mg/L H_3PO_4, 250 mg/L $CaCl_2$, 185 mg/L $MgSO_4 \cdot {}_7$H2O, 179 mg/L KCl, 58 mg/L NaCl, 2.86 g/L H_3BO_3, 1.81g/L $MnCl_2 \cdot 4H_2O$, 220 mg/L $ZnSO_4 \cdot 7H_2O$, 51 mg/L $CuSO_4$, and 120 mg/L$NaMoO_4 \cdot 2H_2O$). The desired N concentrations were adjusted using KNO_3, NH_4Cl or NH_4NO_3, and pH was subsequently adjusted to 5.8. Plants were floated on the nutrient solution using Styrofoam rafts with holes and anchored with a strip of sterilized sponge so the roots were fully submerged in the solution. Four plants (same genotype) were grown in each Magenta^{TM} box. Plants from each Magenta^{TM} box were pooled as 1 biological replicate. Magenta^{TM} boxes were randomized and grown in the same growth room as described above for up to 5 weeks, and the hydroponic solution was renewed weekly at 3 pm on each Friday.

2.2.2 Vector construction and plant transformation

To generate BdNRT2.1 rescue lines, the full coding region of BdNRT2.1 was fused with its 2 kb upstream sequence (native promoter) and flanked by Gateway® attachment site sequences, then inserted into pEarleyGate 301 (Earley *et al*., 2006) using Gateway®

Technology (Hartley *et al*., 2000). Construct diagrams are detailed in Appendix 2.3. Transgenic *Brachypodium* plants were generated using the *Agrobacterium tumefaciens* mediated transformation method, as described previously (Alves *et al*., 2009) with slight modification. Calli of the *bdnrt2.1* were induced from immature embryos on MSB3+Cu0.6 medium for 6 weeks, and co-cultured with Agrobacterium strain AGL1 harboring the pEarleyGate 301 binary vector including BdNRT2.1 gene for 2 d, then transferred onto MSB3 + H100/B100 + T225 selective medium supplemented with 100μg/L BASTA for pEarleyGate 301 for 4 weeks. Resistant calli were subsequently transferred onto MSR26 regeneration medium for shoot induction then MSR63 medium for root regeneration. The transgenic plantlets were then transferred to the growth room in pots of Pro-mix® BX Mycorrhizae growing medium (Premier Tech Horticulture) to grow and produce seeds. T4 homozygous seeds containing the transgene were harvested and three lines (RE7, RE8 and RE10) were used for further analysis.

2.2.3 HATS determination

Influx rate of $^{15}NO_3$ was carried out based on the method previously described by Li *et al*. (2007). *Brachypodium* plants were grown hydroponically in nutrient solution containing 1 mM NH_4NO_3 for 4 weeks and then deprived of N for 1 week to deplete the internal N reservoir. Plants that had been starved for 7 d were transferred into nutrient solution containing 1 mM KNO_3 for 6 h to induce nitrogen response, and then the flux upon immersion into 0.1 mM $K^{15}NO_3$ was measured (the plants were washed with 0.1 mM CaSO4 for 1 min, then immersed in nutrient solution containing 0.1 mM $K^{15}NO_3$ (atom%^{15}N: 99%) for 10 min and finally to 0.1 mM CaSO4 for 1 min). This flux represented the HATS activity. Roots of tested plants were oven dried for 48 h at 60 ^{o}C and grounded into powder. Two and half mg of each subsample was shipped to the UC Davis Stable Isotope Facility (Davis, CA, USA) for ^{15}N abundance determination, where samples were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The ^{15}N analyses were performed on 4 biological replicates.

2.2.4 RNA extraction, semi-quantitative real-time PCR and quantitative real-time PCR

Total RNA were extracted using TRIzol® reagent (Thermo Fisher Scientific) from target tissues obtained from hydroponic culture at 3 pm on each Friday. The RNA concentration was determined using a Nanodrop™ 1000 spectrophotometer. First-strand cDNA was synthesized from 1 μg of DNase I (Thermo Fisher Scientific) treated total RNA using the iScript™ Reverse Transcription Supermix (BIO-RAD) according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed using GoTaq® DNA polymerase (Promega). Quantitative Real-Time PCR was performed on the cDNA templates using the qPCR reaction mix SsoFast™ EVAGreen® supermix (BIO-RAD) according to the manufacturer's instructions in a CX96™ Real Time system – C1000 touch thermal cycler. *BdSamDC* was used as a housekeeping gene for the nitrogen treatment experiments, and *BdUbi4* was used as a housekeeping gene for the different tissue analyses (Hong *et al*., 2008). The specific primers used for quantitative real-time PCR are detailed in Appendix 2.2. The specificity of the primer pairs was validated through sequencing the amplified products (Eurofins). The analyses were performed on 3 biological replicates, each with 3 technical replicates.

2.2.5 Statistical analysis

The difference between two subjects was assessed using the two-tailed student t tests with equal variance. Differences among multiple subjects were assessed using one-way analysis of variance (ANOVA) followed by multiple comparisons using Fisher's LSD method. Subsequently, the effect of nitrogen concentrations/nitrate resupply, including the interaction effect between nitrogen treatment and plant genotype, was examined using a general linear model with plant genotype, and nitrogen treatment as a continuous variable. All statistical analyses were conducted using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL, USA).

2.3 Results

2.3.1 Retrieval of *Brachypodium* NRT2 genes and construction of a phylogenetic tree

With the assistance of previously identified *Brachypodium* NRT2 genes, and using the modified reciprocal best hit (RBH) approach against *Arabidopsis* NRT2 genes (Plett *et al*., 2010), another extensive BLAST search was carried out to identify all putative orthologues of the Arabidopsis *NRT2* genes in the fully sequenced *Brachypodium* genome (Phytozome v11.0). In addition to the five previously identified BdNRT2 genes, I found the sixth member, which shared high amino acid similarity (66.1%) and contained the conserved major facilitator superfamily (MFS) domain (Pfam 31.0). Six genes encoding putative high-affinity nitrate transporters (*BdNRT2*) were named: *BdNRT2.1* (Bradi3g01270.1), *BdNRT2.2* (Bradi3g01250.1), *BdNRT2.3* (Bradi3g01277.1), *BdNRT2.4* (Bradi3g01290.1), *BdNRT2.5* (Bradi2g47640.1), and *BdNRT2.6* (Bradi2g26210.1). The six genes were distributed on two out of five chromosomes in *Brachypodium*: *BdNRT2.5* and *BdNRT2.6* on chromosome 2, and the rest on chromosome 3 (Fig 2.1). A phylogenetic tree of NRT2s was created after alignment of the seven NRT2 members from *Arabidopsis* (Plett *et al*., 2010), the four Maize sequences (Garnett *et al*., 2013), the four Rice sequences (Feng *et al*., 2011), and the six *Brachypodium* sequences identified here (Fig2.2). NRT2 genomic sequence data of the four model plants were compared using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), with the neighbour-joining analyses performed using FigTree (V1.4). Structural features of BdNRT2 genes and proteins are listed in Appendix 2.1.

2.3.2 Tissue specificity of BdNRT2 gene expression

To examine the tissue specificity of *BdNRT2* gene expression, the wild type *Brachypodium* seeds were germinated and grown hydroponically under 1 mM NH_4NO_3 (nitrogen non-limiting) for 25 d (after anthesis), then various organs were collected at 3 pm for gene expression analysis. Expression of all six *BdNRT2* gene members were analyzed using quantitative Real-Time PCR (data not shown) and semi-quantitative PCR (Fig. 2.3). *BdNRT 2.1* and *BdNRT 2.2* were strongly expressed in the root, consistent with *AtNRT2.1*.

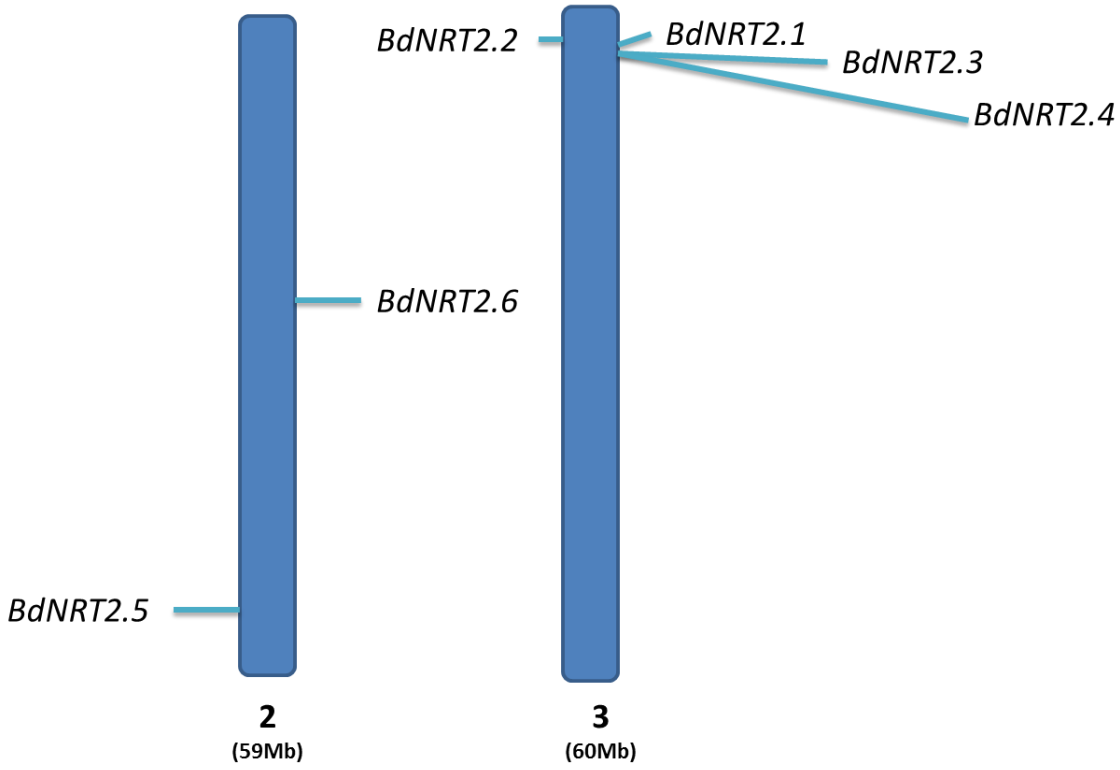


Figure 2.1 Genomic localization and orientation of BdNRT2 genes on chromosomes of *Brachypodium distachyon.*

The blue bars represent chromosome 2 (left) and 3 (right) of the *Brachypodium* genome. The genes shown to the left of the chromosome are transcribed in the positive orientation (from the top down); the genes shown to the right of the chromosomes are transcribed in the negative orientation (from the bottom up).

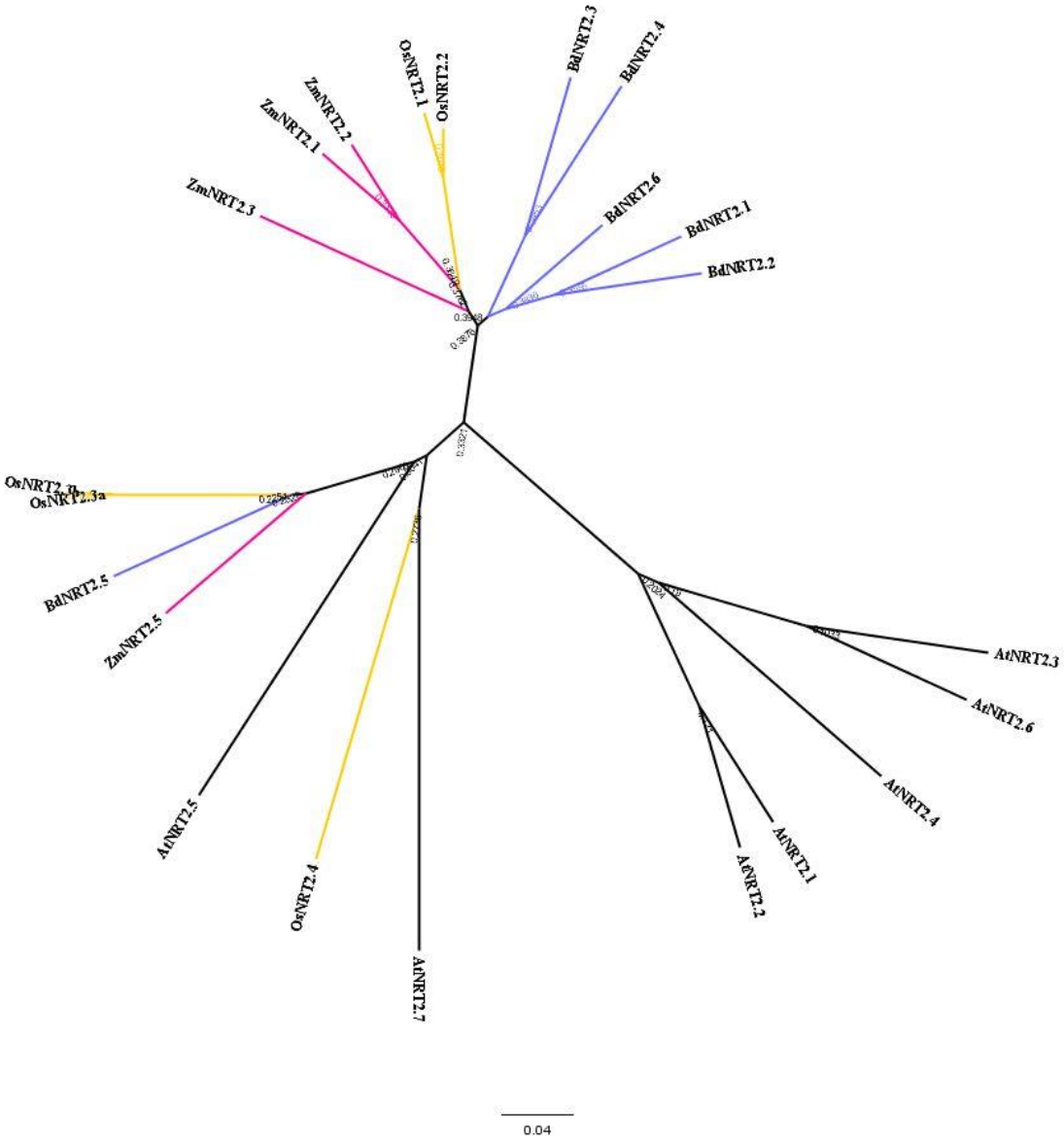


Figure 2.2 Phylogenetic relationship of the NRT2 family.

Unrooted Neighbour-joining tree of NRT2 transporters in Arabidopsis (Black), *Brachypodium* (purple), Maize (Red), and Rice (Yellow). Bootstrap values were used to estimate the confidence limits of the nodes. The scale bar represents a 0.04 estimated nucleotide substitution per residue.

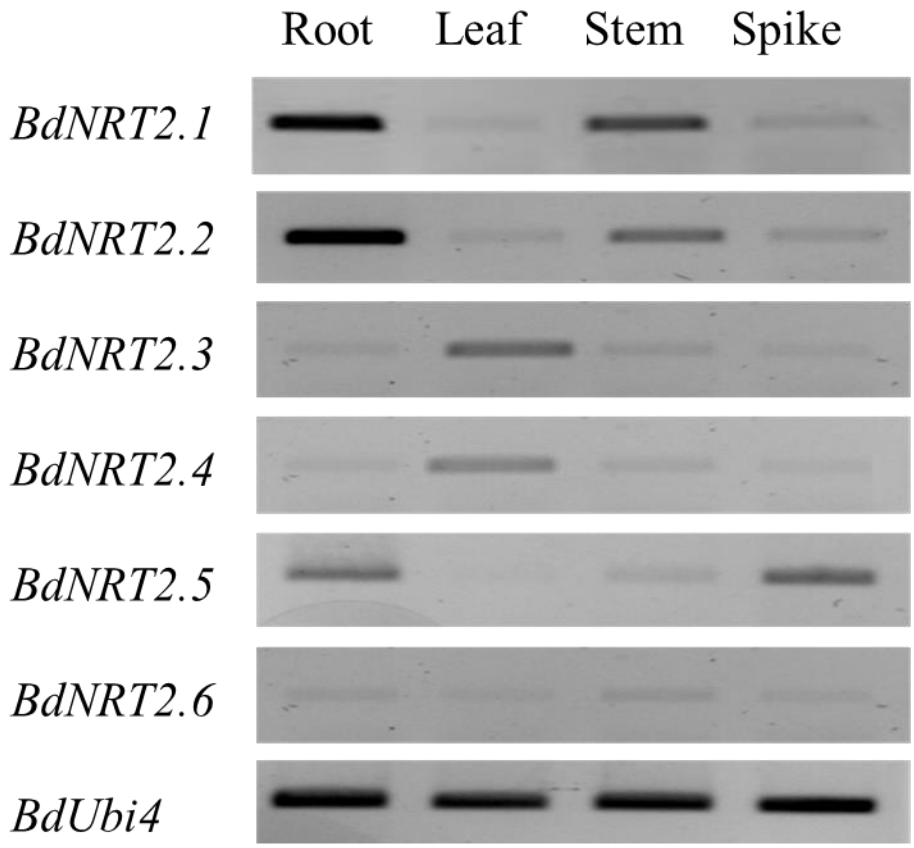


Figure 2.3 Semi-quantitative RT-PCR (30 cycles) used to detect the mRNA levels of *BdNRT2s.*

Samples from root, leaf, stem, and spike were collected 25 DAG under 1 mM NH_4NO_3 (N none-limiting) pooled with 3 biological replicates. *BdUbi4* was used as a housekeeping gene.

However, *BdNRT 2.1* also was strongly expressed in the stem, indicating it has versatile roles other than nitrate uptake. *BdNRT 2.3* and *BdNRT2.4* were preferably expressed in the leaf, with *BdNRT2.5* mostly found in the spikelet, while *BdNRT2.6* was weakly expressed across all organs.

2.3.3 Expression of BdNRT2 genes in response to nitrate

As a first step to characterize the six members of the *Brachypodium* NRT2 family, I analyzed their response to nitrate in a time-course experiment. Wild-type *Brachypodium* seedlings were grown hydroponically in nutrient solution containing 1 mM NH_4NO_3 for 22 d and then deprived of N for 7 d to deplete the internal N reservoir. Then plants were re-supplied with 1 mM nitrate for up to 6h. Root samples were collected at T0 (right before nitrate resupply), T1 (10 min after nitrate resupply) and T2 (6 h after nitrate resupply); this time was picked because plant nitrogen response will be fully induced within 6 h (Li *et al*., 2007). The time-course experiment shown in Fig. 2.4 allowed the classification of the six tested BdNRT2 genes in three groups, namely nitrate inducible, nitrate repressible and nitrate constitutive (Criscuolo *et al*., 2012). The results showed that *BdNRT 2.1* and *BdNRT 2.*2 were nitrate inducible genes ($p=0.030$ and $p=0.012$) and *BdNRT 2.5* was a nitrate repressible gene ($p=0.004$). The rest were nitrate constitutive genes.

