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BdNRT2A and BdNRT3.2 are the major components of the High-Affinity nitrate Transport System in *Brachypodium distachyon*

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1 **Title : BdNRT2A and BdNRT3.2 are the major components of the High-Affinity nitrate Transport**
2 **System in *Brachypodium distachyon***

3
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38 **Summary (284 words)**

39

- 40 • An efficient nitrate uptake system contributes to the improvement of crop nitrogen use
41 efficiency under low nitrogen availability. The High Affinity nitrate Transport System (HATS)
42 in plants is active in low external nitrate and is mediated by a two-component system [high
43 affinity transporters NRT2 associated to a partner protein NRT3 (NAR2)].
- 44 • In *Brachypodium*, the model plant for C3 cereals, we investigated the role of *BdNRT2A* and
45 *BdNRT3.2* through various experimental approaches including gene expression profiling,
46 functional characterisation in heterologous system, intracellular localization by imaging, and
47 reverse genetics via gene silencing.
- 48 • Expression of *BdNRT2.A* and *BdNRT3.2* genes in response to nitrate availability fits with the
49 characteristics of the HATS components. Co-expression of *BdNRT2A* and *BdNRT3.2* is required
50 for an effective nitrate transport in the heterologous expression system *Xenopus* oocytes.
51 Functional interaction between BdNRT2A-GFP and BdNRT3.2-RFP fusion proteins has been
52 observed at the plasma membrane in *Arabidopsis* protoplasts in transient expression
53 experiments. BdNRT3.2 appeared to be necessary for the plasma membrane localization of
54 BdNRT2A. ¹⁵Nitrate influx measurements with *bdnrt2a* mutants (two amiRNA mutants and
55 one NaN₃ induced mutant with a truncated NRT2A protein), confirmed that BdNRT2A is a
56 major contributor of the HATS in *Brachypodium*.
- 57 • Directed mutagenesis in BdNRT2A of a conserved Ser residue (S461) specific to
58 monocotyledons has been performed to mimic a non-phosphorylated S461A or a constitutively
59 phosphorylated S461D, in order to evaluate its potential role in the BdNRT2A and BdNRT3.2
60 interaction leading to plasma membrane targeting. Interestingly, the phosphorylation status of
61 S461 did not modify the interaction, suggesting on a more complex mechanism.
- 62 • In conclusion, our data show that BdNRT2A and BdNRT3.2 are the main components of the
63 nitrate HATS activity in *Brachypodium* (Bd21-3) and allow an optimal growth in low N
64 conditions.

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67 **Keywords :** *Brachypodium distachyon* (Bd21-3), High Affinity nitrate Transport System (HATS), low
68 nitrogen supply, nitrate influx, NRT2/NRT3

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75 **Introduction**

76 Nitrogen (N) is an essential nutrient for the growth and development of plant, and is present in the soil
77 in the form of nitrate (NO_3^-) and ammonium (NH_4^+). To ensure high crop yields, N - containing
78 chemicals fertilizers have largely been used for 60-70 years to provide NO_3^- and NH_4^+ to plants and thus
79 to ensure enough food to the constantly increasing human population. Attempts to improve the Nitrogen
80 Use Efficiency (NUE) of crops are considered as a priority goal for the near future in order to limit the
81 fertilizer over-use and the related environmental problems, such as eutrophication. It is assumed that
82 NUE can be improved by enhancing NO_3^- uptake capacity, the major N source for most plants in aerobic
83 conditions (Bogard *et al.*, 2010; Chen *et al.*, 2016; Wang *et al.*, 2018). Depending on the external NO_3^-
84 concentrations, two different uptake systems occur within the plant: the high-affinity (HATS) and low-
85 affinity (LATS) systems operate when NO_3^- is present in the soil at low (< 1 mM) or high concentration
86 (> 1 mM), respectively. NO_3^- is taken up by roots from the soil by members of NRT2 (Nitrate
87 Transporter 2) and NPF (Nitrate Transporter 1/Peptide Transporter Family) families. NRT2 proteins are
88 active when the soil NO_3^- concentration is low (below 1mM), thus contributing to the HATS for NO_3^-
89 uptake. Molecular characterization of NRT2 genes have been largely performed in Arabidopsis. Among
90 the seven genes encoding NRT2 transporters (*AtNRT2.1* to *2.7*) (Wang *et al.*, 2018), four have been
91 characterized *in planta* as involved in root NO_3^- uptake, *AtNRT2.1* being more active than *AtNRT2.2*
92 (Filleur *et al.*, 2001; Li *et al.*, 2007) and *AtNRT2.4* and *AtNRT2.5* being effective at very low NO_3^-
93 concentrations (Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). Two other *AtNRT2* have functions in aerial
94 parts of the plants: *AtNRT2.6* is involved in biotic interaction in leaves (Dechorgnat *et al.*, 2012) and
95 *AtNRT2.7* is responsible for vacuolar NO_3^- loading in seeds (Chopin *et al.*, 2007).

96 Plants modulate root NO_3^- uptake by regulating transcript abundance and protein activity, depending on
97 their carbon and N status. Thereby, expression of *AtNRT2.1* is stimulated by low NO_3^- concentration,
98 light and sugars (Filleur and Daniel-Vedele, 1999; Lejay *et al.*, 1999; Okamoto *et al.*, 2003) and
99 repressed by NH_4^+ and amino acids (Lejay *et al.*, 1999; Zhuo *et al.*, 1999; Nazoa *et al.*, 2003).

100 Nevertheless, studies of plants overexpressing *AtNRT2.1* reveal that despite a constitutively high
101 expression, HATs activity can be repressed (Laugier *et al.*, 2012) indicating that a post translational
102 regulation interferes, leading to an inactivation of the transporter. More recently, post-translational
103 regulation of *AtNRT2.1* have been identified at the N and C-terminal ends. Phosphorylation of Ser28 in
104 the N-terminus of *AtNRT2.1* results in the protein stabilisation in response to NO_3^- limitation (Zou *et al.*,
105 2020) and phosphorylation of Ser501 inactivates NRT2.1 and thus NO_3^- transport activity in
106 response to NH_4^+ supply (Jacquot *et al.* 2020). Some NRT2s were shown to require a partner protein
107 NRT3 (also known as NAR2) for function (Tong *et al.*, 2005).

108 Proteomic studies in Arabidopsis have revealed the functional structure of the high-affinity NO_3^-
109 transport, composed of two *AtNRT2.1* and two *AtNRT3.1* (NAR2) subunits (Yong *et al.*, 2010). Two
110 *AtNRT3* genes have been identified but *AtNRT3.1* expression is predominating and is induced by NO_3^-
111 (Okamoto *et al.* 2006; Orsel *et al.*, 2006). Analysis of *atnrt3.1* mutants revealed that the absence of

112 AtNRT3.1 protein affects the NO₃⁻-inducible component of HATS (Okamoto *et al.* 2006) and the
113 localization of AtNRT2.1 at the plasma membrane (pm) (Wirth *et al.*, 2007). The molecular mechanism
114 of NRT2 and NRT3 interaction has started to be elucidated. NRT2 protein structure is predicted to
115 contain 12 transmembrane domains and N and C-terminal ends directed towards the cytosol (Jacquot *et*
116 *al.* 2020) and the NRT3 protein contains only one transmembrane domain (Tong *et al.* 2005). The
117 AtNRT2.1/AtNRT3.1 association is related to the N-terminal, the C-terminal ends of AtNRT2.1, and to
118 the central part of NRT3. Leu85 located in the first transmembrane domain of AtNRT2.1 is critical for
119 the association between AtNRT2.1 and AtNRT3.1 (Kotur *et al.*, 2017). In addition to Arabidopsis,
120 capacity of transporting NO₃⁻ through the interaction with NRT3 protein have been proved in various
121 dicotyledonous and also monocotyledonous species, as for HvNRT2.1 (Tong *et al.*, 2005), and
122 OsNRT2.1 and OsNRT2.3a (Yan *et al.*, 2011). In rice 30 amino acids (from 65 to 95) at the N-terminal
123 end of OsNRT2.3a are required for the interaction with OsNAR2.1 and for the NO₃⁻ transport activity
124 (Feng *et al.*, 2011). The central region of NRT3 has been indicated to interact with NRT2 in rice, where
125 two residues (R100 and D109) placed in the middle region of OsNAR2.1 are necessary for the
126 interaction with OsNRT2.3a at the pm (Liu *et al.*, 2014). In Arabidopsis the replacement of D105 in
127 AtNRT3.1 markedly reduced NO₃⁻ uptake (Kawachi *et al.*, 2006). But the C-terminus region of NRT2
128 has been also indicated to interact with central region of NRT3 in barley, where the Ser463 of HvNRT2.1
129 was shown to control the interaction between HvNRT2.1 and HvNAR2.1, and it was suggested that the
130 interaction is regulated by the phosphorylation/dephosphorylation of Ser463 (Ishikawa *et al.*, 2009). The
131 phosphorylation site Ser501 at the C-terminus of AtNRT2.1 was shown to inactivate the transporter
132 leading to a decrease in HATS activity, but clearly independently of the breaking of AtNRT2.1 and
133 AtNRT3.1 interaction (Jacquot *et al.*, 2020). Intriguingly, this Ser is replaced by a Gly in
134 monocotyledons suggesting distinct regulation mechanisms between monocotyledons and dicotyledons.
135 (Jacquot *et al.*, 2017).

136 NRT2.1 orthologs have been identified in numerous species as rice (Cai *et al.*, 2008), barley (Vidmar *et*
137 *al.*, 2000), wheat (Yin *et al.*, 2007), tomato (Ono and Frommer, 2000), tobacco (Alberto *et al.*, 1997),
138 rapeseed (Faure-Rabasse *et al.*, 2002) and peach (Nakamura *et al.*, 2007). In *Brachypodium distachyon*,
139 the model plant for C3 cereals, we have previously identified seven NRT2 and two NRT3 (Girin *et al.*,
140 2014). In the phylogenetic tree of the *NRT2* family, we identified five *Brachypodium* genes that cluster
141 together in the same clade as AtNRT2.1 and HvNRT2.1 (Girin *et al.*, 2014). It was impossible to predict
142 which one was a functional orthologous of AtNRT2.1 among the five *BdNRT2*. We previously observed
143 that the HATS activity was regulated by N availability and correlated with the expression of two
144 *BdNRT2* (*BdNRT2A/2B*) and one *BdNRT3* (*BdNRT3.1*) genes, suggesting these *BdNRTs* are good
145 candidates for elements of HATS activity (David *et al.*, 2019). In this study, we demonstrated the major
146 role of *BdNRT2A/BdNRT3.1* in the HATS activity by using *bdnrt2a* mutants. We also studied the role
147 of a Ser residue (S461) conserved in monocotyledons, but not in dicotyledons, for the interaction
148 between *BdNRT2A* and *BdNRT3.2*.

149 **Materials and Methods**

150 **Plant material and culture conditions**

151 *Brachypodium distachyon* accession Bd21-3 was used for all experiments.

152 For the NO₃⁻ influx measurements, *Brachypodium* seeds were germinated for four days in water at room
153 temperature conditions. Seedlings plants were then transferred to hydroponic conditions in a growth
154 chamber (18 h light at 22° C /6 h dark at 18°C cycle and 250 μmol photons m⁻². s⁻¹ irradiation, OSRAM
155 Lumilux L36W865 cool day light). Plants were provided with media containing 0.2 mM NO₃⁻ : 0.05
156 mM Ca(NO₃)₂, 0.1 mM KNO₃, 1 mM KH₂PO₄, 3.25 mM CaCl₂, 5.45 mM KCl, 2 mM MgSO₄, 4.5 μM
157 MnCl₂, 10 μM H₃BO₃, 0.7 μM ZnCl₂, 0.4 μM CuSO₄, 0.22 μM MoO₄Na₂, 50 μM iron-EDTA. For 1
158 mM and 0.1mM of nitrate, media contain 0.5 mM Ca(NO₃)₂ or 0.05 mM Ca(NO₃)₂ respectively and
159 potassium was compensated with 5 mM and 5.45 mM of KCl respectively. For the nutrient solution
160 containing 0.02 mM NO₃⁻, nitrate was supplemented with 0.01 mM KNO₃ and 0.005 mM Ca(NO₃)₂
161 and with 5 mM KNO₃ and 2.5 mM Ca(NO₃)₂ for the 10 mM NO₃⁻ nutrient solution.

162 For the NO₃⁻ induction experiments, *Brachypodium* plants were grown in hydroponics, as described
163 above, for 18 days on 0.1 mM NO₃⁻, then they were NO₃⁻ starved for 4 days (KCl was supplemented
164 instead of KNO₃) and finally re-supplied with 1 mM NO₃⁻ for 2h or 3 h before harvest.

165 For the complementation study and protoplasts transfection study, WT and mutant seeds of *Arabidopsis*,
166 ecotype Wassilewskija (Ws) (*A. thaliana*), were used. For the complementation study plants were
167 grown hydroponically in a growth chamber with 8 h light at 21° C /16h dark at 17°C cycle, 80% relative
168 humidity and 150 μmol photons m⁻² s⁻¹ irradiation. Seeds were sterilized and stratified in water at 4 C
169 for 5 days before sowing. Each seed was sown on top of a cut Eppendorf tube filled with medium
170 consisting of half-strength nutrient solution containing 0.8% agar. Plants were supplied with media
171 containing 0.2 mM NO₃⁻ : 0.1 mM KNO₃, 0.05 mM Ca(NO₃)₂ and 2.45 mM K₂SO₄, 2.15 mM CaCl₂, 2
172 mM MgSO₄, 2 mM KH₂PO₄, 10 μM MnSO₄, 24 μM H₃BO₃, 3 μM ZnSO₄, 0.9 μM CuSO₄, 0.04
173 μM(NH₄)₆Mo₇O₂₄, iron-EDTA 10 mg l⁻¹. The nutrient solution was changed every 3 days and, during
174 the first 2 weeks was used at half-strength media. Plants were harvested 40 days after sowing, 1 h after
175 illumination had started and analyzed for nitrate influx. Shoots and roots were weighed separately and
176 frozen in liquid nitrogen. Influxes of ¹⁵NO₃⁻ were performed on wild type genotype (Ws), mutant
177 (*atnrt2.1-1*) and *atnrt2.1-1* mutant lines overexpressing *Pro35S::BdNRT2A* or *Pro35S::BdNRT2A-GFP*
178 or *Pro35S::GFP-BdNRT2A* after 42 days of hydroponic growth.

