Characterization of the signalling pathways involved in the repression of root nitrate uptake by nitrate in Arabidopsis thaliana

Valentin Chaput, Jianfu Li, David Séré, Pascal Tillard, Cécile Fizames, Tomas Moyano, Kaijing Zuo, Antoine Martin, Rodrigo A Gutiérrez, Alain Gojon, et al.

To cite this version:

Valentin Chaput, Jianfu Li, David Séré, Pascal Tillard, Cécile Fizames, et al.. Characterization of the signalling pathways involved in the repression of root nitrate uptake by nitrate in Arabidopsis thaliana. Journal of Experimental Botany, 2023, 74 (14), pp.4244 - 4258. 10.1093/jxb/erad149 . hal-04192715

HAL Id: hal-04192715
https://hal.inrae.fr/hal-04192715

Submitted on 26 Oct 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Characterisation of the signalling pathways involved in the repression of root nitrate uptake by nitrate in *Arabidopsis thaliana*

Valentin Chaput[1], Jianfu Li[2], David Séré[1], Pascal Tillard[1], Cécile Fizames[1], Tomas Moyano[3], Kaijing Zuo[2], Antoine Martin[1], Rodrigo A. Gutiérrez[3], Alain Gojon[1], and Laurence Lejay[1*]

[1]IPSiM, Univ Montpellier, CNRS, INRAE, Institut Agro, 34060, Montpellier, France

[2]Plant Biotech Center: Center of Single Cell Research, School of Agriculture and Life Sciences, Shanghai Jiao Tong University, Shanghai 200240, China.

[3]Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Millennium Institute for Integrative Biology, Millennium Institute Center for Genome Regulation, Institute for Ecology and Biodiversity, Pontificia Universidad Católica de Chile, Santiago 8331150, Chile

e-mail :

valentin.chaputmontagnac@hotmail.fr

lijianfu120109@hotmail.com

david.serefily@gmail.com

tillardp@laposte.net

cecile.fizames@inrae.fr

tcmoyano@uc.cl

kjzuo@sjtu.edu.cn

antoine.martin@cnrs.fr

rgutierrez@bio.puc.cl

alain.gojon@inrae.fr

laurence.lejay@inrae.fr

© The Author(s) 2023. Published by Oxford University Press on behalf of the Society for Experimental Biology. All rights reserved. For permissions, please email: journals.permissions@oup.com

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

*Corresponding author: Laurence Lejay; Phone: +33-(0)4-99-61-26-12

Statement:

Characterisation and identification of molecular elements involved in the signalling pathways repressing NRT2s transporters and root nitrate uptake in response to nitrate.

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Abstract

In *Arabidopsis thaliana*, root high-affinity nitrate (NO_3^-) uptake depends mainly on NRT2.1, 2.4 and 2.5, which are repressed by high NO_3^- supply at the transcript level. For *NRT2.1*, this regulation is due to the action of (i) feedback downregulation by N metabolites and (ii) repression by NO_3^- itself mediated by the transceptor NRT1.1(NPF6.3). However, for *NRT2.4* and *NRT2.5* the signaling pathway(s) remain unknown along with the molecular elements involved. Here we show that unlike *NRT2.1*, *NRT2.4* and *NRT2.5* are not induced in a NO_3^- reductase mutant but are strongly upregulated following replacement of NO_3^- by ammonium (NH_4^+) as the N source. Moreover, increasing NO_3^- concentration in a mixed nutrient solution with constant NH_4^+ concentration results in a gradual repression of *NRT2.4* and *NRT2.5*, which is suppressed in a *nrt1.1* mutant. This indicates that *NRT2.4* and *NRT2.5* are subjected to repression by NRT1.1-mediated NO_3^- sensing, and not to feedback repression by reduced N metabolites. We further show that key regulators of NRT2s transporters, such as HHO1, HRS1, PP2C, LBD39, BT1 and BT2, are also regulated by NRT1.1-mediated NO_3^- sensing, and that several of them are involved in NO_3^- repression of *NRT2.1*, *2.4* and *2.5*. Finally, we provide evidence that it is the phosphorylated form of NRT1.1 at the T101 residue, which is most active in triggering the NRT1.1-mediated NO_3^- regulation of all these genes. Altogether, these data led to propose a regulatory model for high-affinity NO_3^- uptake in *Arabidopsis*, highlighting several NO_3^- transduction cascades downstream the phosphorylated form of the NRT1.1 transceptor.

Key Words: NO_3^- uptake, NRT2 transporters, BTB genes, NIGTs, LBDs, NO_3^- signaling, NRT1.1

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Introduction

The nitrogen (N) nutrition of most herbaceous plants relies on the uptake of nitrate (NO_3^-), which is ensured in root cells by two classes of transport systems. The High-Affinity Transport System (HATS) is predominant in the low range of NO_3^- concentrations (< 1 mM), whereas the Low-Affinity Transport System (LATS) makes an increasing contribution to total NO_3^- uptake with increasing external NO_3^- concentration (Crawford and Glass, 1998). In all species investigated to date, genes encoding the various transporter proteins involved in either HATS or LATS have mostly been identified in the *NRT2* and *NPF* (formerly *NRT1/PTR*) families, respectively (Nacry *et al.*, 2013; O'Brien *et al.*, 2016). With the exception of agricultural soils, where NO_3^- concentration can rise up to several millimolar after fertilizer application, it is generally assumed that root NO_3^- uptake is mostly determined by the activity of the HATS (Crawford and Glass, 1998; Malagoli *et al.*, 2004). In *Arabidopsis thaliana*, almost all the HATS activity in roots depends on three NRT2 transporters, namely NRT2.1, NRT2.4 and NRT2.5 (Filleur *et al.*, 2001; Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). Under most conditions, NRT2.1 is the main contributor to the HATS (Cerezo *et al.*, 2001; Filleur *et al.*, 2001). However, NRT2.4 and NRT2.5 display a very high-affinity for NO_3^- and are important for taking up this nutrient when present at very low concentration (<50 µM) in the soil solution (Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). Furthermore, unlike NRT2.1 and NRT2.4, NRT2.5 does not require the presence of NO_3^- to be expressed, and is therefore considered crucial for ensuring the initial uptake of NO_3^- as soon as it appears in the external medium (Kotur and Glass, 2015).