2.3.4 Expression of BdNRT2 genes was governed by internal N status regardless of external nitrate and ammonium

To further characterize the six members of the *Brachypodium* NRT2 family, I analysed their responses to nitrate and ammonium in gradient experiments. Wild-type *Brachypodium* seedlings were grown hydroponically in nutrient solution containing various concentration of nitrate or ammonium (ranging from 0 mM to 3.2 mM) for 15 d, root samples were collected at 3 pm. For the nitrate response (Fig. 2.5A), agreeing with the nitrate resupply experiment, the increased nitrate concentration induced *BdNRT 2.1* by 3.6 fold ($p<0.001$) and *BdNRT 2.2* by 2.2 fold ($p=0.001$) expression and repressed *BdNRT2.5* expression (10-fold repression, $p<0.001$). The rest were constitutively expressed across all nitrate concentrations. Interestingly, the same patterns were also observed if the nitrate was replaced by the same concentrations of ammonium (Fig. 2.5B)

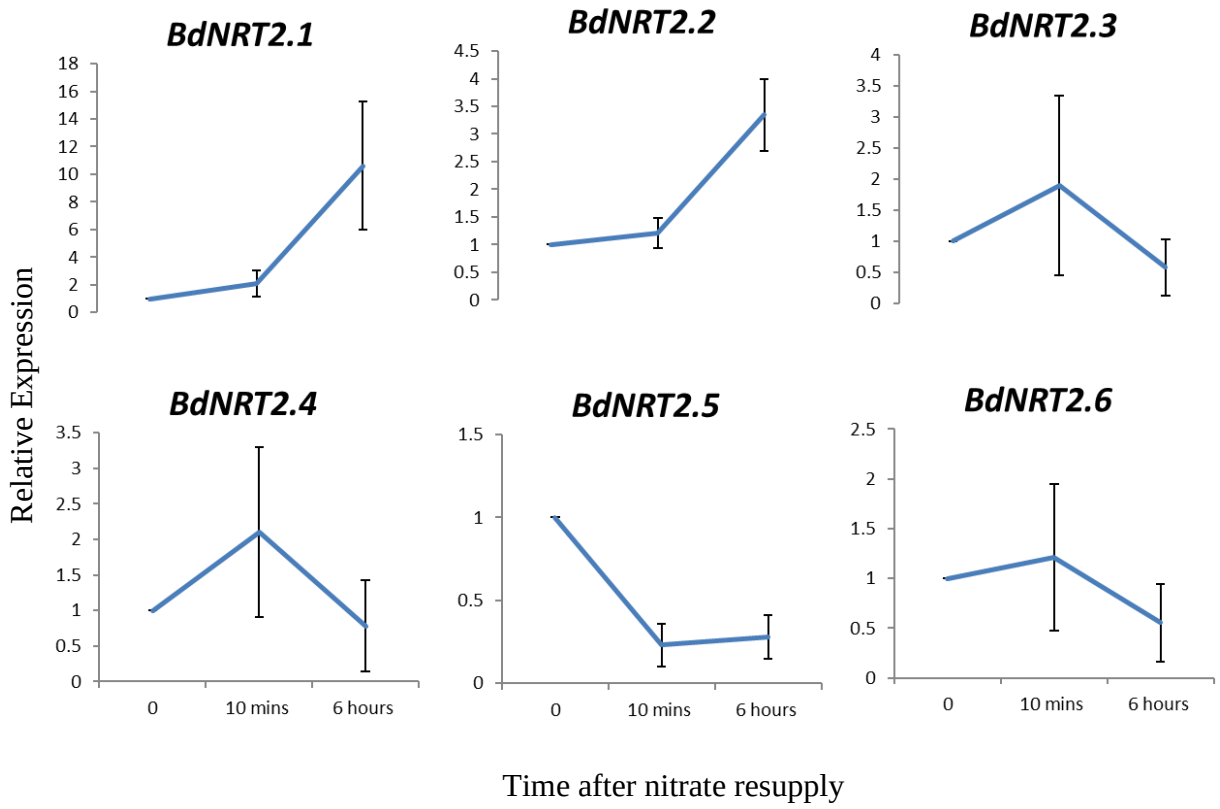
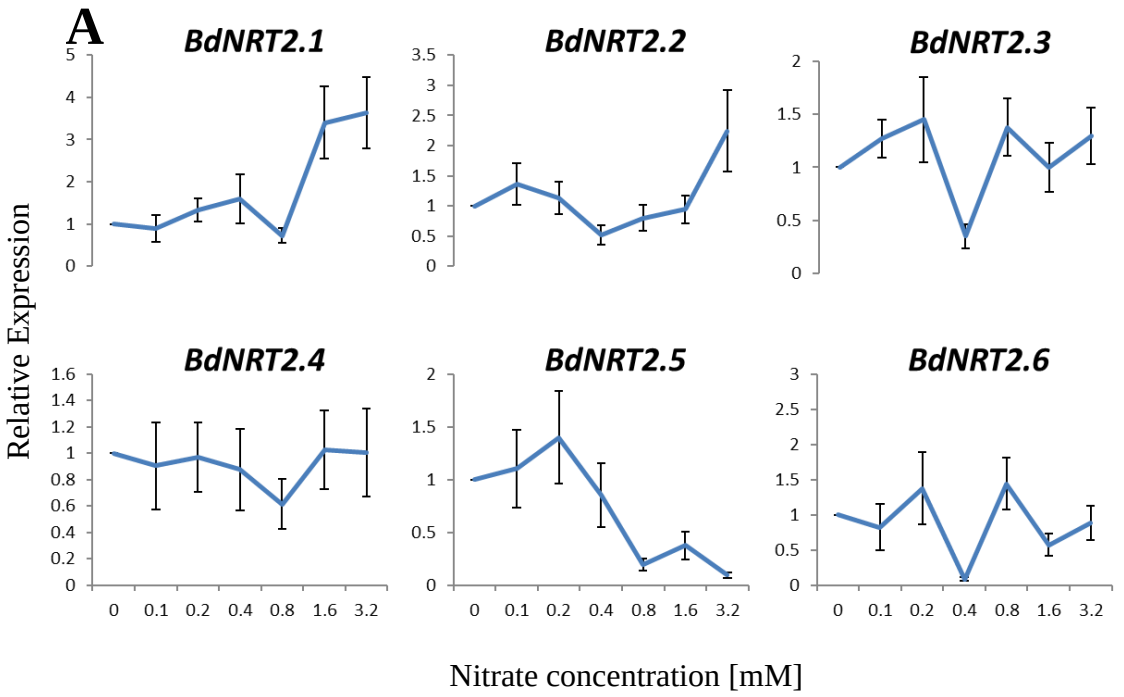


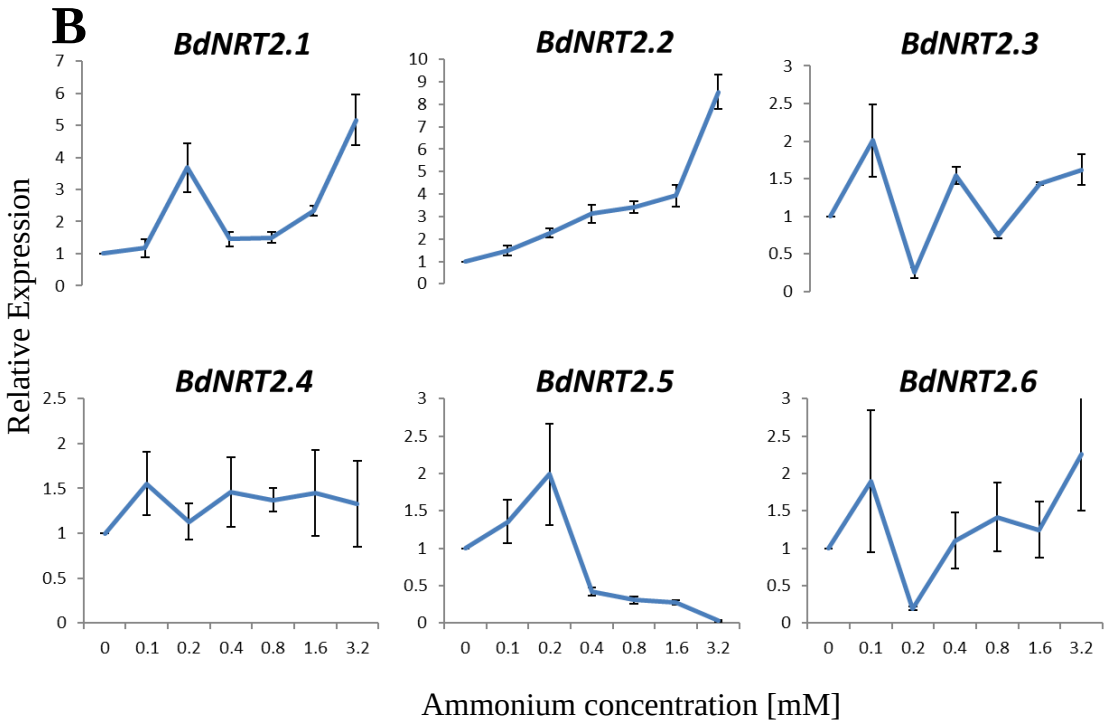
Figure 2.4 Expression patterns of BdNRT2 genes in response to nitrate provision.

RT-qPCR products were obtained from roots of 4-week-old *Brachypodium* plants, which were grown hydroponically for 3 weeks in solution containing 1 mM NH_4NO_3. Plants were N deprived for 1 week (0 h), and then re-supplied with 1 mM KNO_3 for 10min–6 h. *BdSamDC* was used as the housekeeping gene. Values are mean ± SE (n=3; 3 trials with the average of 3 biological replicates in each trial). For the q-PCR analysis of each gene, expression data of WT-T_0 was normalized as 1 in each trial.

Figure 2.5 Expression patterns of BdNRT2 genes at a series of nitrate (A) and ammonium (B) concentrations.

RT-qPCR products were obtained from roots of *Brachypodium* plants, which were grown hydroponically for 15 d in solution containing a range of KNO_3 or NH_4Cl concentrations (0-3.2 mM). BdSamDC was used as the housekeeping gene. Values are mean ± SE (n = 3). For the q-PCR analysis of each gene, expression data of WT-N_0 was normalized as 1 in each replicate.


A
BdNRT2.1
BdNRT2.2
BdNRT2.3
BdNRT2.4
BdNRT2.5
BdNRT2.6
Relative Expression
0 0.1 0.2 0.4 0.8 1.6 3.2
Nitrate concentration [mM]


B
BdNRT2.1
BdNRT2.2
BdNRT2.3
BdNRT2.4
BdNRT2.5
BdNRT2.6
Relative Expression
0 0.1 0.2 0.4 0.8 1.6 3.2
Ammonium concentration [mM]

; that is, increased ammonium concentration also induced *BdNRT 2.1* (5.2-fold increase, $p<0.001$) and *BdNRT 2.2* (8.6-fold increase, $p<0.001$) expression and repressed *BdNRT 2.5* expression (33.3-fold repression, $p<0.001$). The rest were constitutively expressed across all ammonium concentrations. These results indicated the expression of *BdNRT2* genes was probably governed by internal N status regardless of external nitrate and ammonium concentrations.

2.3.5 HATS was decreased in the *BdNRT2.1* mutant

The *bdnrt2.1* mutant has a single T-DNA insertion on the only exon of *BdNRT2.1*. *BdNRT2.1* transcript levels were minimal in both shoots and roots for the *bdnrt2.1*, and expression of truncated *BdNRT2.1* also was undetectable using primers specific to the 5'-UTR of the gene. Three representative homozygous *BdNRT2.1* rescue lines, named RE-7, RE-8, RE-10, were obtained with the expression of *BdNRT2.1* fully restored in the roots (Appendix 2.4). The $^{15}NO_3$ Influx experiment (Fig. 2.6) showed HATS activity was about 25 $\mu molg^{-1}DWh^{-1}$ in the *Brachypodium* root. Like the results of Li *et al*. (2007) on Arabidopsis *NRT2.1,* I also found that HATS were significantly reduced for the *bdnrt2.1* compared with the wild type (Fig. 2.6, 30% reduction, $p=0.03$), and the nitrate influx was restored to the similar level as the wild type in the rescue lines, indicating that BdNRT2.1 is responsible for HATS in *Bachypodium*. However, this reduction is observed to a much lesser extent compared to the 75% decrease of HATS in the *AtNRT2.1* mutant (Filleur *et al*., 2001).

2.3.6 *BdNRT2.6* was up-regulated to compensate the loss of *BdNRT2.1*, and *BdNRT2.2* was not inducible in the *BdNRT2.1* mutant

To investigate the impact of the absence of *BdNRT2.1* on the other family members, I compared the expression of BdNRT2 genes between wild-type and the *BdNRT2.1* mutant in response to nitrogen in the same nitrate resupply and gradient experiment condition described previously. As Fig. 2.7 shows, *BdNRT2.1* expression was not detectible at all conditions in the *BdNRT2.1* mutant (MU-red). *BdNRT2.6* was up-regulated by 6.1-fold (Fig. 2.7A, $p=0.01$), 7.5-fold (Fig. 2.7B, $p=0.04$), and 35-fold (Fig. 2.7C, $p<0.001$) in the mutant compared with the wild type (WT-blue) regardless of the treatment. The

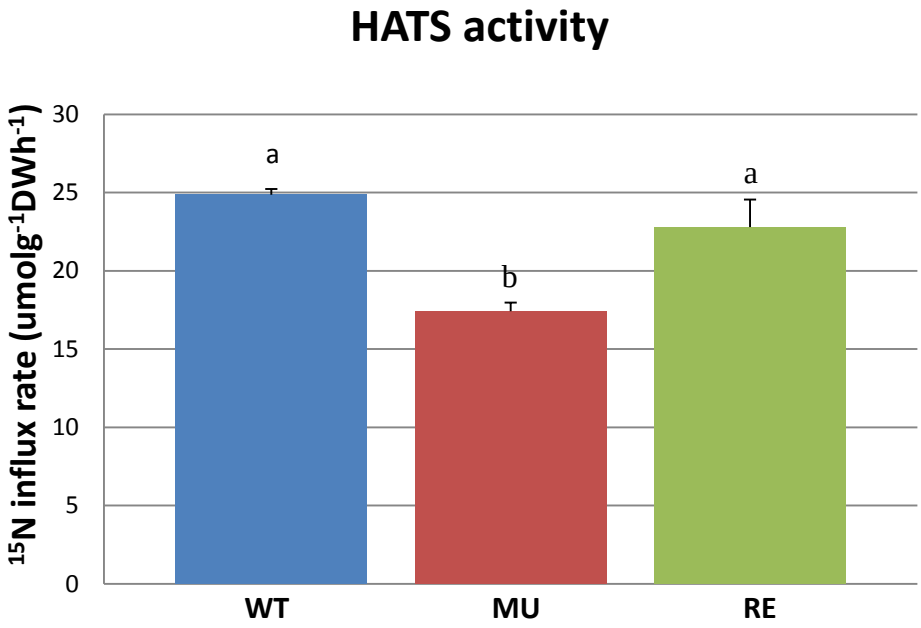
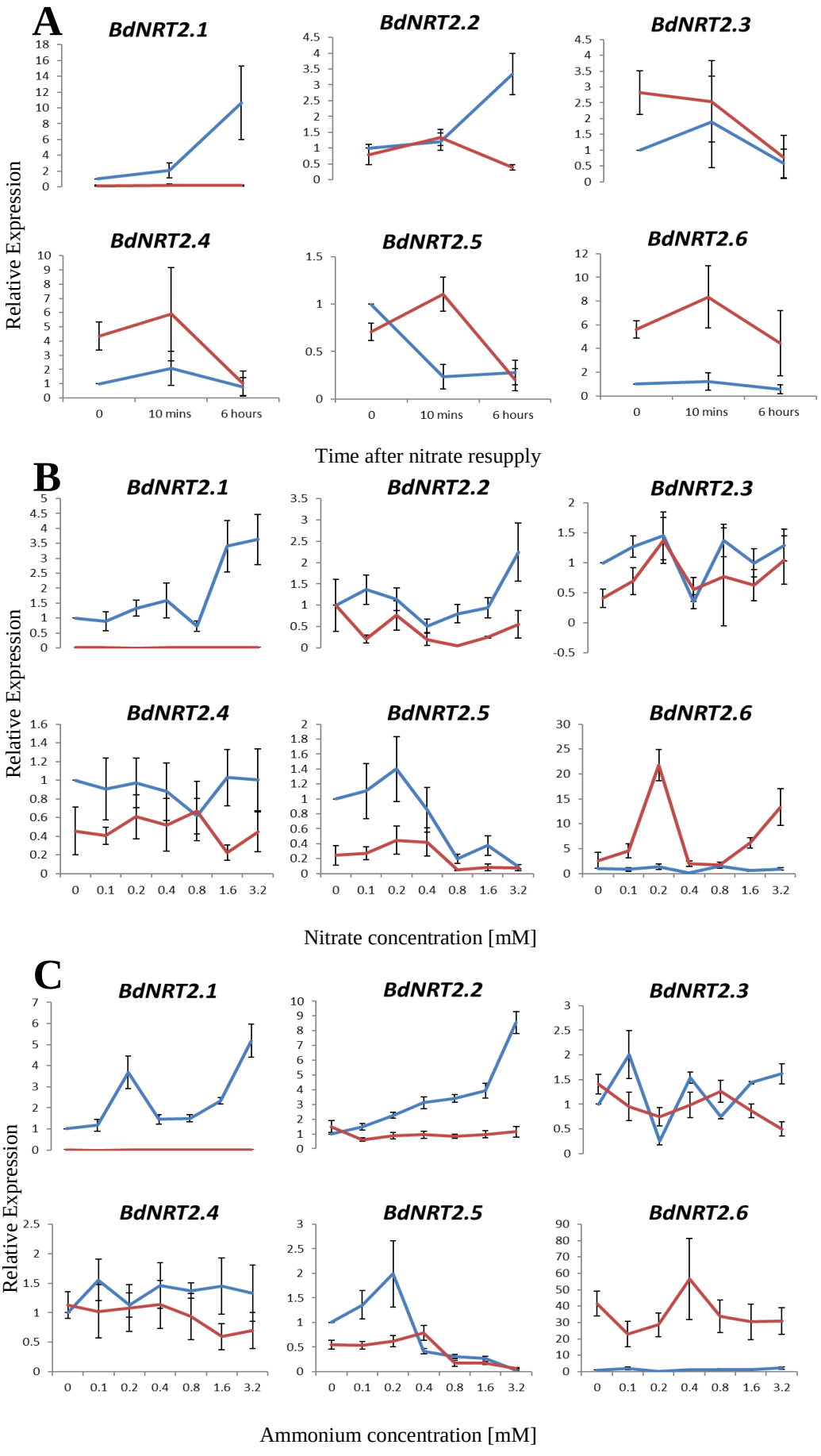


Figure 2.6 Root influx of ^{15}N in wild type (WT), the *bdnrt2.1* mutant (MU), and rescue lines (RE, equally pooled with RE-7, RE-8, RE-10).

Values are mean ± SE (n = 4). Different letters above bars indicate significant differences ($p<0.05$).

Figure 2.7 Expression patterns of BdNRT2 genes under different N conditions in wild-type (WT-blue) and *bdnrt2.1* (MU-red).

(A) Relative expression in N resupply experiment; (B) Relative expression in gradient nitrate experiment; (C) Relative expression in gradient ammonium experiment. *BdSamDC* was used as the housekeeping gene. Values are mean ± SE (Panel A was based on 3 trials with the average of 3 biological replicates in each trial; Panel B, C were based on 3 biological replicates from 1 trial). For the q-PCR analysis of each gene, expression data of WT_0 was normalized as 1 in each replicate.


A
BdNRT2.1
BdNRT2.2
BdNRT2.3
BdNRT2.4
BdNRT2.5
BdNRT2.6
Relative Expression
0
10 mins
6 hours
Time after nitrate resupply
B
BdNRT2.1
BdNRT2.2
BdNRT2.3
BdNRT2.4
BdNRT2.5
BdNRT2.6
Relative Expression
0 0.1 0.2 0.4 0.8 1.6 3.2
Nitrate concentration [mM]
C
BdNRT2.1
BdNRT2.2
BdNRT2.3
BdNRT2.4
BdNRT2.5
BdNRT2.6
Relative Expression
0 0.1 0.2 0.4 0.8 1.6 3.2
Ammonium concentration [mM]

BdNRT2.6 expression level was restored to the same level of wild-type in the *BdNRT2.1* rescue line (Appendix 2.5B). Intriguingly, regarding *BdNRT2.2* expression, there were significant interactions between nitrogen treatment and plant genotype (Fig. 2.7A, $p=0.001$, Fig. 2.7B, $p=0.002$, Fig. 2.7C, $p<0.001$). *BdNRT2.2* lost its nitrogen inducible property in the genetic background of the *BdNRT2.1* mutant, and it could not be recovered in the *BdNRT2.1* rescue line (Appendix 2.5A). *BdNRT2.1* and *BdNRT2.2* were tandem repeats on chromosome 3 with only 5.3 kb separating them. The regulatory change of *BdNRT2.2* expression in the *bdnrt2.1* could be explained by the possibility that the nitrogen responsive elements of *BdNRT2.2* may be physically disrupted by T-DNA insertion on the *BdNRT2.1* exon of the *bdnrt2.1* mutant.

2.4 Discussion

The gene expression profiling of *NRT2* has been well described in Arabidopsis (Orsel *et al*., 2002; Okamoto *et al*., 2003) and rice (Cai *et al*., 2008; Feng *et al*. 2011). Although rice is a monocot plant, its roots are usually grown in flooded paddy soils and only a trace amount of nitrate exists in its rhizosphere. *Brachypodium* is a typical monocot model plant that is closely related to many important cereal crops in various aspects. Six members were identified as NRT2 genes in *Brachypodium*. To gain more insight into those *BdNRT2s*, I first characterized their tissue specificity. Unlike the *AtNRT2s*, which were all highly expressed in the roots, except for *AtNRT2.7* (mainly in seeds) (Orsel *et al*., 2002; Okamoto *et al*., 2003; Chopin *et al*., 2007), I discovered that only *BdNRT2.1* and *BdNRT2.2* were strongly expressed in the root (Fig. 3), suggesting they are likely the main contributors to root nitrate uptake. *BdNRT2.3* and *BdNRT2.4* were preferably expressed in the leaf, *BdNRT2.5* was mostly found in the root and spikelet, while *BdNRT2.6* was weakly expressed across all organs. This difference in tissue specificity illustrated the phylogenetic divergence between Arabidopsis and *Brachypodium* (Fig. 2.2) which has also been demonstrated by Plett *et al*. (2010). Additionally, *BdNRT2.1* also was strongly expressed in the stem, indicating that it has versatile roles other than nitrate uptake. This will be confirmed in Chapter 3, which demonstrates that *BdNRT2.1* is responsible for N remobilization during leaf senescence. *BdNRT2.3* and *BdNRT2.4* were

tandem repeats on chromosome 3, 8.8kb apart from each other, and they exhibit the same tissue expression pattern. As the only member of *BdNRT2* that shared the same branch with *AtNRT2* on the phylogenetic tree, *BdNRT2.5* may have similar functions as *AtNRT2.5*, which is likely responsible for phloem loading and is inducible under nitrogen starvation (Lezhneva *et al*., 2014). The latter was confirmed in my nitrogen provision experiments; that is, *BdNRT2.5* was repressible under nitrate resupply (Fig. 2.4) and high nitrate (Fig. 2.5A) and ammonium (Fig. 2.5B) concentrations. Moreover, given that *BdNRT2.5* had strong expression in spikes (Fig. 2.3) and is located on the same clade as *AtNRT2.7* (Fig. 2.2), it could serve the same role as *AtNRT2.7* (Chopin *et al*., 2007), and could be responsible for seed nitrogen filling in *Brachypodium*. Overall, the tissue specificity of *BdNRT2* members differs from *AtNRT2s*. Among *BdNRT2s*, the tissue specificity varied with their genomic localization (Fig.2.1), and the neighboring genes tend to exhibit the same expression pattern, while *BdNRT2.5* and *BdNRT2.6*, which were located on a different chromosome, had a unique expression pattern.