179 For the protoplast transfection study *Arabidopsis* seeds were surface sterilized and sown on *in vitro*
180 plates containing 1 % agar (Sigma-Aldrich France) as previously described (David *et al.*, 2016) and
181 supplemented with a 9 mM NO₃⁻ medium : 2 mM Ca(NO₃)₂, 5 mM KNO₃, 2.5 mM KH₂-PO₄, 2 mM
182 MgSO₄, 0.07 % MES (pH 6), 0.005 % (NH₄)₅Fe(C₆H₄O₇)₂, 70 μM H₃BO₃, 14 μM MnCl₂, 0.5 μM CuSO₄,
183 10 μM NaCl, 1 μM ZnSO₄, 0.001 μM CoCl₂, and 0.2 μM NH₄MoO₄. After 3 days of stratification at 4
184 °C in the dark, plates were placed in a growth chamber at 18 °C with a 16/8 h light photoperiod, 60 %

185 of humidity, and a light intensity of 50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Seedlings were harvested 2 weeks after the
186 transfer in light conditions for protoplast purification and transfection.

187

188 $^{15}\text{NO}_3^-$ uptake in heterologous expression system (*Xenopus laevis* oocytes)

189 The coding sequences of BdNRT2A and BdNRT3.2 were cloned into pGEMT easy vector (Promega)
190 and then digested with NotI enzyme. cDNA fragments were blunted using the Klenow fragment and
191 subcloned in the EcoRV site of the pT7TS expression vector containing the 5'-untranslated region
192 (UTR) and 3'-UTR of the *Xenopus* β -globin gene (Cleaver *et al.*, 1996). Clones with correct sequence
193 were used for *in vitro* synthesis of RNA, pT7TS clones were linearized by digestion with XbaI. Capped
194 full-length cRNAs were synthesized using a T7 RNA transcription kit (mMESSAGE mMACHINE;
195 Ambion). As described in Orsel *et al.*, (2006), we used a heterologous expression system *Xenopus laevis*
196 oocytes. *Xenopus* oocytes were prepared as described previously (Zhou *et al.*, 1998) and stored in ND96
197 solution (96 mM NaCl, 2 mM KCl, 1.80 mM CaCl_2 , 1mM MgCl_2 , 15 mM MES, adjusted at pH 6 with
198 NaOH). Healthy oocytes at stage V or VI were injected with 50 nL of water (nuclease free) or different
199 cRNAs at 1mg/mL each. After 3-days incubation at 18°C, five to 10 oocytes were incubated in 3 mL of
200 ND96 solution enriched with 0.5 mM $\text{Na}^{15}\text{NO}_3$ (atom% ^{15}N : 98%) during 16 h at 18°C. The oocytes
201 were then thoroughly washed four times with ice-cooled 0.5 mM NaNO_3 ND96 solution and dried at
202 60°C. The ^{15}N to ^{14}N ratio of single dried oocyte was measured using an isotope ratio mass spectrometer
203 (model Integra CN; PDZ Europa). The delta ^{15}N was calculated as described previously (Tong *et al.*,
204 2005).

205

206 Identification of chemical mutagenesized mutants with mutations in the coding sequence of 207 *BdNRT2A* in the TILLING collection of Versailles

208 The NaN_3 -induced mutant collection from Versailles (Dalmais *et al.*, 2013) was used to search point
209 mutations by a TILLING method in the coding sequence of *BdNRT2A*. The genomic DNA pools
210 corresponding to 5530 M2 families were screened by PCR using *BdNRT2A* specific primers fused to
211 fluorochromes. Mutations were then identified by sequencing the PCR products after digestion by
212 restriction endonuclease ENDO1 and electrophoresis detection by laser of the cleaved amplicons. The
213 effect of each point mutation was analysed and predicted using SIFT (Sorting Intolerant From Tolerant)
214 program (<http://sift.jcvi.org/>). A SIFT score lower than 0.05 predicted a deleterious amino acid
215 substitution for a point mutation.

216

217 *BdNRT2A* amiRNA mutants

218 The amiRNA constructs were engineered using the online microRNA designer WMD3
219 (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>). Specific sequences were designed to target
220 *BdNRT2A* (TAAAGACAGCAGCAGTCGCGG) or the five *NRT2* genes BdNRT2A/B/C/D/F
221 (TATCATGATGCGCACCTACTA). DNA fragments containing the specific sequences, the

222 microRNA structure of pNW55 (based on the rice osa-MIR528; Warthmann *et al.*, 2008) and Gateway
223 attL1/attL2 borders were synthesized and cloned at the EcoRV site of pUC57-Kan (GenScript Biotech
224 Corporation) (Supplementary data S1). These entry clones were subsequently recombined into the
225 destination vector pIPKb002 (Himmelbach *et al.*, 2007) using the Gateway LR Clonase II enzyme
226 (Invitrogen). This vector contains the Hpt plant selection marker (hygromycin resistance) and drives the
227 expression of amiRNAs under the constitutive *ZmUbi1* promoter. Transgenes were integrated into the
228 *B. distachyon* accession Bd21-3 genome by Agrobacterium-mediated transformation (Agrobacterium
229 tumefaciens strain AGL1) of embryogenic calli following the protocol described by Vogel and Hill
230 (2008). Homozygous lines were selected based by hygromycin resistance.

231

232 **GFP fusions and functional complementation of *atnrt2.1-1* with *BdNRT2A***

233 The cDNA of *BdNRT2A* was first amplified by PCR using specific forward primer *70F2* and reverse
234 primer *70R2* before being cloned into the pGEMT-easy vector (Promega). Clones with correct sequence
235 were used to produce appropriate PCR product for Gateway cloning. First primers Bd70start and
236 Bd70end or Bd70stop were used and PCR products were amplified with the universal *U3endstop* and
237 *U5* primers to create the recombinant sites AttB. The product of recombination reactions (BP reactions)
238 was used to transform competent *Escherichia coli* strain TOP10 (Invitrogen), by heat shock. LR clonase
239 reactions to transfer fragments from the entry clone to the destination binary vector pMDC32
240 (*Pro35S::BdNRT2A*), pMDC43 (*Pro35S::GFP-BdNRT2A*) and pMDC83 (*Pro35S::BdNRT2A-GFP*)
241 were performed. The vectors containing the different constructs were sequenced before transformation
242 of *A. tumefaciens*. The *atnrt2.1-1* mutants (Ws background) (Filleur *et al.*, 2001) was transformed with
243 each construct by the *in planta* method using the surfactant Silwet L-77 (Clough and Bent, 1998) and
244 transformants were selected on 20 mg L⁻¹ of hygromycin B (Sigma). Three independent homozygous
245 mono-insertional T3 lines of were selected per construct, and over-expression was confirmed q-PCR.
246 Roots of seven-days-old plantlets grown *in vitro* on Arabidopsis media (Duchefa Biochemie B.V; The
247 Netherlands) were observed with the confocal microscope SP5 (Leica) or tested for the *BdNRT2A*
248 overexpression by qRT-PCR.

249

250 **RFP fusions with *BdNRT3.2***

251 The cDNA of *BdNRT3.2* was amplified from genomic DNA using *EcoR1-BdNRT3_qL* and
252 *BdNRT3_20_qR-Sal1*, and was cloned into the pGEMT-easy vector. Then, pGEMT containing
253 *BdNRT3.2* was digested by EcoR1 and Sal1, and the product of digestion was cloned by using T4 ligase,
254 into pSAT6-RFP-C1 (NovoPro) and used for transitory expression of *p35S::RFP-BdNRT3.2* into
255 mesophyll protoplasts of Arabidopsis.

256

257 **Directed mutagenesis of *BdNRT2A***

258 Directed mutagenesis of the conserved Ser specific to monocots (supplementary data S3) were
259 performed using the protocol of ‘QuickChange II XL Site-Directed Mutagenesis’ (Stratagen). Sequence
260 of *BdNRT2A* was amplified from pDONR207 containing *BdNRT2A* with the forward and reverse
261 primers *BdNRT2A*^{S461D} for *BdNRT2A*^{S461D} and the forward and reverse primers *BdNRT2A*^{S461A} for
262 *BdNRT2A*^{S461A}. The amplification products were digested with *Dpn* I and then cloned into *E. coli* One
263 Shot TOP10. The different constructs were sequenced, and those with desired mutations were re-
264 introduced into pMDC43 (*Pro35S::GFP-BdNRT2A*^{S461D}) and pMDC43 (*Pro35S::GFP-BdNRT2A*^{S461A})
265 for transitory expression in *Arabidopsis* mesophyll protoplasts.

266

267 **Transfection of Arabidopsis protoplasts**

268 The protoplast were isolated from seedlings of *Arabidopsis* and transfected as described in Zhai *et al.*,
269 (2009). We used wild type genotype (Ws), *atnrt2.1-1*, *atnrt2-1.1xatnrt3.1*, and *atnrt2.1-1*
270 overexpressing *Pro35S::GFPBdNRT2A* and *Pro35S::BdNRT2A-GFP* for isolation and transfection of
271 protoplasts. Transfection were performed with pMDC43 containing *Pro35S::GFP-BdNRT2A* or
272 pMDC83 containing *Pro35S::BdNRT2A-GFP*, and co-transfection were performed with pSAT6
273 containing the construction *Pro35S::GFPBdNRT3.2* and pMDC43 containing *Pro35S::GFP-BdNRT2A*
274 or pMDC83 containing *Pro35S::BdNRT2A-GFP*. Co-transfection were also performed with pSAT6
275 (Citovsky *et al.*, 2006) containing the construction *Pro35S::GFPBdNRT3.2* and pMDC43
276 (*Pro35S::GFP-BdNRT2A*^{S461D}) or pMDC43 (*Pro35S::GFP-BdNRT2A*^{S461A}).

277

278 **Confocal imaging microscopy analyses**

279 Confocal imaging microscopy analyses were performed using the Leica SP2 and SP5 microscope
280 equipped with an argon laser (488 nm for GFP excitation and 543 nm for RFP). Emission was collected
281 at 495-525 nm (GFP) and 580-650 nm (RFP). Autofluorescence was detected using the argon laser (488
282 nm) and emission was collected at 675-750nm. Images were processed in ImageJ.

283

284 **Root ¹⁵NO₃⁻ influx**

285 Root influxes of ¹⁵NO₃⁻ were performed two weeks after growth in hydroponic conditions in order to
286 measure HATS and ‘LATS plus HATS’ activities. First, plants were transferred to 0.1 mM CaSO₄ for
287 1 min, then to a complete nutrient solution containing 0.2 mM of ¹⁵NO₃⁻ for the HATS and 6 mM ¹⁵NO₃⁻
288 for the ‘LATS plus HATS’ (atom% ¹⁵N : 99%) for 5 min and finally to 0.1 mM CaSO₄ for 1 min. Roots
289 were separated from the shoots immediately after the final transfer and frozen in liquid nitrogen. After
290 grinding, an aliquot of the powder was dried overnight at 80°C and analyzed using a FLASH 2000
291 Elemental Analyzer coupled to an IRMS Delta IV (Thermo Fisher Scientific, Villebon, France). Influxes
292 of ¹⁵NO₃⁻ were calculated from the ¹⁵N content of the roots.

293

294 **NO₃⁻ content**

295 The NO_3^- content was measured by a spectrophotometric method adapted from Miranda *et al.*, (2001)
296 and described in David *et al.*, (2019). The principle of this method is a reduction of NO_3^- by vanadium
297 (III) combined with detection by the acidic Griess reaction.

298

299 **RNA extraction and qRT-PCR**

300 Total RNAs were isolated using Trizol[®] reagent (Ambion, Life Technologies) and RT-qPCR were
301 performed as described in David *et al.*, (2019).

302

303 **Statistical analyses**

304 Statistical analyses were performed using one-way ANOVA and the means were classified using Tukey
305 HSD test. ($P < 0.05$)

306

307 **Results**

308 ***BdNRT2A*, *BdNRT2B* and *BdNRT3.2* were induced in response to NO_3^-**

309 We previously observed that the HATS activity in *Brachypodium* (Bd21-3 accession) decreased with
310 increasing availability of NO_3^- from 0.1 to 10 mM or 1 mM NH_4NO_3 supply, in correlation with the
311 expression of *BdNRT2A/B* and *BdNRT3.2* in roots (David *et al.*, 2019). The main component of the
312 HATS activity in *Arabidopsis*, *AtNRT2.1*, is also repressed by high NO_3^- and induced upon initial NO_3^-
313 supply (Filleur and Daniel-Vedele 1999; Lejay *et al.*, 1999), similarly to *TaNRT2.1* which is induced by
314 NO_3^- in wheat (Yin *et al.*, 2007). Since we found seven *BdNRT2* genes (Girin *et al.*, 2014), we further
315 investigated the effect of 1 mM NO_3^- re-supply ('+N') after 4 days of N deprivation ('-N') on root
316 expression levels of the 5 members of the *BdNRT2* family which are most phylogenetically related to
317 *AtNRT2.1*. *BdNRT2A* was induced by a factor 5 (Fig 1A), *BdNRT2B* and *BdNRT2F* were induced by a
318 factor 2 (Fig 1B, Fig S1B), while *BdNRT2D* was extremely weakly expressed in both '-N' and '+N'
319 conditions (Fig S1A) and *BdNRT2C* expression level was not modified by NO_3^- availability (David *et*
320 *al.*, 2016). Besides, *BdNRT2.E* which is more phylogenetically related to *AtNRT2.5* (Girin *et al.*, 2014)
321 was repressed by NO_3^- treatment (Fig S1C), while *BdNRT2.G*, phylogenetically related to *AtNRT2.7*
322 (Girin *et al.*, 2014), was not expressed in roots (data not shown). Expression of *BdNRT3.2* was also
323 induced by a factor 1.4 (Fig. 1C), whereas expression of *BdNRT3.1* was not affected by NO_3^- availability
324 (David *et al.*, 2016). Thus, *BdNRT2A* was the *BdNRT2* most induced in response to NO_3^- induction
325 (concomitantly with *BdNRT3.2*), an expression profile that fitted exactly with the characteristics of HATS
326 activity.