Plants have evolved to respond to a challenging environment where NO_3^- concentration in the soil is highly variable in both time and space. Root NO_3^- uptake is strongly regulated in response to changes in the external NO_3^- availability or in the N demand of the whole plant (Crawford and Glass, 1998; Gojon *et al.*, 2009). On the one hand, the HATS activity is quickly stimulated following first NO_3^- supply or re-supply, as a consequence of the so-called primary NO_3^- response (PNR), which is characterized by a rapid induction of *NRT2.1* in the roots shortly (*e.g.*, 30 min) after NO_3^- treatment (Filleur and Daniel-Vedele, 1999; Lejay *et al.*, 1999; Okamoto *et al.*, 2003; Tsay *et al.*, 1993; Zhuo *et al.*, 1999). On the other hand, the HATS activity is subjected to a repression exerted on a longer term (*e.g.*, several days) by high N status of the whole plant and/or high NO_3^- supply, that down-regulates *NRT2.1*, *NRT2.4* and *NRT2.5* expression in roots under N satiety conditions (Kiba *et al.*, 2012; Lejay *et al.*, 1999; Lezhneva *et al.*, 2014; Zhuo *et al.*, 1999). This repression is relieved when plants experience N starvation, resulting in a strong increase in HATS capacity that improves NO_3^- uptake efficiency under N limiting conditions (Lejay *et al.*, 1999; Nazoa *et al.*, 2003; Ohkubo *et al.*, 2017; Zhuo *et al.*, 1999; Ota *et al.*, 2020). For *NRT2.1*, repression by high NO_3^- supply is a complex process that requires the concurrent action of two different signaling mechanisms (Krouk et al. 2006). The

Accepted Manuscript
Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

first one is a feedback downregulation induced by N metabolites that are products of NO_3^- assimilation. This is evidenced by the facts that *NRT2.1* is strongly upregulated in a nitrate reductase (NR) deficient mutant (called g'4.3) fed with NO_3^- as compared to the wild-type, but is downregulated following ammonium (NH_4^+) or amino acids provision (Lejay *et al.*, 1999; Zhuo *et al.*, 1999; Nazoa *et al.*, 2003). The second mechanism is a repression induced by the perception of high external NO_3^- availability by the roots, mediated by the NRT1.1(NPF6.3) transporter acting as a NO_3^- sensor, which is thus referred to as a 'transceptor' (transporter/receptor) (Gojon *et al.*, 2011; Maghiaoui *et al.*, 2020). Indeed, repression of *NRT2.1* by high NO_3^- supply is suppressed or strongly attenuated in *nrt1.1* mutants, even in conditions where root N uptake is not reduced by *NRT1.1* deficiency (Munos *et al.*, 2004). This results in the overexpression of *NRT2.1* in normally suppressive conditions (e.g. in NH_4NO_3-fed *nrt1.1* plants) along with a lack of stimulation of *NRT2.1* expression by N starvation in *nrt1.1* mutants. Importantly, both repressive mechanisms mediated by NO_3^- and reduced N metabolites signaling need to be active to downregulate *NRT2.1* (Krouk et al. 2006). This explains why high NO_3^- supply fails to lower *NRT2.1* expression in roots of the NR-deficient mutant g'4.3 (the repression by reduced N metabolites is suppressed), and conversely why high NH_4^+ supply also fails to lower *NRT2.1* expression under mixed NH_4NO_3 nutrition if the NO_3^- concentration is low, or if NRT1.1 is deficient (the repression by high NO_3^- is suppressed). For *NRT2.4* and *NRT2.5*, the available data do not allow for now to determine whether they obey to the same regulatory model, or not. Both genes are induced by N starvation (Kiba *et al.*, 2012; Lezhneva *et al.*, 2014), but it is not known if this is due to the relief of repression by NO_3^- or reduced N metabolites, or both.

Several genes, mainly transcription factors, have been found to encode regulators of *NRT2.1* repression by high N such as *LBD37-39* (Rubin *et al.*, 2009), members of NIGT1 family (HRS1/NIGT1.4; HHO1/NIGT1.3; HHO2/NIGT1.2; HHO3/NIGT1.1) (Kiba *et al.*, 2018; Maeda *et al.*, 2018; Medici *et al.*, 2015) and members of BTB family namely BT1 and BT2 (Araus *et al.*, 2016). All these regulators are repressors of *NRT2.1* expression in high N conditions, but once again the experiments performed do not allow to distinguish if they are involved in the regulation by reduced N metabolites and/or by high NO_3^-. In this context, our study aimed at (i) characterising the regulatory mechanism involved in the repression of *NRT2.4* and *NRT2.5* by high N, and (ii) find regulatory elements involved. By performing experiments on different NH_4^+/NO_3^- regimes combined with the analysis of transcriptomic experiments and the use of mutants for the known regulatory elements we were able to clarify the regulation of *NRT2.4* and *NRT2.5* and to refine our knowledge of the NRT1.1 dependent signaling pathway in response to high NO_3^-.

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Materials and Methods

Plant Material

Arabidopsis thaliana genotypes used in this study were the wild-type Col-0 ecotype and the mutants *chl1-5* (Tsay *et al.*, 1993), *g'4.3* (NR mutant) (Wilkinson and Crawford, 1993), *hho1/hrs1* (*hh*) (Medici *et al.*, 2015), *bt1/bt2* (Sato *et al.*, 2017) and *lbd37/lbd39* (*lbd37-1*, SALK_097991; *lbd39-1*, SALK_049910).