The time-course nitrate resupply experiment shown in Fig. 2.5 allowed me to characterize *BdNRT2.1* and *BdNRT2.2* as nitrate inducible genes (10.6-fold and 3.3-fold induction, respectively, within 6 h), *BdNRT2.5* as nitrate repressible gene (3.6-fold repression within 6 h), and the rest as nitrate constitutive genes. The inductions of *NRT2.1* and *NRT2.2* in response to nitrate provision also were reported in Arabidopsis (Okamoto *et al*., 2003), and they were found to be responsible for the inducible high-affinity nitrate transport activity (Li *et al*, 2007). *AtNRT2.4* was strongly repressed with nitrate provision (Okamoto *et al*., 2003; Kiba *et al*., 2012) and this was not observed in *Brachypodium*. *BdNRT2.3* and *BdNRT2.4* shared the same tissue specific expression pattern and were both constitutively expressed in response to nitrate provision. Interestingly, like *AtNRT2.5*, *BdNRT2.5* is a nitrate repressible gene, however, none of the *NRT2s* in rice were found to be repressible (Cai *et al*., 2008; Feng *et al*., 2011). Nitrate is a signaling molecule. Within minutes, nitrate can trigger thousands of genes in primary N assimilation pathways, including *NRT2.1* and *NRT2.2* in Arabidopsis (reviewed by Wang *et al*., 2012). *AtNLP7* has been proposed as the master regulator of early nitrate signaling (Marchive *et al*., 2013). It can directly bind both the 3' and 5' genomic regions of *AtNRT2.1* and *AtNRT2.2*, and *AtNRT2.1* was activated by nitrate in an *AtNLP7* dependent

way (Marchive *et al*., 2013). Although, a so-called NRE (nitrate responsive element) is involved in nitrate regulation of gene coding for NIR1 (nitrite reductase) (Konishi and Yanagisawa, 2010), no conserved regulatory elements have been revealed in other nitrate responsive genes.

To further confirm the nitrate response of each *BdNRT2s*, I analyzed their expression change in nitrate gradient experiments. As predicted, similar expression patterns were observed; that is, the mRNA level of BdNRT2.1 and BdNRT2.2 increased (3.6-fold induction and 2.2-fold induction, respectively) when external nitrate concentrations were high, and the expression patterns conformed to the general linear model. Similarly, the mRNA expression level of *BdNRT2.5* decreased (10-fold repression) when external nitrate was high. To my knowledge, previous studies of *NRT2s*'s nitrogen response to a gradient of nitrate concentrations were performed on the basis of resupplying nitrate to nitrogen-starved plant in a time-course experiment (Orsel *et al*., 2002; Okamoto *et al*., 2003; Cai *et al*., 2008; Feng *et al*. 2011; Criscuolo *et al*., 2012; Pellizzaro *et al*., 2015). My gradient experiment was carried out by growing the *Brachypodium* plant with different nitrate concentrations. The stabilized *BdNRT2s* expression change against varying concentrations was then investigated at 15 DAG. The fact that similar expression patterns were observed in such gradient experiments as in nitrate resupply experiments indicates that the expression of *BdNRT2s* can be regulated by nitrate provision and the regulation stays in effect for at least 15 DAG. In my gradient experiments using the same set of concentrations of ammonium to replace nitrate, similar gene expression patterns were observed. The mRNA expression level of *BdNRT2.1* and *BdNRT2.2* increased (5.2-fold induction and 8.6-fold induction, respectively) when external ammonium concentration was high. Also, the mRNA expression level of *BdNRT2.5* decreased (33.3-fold repression) when external ammonium was high. These results indicate that the regulation of *BdNRT2s* is probably governed by its internal N status, because it is known that NRT2 does not transport ammonium. The linear nitrogen response makes *BdNRT2.1* and *BdNRT2.2* two suitable genes to serve as nitrogen markers in *Brachypodium*. It has been suggested that the internal N metabolites pool, such as amino-acids within the plant, can provide a signal that can regulate N uptake and assimilation (Nunes-Nesi *et al*., 2010). However, mainly negative regulations such as exogenous asparagine and

ammonium repression on N uptake and assimilation have been observed (Nunes-Nesi *et al*., 2010). *NRT2.1* and *NRT2.4* in Arabidopsis have been shown to be repressed by high external ammonium concentrations in different reports (reviewed by Wang *et al*., 2012). Our results did not reflect any ammonium repression effect in *Brachypodium*, which can be explained by the methodological difference, I grew the plant with various ammonium concentrations from germination for 15 d instead of treating N starved plants with various ammonium concentrations for a time course experiment. Furthermore, the highest ammonium level was 3.2 mM, which is much lower than the high ammonium treatment (~10 mM) of previous studies. What I discovered from the gradient experiments was a long-term regulation of BdNRT2s in response to two different nitrogen forms, and together with the nitrate provision results, I conclude that *BdNRT2.1* and *BdNRT2.2* was induced by high nitrogen condition and *BdNRT2.5* was repressed by high nitrogen condition, while the other three members were not found to be regulated by different nitrogen treatment. It is still unknown how the perception of N by *BdNRT2s* works based on current understanding.

In a BdNRT2.1 T-DNA mutant (*bdnrt2.1*), $^{15}NO_3$ influx under low nitrate (0.1 mM) was reduced by 30%, indicating that BdNRT2.1 is responsible for HATS activity. However, this reduction is much less than the 75% decrease of HATS in the AtNRT2.1 mutant (Filleur *et al*., 2001). It can be inferred that members in *Brachypodium NRT2s* other than *BdNRT2.1* are also playing a key role in HATS. Perhaps, *BdNRT2.2* will contribute significantly to HATS due to its strong expression in the roots, and its protein product is almost identical (93.8% at amino acids level, Appendix 2.1) to *BdNRT2.1*. I further examined the N response of *BdNRT2s* in the background of the *bdnrt2.1* mutant, trying to find the impact of losing *BdNRT2.1* on the rest of the family members. *BdNRT2.6* was strongly up-regulated regardless of N conditions in the *bdnrt2.1* mutant (Fig. 2.7ABC). This result indicates that *BdNRT2.6* was up-regulated to compensate for the loss of *BdNRT2.1*. In a gene family with multiple members, the lack of one member often leads to compensation by another member from the family. However, BdNRT2.6 can only partially, if at all, compensate the root nitrate uptake, given the results that the HATS was still down-regulated in the root of the *bdnrt2.1* mutant. Surprisingly, *BdNRT2.2* lost its nitrogen inducible property in the background of the *bdnrt2.1* mutant (Fig. 2.7ABC).

BdNRT2.1 rescue lines cannot recover this loss (Appendix 2.5A), suggesting that the T-DNA insertion in the *bdnrt2.1* mutant may physically disrupt the nitrogen regulatory elements of *BdNRT2.2*. Further research is needed to identify this nitrogen responsive motif of *BdNRT2.2*.

In conclusion, I presented a molecular characterization of the NRT2 family in the monocot model plant *Brachypodium*, which differs from the NRT2 family in *Arabidopsis*. I showed a diversified picture of their expression patterns in response to various nitrogen conditions and in specific tissues, suggesting multifaceted connections between plant nitrogen status and individual *BdNRT2* members. Although redundancy exists among members in the family, *BdNRT2.1* has emerged to be the key member, the lack of which results in an altered nitrogen response of other family members. My work gives a sound foundation for future experiments that will help to elucidate the specific roles of each transporter in monocot plants.

Study limitations:

Due to the high experimental workload and time limitation, the nitrogen gradient experiments and HATS determination experiment were conducted only with 1 trial each, while the nitrate resupply experiment was conducted with three trials. Thus the interpretation of results generated by a single trial should be cautious. Also, reader should keep in mind that the *bdnrt2.1* mutant was not out-crossed to eliminate the possibility of multiple mutations, although the *bdnrt2.1* mutant was reported to have a single T-DNA insertion according to *Brachypodium* T-DNA collection's information sheet.

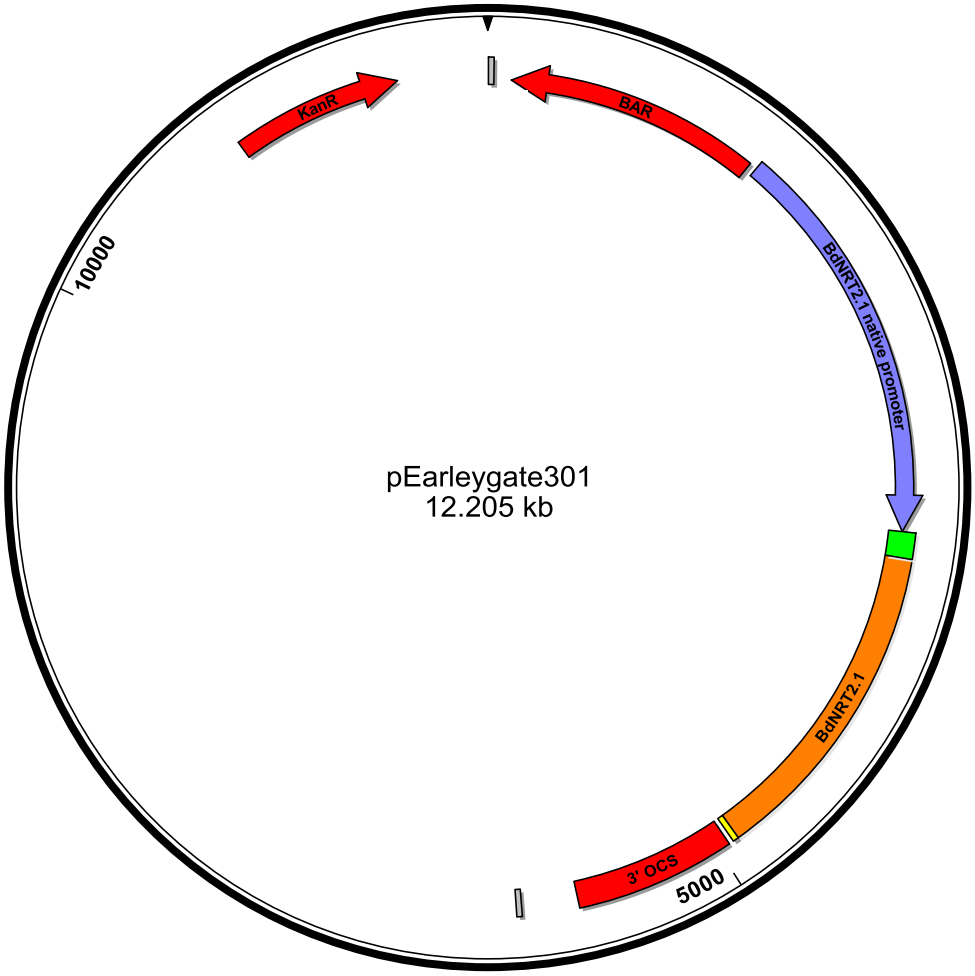
2.5 Appendices

Appendix 2.1 Structural features of BdNRT2 genes and proteins

Gene	Locus ID	Length(aa)	% similarity to BdNRT2.1
BdNRT2.1	Bradi3g01270	498	100
BdNRT2.2	Bradi3g01250	509	93.8
BdNRT2.3	Bradi3g01277	507	86.1
BdNRT2.4	Bradi3g01290	503	85.7
BdNRT2.5	Bradi2g47640	515	68.5
BdNRT2.6	Bradi2g26210	433	66.1

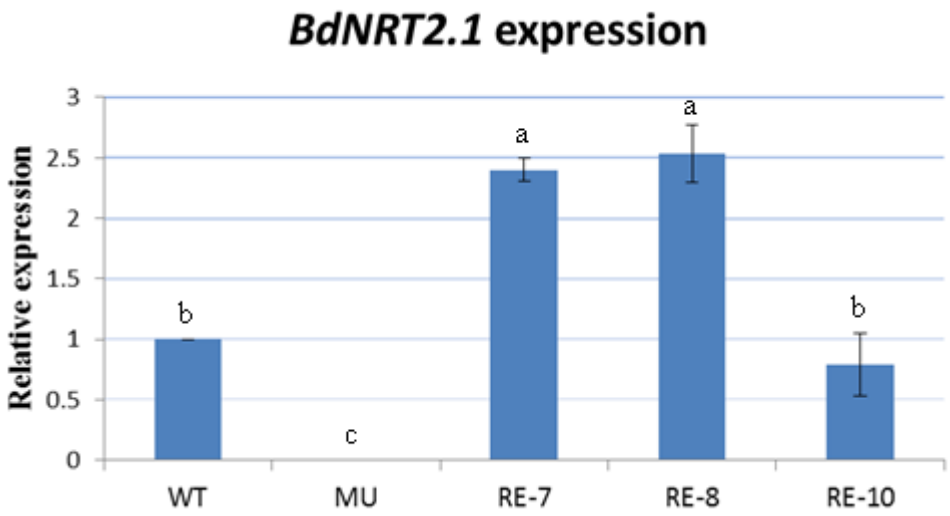
Appendix 2.2 Primers used for RT-qPCR of NRT2 genes.

Gene name	Primers
BdNRT2.1	F: 5'- TTTCCTCTGTCCCAGCTACG -3'
	R: 5'- CGCAGCAAATGTGTAGTACCTC -3'
BdNRT2.2	F: 5'- AAGCCGGCGCTCATGGAGAC -3'
	R: 5'- GAGGTTGTCGCGGATGATAG -3'
BdNRT2.3	F: 5'- ATGGAGGTCGGCACTTCA -3'
	R: 5'- TACATGTTGGGGCGTGTTG -3'
BdNRT2.4	F: 5'- ATGGAGGCCGGCTCTGCT -3'
	R: 5'- TATGTGCTCGGGCGACCCG -3'
BdNRT2.5	F: 5'- ATGGGGGGGGAGTCGAAG -3'
	R: 5'- CACGTCGGCCGGCGGTGAT -3'
BdNRT2.6	F: 5'- ATGGAACTGGAGGTGGGC -3'
	R: 5'- CTCCGAAGCGTAGTAGTCC -3'
BdSamDC	F: 5'- TGCTAATCTGCTCCAATGGC-3'
	R: 5'- GACGCAGCTGACCACCTAGA-3'
BdUbi4	F: 5'- TGACACCATCGACAACGTGA -3'
	R: 5'- GAGGGTGGACTCCTTCTGGA -3'



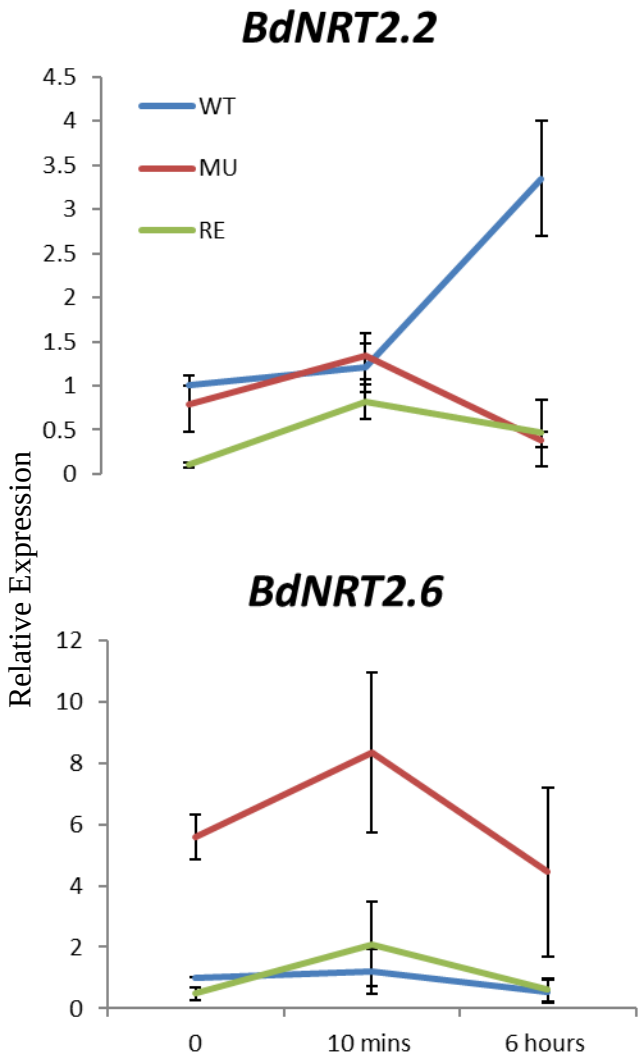
Appendix 2.3 Circular map of vector constructs (pEarleygate301) used for BdNRT2.1 complementation.

Map was constructed using SeqBuilder (Lasergene 10)



Appendix 2.4 qPCR expression analyses of BdNRT2.1 gene in root of the wild type, mutant and rescue lines.

Values are mean ± SE (n = 3). *BdSamDC* was used as the housekeeping gene. Different letters above bars indicate significant differences ($p<0.05$). For the q-PCR analysis of the gene, expression data of WT was normalized as 1 in each replicate.



Appendix 2.5 Expression patterns of BdNRT2.2 (A) and BdNRT2.6 (B) in response to nitrate provision of wild-type (WT-blue), *bdnrt2.1* (MU-red), and *bdnrt2.1* rescue line (RE-green).

RT-qPCR products were obtained from roots of 4-week-old *Brachypodium* plants, which were grown hydroponically for 3 weeks in solution containing 1 mM NH_4NO_3. Plants were N deprived for 1 week (0 h), and then re-supplied with 1 mM KNO_3 for 10min–6 h. *BdSamDC* was used as the housekeeping gene. Values are mean ± SE (n = 3). For the q-PCR analysis of each gene, expression data of WT_0 was normalized as 1 in each trial.

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3 The High-affinity Nitrate Transporter BdNRT2.1 Controls Nitrogen Remobilization and Increases Yield of *Brachypodium Distachyon*

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3.1 Introduction

As described in Chapter 1.1, the world is experiencing many problems resulting from excess agricultural nitrogen supply. The global annual amount of synthetic nitrogen (N) fertilizer applied to crops has risen dramatically in the past few decades, resulting in significantly increased crop yields and decreased world hunger (Good *et al*., 2004). However, only a small portion of applied nitrogen is utilized (McAllister *et al*., 2012). More than half of the applied nitrogen is lost through a combination of different processes, including ground run-off, leaching, denitrification and ammonia volatilization (Garnett *et al*., 2009) which adversely affect our human health and eco-systems (Townsend *et al*., 2003;Vitousek *et al*., 2009; Wuebbles, 2009). In addition, the cost of N fertilizer is increasing, because it is derived from the energy intensive Haber-Bosch process (Xu *et al*., 2012). Together, this has created an urgent need to enhance nitrogen use efficiency (NUE) in crops; that is, crops that are better able to take up, utilize and remobilize the nitrogen available to them. For crops, NUE is defined as grain yield per unit of applied N in the soil; for *Arabidopsis*, NUE is expressed as fresh or dry biomass per nitrogen content in the plant (Good *et al*., 2004). In both cases, NUE is the combination of nitrogen uptake efficiency (NUpE)/nitrogen assimilation efficiency (NAE) and nitrogen utilization efficiency (NUtE)/nitrogen remobilization efficiency (NRE) (Xu *et al*., 2012).

Nitrate is the major form of inorganic N that can be utilized by crop plants in aerobic soils (Xu *et al*., 2012). Plant roots generally have two systems for nitrate uptake, a high-affinity nitrate transport system (HATS), which is activated under low external nitrate concentrations (< 500μM) and a low-affinity nitrate transport system (LATS), which is activated under high external nitrate concentrations (>500μM) (Noguero and Lacombe, 2016). Both constitutive and inducible forms co-exist within each of these nitrate transport systems (Miller *et al*., 2007; Noguero and Lacombe, 2016). These four systems together ensure the efficient uptake of nitrate from the soil and distribution within the whole plant in response to various environmental and developmental conditions (reviewed in Tsay *et al*., 2007; Masclaux-Daubresse *et al*., 2010; Wang *et al*., 2012; Krapp *et al*., 2014; Krapp, 2015). There are seven members in the NRT2 family in *Arabidopsis thaliana* that are high-affinity nitrate transporters (Orsel *et al*., 2002).

AtNRT2.1, mainly expressed in the epidermal and cortical cells of mature roots (Wirth *et al*., 2007), has been demonstrated as the main component of HATS (Filleur *et al*., 2001; Li *et al*., 2007), and it is responsible for efficient use of nitrate when nitrate is limiting (Krapp, 2015). Besides its essential role in root nitrate influx, *AtNRT2.1* is also involved in nitrate sensing independently; the loss of function mutant *lin1* has the ability to initiate large numbers of lateral roots (Little *et al*., 2005). However, little or no impact of *AtNRT2.1* on the NUE related phenotype was detected under N non-limiting conditions.

The small monocot plant, *Brachypodium distachyon* (*Brachypodium*), is rapidly emerging as a powerful model system (reviewed in Girin *et al*., 2014; Kellogg, 2015) to study questions unique to monocot crops (wheat, maize, rice, etc.), due to its small genome (270 Mbp; Vogel *et al*., 2010) and plant size, short life cycle, efficient transformation and ease of cultivation. There are fundamental differences between *Arabidopsis* and grass species in gene number, family structure and the phylogenetic tree of NRT2 (Plett *et al*., 2010), which does not make it feasible to determine the functions of NRT2 genes in monocot plants simply based on homologuous genes identified in *Arabidopsis*. More importantly, like common monocot crops, *Brachypodium* can generate grain yield which is a desirable and inheritable trait for NUE research, and makes it an ideal system for study of such specific processes.

In this study, I analyzed the function of a putative high-affinity nitrate transporter, *BdNRT2.1*, in *Brachypodium* with the aid of the *bdnrt2.1* mutant, transgenic lines including *BdNRT2.1* cDNA rescue lines and *BdNRT2.1* over-expression lines. I showed that *BdNRT2.1* is indispensable for NUE and also controls the nitrogen remobilization process during leaf senescence in *Brachypodium* under N non-limiting conditions. This higher expression of *BdNRT2.1* can result in better NUE.