327

328

329

330 **Co-expression of *BdNRT2A* and *BdNRT3.2* was required for an effective NO₃⁻ transport in the**
331 **heterologous expression system *Xenopus* oocytes**

332 The two component system NRT2/NRT3 has been described as a hetero-oligomer in many species
333 (Yong *et al.*, 2010). Thus, we further investigated whether *BdNRT2A* needs to interact with *BdNRT3.2*
334 to transport NO₃⁻. As already described in Orsel *et al.*, (2006), we used the heterologous expression
335 system in *Xenopus laevis* oocytes to express *BdNRT2A*, *BdNRT3.2* or both genes and then we measured
336 the NO₃⁻ uptake into oocytes. Only oocytes co-injected with cRNAs corresponding to *BdNRT2A* and
337 *BdNRT3.2* were able to accumulate ¹⁵NO₃⁻ after 16 h of incubation in 0.5 mM Na¹⁵NO₃, indicating that
338 *BdNRT2A* and *BdNRT3.2* interaction was required for an active transport system (Fig. 2) similarly to
339 other species (Orsel *et al.*, 2006).

340

341 **HATS activity and growth were lowered in *Brachypodium* mutants deficient in *BdNRT2A***

342 *AtNRT2.1* is the main actor of HATS activity in Arabidopsis and *atnrt2.1* mutants have a HATS activity
343 reduced by 56% (Yin *et al.*, 2007). In the double mutant *atnrt2.1 atnrt2.2* more than 70% of the HATS
344 was impaired (Filleur *et al.*, 2001) while LATS activity was unaffected in both single and double
345 mutants. In order to confirm that *BdNRT2A* and/or *BdNRT2B* are functional orthologs of *AtNRT2.1* and
346 *AtNRT2.2* we searched for *Brachypodium* mutants affected in *BdNRT2A* and/or *BdNRT2B*. In the JGI
347 *Brachypodium* collection no true T-DNA insertion mutant in *BdNRT2A* is available following our own
348 testing experiments (Supplementary data S1). We used the NaN₃ mutant collection from Versailles
349 (Dalmais *et al.*, 2013) to search for point mutations in the coding sequence of *BdNRT2A*. We identified
350 seven independent lines corresponding to three silent and four non-silent point mutations leading to
351 amino acid changes (Table 1). The SIFT scores for three of the non-silent point mutation were high (>
352 0.2) and thus with very low chance to impede the protein structure, but one mutation resulted in a stop
353 codon (W248*) that likely resulted in a truncated protein deprived of 257 amino acids (out of 505 total
354 amino acids) at the C-terminal. We further selected at the M₃ generation one *bdnrt2a-W248**
355 homozygous plant (9.2) on the one hand, and on the other hand two lines having lost by segregation the
356 stop codon but potentially keeping other point mutations “azygotes” (az1 and az2).

357 We also obtained *Brachypodium* mutant lines using artificial micro RNA technology (amiRNA). The
358 amiRNAs sequences were designed to target specifically *BdNRT2A* (*amiR n3*) or five out of the seven
359 *BdNRT2* (*2A*, *2B*, *2C*, *2D*, *2F*) (*amiR j2*). Expression of the amiRNA transgenes were verified in both
360 independent transformed lines. *BdNRT2A* expression level was measured by qRT-PCR in order to verify
361 the silencing effect of the amiRNA constructs. *BdNRT2A* and *BdNRT2B* mRNA level were decreased
362 respectively by 26% and 66% in *amiRn3* line and by 45% and 10% in *amiRj2* line (Fig. S2).

363 The amiRNA mutant lines *amiRj2*, *amiRn3* and the TILLING mutant 9.2 line were grown in
364 hydroponics under 0.2 mM NO₃⁻ for three-week in order to study the impact of the decrease in *BdNRT2A*
365 and *BdNRT2B* expression and of the presence of a truncated *BdNRT2A* protein on growth and NO₃⁻
366 influx in comparison to WT and two azygotic (az) lines (az1, az2). All the mutants showed significant

367 decreases in shoot/root ratio (Fig. 3A) resulting from a stronger decrease in shoot than in root biomass
368 (Fig. S3). The 9.2 mutant line with a truncated BdNRT2A was the most affected with a decrease of 57%
369 in shoot/root ratio, while decreases of 24% and 27% were observed for the shoot/root ratio in *amiRj2*
370 and *amiRn3* respectively. Other developmental features such as increase in root length and decrease in
371 tiller numbers were observed only in the mutant 9.2. The mutant 9.2 showed a 1.7-fold increase in root
372 length (Fig. 3B) and a 68% decrease in tiller number. The azygotes lines were not statistically different
373 from the WT for the shoot/root ratio, root length and tiller numbers (Fig. 3A, 3B, 3C). However, the
374 azygotes lines displayed reduced root and shoot biomasses compared to the WT, that could likely be
375 due to the remaining point mutations in these lines, potentially disturbing the plant growth.

376 HATS and the combined LATS and HATS activities were measured at 0.2 mM $^{15}\text{NO}_3^-$ and 6 mM $^{15}\text{NO}_3^-$
377 respectively on three-week-old plants grown in hydroponics. HATS was reduced significantly (up to
378 43% compared to WT) in the mutant 9.2, and only a tendential decrease was observed in the *amiRj2* and
379 *amiRn3* (up to 14% and 17% respectively compared to WT) (Fig. 4A) while the nitrate uptake at 6mM
380 nitrate corresponding to the combined LATS and HATS was not significantly affected in all the mutants
381 (Fig. 4B). The $^{15}\text{NO}_3^-$ influx of the azygotes lines was similar to the wild type for the $^{15}\text{NO}_3^-$ influx,
382 suggesting that the decrease in $^{15}\text{NO}_3^-$ influx observed in the 9.2 mutant line was specifically due to the
383 truncated BdNRT2A.

384 Root total N contents were slightly but significantly decreased (by 6%) in the 9.2 mutant line in
385 comparison to WT and az lines, while no differences have been observed for the *amiR* lines (Fig. 5A).
386 In roots, nitrate content (Fig. 5B), total C content, C/N ratio were not changed for neither line compared
387 to WT and az lines (S4A, S4B). Shoot nitrate content were not significantly affected either (Fig S4C).
388 All together, these results showed that the 9.2 mutant line was the most affected line for HATS activity
389 and growth, likely due to the loss of function of a truncated BdNRT2A. These results confirmed the
390 conserved functional role for BdNRT2A similar to AtNRT2.1, since *atnrt2.1-1* mutant shows a reduced
391 HATS activity when grown on 0.2 mM of nitrate as sole nitrogen source, resulting in a lower biomass
392 compared to the wild type (Filleur *et al.*, 2001).

393 We further performed hydroponic cultures using this mutant in order to study the role of BdNRT2A
394 under a range of NO_3^- supplies from 0.02 mM to 10 mM nitrate for 3 weeks. The 9.2 mutant line showed
395 a significant increase in root/shoot ratio whatever the nitrate supply, and an increase in root length under
396 0.2 and 10mM nitrate, while the tiller number was reduced only under 10 mM nitrate (Fig. S5A, S5B,
397 S5C). These results suggest that BdNRT2A modulated Brachypodium growth even under a large range
398 nitrate supply.

399

400 **Overexpression of *BdNRT2A* in *atnrt2.1-1* was not sufficient for a functional complementation**

401 In order to further investigate the function of BdNRT2A *in planta*, we produced *atnrt2.1-1* plants
402 overexpressing *BdNRT2A* translationally fused or not with Green Fluorescent Protein (GFP) coding
403 sequence (in C or N terminal position) under the control of 35S promoter. For each construct, three

404 independent over-expressing lines were selected (Fig. S6). Then, functional complementation test was
405 performed on plants grown under hydroponic conditions at 0.2 mM of nitrate. Shoot biomass of the
406 complemented lines were reduced as compared to the WT line, and similar to the *atnrt2.1-1* mutant (Fig.
407 6A). Root $^{15}\text{NO}_3^-$ influx was measured at 0.2 mM NO_3^- and showed that HATS activity was not restored
408 to the wild type level in any of the tested complemented lines (Fig. 6B). Moreover, a cytosolic sub-
409 cellular localization of BdNRT2A fused to GFP indicated that BdNRT2A was not targeted at the pm in
410 the *atnrt2.1-1* lines overexpressing *Pro35S::BdNRT2A-GFP* or *Pro35S::GFP-BdNRT2A*, for both C and
411 N terminal GFP fusions (Fig. 6C). Unexpectedly, these results demonstrate that heterologous
412 overexpression of *BdNRT2A* in *atnrt2.1-1* was not sufficient for a functional complementation of the
413 mutant, likely due to the lack of BdNRT2A targeting to the pm.

414

415 **BdNRT2A was targeted to plasma membrane in presence of BdNRT3.2 but not in the presence of** 416 **AtNRT3.1**

417 In Arabidopsis, HATS is mediated by two component systems. The interaction between NRT2 and
418 NRT3 proteins is required for the pm targeting of the complex and for an active NO_3^- transport (Wirth
419 *et al.*, 2007). However, we did not observe BdNRT2A targeting to the pm in *atnrt2.1-1* complemented
420 with *BdNRT2A*, suggesting that BdNRT2A could not interact with AtNRT3.1. Besides, we observed
421 that co-expression of *BdNRT2A* and *BdNRT3.2* was required for an effective NO_3^- transport in the
422 heterologous expression system *Xenopus* oocytes, validating the two-component system in
423 Brachyopodium. Then, to further investigate the sub-cellular localization of the BdNRT2A/BdNRT3.2
424 complex in Arabidopsis by using another expression system, we used transient expression in mesophyll
425 protoplasts from Arabidopsis. We transfected mesophyll protoplasts from Arabidopsis seedlings with
426 *Pro35S::GFP-BdNRT2A* and/or *Pro35S::RFP-BdNRT3.2*.

427 Interestingly, when *Pro35S::GFP-BdNRT2A* was expressed transiently in protoplasts from the single
428 mutant *atnrt2.1*, the localization of BdNRT2A fusion protein was cytosolic (Fig. 7A), similarly to what
429 was observed in leaves or in protoplasts obtained from an *atnrt2.1-2* mutant stably transformed with
430 *Pro35S::GFP-BdNRT2A* line (Fig. 6C, Fig.S7). Conversely, when *Pro35S::GFP-BdNRT2A* and
431 *Pro35S::RFP-BdNRT3.2* were co-expressed transiently in protoplasts of *atnrt2.1-1* mutant, BdNRT2A
432 and BdNRT3.2 fusion proteins were co-localized at the pm (Fig. 7B). Moreover, when mesophyll
433 protoplasts from *atnrt2.1-1 Pro35S::GFP-BdNRT2A* line (B3) were transfected with *Pro35S::RFP-*
434 *BdNRT3.2*, we observed a pm colocalization of BdNRT2A and BdNRT3.2 fusion proteins (Fig. 8),
435 corroborating that BdNRT2A could not interact with AtNRT3.1 and that the interaction with BdNRT3.2
436 allows its targeting to the pm. Besides, when we used transient expression of *Pro35S::GFP-BdNRT2A*
437 and *Pro35S::BdNRT2A-GFP* in *Nicotiana benthamiana* leaves, we also observed a cytosolic
438 localization for BdNRT2A fusion protein in epidermal cells (Fig. S8). These results revealed a species-
439 specific interaction between NRT2 and NRT3 proteins that ensures BdNRT2A targeting to the pm.

440

441 **The conserved BdNRT2A S461 residue is not required for the interaction between BdNRT2A and**
442 **BdNRT3.2 in Brachypodium**

443 The molecular mechanism involved in NRT2 and NRT3 interaction is not completely elucidated.
444 However, in barley the interaction between HvNRT2.1 and HvNRT3.1 is impaired when HvNRT2.1
445 Ser463 in the C-terminus is changed to alanine that mimics a non-phosphorylated residue (Ishikawa *et*
446 *al.*, 2009). This residue is conserved in NRT2 proteins from monocotyledons and algae only (Jacquot *et*
447 *al.*, 2017) (supplementary data S3) and not in dicotyledons as for AtNRT2.1. We investigated whether
448 this Ser residue is required for the interaction between BdNRT3.2 and BdNRT2A similarly to barley.
449 We thus co-transfected mesophyll protoplasts of *atnrt2 atnrt3.1* with *Pro35S::RFP-BdNRT3.2* and
450 *Pro35S::GFP-BdNRT2A^{S461A}* or *p35S::GFP-BdNRT2A^{S461D}* that mimicked a non phosphorylated and a
451 constitutively phosphorylated Ser, respectively. Surprisingly both mutagenized constructs allowed the
452 targeting of BdNRT2A/BdNRT3.2-GFP/RFP fusion proteins at the pm, suggesting that the
453 phosphorylated state of S461 (S461A or S461D) had no impact on the capacity of BdNRT2A to interact
454 with BdNRT3.2 (Fig. 9) unlike in barley. Thus, the absence of the conserved S461 specific to monocots
455 in AtNRT2.1 seems to not be responsible for the impaired pm targeting of BdNRT2A in Arabidopsis,
456 that is likely due to lack of interaction between BdNRT2A and AtNRT3.1.
457 These results did not allow to explain the impaired targeting of BdNRT2A to pm in absence of
458 BdNRT3.2 in Arabidopsis, but emphasized the complexity of this protein-protein interaction and need
459 for further investigation of this interaction.