In all experiments plants were grown hydroponically under non sterile conditions as described by Lejay *et al*. (1999). Briefly, the seeds were germinated directly on top of modified Eppendorf tubes filled with pre-wetted sand. The tubes were then positioned on floating rafts and transferred to tap water in a growth chamber under the following environmental conditions: light/dark cycle of 8 h/16 h, light intensity of 250 µmol·m^{-2}·s^{-1}, temperature of 22/20°C, and RH of 70%. After 1 week, the tap water was replaced with a complete nutrient solution containing 1 mM KH_2PO_4, 1 mM $MgSO_4$, 0.25 mM K_2SO_4, 0.25 mM $CaCl_2$, 0.1 mM FeNa-EDTA, 50 μM KCl, 30 μM H_3BO_3, 5 μM $MnSO_4$, 1 μM ZnSO4, 1 μM $CuSO_4$, and 0.1 μM $(NH_4)_6Mo_7O_{24}$. For growth of the plants, 1 mM NH_4NO_3 or 1 mM NO_3^- was added to the medium as the N source as indicated in the text and figures. The plants were allowed to grow for 3 additional weeks before the experiments. Nutrient solutions were renewed weekly and on the day before the experiments. Depending on the experiments, 1 mM NH_4NO_3 or 1 mM NO_3^- was replaced as a N source by either KNO_3 or NH_4Cl, or mixtures of these salts, as indicated in the text and figures.

RNA Extraction and Gene Expression Analysis

Root samples were frozen in liquid N_2 in 2-mL tubes containing one steel bead (2.5 mm diameter). Tissues were disrupted for 1 min at 30 s^{-1} in a Retsch mixer mill MM301 homogenizer (Retsch, Haan, Germany). Total RNA was extracted from tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently 2 µg of RNA were used to perform reverse transcription in the presence of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) after annealing with an anchored oligo$(dT)_{18}$ primer as described by (Wirth *et al.*, 2007). The quality of the cDNA was verified by PCR using specific primers spanning an intron in the gene *APTR* (At1g27450) forward 5'-CGCTTCTTCTCGACACTGAG-3' ; reverse 5'-CAGGTAGCTTCTTGGGCTTC-3'.

Gene expression was determined by quantitative real-time PCR (qRT-PCR; LightCycler 480, Roche Diagnostics, Rotkreuz, Switzerland) using SYBR Premix Ex Taq™ (TaKaRa, Kusatsu, Japan) according to the manufacturer's instructions. Conditions of amplifications were performed as described by

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Accepted Manuscript

Wirth *et al*. (2007), except the first 10 min at 95°C was changed to 30 s. All the results presented were standardized using the housekeeping gene Clathrin (At4g24550). Gene-specific primer sequences are presented in Table 1.

Table 1: Gene-specific primer sequences used for qRT-PCR experiments

Gene Name	AGI	Forward sequence	Reverse Sequence
Clathrin	At4g24550	AGCATACACTGCGTGCAAAG	TCGCCTGTGTCACATATCTC
NRT2.1	At1g08090	AACAAGGGCTAACGTGGATG	CTGCTTCTCCTGCTCATTCC
NRT2.4	At5g60770	GAACAAGGGCTGACATGGAT	GCTTCTCGGTCTCTGTCCAC
NRT2.5	At1g12940	TGTGGACCCTCTTCCAAAAA	TTTGGGGATGAGTCGTTGTGG
NRT1.1/NPF6.3	At1g12110	GCACATTGGCATTAGGCTTT	CTCAATCCCCACCTCAGCTA
HHO1/NIGT1.3	At3g25790	GTAGGAAGATTTCGGAAGATAGAT	TTTGTACGAGTAGAACAAGACATAG
HHO2/NIGT1.2	At1g68670	AAACCAAAAAGCGGTGCGTT	ACTAGCTACTTTCACCGCCG
HHO3/NIGT1.1	At1g25550	ACTAATAATAGAGTTTACGCTCCTG	GGTGTGTGTGTAGTAGTAGAAGATG
HRS1/NIGT1.4	At1g13300	TTATAGACCGTCGATTATTGTGGA	TAATGATTACGGGTAGAAGAAGAC
LBD37	At5g67420	TGGATTGAAACCGCCGATGCTC	CGACTGAAACAAAGCAGGACGTTG
LBD38	At3g49940	TCAATGCCCTGCTTTGTTTCAGTC	AACCGCCGCTTGACAAACATTC
LBD39	At4g37540	CCTGAACTCCAACGTCCTGCTTTG	TTGGCATACGTGCCAGTTCCTG
BT1	At5g63160	CCGTTGAACAGACAGAAGGA	CTGCATCGTCGATGAATTGG
BT2	At3g48360	TCCATTCGCAGTTTAAGACC	AACTGGAGAATGTCGAGCTC
PP2C	At4g32950	TGCTGTTCTCGCCGTTAAA	TCCATCCTCACTTGTTCCAATC

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

NO_3^- influx studies

Root NO_3^- influx was assayed as described by Delhon *et al.* (1995). Briefly, the plants were sequentially transferred to 0.1 mM $CaSO_4$ for 1 min, to a complete nutrient solution, pH 5.8, containing 0.05 mM or 0.01 mM $^{15}NO_3^-$ (99 atom % excess^{15}N) for 5 min, and finally to 0.1 mM $CaSO_4$ for 1 min. Roots were then separated from shoots, and the organs dried at 70 °C for 48 h. After determination of their dry weight, the samples were analyzed for total nitrogen and atom % ^{15}N using a continuous flow isotope ratio mass spectrometer coupled with a C/N elemental analyzer (model Euroflash Eurovector, Pavia Italy) as described in Clarkson (1986).