3.2 Materials and Methods

3.2.1 Plant material and growth conditions

The *BdNRT2.1* (Bradi3g01270) T-DNA insertion mutant line was obtained from the JGI *Brachypodium* collection (JJ12084). Wild type (Bd21-3), T3 homozygous mutant (based on Bd21-3) seeds and T4 homozygous transgenic seeds (based on Bd21-3) were grown in Pro-mix or hydroponic solution. Three lines of T4 *BdNRT2.1* rescue plants (RE-7, RE-8,

RE-10) and three lines of T4 *BdNRT2.1* overexpressing plants (O-2, O-4, O-13) were used in the experiments. A T-DNA insertion mutant line was obtained from the JGI *Brachypodium* collection (JJ12084). Homozygous T3 lines for the mutants were selected by genomic PCR using specific primers (Appendix 3.4). Wild type (Bd21-3), T3 homozygous mutant seeds and T4 homozygous transgenic seeds were germinated on wet filter paper in a petri dish sealed with 3M Micropore tape and incubated for 4 days in the dark at 4^{o}C to synchronize germination. To initiate germination, seeds were transferred to a growth room with a 20 h photoperiod, a temperature of 23^{o}C, a light intensity of 75 μmol/m^{2}/s, for 5 days. Successfully germinated seedlings with similar size were planted (1 seedling per pot) in pots of Pro-mix® BX Mycorrhizae growing medium (500 g each pot, Premier Tech Horticulture) in the growth room (pots with different genotype were randomized on the shelf in the growth room) to grow and produce seeds (~2 months for each generation). The aerial parts of the plants were harvested for the measurement of yield, harvest index, spike number, number of seeds per spike and 100-seeds weight. Each pot was considered as 1 biological replicate. The measurements were performed on 8 biological replicates.

The hydroponic growth condition was described in chapter 2.2.2. The transformation process was described in Chapter 2.2.3. Over expressing DNA construct was generated by inserting the full coding region of *BdNRT2.1 (Bradi3g01270)* flanked by Gateway® attachment site sequences into pMDC99-101(Modified by Dr. Gang Tian) using Gateway® Technology (Hartley *et al*., 2000). One hundred μg/L hygromycin was used to select the resistant calli. Construct diagrams are detailed in Appendix 3.3. The specific primers used in vector construction are detailed in Appendix 3.5.

3.2.2 RNA extraction and quantitative real-time PCR

The methods were described in chapter 2.2.5. The specific primers used for quantitative real-time PCR are detailed in Appendix 3.6 & 3.7.

3.2.3 Protein extraction and Western blot

For protein immunoblot analysis, 100 mg of *Brachypodium* whole plant tissue was collected at 3 pm on Friday of the 4th week of hydroponic culture. Samples were

homogenized using Tissuelyser (QIAGEN) and then direct lysis in laemli buffer consisting of 4% SDS, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl pH 6.8 and 10% (v/v) beta-mercaptoethonol. The lysate was boiled for 5min before loading. An equal amount of protein (~30 µg) was seprated by SDS-PAGE (8%). The gel was blotted for 30 minutes to PVDF using a semi-dry transfer cell (BIO-RAD). Blots were blocked immediately following transfer in 5% (w/v) non-fat milk dissolved in Tris Buffered Saline pH 7.4 (Fisher, cat. no. 166103) with Tween-20 (TBST) for 1 h at room temperature with agitation. Blots were incubated in the primary antibodies (anti-GS, CEDARLANE cat. no. AS08292, at a dilution of 1:10000; anti-GOGAT, CEDARLANE cat. no. AS07242, at a dilution of 1:5000) for 1 h at room temperature with agitation. The anti-body solution was decanted and the blot was rinsed 3b × 15 min with TBST. Blots were incubated in secondary antibody (anti-Rabbit, Cell Signaling and Millipore, at a dilution of 1:5000) for 1 h at room temperature with agitation. Blots were washed as above. Proteins were detected using ECL Prime Western Blot detection reagents (GE health care Life Sciences, VWR cat. no. CA89168-782) and exposed to Classic Single-Emulsion Autoradiography Film (Mandel Scientific). The films were exposed for 30 seconds and 1 min for anti-GS and anti-GOGAT, respectively, and then automatically developed by an AGFA CP1000 X-Ray Film Processor and scanned with an UMAX Powerlook 1120 scanner. The blot was based on two trials; each trial was based on a pooled sample from 3 biological replicates.

3.2.4 Metabolites analysis

Fifty milligrams of freeze-dried material (both root and shoot, excluding spikelet) were homogenized using a tissue lyser and dissolved in 1 ml ice-cooled methanol:water (4:1, v/v) followed by vigorous vortexing. Samples were then sonicated for 15 mins in a water bath followed by gentle shaking @ 4^{o}C for 15 mins before centrifuging at 13000 rpm for 15 min at 4^{o}C. Eight hundred µL of the supernatant was transferred into a fresh Eppendorf tube and evaporated to dryness using a vaccufuge at ambient temperature. The dried residue was re-dissolved in 400 µL of methanol by vigorous vortexing and diluted with 400 µL of water. Processed samples were then filtered using a 0.45 µm PTFE syringe filter (Whatman) and spiked with 5 µL of 1 µg/mL $^{13}C_6$ phenylalanine internal

standard (Cambridge Isotopes, Tewksbury, USA). Samples were then subjected to LC-MS/MS analysis. The measurements were performed on 3 biological replicates.

3.2.5 $^{15}NO_3$ influx

The influx rate of $^{15}NO_3$ was quantified as previously described by Li *et al*. (2007). *Brachypodium* plants were grown hydroponically in nutrient solution containing 1 mM NH_4NO_3 for 4 weeks and then deprived of N for 1 week to deplete the internal N reservoir. When such plants were first exposed to 0.1 mM $K^{15}NO_3$, the flux measured was due to cHATS. The plants were washed with 0.1 mM CaSO4 for 1 min, then immersed in nutrient solution containing 0.1 mM $K^{15}NO_3$ (atom%15N: 99%) for 10 min then finally in 0.1 mM CaSO4 for 1 min. Afterwards, plants that had been starved for nitrate for 7 d were transferred into nutrient solution containing 1 mM KNO_3 for 6 h to induce a nitrogen response, and then exposed to 0.1 mM $K^{15}NO_3$. This flux represented the combination of cHATS and iHATS. By subtracting cHATS from the latter, iHATS activity was determined. Similarly, plants that had been nitrate induced for 6 h were treated with 5 mM KNO_3 before influx measurement; this influx represented the total of cHATS, iHATS and LATS. Roots of the tested plants were oven dried for 48 h at 60^{o}C and ground into powder, and 2.5mg of each subsample was shipped to UC Davis Stable Isotope Facility (Davis, CA, USA) for ^{15}N abundance determination, where samples were analyzed using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The ^{15}N analyses were performed on 4 biological replicates.

3.2.6 N remobilization test

Brachypodium plants were grown hydroponically containing 5 mM $K^{14}NO_3$ until anthesis (3.5 weeks after germination). Plants were subsequently transferred into pots of vermiculite and sand growth medium (4 seedlings per pot, 500 g, contained no nutrients), and each pot was watered with nutrient solution containing 5 mM $K^{15}NO_3$ only weekly until maturation. Pots watered with 5 mM $K^{14}NO_3$ were used as control. Each pot was considered as 1 biological replicate. Mature seeds were harvested and ground (~2.5mg each sample) before being shipping to the UC Davis Stable Isotope Facility (Davis, CA,

USA) for $^{15}N/^{14}N$ abundance determination. The measurements was performed on 2 trials with 3 biological replicates in each trial (n=6).

3.2.7 Statistical analysis

The differences between two subjects were determined using the two-tailed student t test with equal variance. The differences among multiple subjects were assessed using one-way ANOVA followed by multiple comparisons tests (Fisher's LSD method). All statistical analysis was performed using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL, USA).

3.3 Results

3.3.1 *BdNRT2.1* is responsible for NUE, and can increase NUE when over-expressed

The *bdnrt2.1* mutant has a T-DNA insertion on the only exon of *BdNRT2.1* (Fig. 3.1A). *BdNRT2.1* transcripts were the minimum in both shoot and root of *bdnrt2.1* (Fig. 3.1B), and truncated expression of *BdNRT2.1* was also undetectable using primers specific to the 5'-UTR of the gene. Three representative homozygous *BdNRT2.1* rescue lines, named RE-7, RE-8, RE-10, were obtained with the expression of BdNRT2.1 fully restored in roots (Fig. 3.1C). RE-7 and RE-8 had approximately 2-fold overexpression of *BdNRT2.1*, and this did not affect the nitrate influx compared with RE-10 (data not shown), but it increased the grain yield compared with WT (Fig. 3.2). Three representative homozygous over-expression lines, named O-2, O-4, O-13, were obtained with the expression of *BdNRT2.*1, which reached 96-fold, 228-fold, and 341-fold increases, respectively, compared with the wild type in roots (Fig. 3.1D).

All *Brachypodium* plants were grown on the same amount of Pro-mix® BX Mycorrhizae growing medium (~2 mM nitrate) until mature. The *bdnrt2.1* showed significant grain yield decrease compared to the wild type (37% decrease, $p<0.001$, Fig. 3.2 & Table 3.1), as well as reduced grain number per spike, 100-grain weight and harvest index ($p<0.0001$, $p=0.012$, $p= 0.0017$, respectively, Table 3.1). However, the number of spikelets per plant

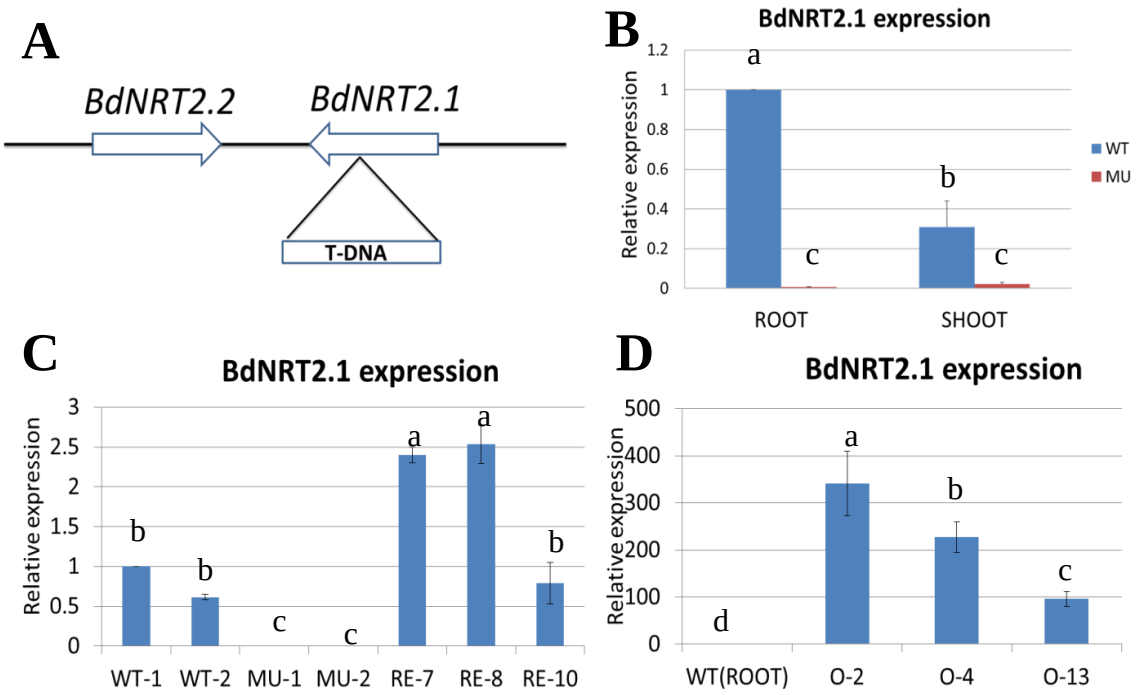


Figure 3.1 (A) Schematic representation of the T-DNA insertion characterized in the bdnrt2.1 mutant. qPCR expression analyses of BdNRT2.1 gene at anthesis (3.5 weeks after germination) (B) in shoot and root of wild type and mutant; (C) in root of wild type, mutant and rescue lines; (D) in root of wild type and over-expression lines.

Values are mean ± SE (n = 3). Different letters above bars indicate significant differences ($p<0.05$). For the q-PCR analysis of each gene, expression data of WT was normalized as 1 in each trial.

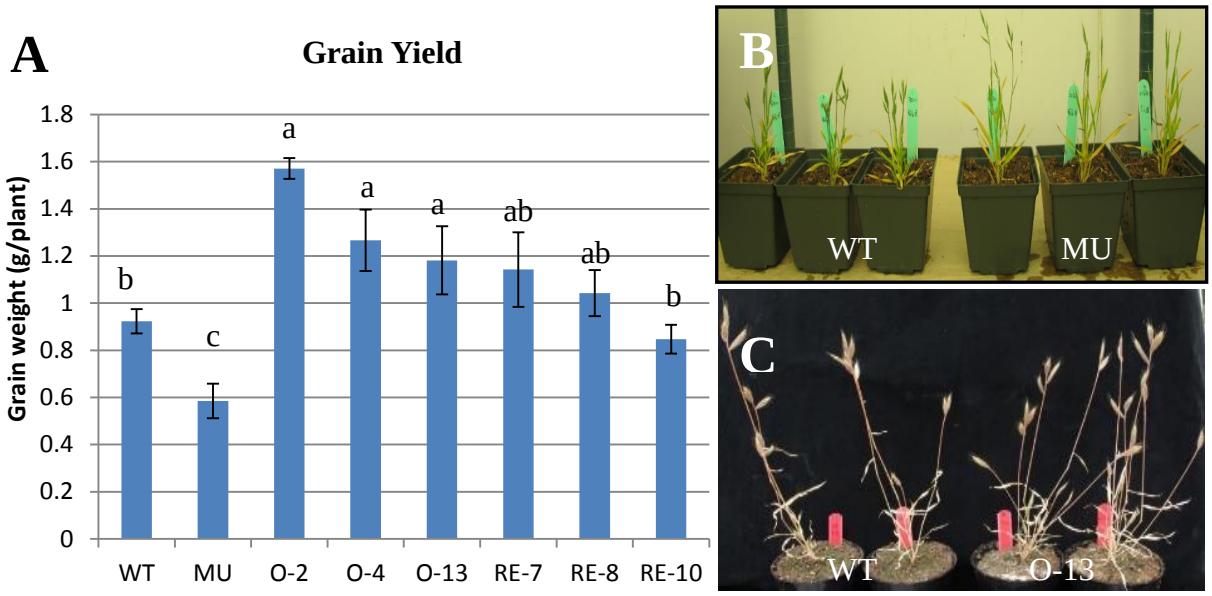


Figure 3.2 Yield and growth of wild type, *bdnrt2.1* mutant, over-expression lines and rescue.

(A) average grain yield of wild type (WT), *bdnrt2.1* mutant (MU), over-expression lines (O-2, O-4, O-13) and rescue lines (RE-7, RE-8, RE-10). Values are mean ± SE (n = 8). Different letters above bars indicate significant differences ($p<0.05$). (B) Phenotypes of WT and MU before maturing. (C) Phenotypes of WT and O-13 (O-2 and O-4 have similar phenotype) after maturing.

Table 3.1 Agronomic traits of the wild type (WT), the *bdnrt2.1* mutant (MU), over-expression lines (O-2, O-4, O-13) and rescue lines (RE-7, RE-8, RE-10) in Pro-mix.

Genotype	Grain yield (g/plant)	p value	Spikelet number (per plant)	p value	Grain number (per spike)	p value	100-grain weight (g)	p value	Harvest index (HI)	p value
WT	0.92±0.05	N/A	20.1±1.2	N/A	9.0±0.2	N/A	0.51±0.01	N/A	0.53±0.02	N/A
MU	0.59±0.07*	0.0020	51.1±4.9*	<0.0001	2.6±0.3*	<0.0001	0.45±0.02*	0.0120	0.39±0.03*	0.0017
O-2	1.57±0.04*	0.0003	36.5±1.5*	<0.0001	9.8±0.2	0.1226	0.44±0.01*	0.0188	0.51±0.03	0.6128
O-4	1.27±0.13*	0.0232	31.7±2.5*	<0.0001	9.5±0.7	0.5288	0.42±0.01*	<0.0001	0.56±0.02	0.2440
O-13	1.18±0.14*	0.0367	31.5±3.5*	0.0031	7.8±0.2*	0.0033	0.48±0.01	0.1532	0.54±0.02	0.7310
RE-7	1.14±0.16	0.1855	47.0±6.0*	0.0004	4.9±0.3*	<0.0001	0.52±0.01	0.5638	0.52±0.01	0.5678
RE-8	1.04±0.10	0.2835	45.5±5.1*	0.0002	4.9±0.1*	<0.0001	0.53±0.04	0.5714	0.51±0.03	0.6274
RE-10	0.85±0.06	0.4369	35.3±4.8*	0.0014	4.8±0.4*	<0.0001	0.51±0.01	0.9229	0.50±0.02	0.2987

Note: Values are mean ± SE (n = 8). * represents $p<0.05$.

was significantly higher in the mutant ($p<0.0001$, Table 3.1). RE-7, RE-8, RE-10 could all restore the grain yield of the *bdnrt2.1* to a similar level as the wild type (Fig. 3.2 & Table 3.1), but with significantly increased spikelet number per plant and reduced grain number per spikelet ($p<0.01$, Table 3.1). Furthermore, O-2, O-4 and O-13 overexpressing lines all could increase the yield significantly ($p<0.001$, $p=0.02$, $p=0.04$, respectively, Table 3.1) compared with the wild type. All three overexpression lines had a significantly increased spikelet number per plant ($p<0.01$, Table 3.1), O-2 and O-4 had a decreased 100-grain weight ($p=0.002$ and $p< 0.0001$, respectively, Table 3.1), which can be explained by the potential negative correlation that the higher yield produced smaller grain. The fact that all rescue lines, including RE-7, RE-8, RE-10, increased spikelet number per plant compared with WT (while still smaller than MU), suggesting that the reduction of BdNRT2.2 (Chapter 2, Fig. 2.7), not the BdNRT2.1 gene itself, which may be responsible for the increase of the spikelet number per plant as BdNRT2.1 rescue lines did not recover the expressions of BdNRT2.2 (Chapter 2, Appedix 2.5).

3.3.2 The *bdnrt2.1* has decreased inducible high-affinity nitrate uptake

The $^{15}NO_3$ influx experiment showed HATS (iHATS+cHATS) and LATS each accounted for about half of the nitrate influx from the *Brachypodium* root (~25 $\mu molg^{-1}DWh^{-1}$ each, Fig. 3.3ABC). Like the results of Li *et al*. (2007) on Arabidopsis *NRT2.1*, I also found the inducible HATS were significantly reduced in the *bdnrt2.1* compared with the wild type (Fig. 3.3B, 35% reduction, $p=0.0002$), indicating BdNRT2.1 is a major component of iHATS in *Bachypodium*. In contrast, the mutant had no effect on cHATS and LATS (Fig. 3.3A and 3.3C). Furthermore, the nitrate influx was restored to a similar level as the wild type in the rescue lines.

3.3.3 BdNRT2.1 is responsible for N remobilization under N non-limiting condition

In this study, nitrogen remobilization was measured in the wild type and the *bdnrt2.1* mutant plants through the "apparent remobilization" method (Masclaux-Daubresse *et al*., 2010) with modifications. All plants were grown hydroponically with sufficient normal ^{14}N-nitrate (5 mM) until anthesis after which they were fed with labeled ^{15}N-nitrate (5

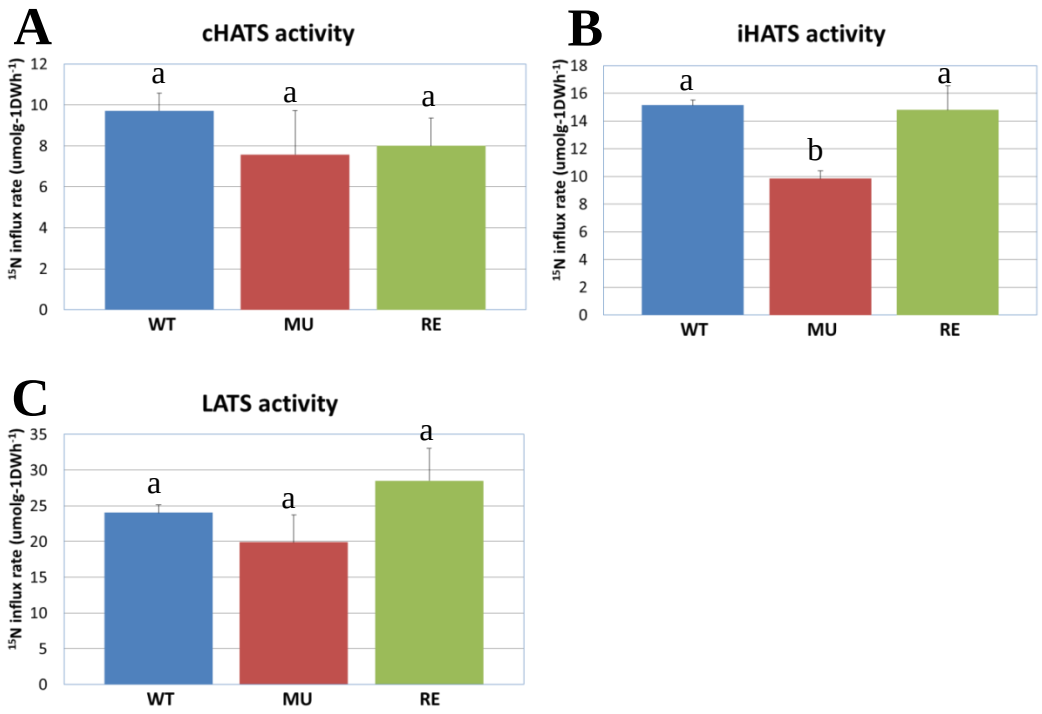


Figure 3.3 Root influx of ^{15}N in the wild type (WT), the *bdnrt2.1* mutant (MU), and rescue lines (RE, equally pooled with RE-7, RE-8, RE-10) representing (A) cHAT activity; (B) iHAT activity; (C) LATS activity.