460

461 **Discussion**

462 HATS and LATS activities for NO₃⁻ in Brachypodium were already characterized in part in our previous
463 paper (David *et al.*, 2019). HATS activity was regulated by N availability and correlated to the
464 expression level of BdNRT2A/2B and BdNRT3.2 suggesting these genes are functional orthologous of
465 AtNRT2.1/2.2 and AtNRT3.1, respectively, and are good candidates involved in HATS activity (David
466 *et al.*, 2019). In order to complete the characterization of the molecular basis of the HATS, we generated
467 two *bdnrt2* mutants (*n3* and *j2* lines) using amiRNA strategy and made a TILLING analysis that allowed
468 to select one NaN₃ induced mutant (line 9.2) with a truncated protein at the C terminus (amino acid
469 W248*). The growth phenotype observed for *bdnrt2a^{W248*}* (line 9.2) was reminiscent to that of the
470 insertional mutant *atnrt2.1* in Arabidopsis (Orsel *et al.*, 2004), with reduced shoot/root ratio, shoot
471 biomass and tiller number, and increased root length, likely due to lack of a functional protein
472 BdNRT2A. Moreover, the NO₃⁻ HATS activity was also reduced (up to 43%) in *bdnrt2a^{W248*}* similar to
473 the decrease in HATS observed in *atnrt2.1* (Orsel *et al.*, 2004, Li *et al.*, 2007). A less marked decrease
474 in HATS was observed for *amiRj2* and *amiRn3* mutants (up to 14% and 17% respectively), in which
475 slight decreases in *BdNRT2A* and *BdNRT2B* transcript levels were observed (but were not statistically
476 significant compared to wild type) and then likely induced only slight decreased BdNRT2A and
477 BdNRT2B protein levels. Besides, a slight reduction of shoot/root ratio and shoot biomass were

478 observed for *amiRj2* and *amiRn3*, indicating that growth of these mutants was limited in correlation with
479 the slight decrease in HATS. Interestingly, *amiRn3* seemed to be more reduced in *BdNRT2B* expression
480 than in *BdNRT2A* but its phenotype was not different from *amiRj2*, suggesting that *BdNRT2A* is the
481 main actor of HATS activity.

482 A slight but significant decrease in root N content was observed in *bdnrt2a*^{W248*} (line 9.2), but no change
483 was observed for root and shoot NO₃⁻ content in this mutant, while N and NO₃⁻ content were not changed
484 in *amiRj2* and *amiRn3*. On the contrary a 3 fold lower NO₃⁻ content has been described in shoots of
485 *atnrt2.1* accompanied by a slight decrease in root and shoot N content (Orsel *et al.*, 2004). We previously
486 observed that at low NO₃⁻ supply (for NO₃⁻ < 2 mM) and its assimilation products were used in priority
487 for growth, and NO₃⁻ storage in shoot occurred only under higher NO₃⁻ supply in *Brachypodium* (David
488 *et al.*, 2019). This strategy seemed to be different from *Arabidopsis*, and that could explain why a lower
489 NO₃⁻ uptake in *bdnrt2a*^{W248*} was accompanied by a reduced growth without changes in NO₃⁻ content.

490 We did not include in our study a *bdnrt2a* insertional mutant, although one line is displayed as available
491 in the insertional mutant collection the JGI *Brachypodium* collection (studied in Wang *et al.*, 2019),
492 because we proved after thorough verifications that the T-DNA insertion was not placed into our gene
493 of interest *BdNRT2A* (supplementary data S1).

494 Next, we overexpressed *BdNRT2A* with or without GFP-tag in the *atnrt2.1-1* mutant background to
495 investigate if *BdNRT2A* can functionally complement *atnrt2.1-1*, but surprisingly the functional
496 complementation was not observed, since the different complemented lines showed a reduced growth
497 phenotype and a lower HATS activity, similar to *atnrt2.1-1*. This lack of functional complementation
498 of *atnrt2.1-1* mutant by overexpression of *BdNRT2A* is probably due to the subcellular localization of
499 the *BdNRT2A*-GFP fusion protein, that was cytosolic and not in the pm as initially expected. Our results
500 remind the diffused fluorescence throughout the cell, that was observed when *AtNRT2.1* fused to GFP
501 was constitutively expressed in *atar2.1-1* (also named *atnrt3.1*) background, while in wild type
502 background, it was clearly associated with pm (Orsel *et al.*, 2007). The authors hypothesized that
503 *AtNRT3.1* is involved in the stability of *AtNRT2.1* and possibly through the pm targeting process,
504 leading to NO₃⁻ uptake, as it was demonstrated later (Okamoto *et al.*, 2006, Wirth *et al.*, 2007, Kotur *et al.*,
505 *et al.*, 2012). Besides, we confirm that *BdNRT2A* and *BdNRT3.2* are responsible for the two-component
506 system of HATS using a heterologous expression system in oocytes, similarly to other species.
507 Consequently, we hypothesized that, in contrast to *NpNRT2.1* (Filleur *et al.*, 2001), *BdNRT2A* could
508 not interact with *AtNRT3.1* in the *atnrt2.1-1* line overexpressing *BdNRT2A* with or without GFP-tag
509 and that was subsequently verified using transfection of *Arabidopsis* mesophyll protoplasts in either a
510 wild type or an *atnrt2.1-1* background. When transfection was performed with *BdNRT2A* fused to *GFP*,
511 *BdNRT2A* was cytosolic, and on the contrary, the co-expression of *BdNRT2A* and *BdNRT3.2*, fused to
512 *GFP* and *RFP* respectively, allowed the targeting of the *BdNRT2A/BdNRT3.2* to the pm in either an
513 *atnrt2.1-1* or an *atnrt2.1-1 x atnrt3.1* background. Post-translational regulations of *AtNRT2* occur in
514 response to variations of N supply (Engelsberger and Schulze 2012; Menz *et al.*, 2016), and

515 phosphorylation of AtNRT2.1-S²⁸ is crucial for the AtNRT2.1 stability in response to NO₃⁻ limitation
516 (Zou *et al.*, 2020). Recently, S501 in the C-terminus of AtNRT2.1 was found to be phosphorylated in
517 NH₄NO₃ conditions leading to inactivation of AtNRT2.1 and decrease of NO₃⁻ influx, but not to a
518 dissociation of the AtNRT2.1/AtNRT3.1 complex (Jacquot *et al.*, 2020). Interestingly this S501 residue
519 is not conserved in monocotyledons, such as Brachypodium, and replaced by a Gly. On the other hand,
520 another residue Ser is present in monocotyledonous and not conserved in dicotyledons, and this residue
521 S463 was required for interaction of HvNRT2.1 and HvNRT3.1 (Ishikawa *et al.*, 2009). Thus, we
522 hypothesized that this residue could play an important role in the species specificity we observed for
523 NRT2.1/NRT3.1 interaction. Using the transfection of protoplasts, we observed no effect of the S461
524 substitution to S461A and S461D mimicking respectively constitutively non-phosphorylated and
525 phosphorylated residues. The plasmalemmic sub-cellular co-localization of BdNRT2A and BdNRT3.2 was
526 not changed by the substitution of Ser461 suggesting that it was not involved in the regulation of
527 BdNRT2A and BdNRT3.2 interaction in Brachypodium unlike in barley.

528

529 In conclusion, we demonstrated that BdNRT2A has a major role in NO₃⁻ uptake at low N availability,
530 and that BdNRT2A and BdNRT3.2 are the main component of the HATS in Brachypodium. The
531 functional complementation experimentations in Arabidopsis suggested that a
532 monocotyledons/dicotyledons species specificity exists for the NRT2A and NRT3 interaction, that was
533 not explained by the presence of specific residue S461 conserved only in monocotyledons.

534

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542

543 **Authors contribution**

544 SFM, LCD designed and planned the experiments at IJPB. LCD, PB, SFM, MG, TG performed or
545 participated to the various experiments. LCD performed the oocyte ¹⁵N uptake experiments at the John
546 Ines Center under the supervision of AJM. TG planned the production of the amiRNA and PB generated
547 the transformed plants. LCD selected the tilling mutants under the supervision of MD and AB. SFM,
548 PB, TG and LCD performed the ¹⁵N influx experiments. SFM, PB and LCD performed the protoplasts
549 transfection, and confocal analyses. AM performed the ¹⁵N and N analyses. SFM, LCD, TG analyzed
550 the data. TG, FDV and AK contributed to the design of this study. SFM wrote the manuscript and AK,
551 TG, LCD, FDV, AJM, AM participated to its critical reading.

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705

List of primers

| | forward | | reverse | |
|---|---------------------|---|----------------------|---|
| Constructions of GFP fusions and functional complementation of <i>atnrt2.1-1</i> with <i>BdNRT2A</i> | | | | |
| | 70F2 | GCGGCGAAG AGCAAGTTC | 70R2 | AGACGTGCTGG GGAGTGTT |
| | Bd70start | GGAGATAGA ACCATGGCG GCGAAGAGC | Bd70end | TCCACCTCCGG ATCAGACGTGC TGGGGAG |
| | U3endstop | AGATTGGGG ACCACTTTGT ACAAGAAAG CTGGGTCTCC ACCTCCG | U5 | GGGACAAGTT TGTACAAAAA GCAGGCTTCGA AGGAGATAGAA CCATG |
| Constructions of RFP fusions with <i>BdNRT3.2</i> | | | | |
| | EcoRI- BdNRT3_qL | GAATTC AAT GGCACGGCA CGGTCT | BdNRT3_2 qR- SalI | ACAGCAATCTA TTGCAAGGCGT CGAC |
| Directed mutagenesis of <i>BdNRT2A</i> | | | | |
| | BdNRT2AS454D | CGCCGCCGA GTGGGCTGA GGAGGAGAA GAG | BdNRT2AS454D | CGCCGCCGAGT GGGCTGAGGAG GAGAAGAG |
| | BdNRT2AS454A | G TACTACGC CGCCGAGTG GGATGAGGA GGAGAAGAG CAAG | BdNRT2AS454A | CTTGCTCTTCTC CTCCTCATCCCA CTCGGCGGCGT AGTAC |
| RT-qPCR | | | | |
| <i>BdNRT2A</i> (<i>Bradi3g01270</i>) | | GTCGGGTTCC ATCTTCTCG | | CCGATGATCTTG CTGTTGAA |
| <i>BdNRT2B</i> (<i>Bradi3g01250</i>) | | TAAGCTAGC TCGGACATG GA | | ATGATAGGCAC CAGGGGC |
| | | | | |
| <i>BdNRT2D</i> (<i>Bradi3g01277.1</i>) | | GCTTCGGCTT GGCTAATAT C | | ACCATGGATAC GATGGAGGT |
| <i>BdNRT2.E</i> (<i>Bradi2g47640</i>) | | CAACTACCA CAAGCTGCA CA | | TAGAAGTACTG CGCCACGAT |
| <i>BdNRT2.F</i> (<i>Bradi2g26210.1</i>) | | CTCCTCTACG GCTACTGCA C | | GGAGGATCCAG ATGTTCCAA |
| <i>BdEF1a</i> (<i>DV482887</i>) | | CCATCGATAT TGCCTTGTGG | | GTCTGGCCATCC TTGGAGAT |
| <i>BdUBC18</i> (<i>DV481689</i>) | | GGAGGCACC TCAGGTCATT T | | ATAGCGGTCAT TGTCTTGCG |
| <i>BdSAMDC</i> (<i>DV482676</i>) | | TGCTAATCTG CTCCAATGG C | | GACGCAGCTGA CCACCTAGA |
| <i>BdUbi10</i> (<i>DV484269</i>) | | TCCCACTCC ACTTGGTGCT | | GAGGGTGGACT CCTTTTGGGA |

| | | | | |
|---|----------------|------------------------------|--------------|-------------------------|
| Selection of homozygous tilling <i>bdnrt2a</i> mutant and Search of T-DNA insertional mutant in <i>BdNRT2A</i> | BdNRT2AspeciF | GTGGAGACC GGCGACATG | BdNRT2A2BRev | AACTTTTGGCCG GGGAGAT |
| | BdNRT2BspeciF | TAAGCTAGC TCGGACATG GA | | |
| RT-PCR | | | | |
| Search of T-DNA insertional mutant in <i>BdNRT2A</i> | BdNRT2AspecifF | GTGGAGACC GGCGACATG | 70R2 | AGACGTGCTGG GGAGTGTT |
| | BdNRT2BspecifF | TAAGCTAGC TCGGACATG GA | | |
| | | | | |

Table 1 : List of mutation sites identified in *BdNRT2A* by screening the Versailles NaN₃ mutant collection (Dalmais *et al.* 2013).

^a Point mutation positions of in mutants relative to the starting ATG on the coding sequence.

^b Position of amino acid substitution in mutants are relative to the starting methionine of the encoded protein.

^c numbers are predictive score from the SIFT software (<http://sift.bii.a-star.edu.sg/>).

| Nucleic acid mutation ^a | Amino acid substitution ^b | SIFT ^c | Name of the mutant line chosen in this study |
|------------------------------------|--------------------------------------|-------------------|--|
| <i>G315A</i> | G105G | - | - |
| <i>T678C</i> | D226D | - | - |
| <i>C672T</i> | L224L | - | - |
| <i>G736A</i> | V336I | 0.2 | |
| <i>G744A</i> | W248* STOP | 0 | 9.2 |
| <i>C818T</i> | T273I | 0.46 | - |
| <i>G862A</i> | D288N | 0.9 | - |

Figure 1. Root expression levels of *BdNRT2A* (1A), *BdNRT2B* (1B) and *BdNRT3.2* (1C) in response to 3h NO₃⁻ re-supply (+N) after 4 days of N deprivation (-N).

Gene expressions were quantified by qRT-PCR and were normalized to the level of a synthetic reference gene (SRG). *BdEF1α*, *BdUBC18* and *BdSAMDC* were used to compose the SRG. Values are means ± SD of 4 biological replicates.