Results

Regulation of *NRT2.4* and *NRT2.5* by products of NO_3^- assimilation

To discriminate between repression by NO_3^- or by reduced N metabolites for the regulation of *NRT2.4* and *NRT2.5*, we used the *g'4.3* mutant of *A. thaliana*. This mutant is impaired in the first step of NO_3^- assimilation, catalysed by nitrate reductase (NR), and is therefore deficient in reduced N metabolites in the presence of NO_3^- as sole N source. Wild-type and *g'4.3* plants were first grown on a nutrient solution containing NH_4NO_3, allowing normal growth of the two genotypes, and then transferred to NO_3^- as sole N source for 24h and 72h (Figure 1). As observed previously, *NRT2.1* level of expression was higher in the roots of the *g'4-3* mutant as compared to the wild-type, especially after transfer to NO_3^- solution (Lejay *et al.*, 1999). In contrast, there is no significant difference between the two genotypes for *NRT2.4* and *NRT2.5* in all conditions (Figure 1). These results indicate that, unlike *NRT2.1*, the transcriptional regulation of *NRT2.4* and *NRT2.5* does not seem to involve feedback repression by reduced N metabolites produced by NO_3^- assimilation.

Regulation of *NRT2.4* and *NRT2.5* by NO_3^-

To investigate the implication of NO_3^- itself in the repression of *NRT2.4* and *NRT2.5*, wild-type plants were grown on 1 mM NO_3^- and transferred for 24h, 48h and 72h on a solution containing 1 mM NH_4Cl as the sole N source (Figure 2). In those conditions, plants are not starved for N and the only difference is the presence or absence of NO_3^- in the solution. As previously described in other studies, *NRT2.1* is strongly repressed after the transfer of the plants on NH_4^+ (Figure 2A) (Krouk *et al.*, 2006; Munos *et al.*, 2004). At the opposite, the expression of both *NRT2.4* and *NRT2.5* transporters is very low on NO_3^- and increased markedly, as soon as 24h, after the transfer of the plants on NH_4^+ (Figure 2B). These results confirm that *NRT2.4* and *NRT2.5*, unlike *NRT2.1*, are not repressed by

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Accepted Manuscript

reduced N metabolites and rather suggest that the presence of NO_3^- itself in the nutrient solution is involved in their regulation. In the same experiment, root $^{15}NO_3^-$ influx was measured at two different concentrations. At 50 µM it was correlated with *NRT2.1* level of expression and it decreased when the plants were transferred on NH_4^+ (Figure 2A). But when $^{15}NO_3^-$ influx was measured at 10 µM, it tended to increase gradually after 72h on the solution containing NH_4Cl 1 mM (Figure 2B).

To confirm these results, suggesting that *NRT2.4* and *NRT2.5* are specifically repressed by NO_3^-, we performed experiments adapted from Krouk *et al*. (2006). Plants were treated with various nutrient solutions that contain 1 mM NH_4Cl, but differ in KNO_3 concentration (from 0.1 mM to 10 mM in Krouk *et al*. (2006)). This experimental design revealed that even with an ample supply of NH_4^+, which is a strongly repressive condition for *NRT2.1*, this gene was ultimately regulated by NO_3^- signalling, being markedly upregulated when NO_3^- concentration was low (0.1 mM) and repressed when NO_3^- concentration increased up to 10 mM (Krouk *et al*., 2006). Furthermore, Munos *et al*. (2004) and Krouk *et al*. (2006) showed that the repression of *NRT2.1* by high NO_3^- was strictly dependent on NRT1.1/NPF6.3, as the lack of this protein in the mutant *chl1.5* was able to lift it almost totally. To determine if *NRT2.4* and *NRT2.5* are specifically repressed by NO_3^- like *NRT2.1*, we grew wild-type plants and *chl1.5* mutants on 1 mM NH_4NO_3 and transferred them during 72h on a mixed solution containing 1 mM NH_4Cl and 0.1 mM, 1 mM or 5 mM KNO_3 (Figure 3A). This experiment confirmed that just like *NRT2.1*, *NRT2.4* and *NRT2.5* are specifically repressed by NO_3^- and that this repression is NRT1.1/NPF6.3-dependent. This is particularly marked for *NRT2.5*, which appeared to be already significantly repressed even at 0.1 mM NO_3^-. Interestingly, this regulation has also a strong functional impact since root $^{15}NO_3^-$ influx, measured at 10 µM, was no longer repressed by increasing NO_3^- concentrations in *chl1.5* mutant, compared to wild-type plants (Figure 3A).

Furthermore, it was previously shown that phosphorylation of NRT1.1/NPF6.3 at the T101 residue modulates both the transport and signalling activity of the transceptor (Ho *et al*., 2009). Because repression of *NRT2.1* expression by high NO_3^- was prevented by T101A substitution, which suppresses phosphorylation, while the phosphomimic T101D mutation did not markedly alter it, Bouguyon *et al*. (2015) suggested that the phosphorylated form of NRT1.1/NPF6.3 is specifically responsible for this repression. We investigated this hypothesis for *NRT2.4* and *NRT2.5*, using NRT1.1/NPF6.3 T101A and T101D mutants. The results showed that T101D mutation was able to phenocopy the repression by high NO_3^- observed in wild-type plants not only for *NRT2.1* but also for *NRT2.4* and *NRT2.5* (Figure 3B). However, compared to *NRT2.1*, T101A mutation was not enough to completely prevent high NO_3^- repression, especially for *NRT2.4*, which was only slightly higher in

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Accepted Manuscript

T101A compared to wild-type plants after 72h on a mixed solution with 1 or 5 mM NO_3^- (Figure 3B). Altogether, these results suggest that like for *NRT2.1*, the phosphorylated form of NRT1.1/NPF6.3 is more specifically active than the non-phosphorylated form for triggering *NRT2.4* and *NRT2.5* repression by NO_3^-. However, compared to *NRT2.1*, it seems that repression of *NRT2.5* and mainly that of *NRT2.4* can also be activated by the non-phosphorylated form.