Values are mean ± SE (n = 4). Different letters above bars indicate significant differences ($p<0.05$).

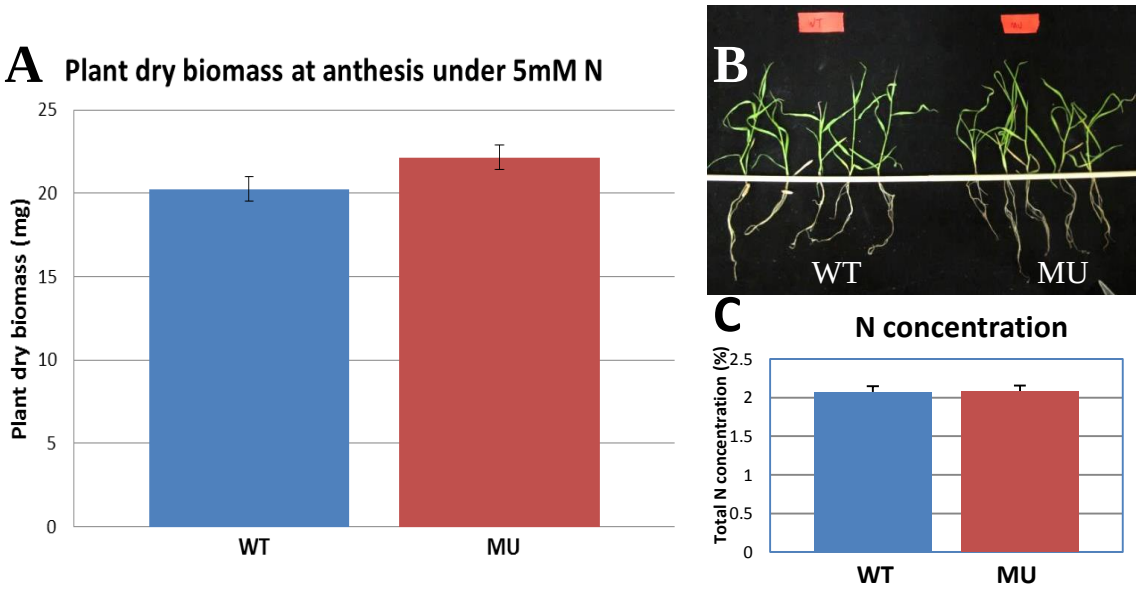


Figure 3.4 (A) Plant dry biomass at anthesis (3.5 weeks after germination) of WT and MU under 5 mM nitrate. (B) Phenotype of WT and MU under 5 mM nitrate. (C) Total N concentration of WT and MU at anthesis.

Values are mean ± SE (n = 8).

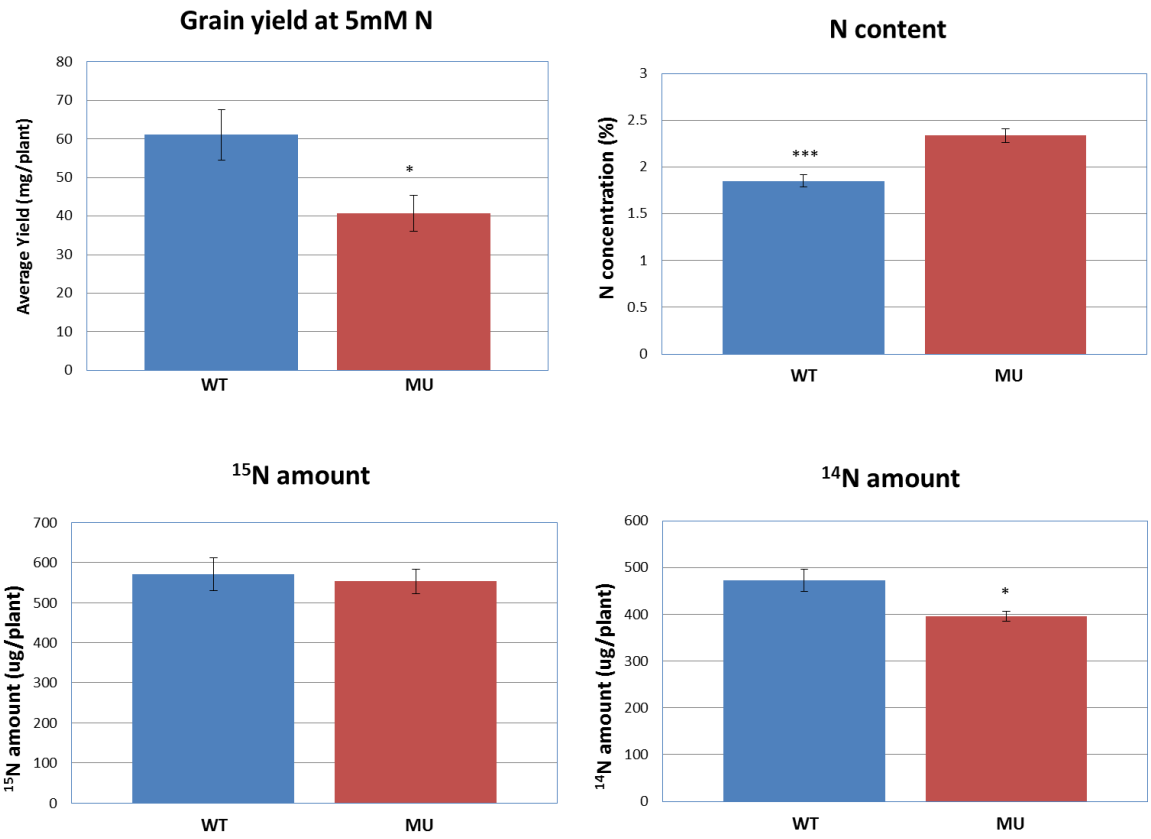


Figure 3.5 N remobilization analysis in the wild type (WT) and *bdnrt2.1* mutant (MU).

(A) Average grain yield in WT and MU at 5 mM nitrate. (B) N content (%) in grains of WT and MU. (C) ^{15}N amount per plant yield of WT and MU. (D) ^{14}N amount per plant yield of WT and MU. Values are mean ± SE (n = 6). * above bars represents $p<0.05$, *** above bars represents $p<0.01$.

mM) until seed maturation. The ^{15}N amount in mature seed reflected the post-flowering nitrate uptake and the ^{14}N amount in mature seed represented N remobilization from vegetative tissues that formed during the pre-flowering stage. There was no difference in whole plant fresh/dry weight at anthesis between the wild type and the *bdnrt2.1* mutant (Fig. 3.4), suggesting NUpE was not affected in the mutant. However, consistent with the NUE results under Pro-mix® described previously, the *bdnrt2.1* mutant had significantly reduced grain yield under 5 mM N-nitrate (Fig. 3.5A, p=0.028). Although the mutant had higher N content in mature seeds (Fig. 3.5B, p=0.0024), total N was lower in the mutant mature seeds, among which the ^{15}N amount per plant was the same between the *bdnrt2.1* mutant and the wild type (Fig. 3.5C). This result indicated post flowering nitrate uptake was not affected in the mutant. In contrast, the ^{14}N amount per plant was significantly reduced in the mutant (Fig. 3.5D, p=0.016), indicating N remobilization was impaired in the *bdnrt2.1* mutant.

3.3.4 BdNRT2.1 may act as a signal transducer to coordinate N remobilization with developmental cues

Brachypodium NAC1 and NAC 71 were down regulated in the *bdnrt2.1* mutant during leaf senescence (Fig. 3.6, p= 0.027 and p<0.001), suggesting a potential feed forward regulation role of BdNRT2.1 on NAC activation. The orthologous wheat TaNAC2-5 (Appendix 3.1) binds to the promoter region of genes encoding nitrate transporter and glutamine synthetase (Garnett *et al*., 2013), providing a putative link between BdNRT2.1 and nitrogen remobilization. Furthermore, cytosolic glutamine synthesis 1 (GS1), which plays a key role during N remobilization (Masclaux-Daubresse *et al*., 2010), also was found to be significantly down regulated during leaf senescence at both the mRNA abundance level (Fig. 3.7ABC, p=0.014, p=0.038 and p=0.028, respectively) and the protein level (Fig. 3.7D). In contrast with GS1, the chloroplastic glutamine synthesis 2 (GS2) isoform was not affected (Fig. 3.7D). Moreover, glutamate synthases (Fd-GOGAT and NADH-GOGAT) also were not changed at either the level of mRNA or protein abundance (Appendix 3.2). Interestingly, glutamine (Gln) and asparagine (Asn), the major forms of N in phloem sap during senescence, significantly accumulated in the vegetative tissues of the *bdnrt2.1* mutant (Table 3.2, p=0.016 and p<0.0001,

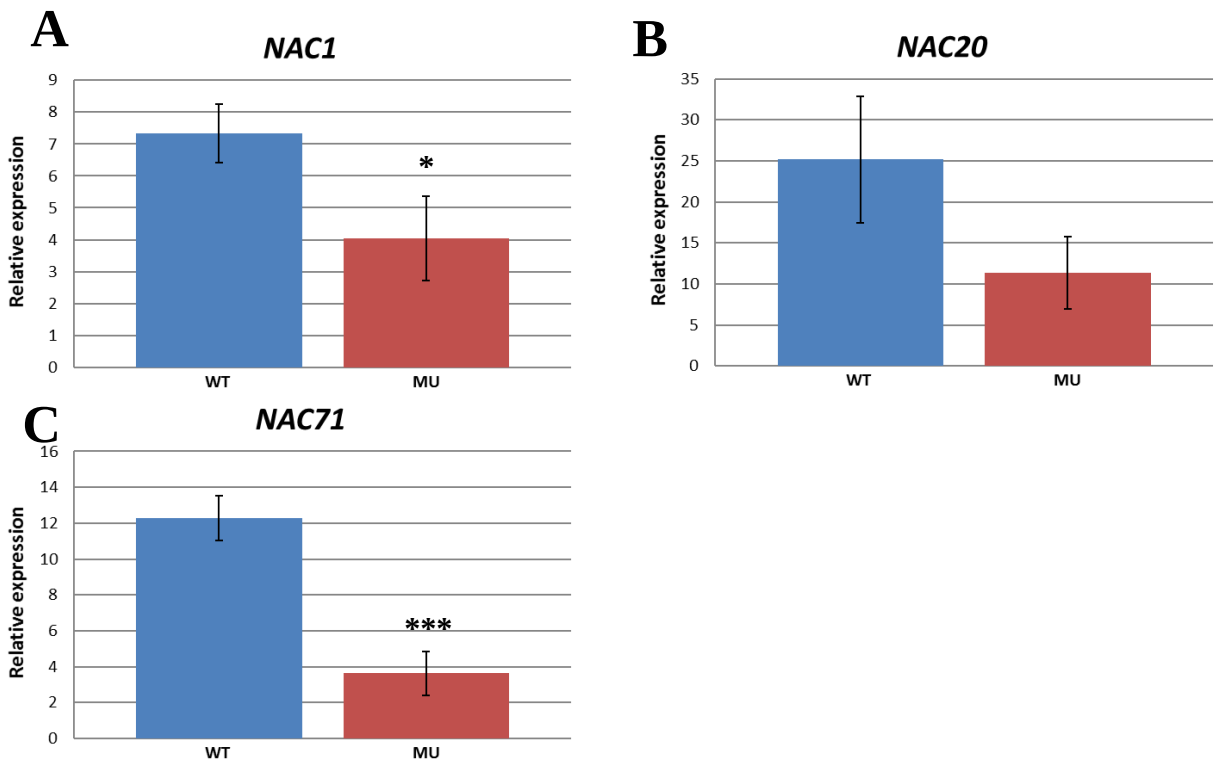


Figure 3.6 Transcriptional analysis of NAC transcription factors in the wild type(WT) and bdnrt2.1 mutant (MU) whole plant tissue during leaf senescence (5 weeks after germination**).**

(A) *NAC1* expression of WT and MU (B) *NAC20* expression of WT and MU. (C) *NAC71* expression of WT and MU. Values are mean ± SE (n = 3; 3 trials with the average of 3 biological replicates in each trial). * above bars represents $p<0.05$, *** above bars represents $p<0.01$.

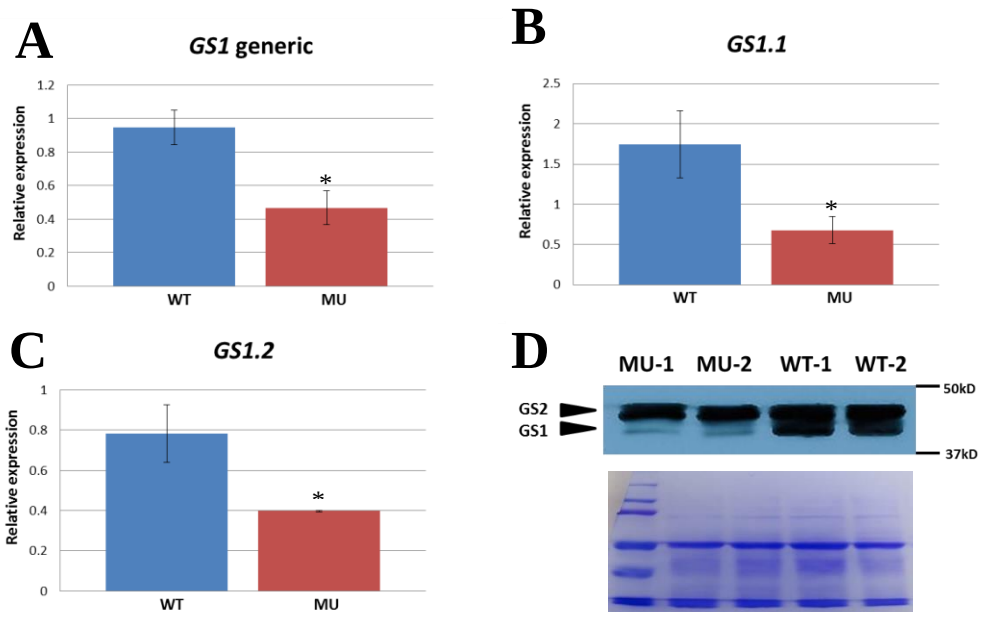


Figure 3.7 Transcriptional and translational analysis of GS1 in wild type (WT) and *bdnrt2.1* mutant (MU) whole plant tissue during leaf senescence (5 weeks after germination).

(A) *GS1* expression of WT and MU using generic primers (B) *GS1.1* expression of WT and MU (C) *GS1.2* expression of WT and MU (D) GS1 and GS2 protein levels of WT and MU. Values are mean ± SE (n = 3; 3 trials with the average of 3 biological replicates in each trial). * above bars represents $p<0.05$. The blot was based on two trials; each trial was based on pooled sample from 3 biological replicates.

Table 3.2 Concentration of amino acids in vegetative tissues of the wild type (WT) and the *bdnrt2.1* mutant (MU)

	Glu (μg $ml^{-1}g^{-1}$)	Gln (μg $ml^{-1}g^{-1}$)	Asp (μg $ml^{-1}g^{-1}$)	Asn (μg $ml^{-1}g^{-1}$)	2OG (μg $ml^{-1}g^{-1}$)
WT	242.3±8	92.1±9.5	85.9±2.9	42.3±3.0	19.4±1.3
MU	253.0±18.4	276.8±44.9	91.2±11.6	164.3±5.6	20.7±2.5
p=	0.622	0.015	0.679	<0.001	0.665

Note: Vegetative tissues including both root and shoot during leaf senescence (5 weeks after germination) excluding spikelet. Values are mean ± SE (n = 3).

respectively), indicating that N-rich amino acids have trouble relocating to the developing seeds in the mutant.

3.4 Discussion

The plant nitrate transporter NRT2.1 plays a major role in high-affinity nitrate uptake in *Arabidopsis* (Filleur *et al*., 2001; Li *et al*., 2007), and also presumably in maize (Garnett *et al*., 2013) and rice (Yan *et al*., 2011). In this study, I showed its role as an inducible high-affinity nitrate transport in the monocot model *Brachypodium* (Fig. 3.3). The fresh/dry weight of the *bdnrt2.1* mutant was no different from that of the wild type at anthesis, suggesting the overall nitrogen uptake efficiency was not affected under non-limiting nitrogen conditions and it had a limited role during vegetative growth. There are 6 members in the NRT2 family in *Brachypodium,* and *BdNRT2.6* was upregulated significantly to compensate for the loss of function of *BdNRT2.1* (Chapter 2.3.5). This, together with the intact LATS, may contribute to the unaffected NUpE under non-limiting N conditions. However, the previous studies of *AtNRT2.1*, which only examined the plant fresh weight in the *atnrt2.1* single mutant, *atnrt2.1-2.2* double mutant or even *atnrt2.1-2.2-2.4* triple mutant of *Arabidopsis* (Cerezo *et al*., 2001; Filleur *et al*., 2001; Li *et al*., 2007; Kiba *et al*., 2012), failed to observe any plant growth reduction of *atnrt2.1* mutant under N non-limiting conditions. I evaluated the seed yield of *Brachypodium* and observed reduced overall NUE under non-limiting N conditions in the *bdnrt2.1* mutant (Fig. 3.2A, Fig. 3.3A). The harvest index (HI), calculated as the ratio of grain yield to whole plant biomass, is an important indicator of nitrogen remobilization. The fact that the *bdnrt2.1* mutant had reduced HI indicates nitrogen remobilization was affected in the mutant. Another nitrate transporter, AtNRT1.7, that controls nitrate remobilization from source to sink has been reported (Fan et al., 2009), however, no biomass reduction was spotted in the *atnrt1.7* under N non-limiting conditions. Conversely, I also report that over-expression of *BdNRT2.1* can enhance the grain yield by 24% on average under N non-limited conditions. The transgenic approach of overexpressing *NpNRT2.1 and OsNRT2.1* was employed in *Arabidopsis* (Fraiser *et al*., 2000) and rice (Katayama *et al*., 2009) with no elevated nitrate uptake observed, and post translational regulation of *NRT2.1* is believed to be very strong in *Arabidopsis* (Fraiser *et al*., 2000). However, only the fresh weight of seedlings was examined in these studies due to the limitations of this

model plant system. Notably, two out of the three over-expression lines showed a decreased seed size (100-grain weight, Table 3.1), suggesting a potential trade-off if *BdNRT2.*1was over-expressed. In the three transgenic *BdNRT2.1* over-expression lines, the HI was not affected, indicating the nitrogen remobilization was not enhanced by overexpression of *BdNRT2.1*. Nevertheless, it is difficult to measure a complicated trait like NUE reliably; multiple field trials with large numbers of plots must be performed (Han *et al*., 2015). Thus, the story behind the yield increase of over-expressing BdNRT2.1 requires further research. Overall, these results indicate that BdNRT2.1 plays an important role during the reproductive stage and makes it indispensable to NUE in *Brachypodium*.

Grain N typically originates from two distinct sources: nitrogen uptake and assimilation during the grain filling period and nitrogen remobilization from senescing vegetative organs (Taulemesse *et al*., 2015). To discriminate these two sources and to find out by which pathway BdNRT2.1 is mainly regulated, I switched the hydropic condition from regular ^{14}N nitrate to ^{15}N labeled nitrate at anthesis. I found each source contributed roughly ~50% to the N in grains of the wild type *Brachypodium* (Fig. 3.5C&D). However, nitrogen remobilization from senescing vegetative organs was reduced in the *bdnrt2.1* mutant (Fig. 3.5D) and the post anthesis nitrate uptake remained at the same level as in the wild type (Fig. 3.5C). Therefore, members in *BdNRT2* may be redundant regarding their roles in nitrate uptake before or after anthesis when external nitrate is not limiting. However, *BdNRT2.1* itself is not only responsible for root nitrate uptake, but also controls the N remobilization from source (vegetative tissue) to sink (grain seeds). *BdNRT2.1* is expressed mainly in the roots, but is also expressed strongly in the stem (Chapter 2.3.1), which again suggests that it may play a versatile role other than root nitrate uptake. Recently, it has been reported that the nitrate-inducible NAC transcriptional factor TaNAC2-5A controls the nitrate response during leaf senescence in wheat and increases crop yield when over-expressed (He *et al*., 2015). I found that its homologous genes in *Brachypodium*, *NAC1*, *NAC20* and *NAC71* (You *et al*., 2015), were down-regulated in the *bdnrt2.1* mutant, implicating a potential role of *BdNRT2.1* as a signal transducer to coordinate the N remobilization during leaf senescence. Additionally, another NAC transcription factor (NAM-B1) also accelerated senescence and increased

nutrient remobilization from leaves to developing grains in wheat (Uauy *et al*., 2006). Thus, the decrease of NACs may have contributed to the reduction of N remobilization in the *bdnrt2.1* mutant. To further investigate the remobilization process, genes encoding enzymes involved in primary nitrogen metabolism, such as cytosolic GS1, chloroplastic GS2 and GOGAT, were analyzed in both the wild type and the *bdnrt2.1* mutant during senescence. Only GS1 was down regulated, whereas GS2 and GOGAT remained unaffected. This result is of great value, because GS1 is the key enzyme in nitrogen management, growth rate, yield and grain filling (Masclaux-Daubresse *et al*., 2010). Over-expressing GS1 had been conducted repeatedly in various plants and increased NUE often was achieved (see review of Xu *et al*., 2012). For example, overexpressing a GS 1 isoform (*Gln1-3*) constitutively in leaves increased maize kernel yield by 30% with unaffected shoot biomass (Martin *et al*., 2006). In the same study, they also reported that *gln1-3, gln1-4 and gln1-3.gln1-4* double mutant showed a sharp decrease of maize yield, whereas nitrogen content was increased. The *gln1-3* and *gln1-3.gln1-4* mutants accumulate large amounts of amino acids in the source leaf below the ear and are dedicated for grain filling. Additionally, the *gln1-2* mutant in *Arabidopsis* also accumulated amides in their old leaves when nitrate was not limiting; however, N-remobilization was not affected in the mutant as demonstrated by a ^{15}N labelling experiment (Lothier *et al*., 2011). Gln and Asn are the two major forms of nitrogen in the phloem sap during remobilization, and I discovered that they accumulated in the N source tissues (old leaves and roots) of the *bdnrt2.1* mutant. This is likely due to the dysfunction in N-export from the phloem to the developing seeds. However, how *BdNRT2.1* coordinates those key players, such as NAC, GS1, during leaf senescence still remains unclear.