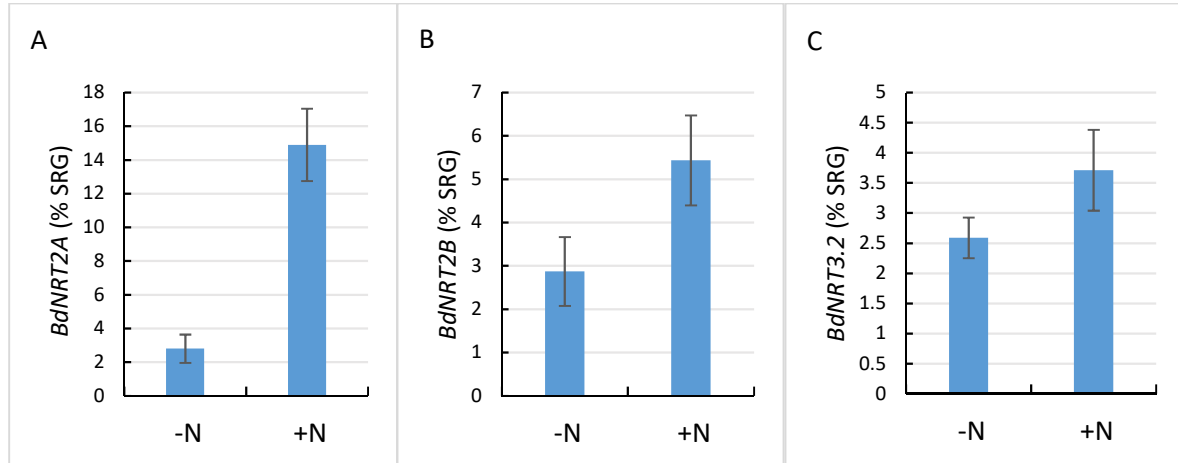


Figure 2. $^{15}\text{NO}_3^-$ influx in heterologous expression system *Xenopus laevis* oocytes.

Xenopus laevis oocytes were injected with nuclease-free water, *BdNRT2A*, *BdNRT3.2* or co-injected with *BdNRT2A* and *BdNRT3.2* cRNAs as indicated. After three days, oocytes were incubated in a solution enriched with 0.5 mM $\text{Na}^{15}\text{NO}_3$ (atom% ^{15}N : 98%) and the ^{15}N enrichment of individual oocytes was measured after 16h. The values are means \pm SD of five replicates from a representative experiment. Asterisk indicates a statistically significant difference (Mann and Whitney, $p < 0.05$).

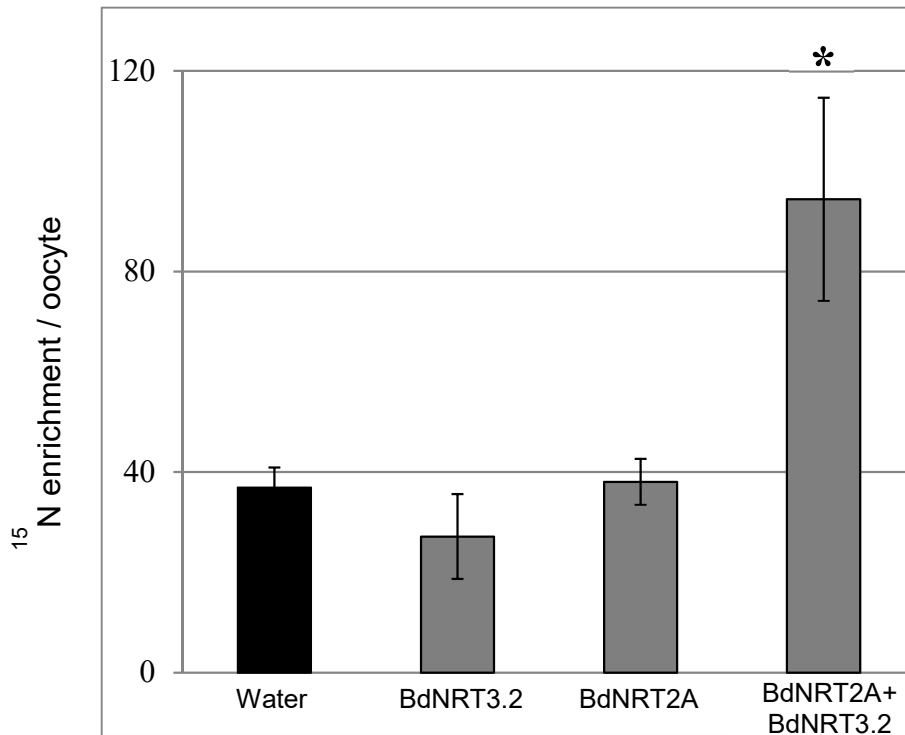


Figure 3. Growth phenotype of *bdnrt2a* mutants.

Wild type and *bdnrt2a* mutants [two amiRNA lines : *amiRj2* and *amiRn3*, and one TILLING *bdnrt2a-W248** mutant (9.2), and two azygotes lines az1 and az2] were grown in hydroponics with 0.2 mM NO₃⁻ in controlled conditions, and the shoot/root biomass ratio (A), root length (B) and tiller number (C) were measured for 3 week-old plants. Values are means ± SD of 12 (A) and 6 replicates (B, C). Statistical analyses were performed using one-ANOVA and the means were classified using Tukey HSD test. (P<0.05).

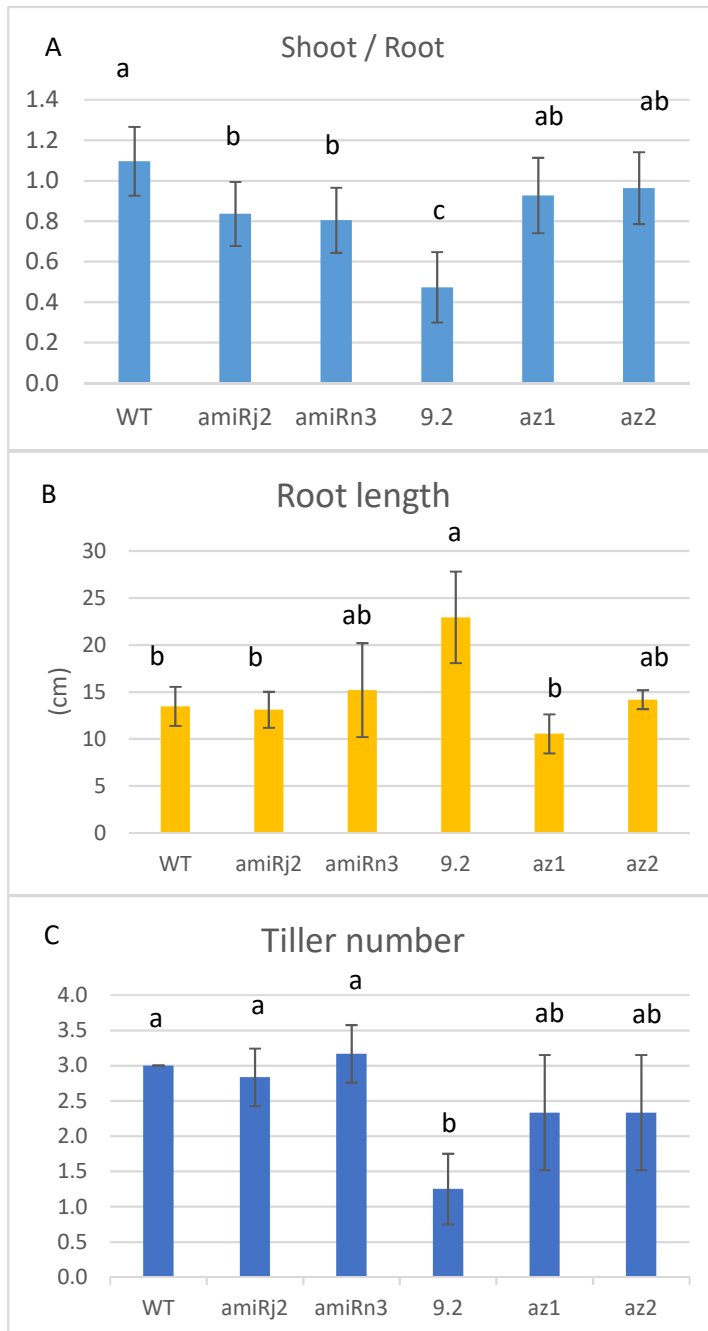


Figure 4. HATS and LATS activities in *bdnrt2a* mutants.

Wild type and *bdnrt2a* mutants were grown in hydroponics with 0.2 mM NO_3^- in controlled conditions, and HATS (A) and LATS (B) were measured at 0.2 mM $^{15}\text{NO}_3^-$ and 6 mM $^{15}\text{NO}_3^-$ respectively for 3 week-old plants. Values are means \pm SD of 12 replicates. Statistical analyses were performed using one-way ANOVA and the means were classified using Tukey HSD test. ($P < 0.05$).

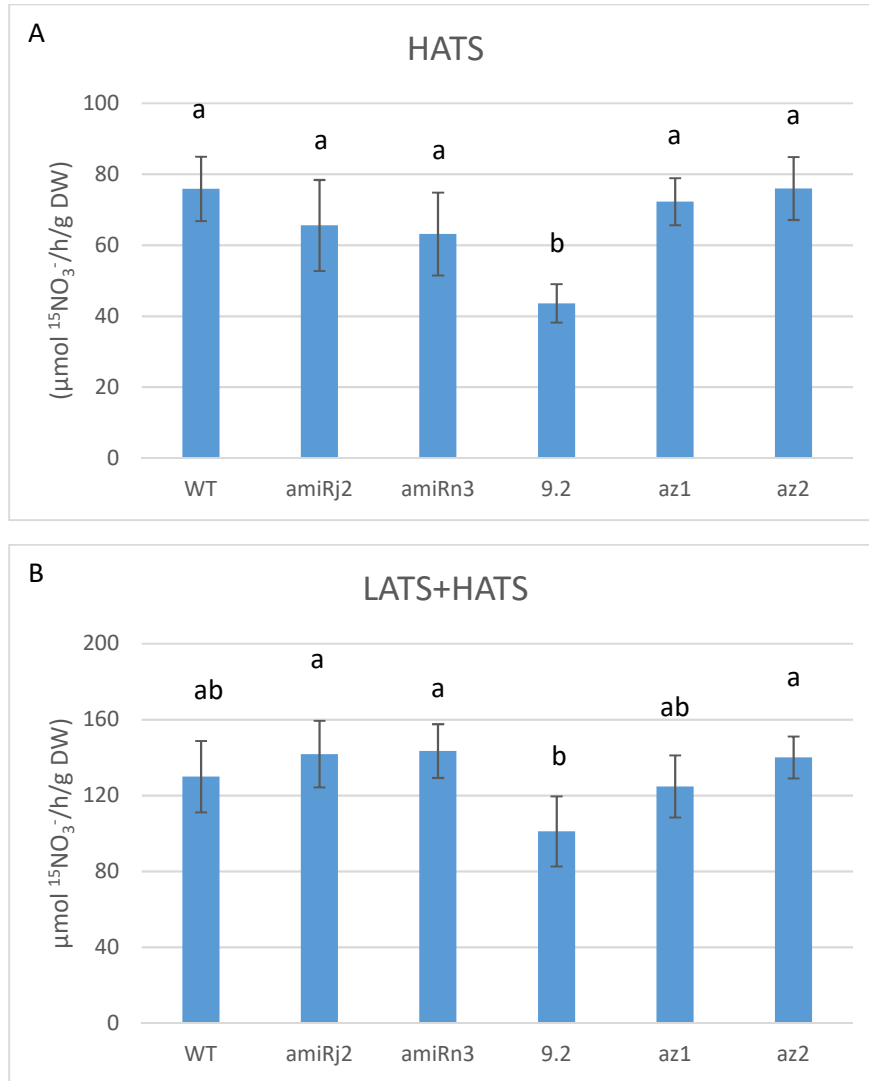


Figure 5. Total N and nitrate contents in roots of *bdnrt2a* mutants.

Wild type and *bdnrt2a* mutants were grown in hydroponics with 0.2 mM NO_3^- in controlled conditions, and the total N content (A) and root nitrate content (B) were measured for 3 week-old plants. Values are means \pm SD of 12 replicates. Statistical analyses were performed using one-way ANOVA and the means were classified using Tukey HSD test. ($P < 0.05$).

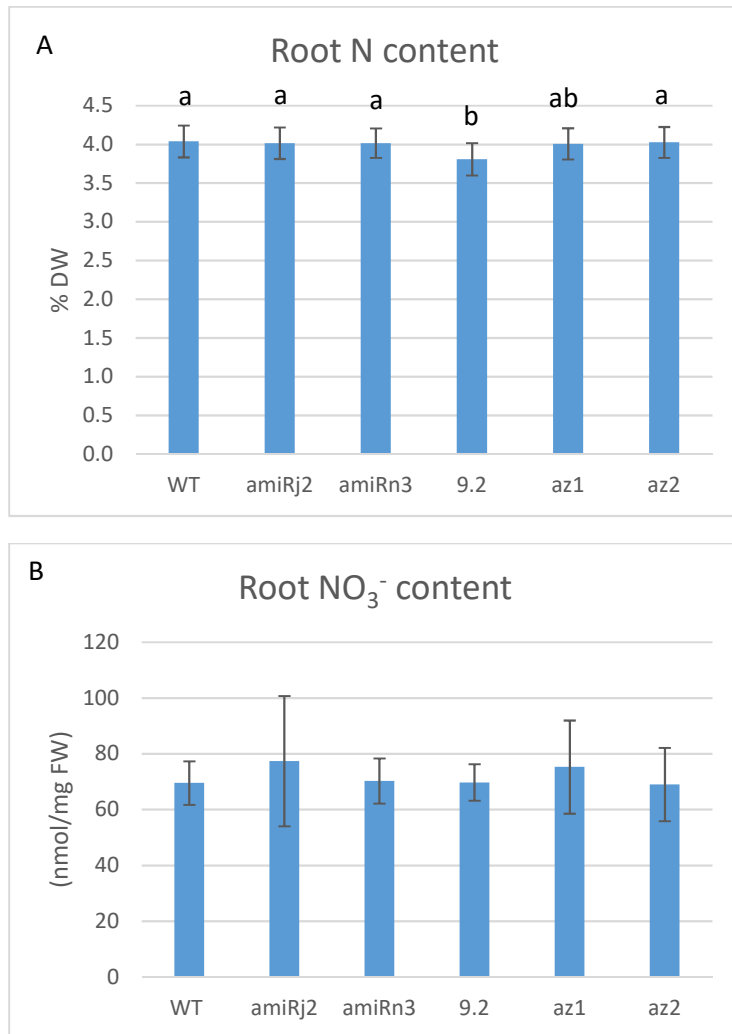


Figure 6. Overexpression of *BdNRT2A* in *atnrt2.1-1* mutant.