Molecular elements involved in the repression of *NRT2s* transporters by NO_3^-

We used transcriptomic data of Bouguyon *et al*. (2015) to go further in the characterisation of the signalling pathway involved in the repression of *NRT2s* transporter by NO_3^-. In this study, wild-type and several NRT1.1/NPF6.3 mutants were grown *in vitro* on 10 mM NH_4NO_3 to find genes regulated by high N concentrations and affected by NRT1.1/NPF6.3 mutation. Analysis of the transcriptomic data revealed, among other things, two particularly interesting clusters (Supplemental Figure 1). A first cluster of 155 genes, whose expression on 10 mM NH_4NO_3 is higher in NRT1.1/NPF6.3 mutants compared to wild-type plants and containing *NRT2.1* and *NRT2.4*, as expected from previous results. *NRT2.5* was not part of this cluster likely because the conditions were too repressive to see its expression. A second cluster of 55 genes, whose expression is lower in NRT1.1/NPF6.3 mutants on 10 mM NH_4NO_3 compared to wild-type plants. Interestingly, the first cluster contains the protein phosphatase PP2C (At4g32950), called CEPD-induced phosphatase (CEPH), which has been involved in the activation of NRT2.1 by directly dephosphorylating Ser501 of NRT2.1 (Ohkubo *et al.*, 2021). The second cluster contains three members of NIGT1 family (HHO1/NIGT1.3; HHO3/NIGT1.1; HRS1/NIGT1.4), one member of LBD family (LBD39) and one member of BTB family (BT1) (Supplemental Figure 1) (Araus *et al.*, 2016; Kiba *et al.*, 2018; Maeda *et al.*, 2018; Medici *et al.*, 2015; Rubin *et al.*, 2009). These elements have been involved in the repression of *NRT2.1* and *NRT2.4* by N, however it was not known if they play a role in the repression activated by the reduced N metabolites or by NO_3^- itself. To address this question, we tested the impact of *chl1.5* mutation as described above, on plants grown on 1 mM NH_4NO_3 and transferred during 72h on a mixed solution containing 1 mM NH_4Cl and 0.1 mM, 1 mM or 5 mM KNO_3. We also included *LBD37*, *LBD38*, *HHO2/NIGT1.2* and *BT2* in the candidate genes list because they have also been involved in the regulation of *NRT2s* by N (Araus *et al.*, 2016; Kiba *et al.*, 2018; Maeda *et al.*, 2018; Rubin *et al.*, 2009). The results showed that among all these elements, *HHO1*, *HRS1*, *LBD37*, *LBD39*, *BT1* and *BT2* were induced by NO_3^-, while the phosphatase PP2C was strongly repressed by NO_3^- (Figure 4). Altogether, regulations depended on NRT1.1 and more specifically on the phosphorylated NRT1.1 like for *NRT2s* transporters (Figure 4 and Figure 5). This was especially true for both the induction of *HHO1*, *HRS1*,

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

BT1, *BT2*, which was significantly impaired on 1 and/or 5 mM NO_3^- in T101A mutant compared to wild-type plants and T101D mutant and for the repression of *PP2C*, which was completely abolished on 1 and 5 mM NO_3^- in T101A mutant (Figure 5). The induction by NO_3^- of both *LBD37* and *LBD39* was less affected by T101A mutation, compared to the other regulatory elements, suggesting that they might be involved in another signalling mechanism dependent on NRT1.1 (Figure 5).

Impact of the regulatory elements on the regulation of root NO_3^- uptake

To determine the impact of the regulatory elements identified above on the repression of root NO_3^- uptake activity by NO_3^-, we measured $^{15}NO_3^-$ influx at 10 µM using the double mutants *hh* (for *HHO1*and *HRS1*), *lbd37/lbd39* and *bt1/bt2*, in the same experimental set up as above, with plants grown on 1 mM NH_4NO_3 and transferred during 72h on a mixed solution containing 1 mM NH_4Cl and 0.1 mM, 1 mM or 5 mM KNO_3. The results showed that the repression of root NO_3^- influx was only affected in the double mutants *hh* and *bt1/bt2* (Figure 6). Compared to wild-type plants, NO_3^- influx was significantly higher in both double mutants after transfer on increasing NO_3^- concentration at 1 and 5 mM (Figure 6). Conversely, no differences were observed between the double mutant *lbd37/lbd39* and wild-type plants. However, it should be noted that the lack of *HHO1*/*HRS1* or *BT1*/*BT2* did not completely prevent NO_3^- repression of root NO_3^- uptake (Figure 6). As expected, the mis-regulation of root NO_3^- uptake activity in the double mutant *hh* was correlated with a complete lack of repression by NO_3^- of *NRT2.1* and a significant higher expression of *NRT2.4* and *NRT2.5* on 1 and 5 mM NO_3^- compared to wild-type plants (Figure 7). But surprisingly, repression of *NRT2.1*, *NRT2.4* and *NRT2.5* was the same in the double mutant *bt1/bt2* compared to wild-type plants and could not explain the higher level of root NO_3^- uptake activity observed in the double mutant (Figure 6 and Figure 7).

Discussion

NRT2.4 and *NRT2.5* are repressed by NO_3^- but not by N metabolites

Previous studies showed that *NRT2.4* and *NRT2.5* are, like *NRT2.1*, upregulated by N starvation (Kiba *et al.*, 2012; Lejay *et al.*, 1999; Lezhneva *et al.*, 2014). Nevertheless, these N starvation experiments, consisting in transferring the plants from a growing solution rich in NO_3^- to a solution with no N, did not allow to determine if NO_3^- itself or N metabolites were involved in their regulation (Kiba *et al.*, 2012; Lezhneva *et al.*, 2014). For *NRT2.1*, it has been shown that both, N metabolites from NO_3^-