In conclusion, *BdNRT2.1 is* not only responsible for inducible high-affinity nitrate uptake, but also controls N remobilization and the lack of *BdNRT2.1* results in reduced NUE under non-limiting N conditions. Through networking with NAC transcriptional factors and cytosolic GS1 during leaf senescence, *BdNRT2.1* may act as a potential signal transducer to coordinate N remobilization with developmental cues. I also showed that constitutively over-expressing *BdNRT2.1* in *Brachypodium* may be a promising approach to improve NUE.

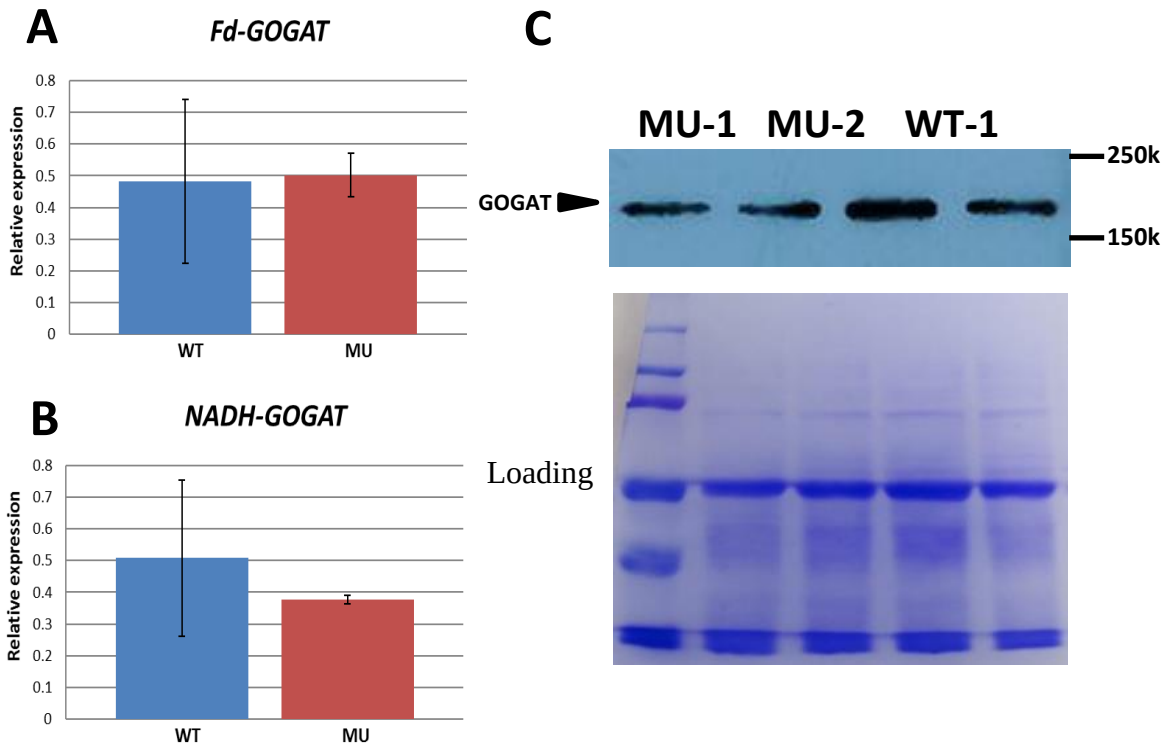
Study limitations:

As detailed in the figure description, some experiments were conducted with only 1 trial although with multiple biological replicates, while some were conducted with 2 trials or 3 trials. Thus, the interpretation of results generated by a single trial should be cautious.

3.5 Appendices

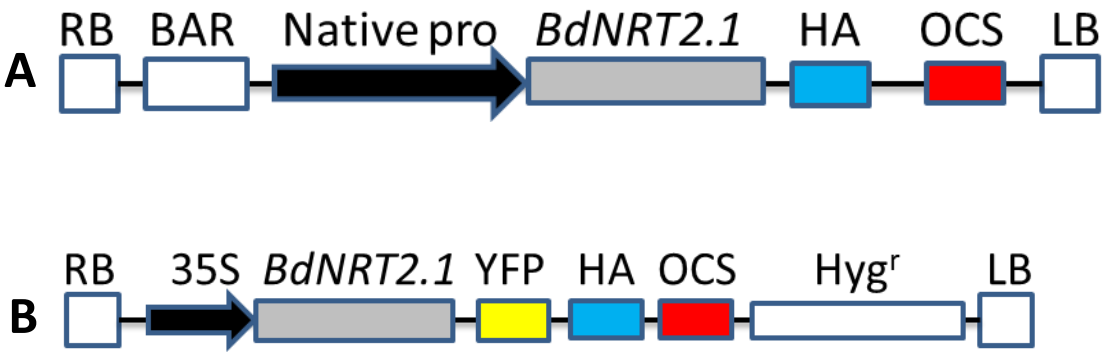
Appendix 3.1 Comparison of NAC proteins.

The amino acid sequences of TaNAC2-5A, BdNAC1, BdNAC20, and BdNAC71 were analyzed with the CLUSTAL Omega (1.2.4) multiple sequence alignment.



Appendix 3.2 Transcriptional and translational analysis of GOGAT in wild type (WT) and the *bdnrt2.1* mutant (MU) whole plant tissue during leaf senescence (5 weeks after germination).

(A) *Fd-GOGAT* expression of WT and MU (B) *NADH-GOGAT* expression of WT and MU. Values are mean ± SE (n = 3; 3 trials with the average of 3 biological replicates in each trial). (C) GOGAT protein levels of WT and MU.



Appendix 3.3 Diagrams of vector constructs used to generate BdNRT2.1 complementation (A) and over-expression lines (B)

Appendix 3.4 Primers used for genotyping bdnrt2.1 mutant

Position	Primers
BdNRT2.1 gene	F: 5'- CGACCACTTCCACCTAGACC -3'
	R: 5'- GACCGATCGGAGTTACATGA -3'
T-DNA-LB	R: 5'- ACACAACATACGAGCCGGAAGCATA -3'

Appendix 3.5 Primers used for creating transgenic constructs through Gateway cloning

Construct	Primers
BdNRT2.1 complementation	F: 5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTACACAGACAGCGTTCTCACC -3'
	R: 5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCGACGTGCTGGGGAGTGTTG -3'
BdNRT2.1 overexpression	F: 5'- GGGGACAAGTTTGTACAAAAAAGCAGGCTACATGGCGGCGAAGAGCAAG -3'
	R: 5'- GGGGACCACTTTGTACAAGAAAGCTGGGTCGACGTGCTGGGGAGTGTTG -3'

Note: Letters underlined are the Gateway att sequence.

Appendix 3.6 Primers used for RT-PCR of BdNRT2.1 gene

Gene	Primers
BdNRT2.1 (WT&MU)	F: 5'- TTTCCTCTGTCCCAGCTACG -3'
	R: 5'- CGCAGCAAATGTGTAGTACCTC -3'
BdNRT2.1 (RE lines)	F: 5'- AGTGGGGCTCCATGCTCT -3'
	R: 5'- AGCGTAATCTGGAACATCGTATG -3'
BdNRT2.1 (OE lines)	F: 5'- CCTGAGCAAAGACCCCAAC -3'
	R: 5'- AGCGTAATCTGGAACATCGTATG -3'
SamDC	F: 5'- TGCTAATCTGCTCCAATGGC -3'
	R: 5'- GACGCAGCTGACCACCTAGA-3'

Note: Members of NRT2 in *Brachypodium* are highly conserved. Gene specific primers need to be designed on the 3'-UTR. Gene specific primers for transgenic constructs were designed on HA tag since the transgenic constructs did not include 3'-UTR. Gene expressions were normalized by primer amplification efficiency.

Appendix 3.7 Primers used for RT-PCR of NAC, GS1 and GOGAT genes.

Gene name	Locus ID	Primers
BdNAC1	Bradi1g01640	F: 5'- GCTCCCAAAGGGGTTAAGAC -3'
		R: 5'- ACCATGTCCGACGACTCC -3'
BdNAC20	Bradi1g58057	F: 5'- ATTCTGCCCAAGGAGGAGTT-3'
		R: 5'- TGGATGTGGAGAGCTCAGAA -3'
BdNAC71	Bradi4g13570	F: 5'- GAACAACCACAACGCTCTCA-3'
		R: 5'- GCCGTAGAACCCGTAGTCTG-3'
GS1-generic	N/A	F: 5'- CGCTCACACCAATTACAGCA-3'
		R: 5'- CCCCATTTGAAGGTGTTGAT-3'
GS1.1	Bradi3g59970	F: 5'- AACCTCGACCTCTCCGACTC-3'
		R: 5'- GCCTGTGGGTAGAGGATGAC-3'
GS1.2	Bradi1g69530	F: 5'- ACCTTAGTGACTGCACCGAC-3'
		R: 5'- TCCTTGAAAATGGCTTGAGG-3'
Fd-GOGAT	Bradi1g19080	F: 5'- ATGATGATGCTCGTCCCTGA-3'
		R: 5'- TGTTCTCCAATAGCGTGCTG-3'
NADH-GOGAT	Bradi2g46670	F: 5'- GCCATGAACAAACTTGGAGG-3'
		R: 5'- CCCCCTGAGCCATTTTTATC-3'

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4 Contrasting impact of a high-affinity nitrate transporter, BdNRT2.1, on nitrogen metabolism at different developmental stages in *Brachypodium distachyon*

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4.1 Introduction

Nitrogen is the essential element for most biological molecules, including nucleotides, amino acids, proteins, cell wall components, hormones, chlorophylls, and vitamins. Nitrate (NO_3^-) is the major nitrogen (N) source for most land plants, and nitrate itself is also an important signaling molecule that can regulate many aspects of plant metabolism, growth, and development (Crawford, 1995; Lejay *et al*., 1999; Miller *et al*., 2007; Miller *et al*., 2008; Wang *et al*., 2012; Krapp *et al*., 2014; Medici and Krouk, 2014; Krapp, 2015). As it was detailed in Chapter 1.2, plants take up nitrate mainly through the NPF and NRT2 families which are substrate concentration dependent (see review of Tsay *et al*., 2007; Masclaux-Daubresse *et al*., 2010;). Nitrate can be assimilated into ammonium through a combination of enzymes, such as nitrate reductase (NR) in the cytosol, and nitrite reductase (NiR) (Meyer and Stitt, 2001). Ammonium, whether originating from nitrate reduction, ammonium uptake, or from photorespiration and amino acids recycling, is mainly assimilated through the GS/GOGAT cycle (Lea and Miflin, 1974). In addition to GS/GOGAT cycle, other enzymes, such as cytosolic asparagine synthetase (AS), and mitochondrial NADH-glutamate dehydrogenase (GDH), probably play important roles in ammonium assimilation. Besides N uptake and assimilation, complex interactions via various biochemical networks of metabolite pathways exist in plant N metabolism (Kusano *et al*., 2011), and primary metabolites, such as amino acids, organic acids, sugars, and sugar phosphates, are key components of such complex biochemical networks (Kusano *et al*., 2011). Metabolomics can be used to take a snapshot of the metabolic status of the plant in a targeted or an untargeted manner (Weckwerth, 2003).

My previous research (Chapter 3) demonstrated that a *Brachypodium* mutant deficient for the expression of a gene coding for a putative high-affinity nitrate transporter NRT2.1 displayed reduced N remobilization efficiency and seed yield at maturity under non-limited N conditions. However, at anthesis, shoot and root biomass were not affected in the *bdnrt2.1* mutant under non-limited N conditions, indicating BdNRT2.1 played distinct roles at vegetative (before anthesis) and reproductive stages (after anthesis). On this basis, I investigated the physiological impact of BdNRT2.1 mutation on primary N metabolism during different developmental stages. mRNA abundance levels and protein levels of key enzymes involving primary N metabolism were examined. Additionally,

both targeted and untargeted metabolite profiling for detection of primary and other metabolites was applied. I hypothesized that BdNRT2.1 has a larger impact on nitrogen metabolism after anthesis than before anthesis.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

Plants were grown hydroponically as described in Chapter 2.2.2 for up to 5 weeks. The hydroponic solution was renewed weekly at 3 pm on each Friday. *Brachypodium* plant flowers (anthesis) approximately 3.5 weeks after germination. At 2 and 5 weeks after germination, samples were collected to measure the change between the two typical developmental stages.

4.2.2 RNA extraction and quantitative real-time PCR

Methods were described in Chapter 2.2.5. The specific primers used for quantitative real-time PCR are detailed in Appendix 4.1.

4.2.3 Protein extraction and Western blot

Methods were described in Chapter 3.4.3. One hundred milligrams of *Brachypodium* root tissues was collected at 3 pm on Friday of the 2^{nd} and 5^{th} week of hydroponic culture. The blots were based on 3 trials; each trial was based on a pooled sample from three biological replicates.

4.2.4 Total metabolites extraction

Methods were described in Chapter 3.4.4.

4.2.5 Metabolite measurements and instrument setup

Samples were processed in triplicate as described in Chapter 3.2.4 and were subjected to metabolomics analysis by injecting 5 µl into an agilent 1290 high performance liquid chromatography (HPLC) system coupled to a Q-Exactive Quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific). Chromatographic separation of compounds was achieved using a SeQuant® ZIC®-HILIC column, 3.5µm, 100 Å, 100 ×2.1 mm (EMD Millipore), with a mobile phase 5 mM ammonium acetate, pH = 4.00 (A), B = 90%

acetonitrile, 0.1% formic acid) and the following gradient: 85% B for 3.5 minutes, decreased to 70% over the next 3.5 minutes followed by 20% within the next 2 minutes and held for 1 minute before returning to 85% over 1 minute. The following heated electrospray ionization (HESI) conditions were used: spray voltage, 3.9 kV (ESI+), 3.5 kV (ESI-); capillary temperature, 250 °C; probe heater temperature, 100 °C; sheath gas, 30 arbitrary units; auxiliary gas, 8 arbitrary units; and S-Lens RF level, 60%. Injections of 5 µl were used with a flow rate of 0.3 mL min^{-1}. Four compounds (glutamine, glutamate, asparagine and aspartate) were detected and monitored using a targeted MS/MS in positive ionization mode while 2-oxoglutarate was monitored in the negative ionization mode. Full MS spectra also were obtained between m/z 70-1150 at 17,500 resolution, automatic gain control (AGC) target of 1e6, maximum injection time (IT) of 64 ms and intensity threshold of 3.8e4. MS/MS spectrum were collected at 17,500 resolution, AGC target 5e5, maximum IT set to auto and isolation window of 3 m/z. Normalized collision energy of 25 was used for the MS/MS method. All theoretical masses were calculated with Xcalibur™ software. The above described compounds were identified and quantified using commercial standards by generating standard curves.

4.2.6 Statistical analysis

The significance of differences between two subjects was determined using two-tailed student t tests with equal variance using SPSS 13.0 statistical software (SPSS Inc, Chicago, IL, USA). Metabolomics data analysis was carried out as follows:

Full MS data files acquired in Thermo .RAW format from the instrument were converted to .mzml format using the peak picking filter in ProteoWizard software (Kessner *et al*., 2008). The XCMS package in R was used for importing these files and features were detected using the Centwave method (Tautenhahn *et al*., 2008) followed by retention time correction using the obiwarp method (Prince and Marcotte, 2006). Parameters for feature detection, retention time correction and grouping were optimized using the IPO package in R. The “fillPeaks” function was used with default settings and remaining zeros were imputed with two-thirds the minimum value on a per mass basis. Salt clusters and other ionization artefacts were removed from the feature list using the McMillan correction without applying the retention time filter (Mcmillan *et al*., 2016). All features were

exported to excel to test for statistical significance between treatments using a student's t-test and to generate volcano plots.

4.3 Results

4.3.1 BdNRT2.1 mutant

The *bdnrt2.1* mutant was characterized in Chapter 2.3.4. There was no significant change in *BdNRT2.1* mRNA abundance levels before and after anthesis in the wild-type (Appendix 4.2, p=0.38).

4.3.2 Contrasting impact of BdNRT2.1 on plant growth under N limited and N non-limited conditions

Previous research uncovered that the *Brachypodium bdnrt2.1* mutant displayed reduced N remobilization efficiency and yield at maturity under non-limited N conditions (Chapter 3.3.3). At anthesis, shoot and root biomass was not affected (Chapter 3.2.3). I further compared the growth of the wild-type and the *bdnrt2.1* mutant between N limited (0.1 mM) and non-limited (5 mM) conditions. Plant biomass and grain yield were measured subsequently (Fig. 4.1). Under N limited conditions, plant biomass at anthesis was significantly reduced in the *bdnrt2.1* mutant (Fig. 4.1-top left, p=0.022), and grain yield at maturity also decreased significantly (Fig. 4.1-bottom left, p=0.010). These results indicate that lack of BdNRT2.1 affects both vegetative growth and reproductive growth under N limited conditions. In contrast, under N non-limited conditions, plant

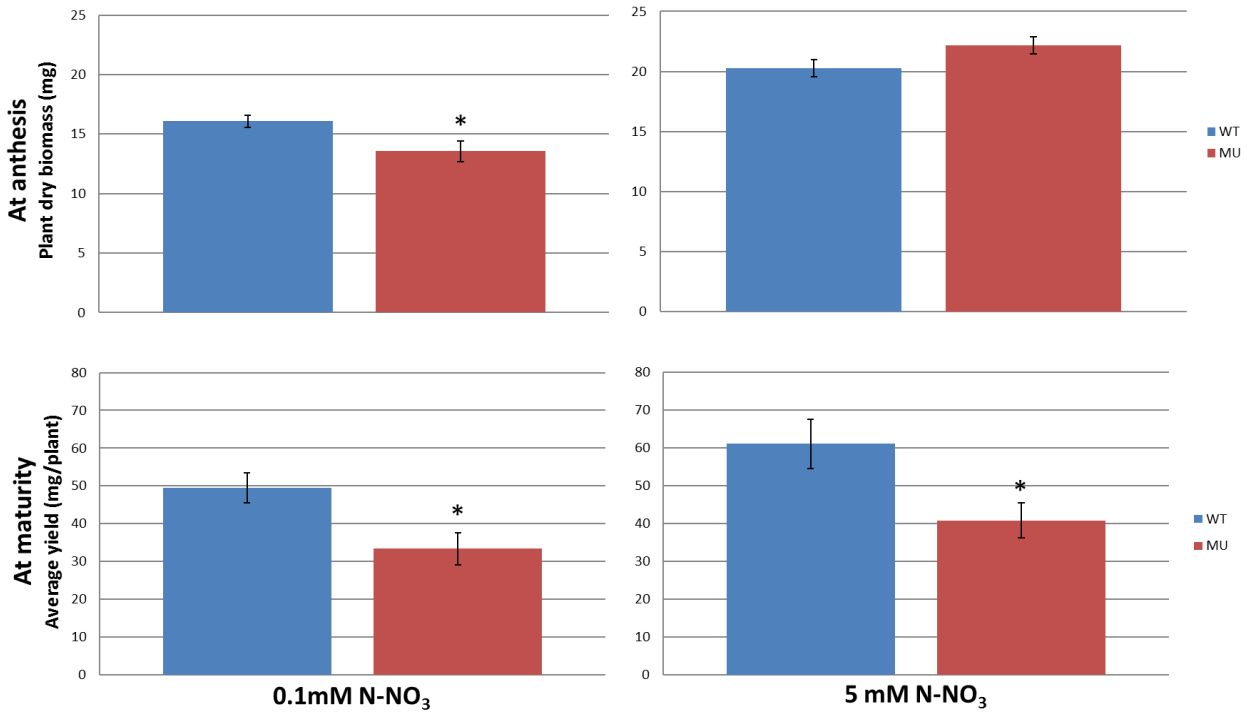


Figure 4.1 Plant biomass (at anthesis) and yield (at maturity) of wild type (WT) and *bdnrt2.1* mutant (MU) under low N (0.1Mm) and high N (5mM) condition.

Values are mean ± SE (n = 8). * above bars indicates $p<0.05$.

biomass at anthesis was not significantly affected in the *bdnrt2.1* mutant (Fig. 4.1-top right), while grain yield at maturity decreased significantly (Fig. 4.1-bottom right, p=0.029). The total N concentrations of wild-type and mutant at anthesis were not affected by either N supply conditions (Appendix 4.3), suggesting N uptake was not affected in the mutant under N non-limited conditions. These results indicate that a lack of BdNRT2.1 only affects reproductive growth, but not vegetative growth, under N non-limited conditions. Thus, N non-limited conditions were used to further elucidate the contrasting phenotype of the *bdnrt2.1* at distinct developmental stages.