(A) Rosette area of wild type (WS), *atnrt2.1-1* and three lines expressing *Pro35S::BdNRT2A*, *Pro35S::GFPBdNRT2A* or *Pro35S::BdNRT2A-GFP* in the *atnrt2.1-1* mutant. Plants were grown hydroponically for 40 days on a nutrient solution containing 0.2 mM NO_3^- .

(B) $^{15}\text{NO}_3^-$ influx (HATS), measured at the external concentration of 0.2 mM $^{15}\text{NO}_3^-$. Values are means of 9 replicates \pm SE.

(C) Sub-cellular localization of *BdNRT2A* fused to GFP in leaves of *atnrt2.1-1* mutant lines expressing *Pro35S::GFP-BdNRT2A*. For each construct, a representative picture of the 3 independent transgenic lines is presented for each construct.

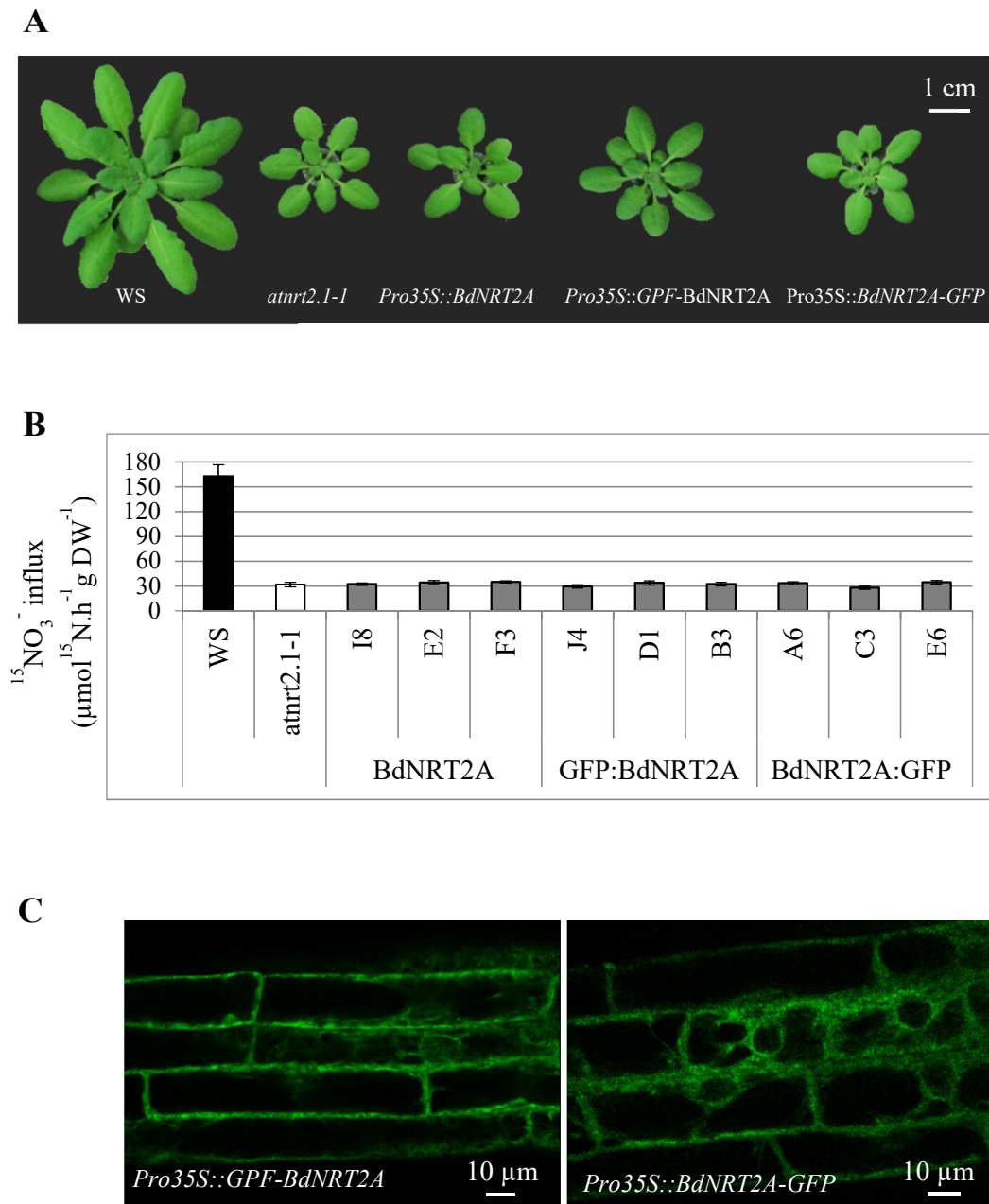


Figure 7. Subcellular localization of BdNRT2A-GFP and BdNRT3.2-RFP fusion proteins in Arabidopsis mesophyll protoplasts. Confocal images from *atnrt2.1-2* protoplasts transiently expressing *Pro35S::GFP-BdNRT2A* (A), or transiently co-expressing *Pro35S::GFP-BdNRT2A* and *Pro35S::RFP-BdNRT3.2* (B). Different images are presented in Fig (A) : GFP fluorescence(a), bright-field image (b), chlorophyll auto-fluorescence indicating position of chloroplasts (c), and merged (d). Different images are presented Fig (B) : GFP fluorescence (a), RFP fluorescence (b), chlorophyll auto-fluorescence indicating position of chloroplasts (c) and merged (d). Scale bar = 5 μ m.

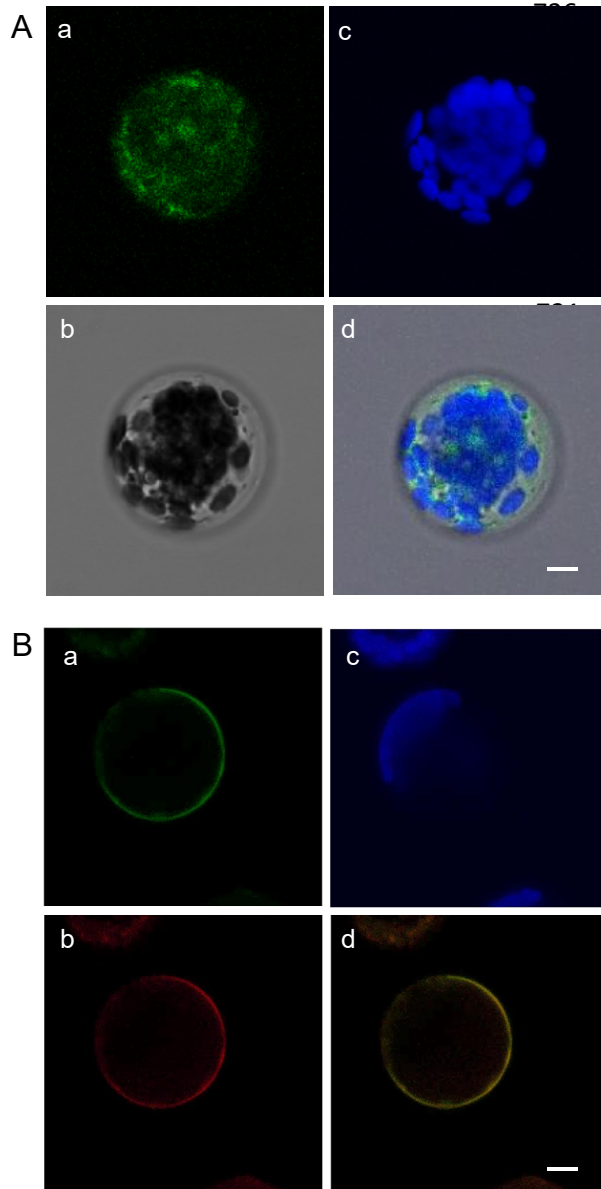


Figure 8. Subcellular localization of BdNRT2A-GFP and BdNRT3.2-RFP fusion proteins in Arabidopsis mesophyll protoplasts. Confocal images of protoplasts from the *atnrt2.1-2* mutant line B3 expressing *Pro35S::GFP-BdNRT2A* and transiently expressing *Pro35S::RFP-BdNRT3.2*. Six images are presented : GFP fluorescence (a), RFP fluorescence (b), chlorophyll auto-fluorescence indicating position of chloroplasts (c), GFP and RFP merged with autofluorescence (d), bright field (e), and merged of all images (f). Scale bar = 5 μ m.

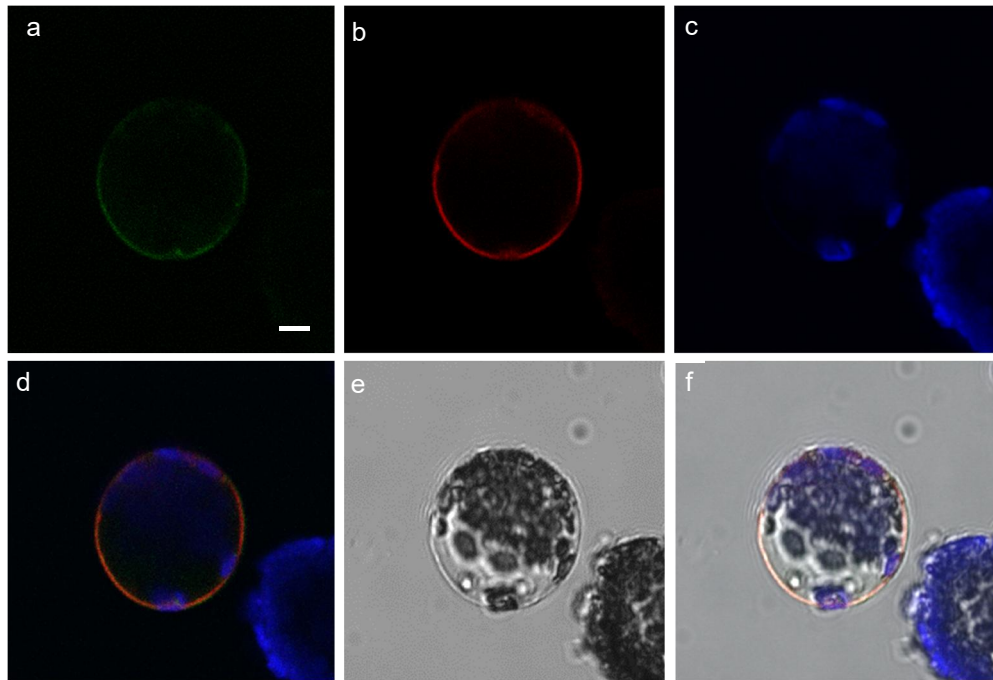


Figure 9. Subcellular localization of BdNRT2A^{S461A}-GFP or BdNRT2A^{461D}-GFP and Bd NRT3.2-RFP fusion proteins in Arabidopsis mesophyll protoplasts. Confocal images of protoplasts of *atnrt2.1-2xatnrt3.1* double mutant transiently co-expressing *Pro35S::GFP-BdNRT2A^{S461A}* and *Pro35S::RFP-BdNRT3.2* (A), or transiently co-expressing *Pro35S::GFP-BdNRT2A^{461D}* and *Pro35S::RFP-BdNRT3.2* (B). Four images are presented : GFP fluorescence (a), RFP fluorescence (b), merged (c), and chlorophyll auto-fluorescence indicating position of chloroplasts (d). Scale bar = 5 μ m.

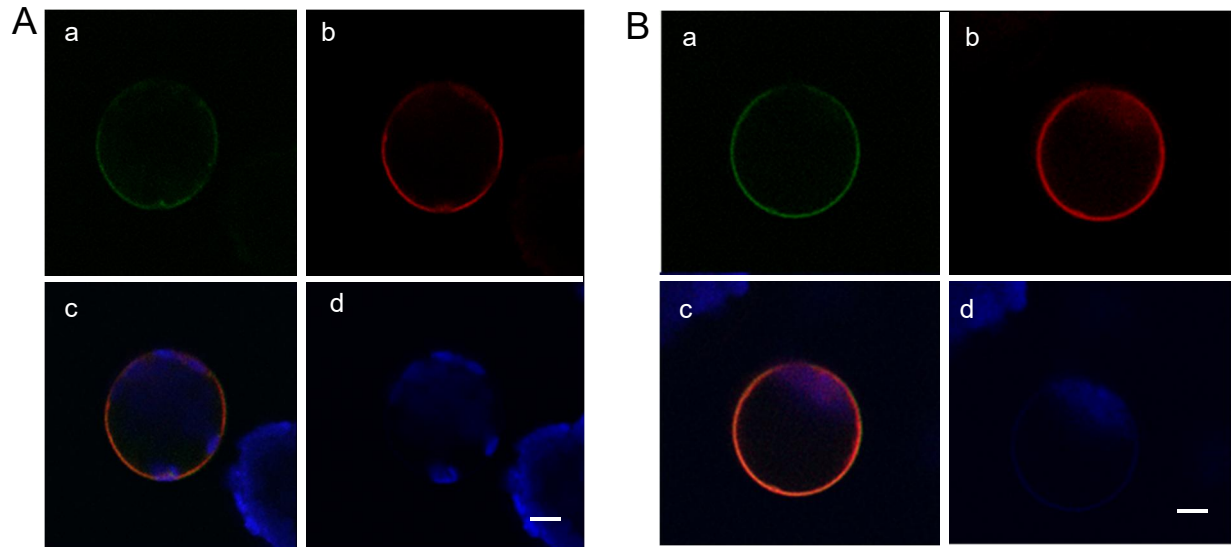


Figure S1. Root expression levels of *BdNRT2D* (S1A), *BdNRT2F* (S1B) and *BdNRT2E* (S1C) in response to 2h NO_3^- re-supply (+N) after 4 days of N deprivation (-N).

Genes expressions were quantified by qRT-PCR and were normalized to the level of a synthetic reference gene (SRG). *BdEF1 α* , *BdUBC18* and *BdSAMDC* were used to compose the SRG. Values are means \pm SD of 4 biological replicates.