Accepted Manuscript
Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

assimilation and high external concentration of NO_3^- itself, are able to repress its expression (Krouk *et al.*, 2006; Lejay *et al.*, 1999). Our results show that this does not hold true for *NRT2.4* and *NRT2.5*, which appeared to be only repressed by high NO_3^-. Indeed, preventing normal NO_3^- assimilation by NR knock-down in *g'4.3* plants failed to increase *NRT2.4* and *NRT2.5* expression compared to WT plants (Figure 1). Moreover, transfer of WT plants from NO_3^- to NH_4^+ as sole N source dramatically increased the expression of *NRT2.4* and *NRT2.5*, while it repressed *NRT2.1* as previously observed (Figure 2) (Lejay *et al.*, 1999). The repressive role of NO_3^- for the regulation of *NRT2.4* and *NRT2.5* was confirmed by transferring the plants from 1 mM NH_4NO_3 to a solution containing 1 mM NH_4^+ but with increasing concentration of NO_3^- (Figure 3A). This experimental protocol has been used previously by Krouk *et al*. (2006) to reveal the specific role of NO_3^- in the repression of *NRT2.1*. Indeed, in those conditions, despite the continuous presence of NH_4^+, *NRT2.1* expression was consistently found to be determined by external NO_3^- concentration, with a strong down-regulation as soon as NO_3^- concentration exceeded 0.2 to 0.5 mM range (Krouk *et al.*, 2006). Furthermore, using NRT1.1/NPF6.3 mutants, it was shown that *NRT2.1* repression by NO_3^- was triggered by NRT1.1/NPF6.3. Our results show that this is also the case for both *NRT2.4* and *NRT2.5* that are, like *NRT2.1*, repressed by the increasing concentration of NO_3^- in the presence of NH_4^+ in WT plants, but not in the *chl1-5* mutant (Figure 3A). In WT plants, the repressive effect of NO_3^- was even stronger for *NRT2.5*, which was already downregulated by 0.1 mM of NO_3^- compared to *NRT2.1* and *NRT2.4*. This is consistent with previous results showing that *NRT2.5* is not induced by NO_3^- compared to *NRT2.1* and *NRT2.*4 (Kotur and Glass, 2015). At the concentration of 0.1 mM NO_3^- it is thus likely that, for *NRT2.1* and *NRT2.4*, the inductive effect of NO_3^- overcome its repressive effect.

Bouguyon *et al*. (2015) suggested that in plants grown on 10 mM NH_4NO_3, repression of *NRT2.1* by high NO_3^- is mediated by NRT1.1/NPF6.3 phosphorylated form on T101 residue. The results we obtained with our experimental setup confirmed this conclusion but also showed that this is not totally the case for both *NRT2.4* and *NRT2.5* (Figure 3B). Indeed, for *NRT2.1*, inhibition of T101 phosphorylation resulted in a complete lack of NO_3^- repression on 1 mM and 5 mM NO_3^-, while for *NRT2.4* and *NRT2.5* the effect was only partial as both genes were still significantly downregulated by increasing NO_3^- concentration in the T101A mutant plants. This suggests that the non-phosphorylated form of NRT1.1/NPF6.3 is somehow also able to mediate repression of *NRT2.1* by high NO_3^-, although less efficiently than the phosphorylated form. Compared with the results obtained with *chl1-5* mutant, it suggests that the regulatory mechanism triggered by NRT1.1/NPF6.3 is more complex and does not only depend on T101 phosphorylation for the repression by NO_3^- of *NRT2.4* and *NRT2.5*.

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

In addition, our data indicate that NO_3^- repression of *NRT2.4* and *NRT2.5* plays a key role in the regulation of high affinity root NO_3^- uptake. Indeed, $^{15}NO_3^-$ influx measurements revealed that, in experiments where *NRT2.1* was not regulated like *NRT2.4* and *NRT2.5*, influx at 50 µM of $^{15}NO_3^-$ was correlated with *NRT2.1* expression, while influx at 10 uM was correlated with *NRT2.4* and *NRT2.5* expression (Figure 2A and 2B). This is in agreement with the role of very high affinity root NO_3^- transporters attributed to both *NRT2.4* and *NRT2.5* (Kiba *et al.*, 2012; Lezhneva *et al.*, 2014).

Repression of root NO_3^- uptake by NO_3^- involves key regulators of NRT2s both at the transcriptional and post-translational level

Analysis of the transcriptomic experiments performed by Bouguyon *et al.* (2015) revealed that several known repressors of *NRT2.1*, *NRT2.4* and *NRT2.5* are induced by 10 mM NH_4NO_3 and that this regulation is triggered by NRT1.1/NP6.3 T101 phosphorylated form (Supplemental Figure 1). Interestingly, a protein phosphatase from PP2C family was also found co-regulated with *NRT2.1* and *NRT2.4*. This phosphatase has recently been involved in the activation of NRT2.1 by directly dephosphorylating S501, a residue that functions as a negative phospho-switch in Arabidopsis (Jacquot *et al.*, 2020; Ohkubo *et al.*, 2021). Using our experimental set up with increasing concentrations of NO_3^- in the presence of 1 mM NH_4^+ in both WT plants and the *chl1-5* mutant, we found that 7 out of the 10 regulators tested were regulated by high NO_3^- in a NRT1.1/NPF6.3 dependent manner (Figure 4). Among them, we confirmed the results obtained by Bouguyon *et al.* (2015) for the regulation of *HHO1*, *HRS1*, *LBD39*, *BT2* and *PP2C*. However, in our hands *HHO3* was not found induced by high NO_3^- in WT plants nor dependent on NRT1.1/NPF6.3 in the *chl1-5* mutant. Conversely, our results show that *LBD37* and *BT1*, which were not selected by Bouguyon *et al.* (2015), are both induced by NO_3^- and dependent on NRT1.1/NPF6.3 signaling pathway (Figure 4). These discrepancies could be explained by the very different conditions between the experiments of Bouguyon *et al.* (2015) and ours, and suggest that the various members of the *HHO/HRS/NIGT* and *LBD* families are differentially regulated. A difference in the regulation of *HHO1*, *HRS1* and *HHO2*, *HHO3* has already been described by Kiba *et al.* (2018). In that case, it has been shown that *HHO2* and *HHO3*, unlike *HHO1* and *HRS1*, are induced by reduced forms of N such as Gln and urea. It supports the hypothesis that *HHO1* and *HRS1* are not involved in the same signaling pathways as *HHO2* and *HHO3*. Concerning LBDs and BTs, the work of Rubin *et al.* (2009) does not allow to identify different roles between LBD37, 38 et 39, while for BT1 and BT2 the work of Araus *et al.* (2016) indicate a functional redundancy suggesting that they are part of the same signaling pathway.