4.3.3 Transcriptional change of genes coding key enzymes in primary N metabolism

To investigate the impact of the BdNRT2.1 mutation on primary N metabolism, I first examined the expression level of genes that code for key enzymes (including GS1, GS2, Fd-GOGAT, NADH-GOGAT, NR, NiR, GDH, and AS) involved in the N assimilation pathway. As it was shown in Fig. 4.1, there were no significant differences between the wild type and the *bdnrt2.1* mutant for all the enzymes at week 2. In contrast, GS1, GS1.1, and GS1.2 transcript levels were all significantly down-regulated in the *bdnrt2.1* mutant at week 5 (Fig. 4.2ABC, p=0.014, 0.035, 0.032, respectively), whereas gene expressions of the other enzymes were not affected. These results suggest that a lack of BdNRT2.1 affects primary N metabolism after anthesis (mainly through the change of GS1), but not before anthesis.

4.3.4 Translational change of genes coding key enzymes in primary N metabolism

In order to confirm the transcriptional change of genes coding those key enzymes, I used the Western blot technique to verify the protein levels of important enzymes including GS1, GS2 and GOGAT. The pixel intensities of the bands were analyzed using image J (Fig. 4.3) and were subsequently compared between wild-type and the *bdnrt2.1* mutant. Both GS and GOGAT were not significantly changed by the BdNRT2.1 mutation at week 2 (Fig. 4.3A, p= 0.44 and 0.39, respectively). In contrast, GS1/GS2 was significantly down-regulated in the *bdnrt2.1* mutant at week 5 (Fig. 4.3B, p=0.04), while GOGAT was not significantly affected (Fig. 4.3B, p=0.29).

Figure 4.2 Transcriptional analyses of genes coding for key enzymes involved in N assimilation pathway in root of wild type (WT) and mutant (MU) before (week 2) and after anthesis (week 5).

(A) GS1 expression change of the WT and MU using generic primers; (B) GS1.1 expression change of the WT and MU; (C) GS1.2 expression change of the WT and MU; (D) GS2.1 expression change of the WT and MU; (E) Fd-GOGAT expression change of the WT and MU; (F) NADH-GOGAT expression change of the WT and MU; (G) NR expression change of the WT and MU; (H) NiR expression change of the WT and MU; (I) GDH expression change of the WT and MU; (J) AS expression change of the WT and MU. Values are mean ± SE (n = 3; 3 trials with the average of 3 biological replicates in each trial). * above dots indicates $p<0.05$ between WT and MU at a specific developmental stage.

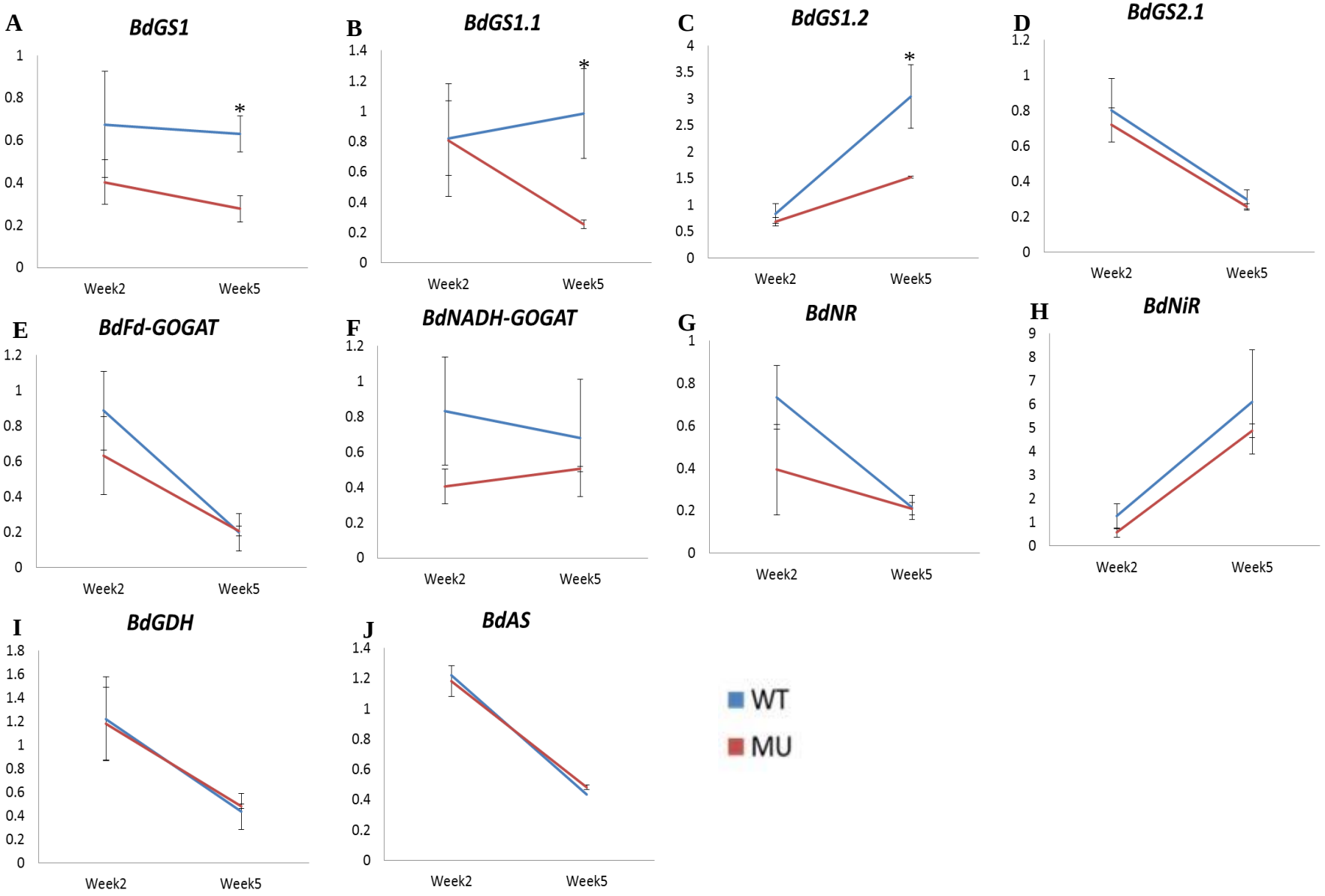
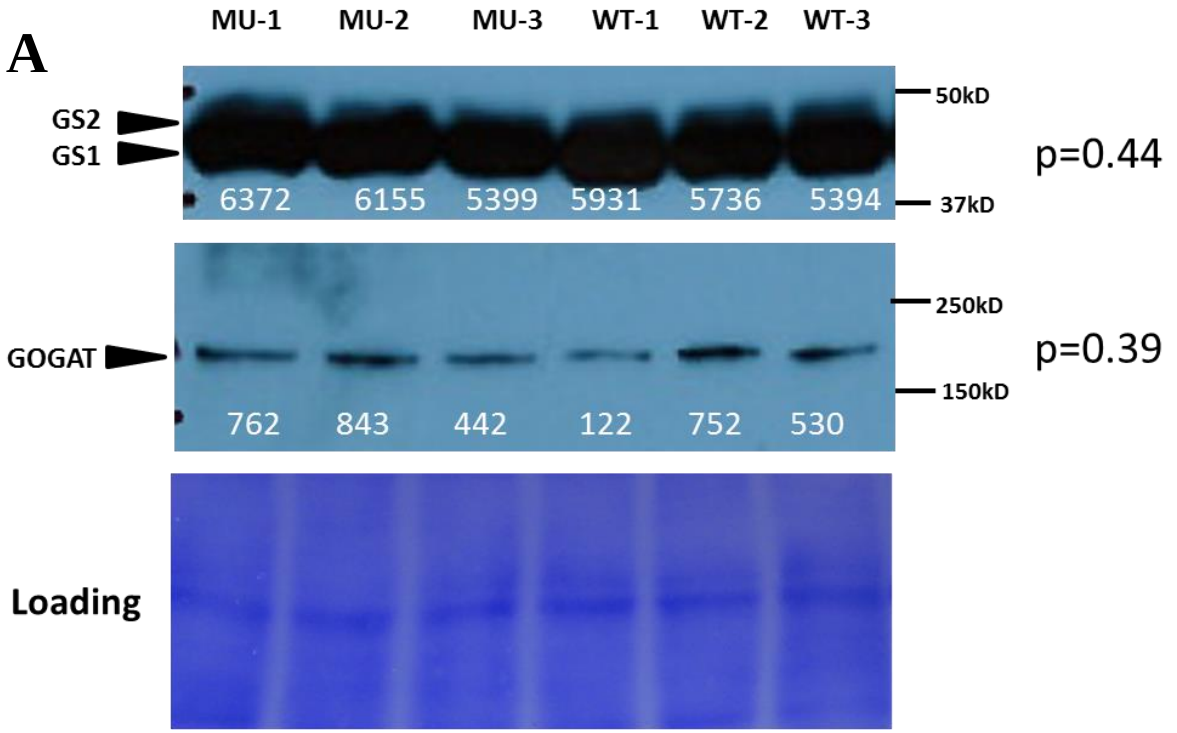
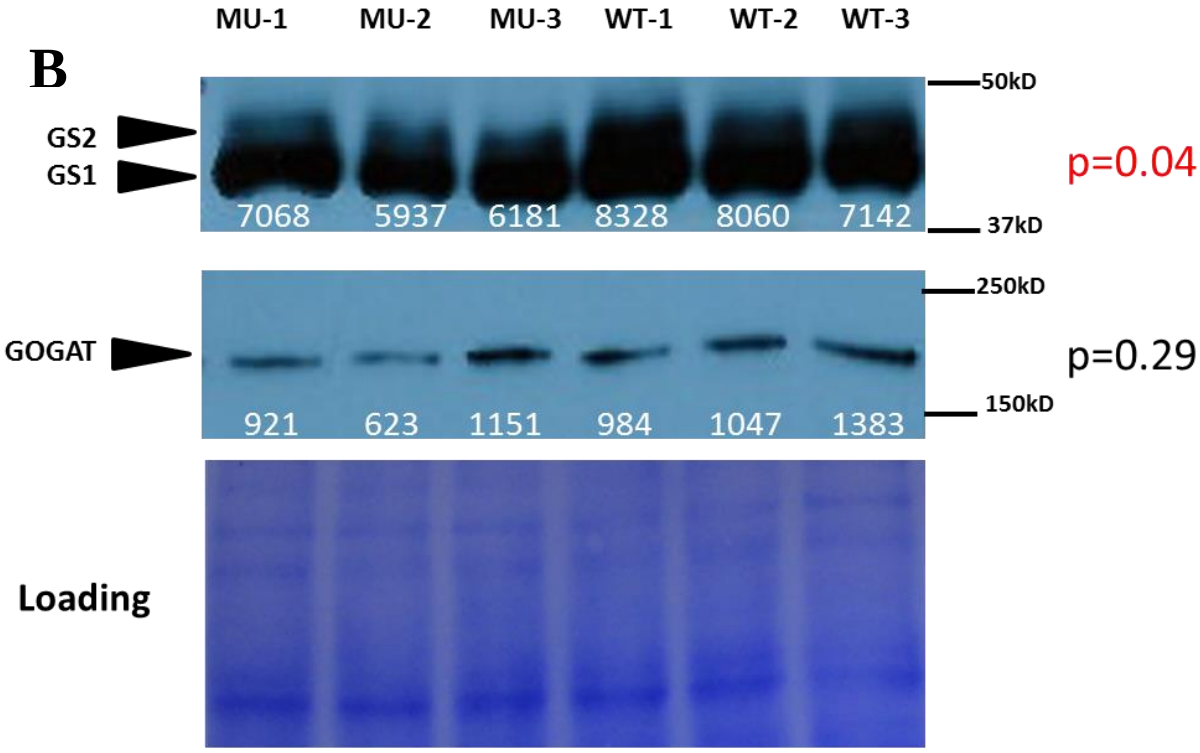

A
BdGS1
1
0.8
0.6
0.4
0.2
0
*
Week2
Week5
B
BdGS1.1
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Week2
Week5
C
BdGS1.2
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Week2
Week5
D
BdGS2.1
1.2
1
0.8
0.6
0.4
0.2
0
Week2
Week5
E
BdFd-GOGAT
1.2
1
0.8
0.6
0.4
0.2
0
Week2
Week5
F
BdNADH-GOGAT
1.2
1
0.8
0.6
0.4
0.2
0
Week2
Week5
G
BdNR
1
0.8
0.6
0.4
0.2
0
Week2
Week5
H
BdNiR
9
8
7
6
5
4
3
2
1
0
Week2
Week5
I
BdGDH
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Week2
Week5
J
BdAS
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Week2
Week5
WT
MU

Figure 4.3 Translational analyses of genes coding for key enzymes (GS1, GS2, and GOGAT) involved in N assimilation pathway in root of the wild type (WT) and mutant (MU).

(A) Protein levels of the WT and MU at week 2 with the loading control. (B) Protein levels change at week 5 with the loading control. The blot was based on 3 trials with 3 plants pooled in each trial. The pixel intensities of the bands were quantified using image J.


A
MU-1
MU-2
MU-3
WT-1
WT-2
WT-3
GS2
GS1
6372
6155
5399
5931
5736
5394
50kD
37kD
p=0.44
GOGAT
762
843
442
122
752
530
250kD
150kD
p=0.39
Loading


B
MU-1
MU-2
MU-3
WT-1
WT-2
WT-3
GS2
GS1
7068
5937
6181
8328
8060
7142
50kD
37kD
p=0.04
GOGAT
921
623
1151
984
1047
1383
250kD
150kD
p=0.29
Loading

4.3.5 Targeted metabolite change

The key metabolites involved in N primary metabolism, including glutamate (Glu), glutamine (Gln), aspartate (Asp), asparagine (Asn), and 2-oxoglutarate (2OG), were targeted and quantified in both the wild type and the *bdnrt2.1* at two developmental stages (Table 4.1). Gln and Asn significantly accumulated in the *bdnrt2.1* at both week 2 ($p=0.036$ and 0.010, respectively) and week 5 ($p=0.015$ and $p<0.001$, respectively). However, they were accumulated to a much greater extend in the mutant at week 5 than that at week 2: Gln and Asn have 1.5 and 2.3-fold accumulation at week 2, and it increased to 3.0 and 3.9-fold accumulation at week 5. Glu, Asp, and 2OG were not changed in the mutant at either developmental stage.

4.3.6 Untargeted metabolite change

Untargeted metabolite profiling allows us to obtain a broader insight into N metabolism involved in a variety of metabolic processes in plants. The same samples from targeted metabolite analysis were used for profiling the primary metabolites involved in N metabolism. As illustrated in the volcano plot in Fig. 4.4A, 142 metabolites were significantly up-regulated in the mutant at week 2, 63 of which passed the 2-fold change threshold; 79 metabolites were significantly up-regulated in the wild-type at week 2, 33 of which passed the 2-fold change threshold. Unfortunately, only three significant accumulated metabolites in the mutant were identified at week 2, including Gln (1.6-fold increase, $p=0.027$), Asn (2.6-fold increase, $p=0.008$), and Maleamic acid (2.6-fold increase, $p=0.008$), two of which had been already shown in the targeted analysis. In contrast, almost all the significantly changed metabolites were accumulating in the *bdnrt2.1* mutant at week 5 (Fig. 4.4B). One hundred and twenty seven metabolites were significantly up-regulated in the mutant, 91 of which passed the 2-fold change threshold; notably, only 2 metabolites were significantly up-regulated in the wild-type, 1 of which passed the 2-fold change threshold. Interestingly, lysine (Lys, 3.3-fold increase, $p=0.003$) and arginine (Arg, 3.7-fold increase, $p=0.004$) in addition to Gln (3.5-fold increase, $p=0.004$) and Asn (4.4-fold increase, $p=0.0007$) also accumulated significantly in the

Table 4.1 Concentration of amino acids in whole plant fresh tissues of the wild type (WT) and the *bdnrt2.1* mutant (MU) during two developmental stages

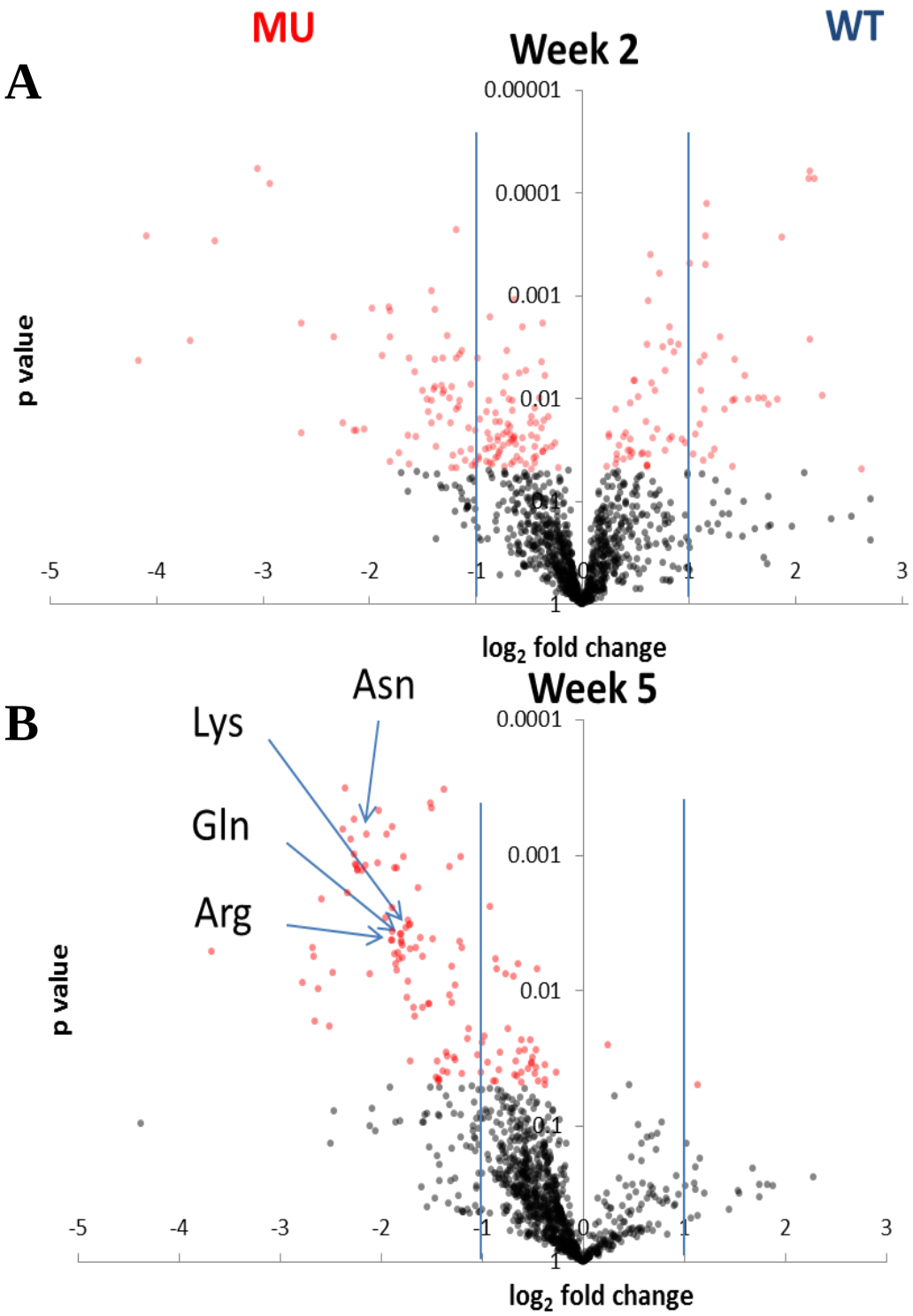
		Glu (μg $ml^{-1}g^{-1}$)	Gln (μg $ml^{-1}g^{-1}$)	Asp (μg $ml^{-1}g^{-1}$)	Asn (μg $ml^{-1}g^{-1}$)	2OG (μg $ml^{-1}g^{-1}$)
	WT	317.9±8.0	62.6±4.9	126.8±3.9	121.6±11.8	16.8±0.5
Week2	MU	317.3±15.2	94.1±8.9	124.5±3.5	275.5±31.5	13.5±1.4
	p=	0.974	0.036	0.685	0.010	0.090
	WT	242.3±8	92.1±9.5	85.9±2.9	42.3±3.0	19.4±1.3
Week5	MU	253.0±18.4	276.8±44.9	91.2±11.6	164.3±5.6	20.7±2.5
	p=	0.622	0.015	0.679	<0.001	0.665

Note: at week 5, vegetative tissues including both root and shoot excluding spikelet were analyzed.

Values are mean ± SE (n = 3).

Figure 4.4 Volcano plot showing metabolomic data at week 2 (A) and week 5 (B).

The arrows indicate points-of-interest that display both large magnitude fold-changes (x axis) and high statistical significance (p value, y axis). The dots colored in red shows significantly changed metabolites (n=3; $p<0.05$). The vertical line marks the 2 ($\log2 = 1$) fold change threshold. The dots to the left side represent metabolites accumulated in the mutant (MU) and the dots to the right side represent metabolites accumulated in the wild-type (WT).