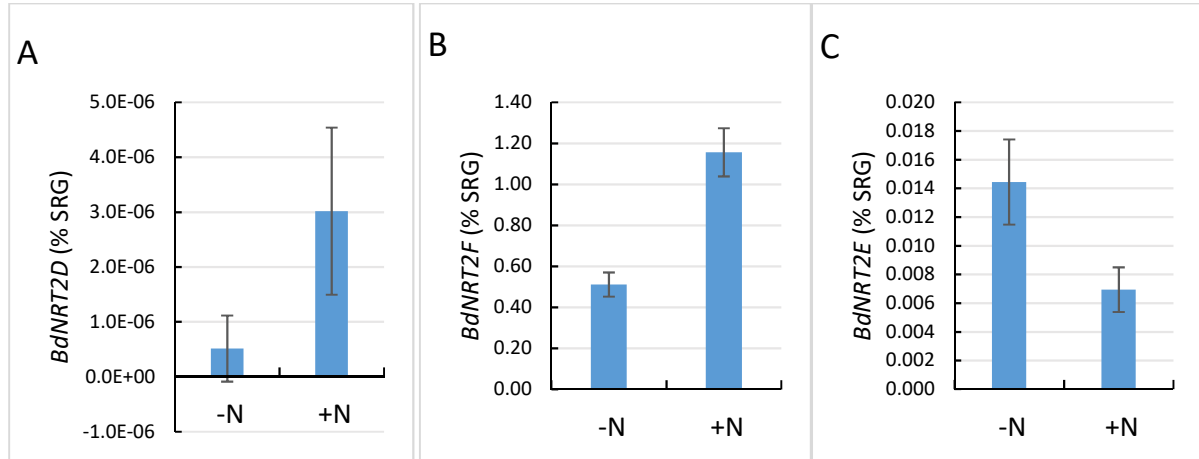


Figure S2. Root expression levels of *BdNRT2A* (4A) and *BdNRT2B* (4B) in amiRNA mutants *amiRj2*, *amiRn3*.

Gene expressions were quantified by qRT-PCR and were normalized to the level of a synthetic reference gene (SRG). *BdUBI10* and *BdSAMDC* were used to compose the SRG. Values are means \pm SD of 4 biological replicates.

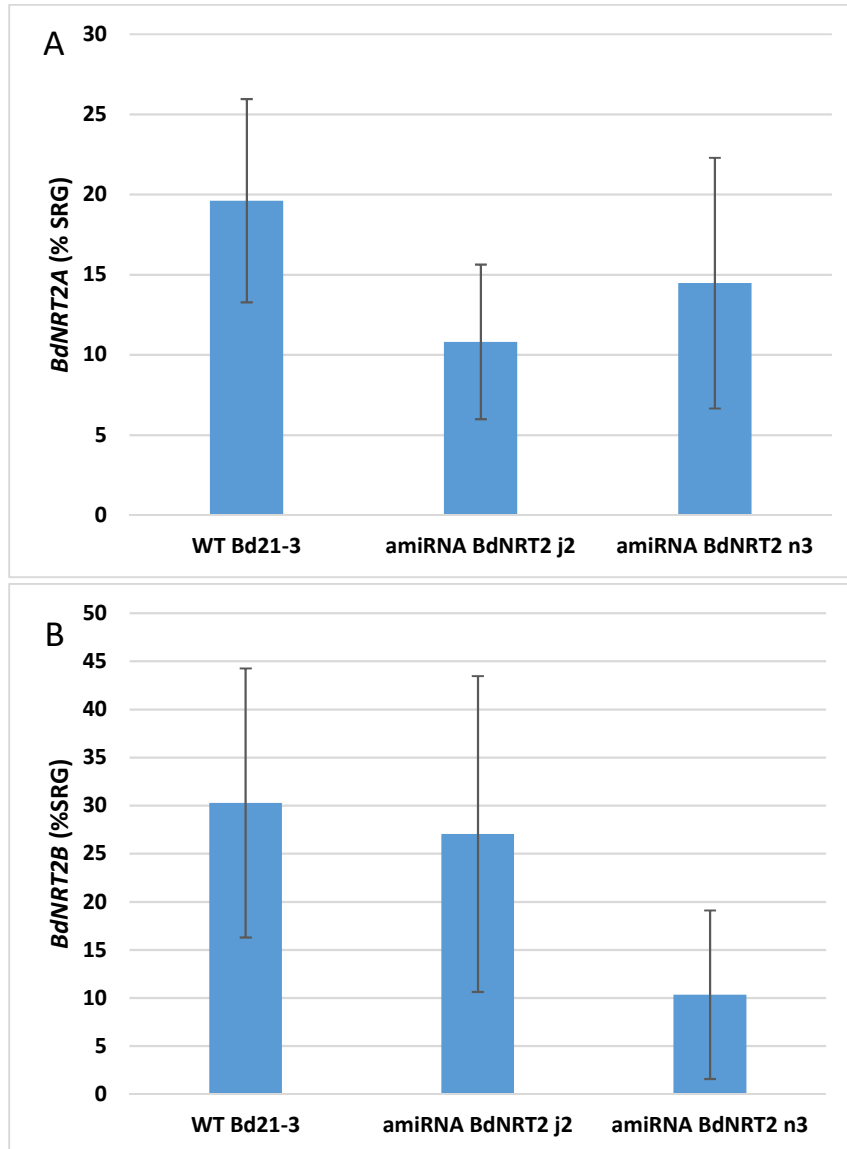


Figure S3. Growth phenotype of *bdnrt2a* mutants.

Wild type and *bdnrt2a* mutants were grown in hydroponics with 0.2 mM NO₃⁻ in controlled conditions, and the root biomass ratio (A) and shoot biomass (B) were measured for 3 week-old plants. Values are means ± SD of 12 replicates. Statistical analyses were performed using one-way ANOVA and the means were classified using Tukey HSD test. (P<0.05).

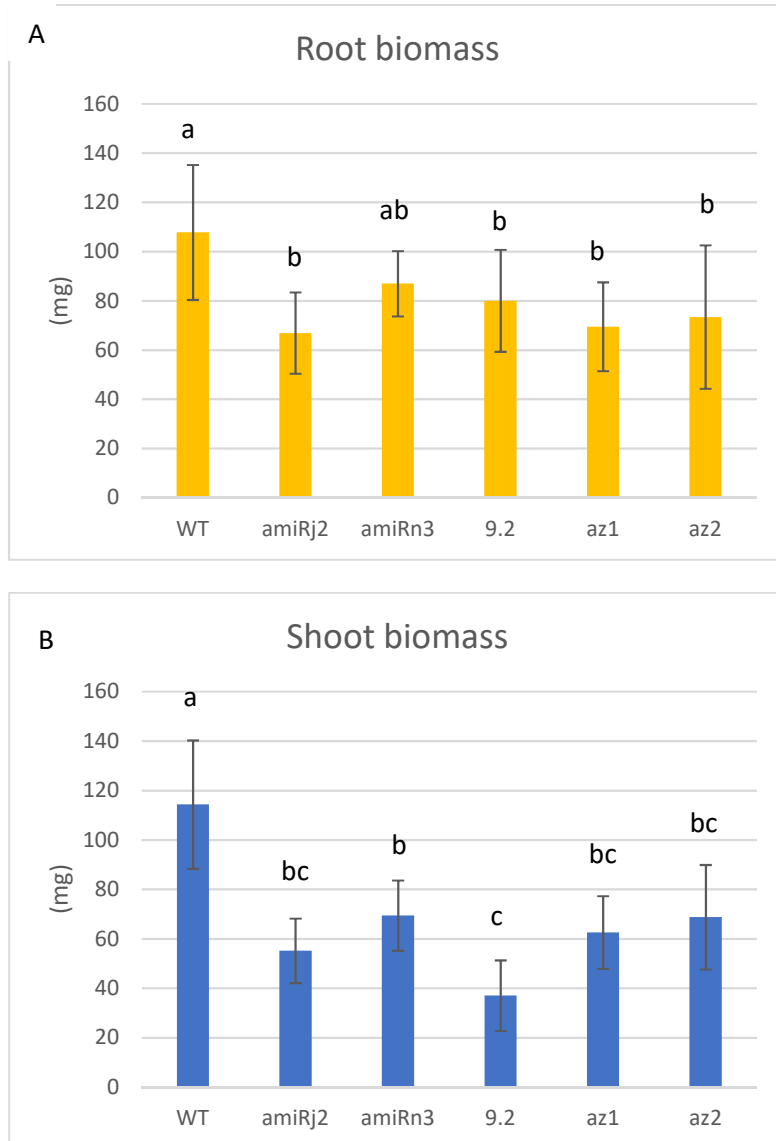


Figure S4. Total C and C/N in roots and shoot nitrate content in *bdnrt2a* mutants.

Wild type and *bdnrt2a* mutants were grown in hydroponics with 0.2 mM NO₃⁻ in controlled conditions. Total C content and C/N in roots (A, B) and shoot nitrate content (C) were measured for 3 week-old plants. Values are means ± SD of 12 replicates. Statistical analyses were performed using one-way ANOVA and the means were classified using Tukey HSD test. (P<0.05).

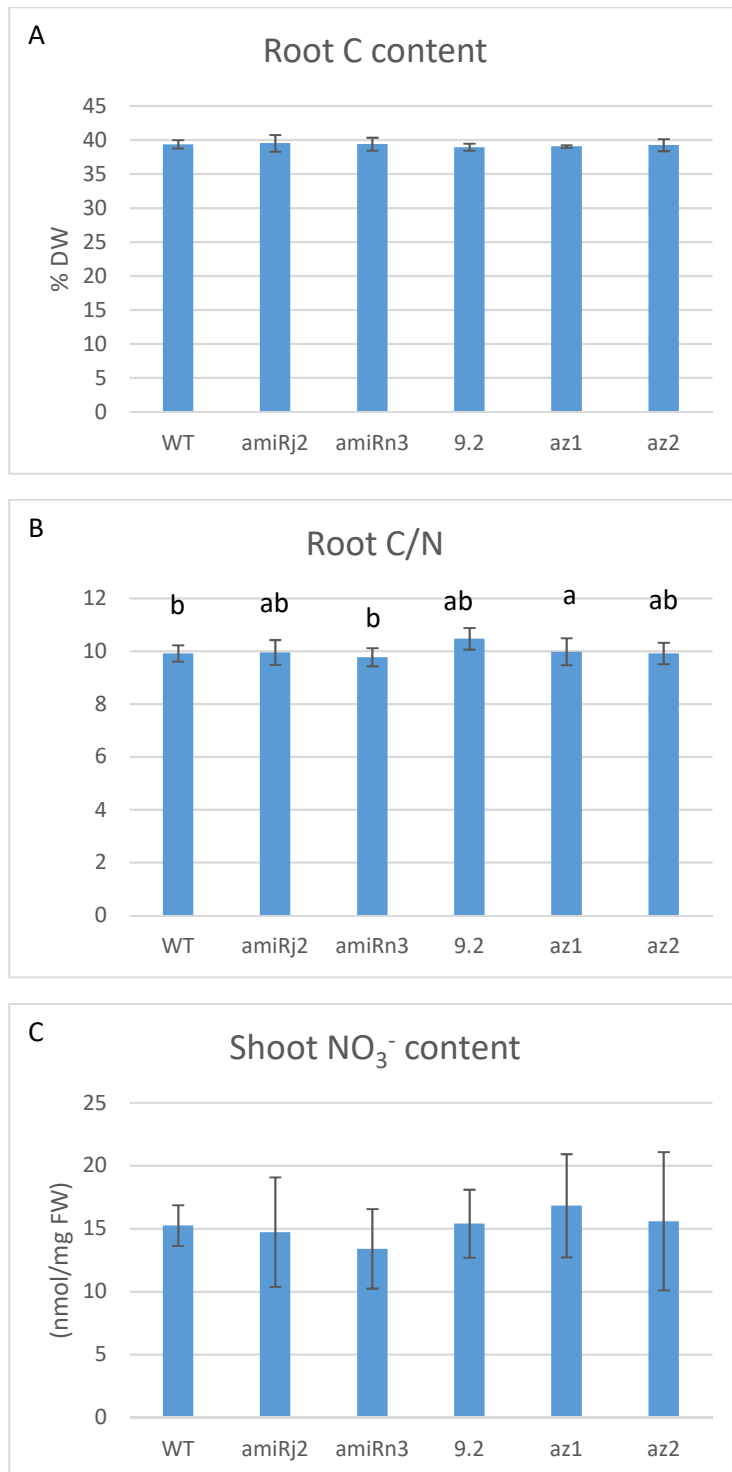


Figure S5. Growth phenotype of *bdnrt2a* mutants under varying nitrate supply.

Wild type and *bdnrt2a* mutants were grown in hydroponics with 0.02, 0.2 and 10 mM NO_3^- in controlled conditions. Shoot/root ratio (A), root length (B), tiller number (D) and were measured for 3 week-old plants. Values are means \pm SD of 12 replicates. Statistical analyses were performed using one-way ANOVA and the means were classified using Tukey HSD test. ($P < 0.05$).