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Surprisingly, despite the fact that *LBD37* and *LBD39* induction by NO_3^- depends on NRT1.1/NPF6.3, it does not seem to specifically involve NRT1.1 phosphorylated form compared to the other molecular elements we identified (Figure 5). It supports the hypothesis, as discussed above, that the regulatory mechanisms triggered by NRT1.1/NPF6.3 are more complex and do not only depend on T101 phosphorylation for the repression by NO_3^- of at least *NRT2.4* and *NRT2.5*. Although the double mutation of *LBD37/LBD39* had no effect of the repression of root NO_3^- influx by high NO_3^-, those of *HHO1/HRS1* and *BT1/BT2* somehow attenuated it (Figure 6). Interestingly, if the impact of the double mutation of *HHO1* and *HRS1* on root NO_3^- influx in *hh* mutant can be explained by the impact of these transcription factors on the expression of *NRT2s* transporters and especially of *NRT2.1*, this is not the case for BT1 and BT2 (Figure 7). It is surprising compared to previous results showing that on low NO_3^-, an increase of NO_3^- uptake in *bt1/bt2* mutant was correlated with an increase in the expression of both *NRT2.1* and *NRT2.4* (Araus *et al.*, 2016). However, once again, the experimental conditions were very different in Araus *et al*. (2016), with plants grown *in vitro* in steady state conditions with two different concentrations of NO_3^-. Furthermore, the molecular function of BT1 and BT2 proteins remains to be elucidated. Indeed, they are found in multisubunit E3 ubiquitin ligase complexes as well as in interaction with the BET10 transcriptional activator (Du and Poovaiah, 2004; Figueroa *et al.*, 2005). It is thus possible that BT proteins are involved in both transcriptional regulation and/or degradation of proteins.

Finally, a particularly interesting result concerns the strong impact of NRT1.1 phosphorylated form on the repression by NO_3^- of the protein phosphatase gene *PP2C* (Figure 5). This may help answering the unresolved question of the respective importance of transcriptional and posttranscriptional regulation of NRT2.1. On the one hand, changes in NO_3^- HATS activity were always found highly correlated with changes in *NRT2.1* transcript accumulation and *NRT2.1* promoter activity in roots, suggesting a major role for transcriptional regulation (Girin *et al.*, 2010; Laugier *et al.*, 2012; Lejay *et al.*, 1999; Wirth *et al.*, 2007). This was further supported by the observations that the mutation or overexpression of key regulators governing *NRT2.1* transcription, such has NRT1.1, NLP7, HHO/HRS/NIGTs or CEPD/CEPDLs also resulted in a deregulation of the NO_3^- HATS activity (Munos *et al*., 2004; Yu *et al*., 2016; Kiba *et al*., 2018; Maeda *et al*., 2018; Ota *et al*., 2020). On the other hand, suppression of the transcriptional regulation of *NRT2.1* using *35S* promoter failed to prevent feedback downregulation of the NO_3^- HATS activity by N satiety or darkness, indicating a predominant role for posttranscriptional control (Laugier *et al*., 2012). Furthermore, several mechanisms have been proposed for posttranslational regulation of NRT2.1 (Wirth *et al*., 2007), among which phosphorylation of the S501 residue was shown to play a crucial role for governing HATS activity (Jacquot *et al*., 2020; Ohkubo *et al*., 2021). Our results showing that both *NRT2.1* and

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

PP2C are common targets of the NRT1.1/NPF6.3-mediated repression of gene expression by high NO_3^- allow to reconcile the above apparently contradictory observations. Indeed, this suggests that transcriptional regulation of *NRT2.1 per se* does not play a predominant role, but that the signalling pathways triggering this regulation are of crucial importance for controlling NO_3^- HATS, because they also govern the expression of posttranslational regulators of NRT2.1 (Figure 3, Figure 4 and Figure 5, Ohkubo *et al.*, 2021). Furthermore, this co-regulation of *NRT2.1* gene expression and NRT2.1 protein activity, through the regulation of the protein phosphatase gene *PP2C* does not only concern the repression by NO_3^- since Ohkubo *et al.* (2021) showed that *PP2C* was regulated like *NRT2.1* in response to NH_4^+, NO_3^- concentration in the media and N starvation. Altogether, these results suggest that the regulation of NO_3^- HATS activity is the result of a redundant regulation of NRT2.1 at the transcriptional and post-translational level. It is interesting to note that redundant regulation at the transcriptional and post-translational level seems to be a general feature of the enzymes involved in N metabolism since it has already been described in plants for Nitrate Reductase (NR), Nitrite Reductase (NiR) and Glutamine synthetase (GS) (Campbell, 1999; Crete *et al.*, 1997; Oliveira and Coruzzi, 1999).

Conclusion

Altogether, as shown in Figure 8, our results allow us to propose a model for the signaling pathway downstream of NRT1.1 phosphorylated form and involved in high NO_3^- repression of root NO_3^- uptake. It involves the transcription factors HHO1 and HRS1, the proteins BT1 and BT2 and the phosphatase PP2C At4g32950. It revealed a complex picture, in which different level of regulation at the transcriptional and post-translational level are involved. HHO1 and HRS1 seem to be directly involved in the transcriptional repression of *NRT2.1*, *NRT2.4* and *NRT2.5* and this is supported by previous results showing that these two transcription factors can bind at least *NRT2.4* and *NRT2.5* promoter (Kiba *et al.*, 2018; Safi *et al.*, 2021). However, based on the results obtained with the mutants for NRT1.1 phosphorylated form, it seems that, for *NRT2.4* and *NRT2.5*, other elements are still missing to fully explained their repression by high NO_3^-. In the meantime, as shown for NRT2.1, the repression by high NO_3^- of the PP2C protein phosphatase At4g32950 leads to an increase in the inactive form of NRT2.1 phosphorylated on S501. It revealed that the regulation of root NO_3^- uptake in response to high NO_3^- is likely the result of both a repression of NO_3^- transporters at the transcriptional level and an inactivation at the protein level. Finally, despite the fact that BT1 and BT2 are involved in the repression of root NO_3^- uptake by high NO_3^-, the molecular function of these proteins remains to be addressed.