A
MU
WT
Week 2
0.00001
0.0001
0.001
0.01
0.1
1
p value
-5
-4
-3
-2
-1
0
1
2
3
log2 fold change
B
Asn
Lys
Gln
Arg
Week 5
0.0001
0.001
0.01
0.1
1
p value
-5
-4
-3
-2
-1
0
1
2
3
log2 fold change

mutant at week 5, and they all belong to the group of N-rich amino acids. Besides, maleamic acid (4.9-fold increase, p=0.0008), serine (Ser, 1.4-fold increase, p=0.05), ectoine (3.8-fold increase, p=0.0007) also accumulated significantly in the mutant at week 5.

4.4 Discussion

BdNRT2.1 is responsible for inducible high-affinity nitrate uptake and N remobilization during leaf senescence in *Brachypodium* (Chapter 3.2.3). Here, I compared the key enzymes in N assimilation as well as related metabolites from two developmental stages of both the wild type and the *bdnrt2.1* mutant plants and proposed BdNRT2.1 may play more important roles in N metabolism during the reproductive stage. Results from different nitrate concentration experiments indicate that BdNRT2.1 has a distinct impact on plant growth before and after anthesis when external nitrate is non-limited (5 mM). The NRT2 family in *Brachypodium* has 6 members. *BdNRT2.6* was strongly up-regulated in the *bdnrt2.1* to compensate the functions of BdNRT2.1 (Chapter 2.3.5). Additionally, under N non-limited conditions (5 mM), both HATS and LATS were activated and resulted in the unchanged overall biomass at anthsis (Fig. 4.1-top right). When N is limited (0.1 mM), only HATS is in effect, reduced growth was shown (Fig. 4.1-top left) due to the fact that BdNRT2.1 is responsible for inducible HATS (Chapter 3.2.2). Thus, BdNRT2.1 may play a redundant role in N metabolism before anthesis when N is not limited. Similarly, no impact of *AtNRT2.1* on plant biomass was detected under N non-limiting conditions in *Arabidopsis* (Li *et al*., 2007). As the plant ages (after anthesis), BdNRT2.1 becomes essential to growth under N non-limited condition (Fig. 2-bottom right) by acting as a potential signal transducer to coordinate N remobilization (Chapter 3.2.4).

In order to further investigate the molecular basis underlying the contrasting phenotype at different developmental stages, the expression change of genes coding for key enzymes in the N assimilation pathway was evaluated. The results are in good agreement with the phenotype; that is, the relative expression of those key genes were not affected in the mutant before anthesis while the genes coding for GS1 were down-regulated in the mutant after anthesis. Expression patterns of genes coding for GS/GOGAT enzymes were

also confirmed at the protein level. At the vegetative stage, the N in plant biomass mainly depends on *de novo* N assimilation. All the key enzymes involved in assimilation were not affected, which explained the unchanged biomass. However, at the reproductive stage, N that consists of the yield mainly originats from two sources: N remobilization from vegetative tissue and *de novo* N uptake and assimilation (Masclaux-Daubresse *et al*., 2010; Taulemesse *et al*., 2015). Key enzymes except for GS1 were not changed, which implies overall N assimilation processes may be not affected in the mutant after anthesis, and my previous results using ^{15}N labelling have shown that *de no* nitrogen uptake is intact in the *bdnrt2.1* mutant during leaf senescence (Chapter 3.2.3), which support such an assumption. Therefore, the lack of BdNRT2.1 mainly influences the remobilization process independently from nitrate uptake during the reproductive stage. As discussed earlier, GS1 is known to be involved in ammonium recycling during particular developmental stages like leaf senescence, and it is also involved in glutamine synthesis for relocation into the phloem sap (Bernard and Habash, 2009). The fact that only GS1 was down-regulated in the mutant after anthesis further supports the idea that the remobilization process is affected by the mutation in *BdNRT2.1*. However, it is still unclear how GS1 was affected by the nitrate transporter BdNRT2.1. My previous work demonstrated that BdNRT2.1 controls N remobilization through networking with the senescence associated transcription factors BdNAC1, BdNAC20, and BdNAC71, and reduced expression of NACs was found in the *bdnrt2.1* mutant (Chapter 3.2.5). Recently, it has been reported that the nitrate-inducible NAC transcriptional factor TaNAC2-5A controls the nitrate response during leaf senescence in wheat, and it can directly bind the promoter regions and activate TaNRT2.1 and GS2, but not GS1 in wheat (He *et al*., 2015). It is possible that BdNRT2.1 may serve as a signal transducer after anthesis and form a feed-forward loop with senescence induced transcription factor NACs, which in turn specifically target GS1 to regulate N remobilization in *Brachypodium*. Of course, further research is needed to test this possibility.

Metabolite analysis showed accumulated levels of Gln and Asn in the *bdnrt2.1* mutant for both of the two developmental stages, but with a stronger accumulation after anthesis. These findings are consistent with the changing pattern of key enzymes. One may expect no changes to metabolites before anthesis due to the unchanged enzyme activity. One

may also expect Gln to decrease after anthesis due to decreased GS1 activity. However, steady-state metabolite concentrations do not directly reflect the rate of associated biochemical reactions, they rather reflect a balance between maximizing enzyme efficiency and minimizing total metabolite load (Tepper *et al*., 2013). Thus, accumulated Gln and Asn indicate that the balances of their assimilations were disrupted in the *bdnrt2.1* mutant at both developmental stages. Also, the feedback regulation of end-product Gln on GS1 enzyme levels cannot be neglected in explaining the reduced GS1 in the mutant after anthesis. In general, Gln and Asn are the major N storage compounds for higher plant, and amides, including Gln and Asn, could be accumulated in response to mineral deficiency stress or salinity stress (Rabe, 1999; Mansour, 2000). The stronger accumulation of Gln and Asn after anthesis may suggest that the mutant plant was experiencing severer stress after anthesis than before. Furthermore, the metabolomics data revealed a dramatic shift of the significantly accumulated metabolite from week 2 (Fig. 4.4A) to week 5 (Fig. 4.4B). Interestingly, an increase in Arg, which is also a major N storage compound and accumulates under mineral stress (Rabe, 1999), was found in the mutant at week 5. This again indicates that the mutant plant was probably experiencing more severe stress at the later stage. Besides, Lys, Arg, Gln, and Asn all belong to the group of six N-rich amino acids, and their accumulation in the vegetative tissue of the mutant at week 5 confirms the hypothesis that N remobilization was impaired in the *bdnrt2.1* mutant during leaf senescence (Chapter 3.2.5). Coincidently, metabolomics results also revealed an accumulation of Ser; this, together with the accumulation of Lys, Arg, Gln, and Asn mimic the metabolites change in response to exogenous ammonia (Magalhaes and Wilcox, 1984). Therefore, it is possible that BdNRT2.1 may serve as a nitrate sensor/signal transducer after anthesis and its malfunction may lead to the plant perceiving a wrong signal such as ammonia stress. However, such a N sensor/signal transducer role would need assistance of certain senescence associated partner, probably NAC, due to its growth stage specificity. Nitrate is not only an essential element for plant growth, but also an important signal molecule that is involved in many physiological processes (reviewed by Wang *et al*., 2012; Krapp, 2015; Noguero and Lacombe, 2016). Nitrate transporters, especially NPF6.3/NRT1.1/CHL1 and NRT2.1, have been reported as nitrate sensors (reviewed by

Noguero and Lacombe, 2016). AtNRT1.1 functions as a nitrate sensor and is able to promote physiological responses in the control of root system architecture (Krouk *et al*., 2010) and to control primary nitrate response independently from nitrate uptake (Ho *et al*., 2009; Bouguyon *et al*., 2015). AtNRT2.1 also can functions as nitrate transducer to regulate lateral root initiation independently from its nitrate uptake property in *Arabidopsis* (Little *et al*., 2005). BdNRT2.1, in this study, could serve as a nitrate transducer mainly during the reproductive stage and coordinate N remobilization independently from *de novo* nitrate uptake.

In conclusion, BdNRT2.1 has contrasting impacts on plant growth, N assimilation enzymes levels, and metabolic status between the vegetative and the reproductive stages. BdNRT2.1 may possess additional roles as a nitrate transducer which would affect N metabolic process, especially N remobilization, if interrupted at the reproductive stage. I believe these results established a good working basis for future research, especially useful to studies on genetic variability of nitrate transporter evolved with developmental changes.

Study limitations:

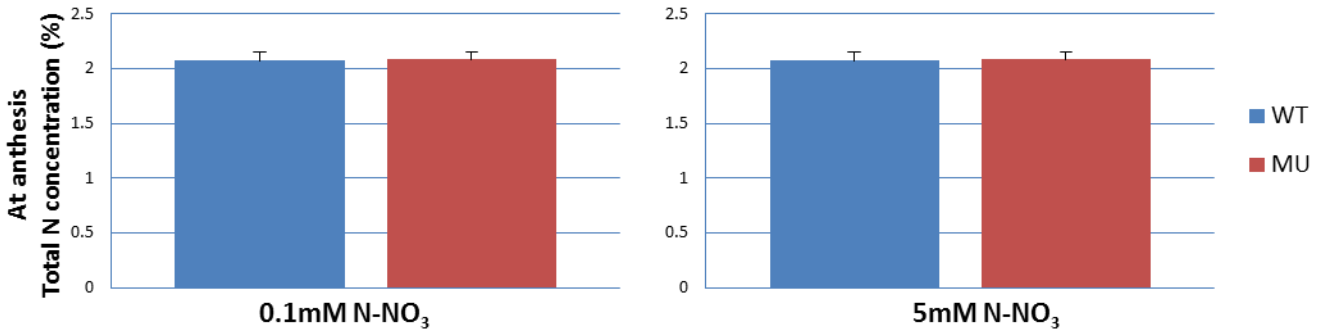
The metabolomics analysis was based on 3 biological replicates from 1 trial. Thus, the interpretation of results generated by a single trial should be cautious.

4.5 Appendices

Appendix 4.1 Primers used for RT-qPCR of genes coding for key enzymes in the N assimilation pathway.

Gene name	Locus ID	Primers
GS1-generic	N/A	F: 5'- CGCTCACACCAATTACAGCA-3'
		R: 5'- CCCCATTTGAAGGTGTTGAT-3'
GS1.1	Bradi3g59970	F: 5'- AACCTCGACCTCTCCGACTC-3'
		R: 5'- GCCTGTGGGTAGAGGATGAC-3'
GS1.2	Bradi1g69530	F: 5'- ACCTTAGTGACTGCACCGAC-3'
		R: 5'- TCCTTGAAAATGGCTTGAGG-3'
GS2.1	Bradi5g24550	R: 5'-AATGAACGGAGGTTGACAGG-3'
		R: 5'- GCTCCCAGAGAAGTGTGGTT-3'
Fd-GOGAT	Bradi1g19080	F: 5'- ATGATGATGCTCGTCCCTGA-3'
		R: 5'- TGTTCTCCAATAGCGTGCTG-3'
NADH-GOGAT	Bradi2g46670	F: 5'- GCCATGAACAAACTTGGAGG-3'
		R: 5'- CCCCCTGAGCCATTTTTATC-3'
NR	Bradi3g37940	F: 5'- CCATCAACGCATTCACGAC-3'
		R: 5'-CAGAAGCACCAGCACCAGTA-3'
NiR	Bradi3g57990	F: 5'- CAGGGACCTCGCCAAGAT-3'
		R: 5'-CCTTCCTCGCCGTACTTGT-3'
GDH	Bradi2g41130	F: 5'- TTTCCGTGTGCAGTTCAGTC -3'
		R: 5'- TGACAAAAACGCATTACCTCA -3'
AS	Bradi4g03827	F: 5'- CTGGTTGCACAATCAGGAAG -3'
		R: 5'- TTTGCCAACACTCTCACAGC -3'
BdSamDC	Bradi5g14640	F: 5'- TGCTAATCTGCTCCAATGGC -3'
		R: 5'- GACGCAGCTGACCACCTAGA -3'

Appendix 4.2 qPCR expression analyses of BdNRT2.1 gene in root of wild type and mutant before and after anthesis. Values are mean ± SE (n = 3; 3 trials with the average of 3 biological replicates in each trial).



Appendix 4.3 Total N concentrations (at anthesis) of the wild type (WT) and *bdnrt2.1* mutant (MU) under low N (0.1 mM) and high N (5 mM) condition.

Values are mean ± SE (n = 8).

4.6 References

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5 General Discussion and Conclusions

As a new monocot model plant, *Brachypodium* has a good potential to study questions that are unique to monocot crops. The fact that *Brachypodium* has significant grain yield makes it more suitable for NUE related studies. In chapter 2, for the first time, I isolated and characterized the six genes coding for NRT2 family in *Brachypodium*, and discovered that they played diverse roles in response to different nitrate and ammonium conditions. For example, *BdNRT2.1* and *BdNRT2.2* are nitrate inducible genes and controlled by internal N status rather than external nitrate, and *BdNRT2.5* is a nitrate repressible gene. Interestingly, *Arabidopsis NRT2.1 NRT2.2* and *NRT2.5* have similar nitrate responses despite the divergence of *NRT2s* between *Arabidopsis* and *Brachypodium* (Plett *et al*., 2010), but they have different tissue specificities. I also reported that *BdNRT2.1, BdNRT2.2* can be used as nitrogen markers to monitor N status in plants. Besides, none of the NRT2s in rice were found to be nitrate repressible (Cai *et al*., 2008; Feng *et al*., 2011). These results indicate that BdNRT2 have distinct roles from *Arabidopsis*, a model plant for dicots, and rice, an ammonium preferring monocot crop. I also observed a clear linkage of *BdNRT2.1* and *BdNRT2.2* regulatory machineries, which indicates that there is a novel cis- regulatory element in controlling nitrate response. Furthermore, the compensation by *BdNRT2.6* in the *BdNRT2.1* knock-out mutant was observed and makes it more interesting to investigate the interconnection among *BdNRT2* members. Results from chapter 2 indicate a potential key role of the *BdNRT2.1* of the *BdNRT2* family, which led to the functional characterization of *BdNRT2.1* in chapter 3.

In chapter 3, The knock-out mutant *bdnrt2.1* showed an impaired inducible high-affinity transport system (iHATS) and reduced overall NUE (37% in average) under N non-limited conditions, whereas the constitutive high-affinity transport system (cHATS), low-affinity transport system (LATS) and nitrogen uptake efficiency (NUpE) were not affected. Reduced iHATS and unchanged cHATS and LATS when knocking out *NRT2.1* were reported in *Arabidopsis* (Li *et al*., 2007). Additionally, intact NUpE in the *NRT2.1* mutant also has been observed in many separate studies in *Arabidopsis* (Cerezo *et al*., 2001; Filleur *et al*., 2001; Li *et al*., 2007; Kiba *et al*., 2012). However, to my knowledge, reducing NUE when knocking out *NRT2.1* under N non-limited conditions has not been

reported elsewhere. This suggests a novel function of BdNRT2.1 that deserves further research. To further investigate the underlying mechanisms that caused the reduced NUE, I used ^{15}N labelling to differentiate N uptake and N remobilization after anthesis and found that the *BdNRT2.1* is involved in N remobilization, whereas the nitrogen uptake after anthesis is unchanged. Moreover, senescence-related NAC transcription factor and cytosolic GS1 are responsible for the reduced N remobilization of the *bdnrt2.1* mutant and they may form a regulatory network with *BdNRT2.1* during leaf senescence. Thus, I propose a model (Fig. 5.1) that illustrates the difference of *BdNRT2.1*'s function between vegetative growth stage (before anthesis) and reproductive growth stage (after anthesis). Based on the results that the *bdnrt2.1* mutant has intact NUpE/NAE, but has reduced NUtE/NRE, I hypothesized that *BdNRT2.1* has contrasting impact on N metabolism related enzyme activity and metabolic status at different developmental stages, which was investigated in chapter 4.

In chapter 4, I measured the mRNA and protein abundance levels of key genes coding for enzymes involved in the N assimilation pathway at two developmental stages: the vegetative stage (before anthesis) and reproductive stage (after anthesis). The results supported my hypothesis that *BdNRT2.1* has contrasting impacts at different developmental stage, as illustrated in the proposed model in Fig. 5.1. Key enzymes were not affected by the mutant before anthesis, explaining the unchanged NUpE/NAE, but GS1, which is known to control N recycling (Bernard and Habash, 2009), was reduced, while other enzymes were unaffected after anthesis, which explained the reduced NUtE/NRE, but still unchanged nitrogen uptake. Metabolomics analysis also presented a contrasting metabolic status at two developmental stages with a significantly altered metabolites distribution in the *bdnrt2.1* after anthesis. Overall, these results suggest a potential role of *BdNRT2.1* as nitrate sensor/signal transducer in a developmental stage dependent and nitrate uptake independent manner. What's more, the accumulation of N-rich amino acids in the vegetative tissues of the *bdnrt2.1* mutant further supports the hypothesis in chapter 3 that N remobilization to grain seeds is impaired by the mutation in *BdNRT2.1*, hence the accumulated N in the vegetative tissue.

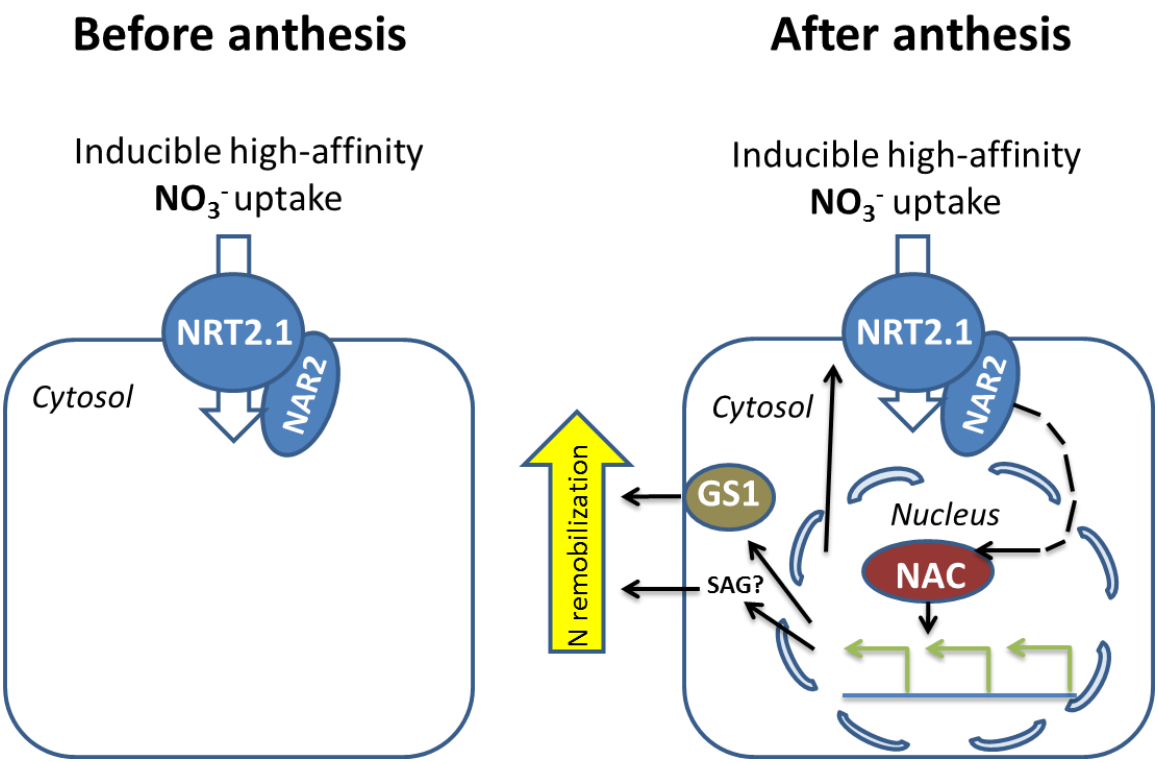

Figure 5.1 Schematic representation of proposed model for BdNRT2.1 before and after anthesis.

NRT2.1 and NAR2 are located on plasma membrane. NAC is senescence induced transcriptional factor and located in the nucleus. GS1 is located in the cytosol and is responsible for N remobilization. SAG represents senescence associated genes that are regulated by NAC. Black arrow represents regulatory direction, green arrow represents gene activation. For simplicity, all players are presented in the same cell, which might be not the case in a plant.

To pursue the goal of increasing NUE, I deployed transgenic tool to overexpress *BdNRT2.1*, as described in chapter 3, and the results were exciting: *BdNRT2.1* over-expression can enhance the grain yield by 24% on average under N non-limited conditions. However, in my three transgenic *BdNRT2.1* over-expression lines, the HI was not affected, implying that the nitrogen remobilization probably was not enhanced by the overexpression of *BdNRT2.1*. The increase in yield was mainly due to the increased number of spikelets per plant. Thus, the question remains with respect to how *BdNRT2.1* over-expression contributed to the enhanced yield. It is plausible that over-expressing *BdNRT2.1* may simply increase nitrate uptake or it may increase N management through its nitrate transducer related functions. Therefore, continuing work is still needed to answer the above-mentioned questions. Also, it would be interesting to find out the linkage of *BdNRT2.1* and *BdNRT2.2* regulatory machineries, Because they were tandem repeats on the same chromosome in many species, including *Brachypodium, Arabidopsis*, rice, maize, and *Medicago truncatula* (Orsel *et al*., 2002; Cai *et al*., 2008; Garnett *et al*., 2013; Pellizzaro *et al*., 2015).

In conclusion, a molecular and physiological characterization of the NRT2 family in the model monocot plant *Brachypodium distachyon* was carried out. Among six close-related, but not completely redundant, family members, BdNRT2.1emerged to play the key role, and it is essential to nitrogen use efficiency. Finally, the distinct role of BdNRT2.1 at different developmental stages was demonstrated, and BdNRT2.1 may serve as a signal transducer to coordinate N remobilization during the reproductive stage. More importantly, constitutively over-expressing *BdNRT2.1* is a promising approach to improve NUE.

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