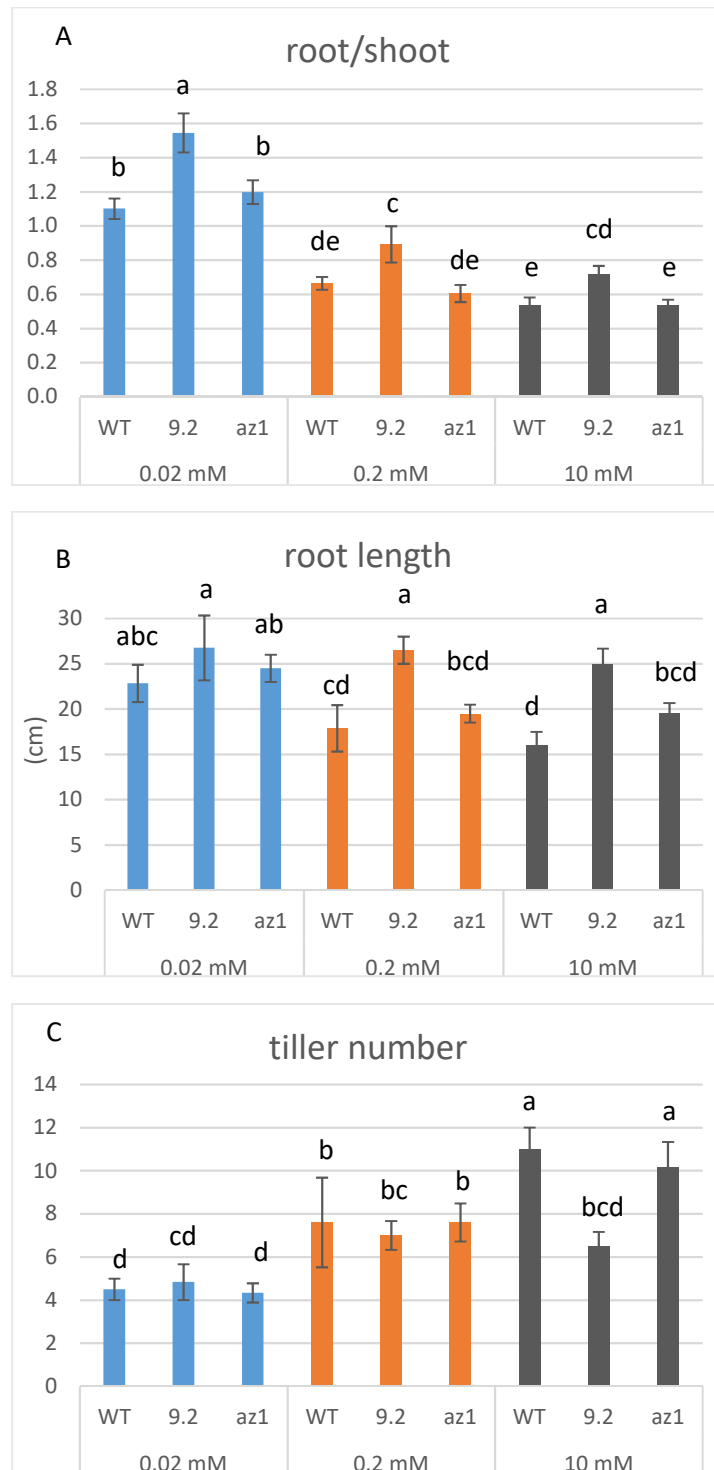


Figure S6. Overexpression of *BdNRT2A* in *atnrt2.1-1* mutant

Overexpression of *BdNRT2A* has been quantified in *atnrt2.1-1* lines overexpressing *Pro35S::BdNRT2A*. Gene expressions were quantified by qRT-PCR and was normalized to the level of a synthetic reference gene (SRG). *BdACT* and *BdPP2A3* were used to compose the SRG.

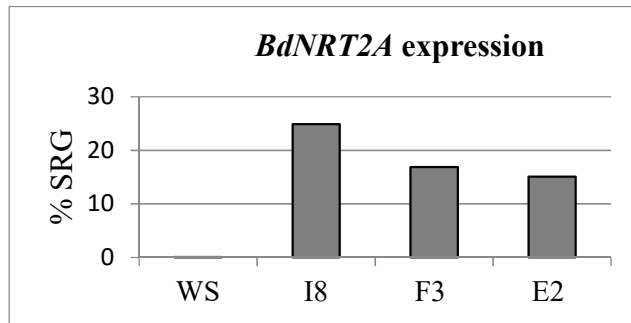


Figure S7. Subcellular localization of BdNRT2A-GFP fusion proteins in Arabidopsis mesophyll protoplasts in *atnrt2.1-2* mutant line B3. Confocal images from protoplasts of *atnrt2.1-2* mutant line B3 expressing *Pro35S::GFP-BdNRT2A*. Four images are presented : GFP fluorescence (a), bright field (b), chlorophyll auto-fluorescence indicating position of chloroplasts (c), and merged (d). Scale bar = 5 μ m.

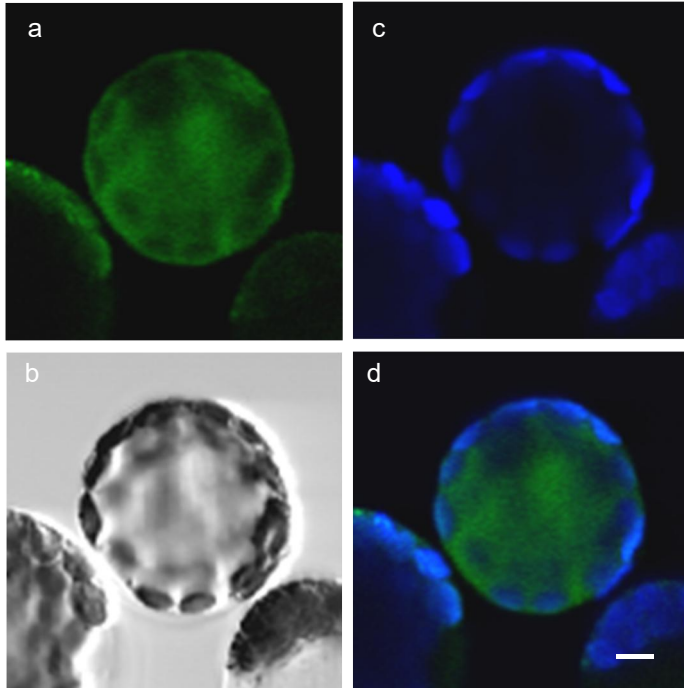
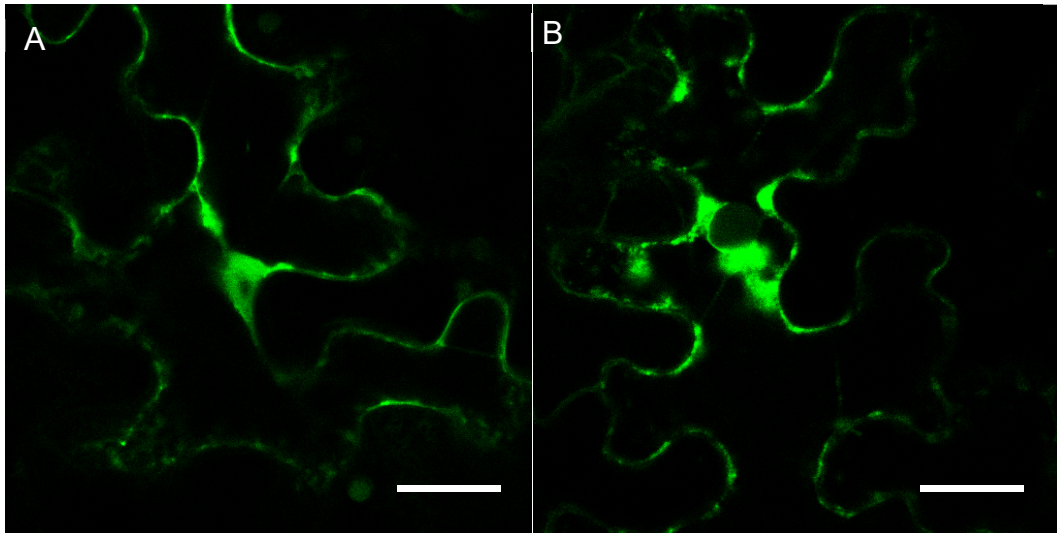


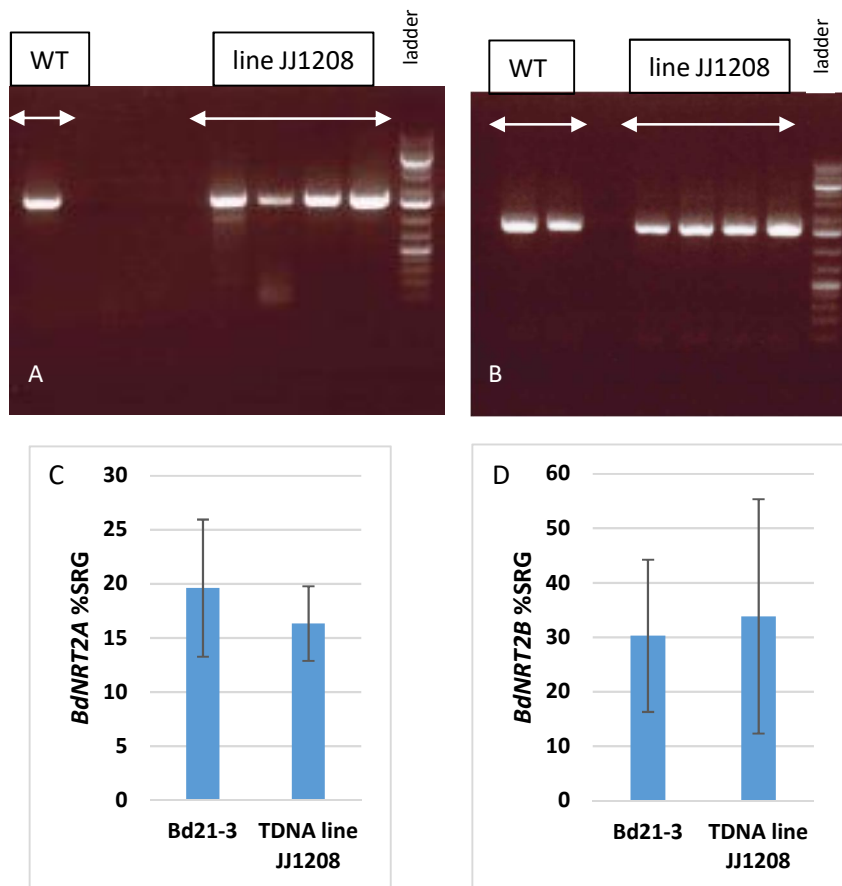
Figure S8. Subcellular localization of BdNRT2A-GFP fusion protein in *Nicotiana benthamiana* leaves. Confocal images from mesophyll tobacco cell transiently expressing *Pro35S::GFP-BdNRT2A* (A), *Pro35S::BdNRT2A-GFP* (B). GFP fluorescence are presented. Scale bar = 50 μ m.



Supplementary data S1 : Absence of a T-DNA insertion in the *BdNRT2A* gene in line JJ1208 (obtained from the JGI Brachypodium collection T-DNA mutants in the Bd21-3 accession) demonstrated by the quantification of *BdNRT2A* and *BdNRT2B* gene expression in roots of plants homozygous for the resistance to hygromycin.

S1A-B. Gene expressions were measured by RT-PCR (using specific primers (*BdNRT2AspecifF* and *70R2* for *BdNRT2A*) (S1A) and (*BdNRT2BspecifF* and *70R2* for *BdNRT2B*) (S1B) (annealing temperature 54°C, 35 cycles of amplification). Products of amplification (1.5kb) were separated by electrophoresis on 1% agar gel, and GeneRuler™ 1kb plus ladder (Fermentas) was used for the size quantification. Four independent plants are shown for the T-DNA line JJ1208.

S1C-D. Gene expressions were quantified by qRT-PCR using specific primers (see list of primers) for *BdNRT2A* (S1C) and *BdNRT2B* (S1D) and were normalized to the level of a synthetic reference gene (SRG). *BdUBI10* and *BdSAMDC* were used to compose the SRG. Values are means \pm SD of 4 biological replicates.



Supplementary data S2 : Sequences of amiRNA constructs in pUC57-Kan.

Target-specific sequences are in bold; *attL1* and *attL2* sequences for Gateway LR reaction are underlined; remains of *EcoRV* sites used to clone the fragments in pUC57-Kan are highlighted in grey.

These constructs allowed the generation of two amiRNA mutant lines (*amiRj2* with *BdNRT2s*-amiR and *amiRn3* with *BdNRT2s*-amiR) as described in the results.

>*BdNRT2A*-amiR2

CGGTACCTCGCGAATACATCTAGATCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTTCGTT
GCAACAAATTGATGAGCAATGCTTTTTATAATGCCAACTTTGTACAAAAAGCAGGCTCCGCGGC
CGCCCCCTTACCTCGGATCCCAGCAGCAGCCACAGCAAAATTTGGTTTGGGATAGGTAGGTGT
TATGTTAGGTCTGGTTTTTTGGCTGTAGCAGCAGCAGT**AAAGACAGCACCCAGACGCGGC**CAGGAG
ATTCAGTTTGAAGCTGGACTTCACTTTTGCCTCTCT**CCGCGACTGCTGCTGCTTTT**ATTCCTGCTG
CTAGGCTGTTCTGTGGAAGTTTGCAGAGTTTATATTATGGGTTTAAATCGTCCATGGCATCAGCATC
AGCAGCGGTACCGAGGGCTGCAGGAATTCAAGGGTGGGCGCGCCG**ACCCAGCTTTCTTGTACA**
AAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAA
ATAAAATCATTATTTGATCGGATCCCGGGCCCGT**CGACTGCAGAGGCC**

>*BdNRT2s*-amiR

CGGTACCTCGCGAATACATCTAGATCAAATAATGATTTTATTTTGACTGATAGTGACCTGTTTCGTT
GCAACAAATTGATGAGCAATGCTTTTTATAATGCCAACTTTGTACAAAAAGCAGGCTCCGCGGC
CGCCCCCTTACCTCGGATCCCAGCAGCAGCCACAGCAAAATTTGGTTTGGGATAGGTAGGTGT
TATGTTAGGTCTGGTTTTTTGGCTGTAGCAGCAGCAGT**ATCATGATGCCACGTA**CTACAGGAGA
TTCAGTTTGAAGCTGGACTTCACTTTTGCCTCTCT**TAGTAGGTGCGCATCATGAT**ATTCCTGCTG
CTAGGCTGTTCTGTGGAAGTTTGCAGAGTTTATATTATGGGTTTAAATCGTCCATGGCATCAGCATC
AGCAGCGGTACCGAGGGCTGCAGGAATTCAAGGGTGGGCGCGCCG**ACCCAGCTTTCTTGTACA**
AAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTGCAACGAACAGGTCACTATCAGTCAAA
ATAAAATCATTATTTGATCGGATCCCGGGCCCGT**CGACTGCAGAGGCC**