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Accepted Manuscript

Acknowledgments

We thank Dr. Gabriel Krouk and Dr. Anna Medicis for providing the seeds for the double mutant *HHO1/HRS1* (*hh*) and Dr. Shuichi Yanagisawa for providing the seeds for the double mutant *BT1/BT2 (bt1/bt2*).

Author contributions

VC performed most of the experiments with the support of JL. DS and AM produced and characterised LBDs double mutants. PT performed ^{15}N measurements. CF, TM and RG performed bioinformatics analysis. LL, AG and RG designed the experiments. LL and AG wrote the manuscript with the support of KZ.

No conflict of interest

Funding

This work was supported by an international grant from Agropolis Fondation (GeneRice 1605-019).

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Figure legends

Figure 1. Impact of nitrate reductase mutation (*g'4.3*) on *NRT2.1*, *2.4* and *2.5* regulation by the N status. Plants were grown on 1 mM NH_4NO_3 for 5 weeks, before being transferred on 1 mM KNO_3 during 24h and 72h. Roots have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col) and the mutant *g'4.3* are significant at **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t* test).

Figure 2. Regulation of *NRT2.1*, *2.4, 2.5* expression and of root NO_3^- influx after transfer of the plants from NO_3^- to NH_4^+. Plants were grown on 1 mM NO_3^- for 5 weeks before being transferred on 1 mM KNO_3 during 24h, 48h or 72h. Roots have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Root NO_3^- influx was measured at the external concentration of **(A)** 50 µM $^{15}NO_3^{-.}$ and **(B)** 10 µM $^{15}NO_3^{-.}$ Plants were treated in the same conditions as for *NRT2s* mRNA level measurements. Values are means of 12 replicates ± SD. Differences between plants on 1 mM NO_3^- and 1 mM NH_4Cl are significant at **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t* test).

Figure 3. Impact of *NRT1.1* mutations on *NRT2.1*, *2.4, 2.5* and root NO_3^- influx repression by NO_3^-. Plants were grown on 1 mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1 mM NH_4Cl with 0.1, 1 or 5 mM KNO_3. Roots of (A) wild type and *chl1-5* knock-out mutant and (B) wild type and T101A and T101D mutants have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Root NO_3^- influx was measured at the external concentration of 10 µM $^{15}NO_3^{-.}$ Plants were treated in the same conditions as for *NRT2s* mRNA level measurements. Values are means of 12 replicates ± SD. Differences between WT (Col) and the mutants are significant at **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t* test).

Figure 4. Impact of *nrt1.1* mutation (*chl1.5*) on *HHO1, HHO2, HHO3, HRS1, LBD37, LBD38, LBD39, BT1* and *BT2* regulation by NO_3^-. Plants were grown on 1 mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1 mM NH_4Cl with 0.1, 1 or 5 mM KNO_3. Roots have been collected to

Accepted Manuscript
Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

assess mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col) and the mutant *chl1.5* are significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test).

Figure 5. Impact of *NRT1.1* mutations (T101A, T101D) on *HHO1, HRS1, LBD37, LBD39, BT1, BT2* and *PP2C* regulation by NO_3^-. Plants were grown on 1 mM NH_4NO_3 for 5 weeks, before being transferred during 72h on 1 mM NH_4Cl with 0.1, 1 or 5 mM KNO_3. Roots have been collected to assess mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col) and the mutant *chl1.5* are significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test).

Figure 6. Impact of *hh* (*hho1/hrs1*), *lbd37/39* and *bt1/bt2* mutations on root NO_3^- influx regulation by NO_3^-. Plants were grown on 1 mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1 mM NH_4Cl with 0.1, 1 or 5 mM KNO_3. Root NO_3^- influx was measured at the external concentration of 10 µM $^{15}NO_3^-$. Values are means of 12 replicates ± SD. Differences between WT (Col) and the mutants are significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test).

Figure 7. Impact of the double mutation *hho1/hrs1* (*hh*) and *bt1/bt2* on *NRT2.1, 2.4, 2.5* repression by NO_3^-.

Plants were grown on 1 mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1 mM NH_4Cl with 0.1, 1 or 5 mM KNO_3. Roots have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD.

Differences between WT (Col) and the mutant *chl1.5* are significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test).

Figure 8. Model for the regulation of root NO_3^- uptake by high NO_3^- concentration

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Accepted Manuscript

Literature Cited

Araus V, Vidal EA, Puelma T, Alamos S, Mieulet D, Guiderdoni E, Gutierrez RA. 2016. Members of BTB Gene Family of Scaffold Proteins Suppress Nitrate Uptake and Nitrogen Use Efficiency. Plant Physiology **171**, 1523-1532.

Campbell WH. 1999. NITRATE REDUCTASE STRUCTURE, FUNCTION AND REGULATION: Bridging the Gap between Biochemistry and Physiology. Annual Revue of Plant Physiology and Plant Molecular Biology **50**, 277-303.

Cerezo M, Tillard P, Filleur S, Munos S, Daniel-Vedele F, Gojon A. 2001. Major alterations of the regulation of root NO(3)(-) uptake are associated with the mutation of Nrt2.1 and Nrt2.2 genes in Arabidopsis. Plant Physiology **127**, 262-271.

Clarkson DT. 1986. Regulation of absorption and release of nitrate by plant cells: a review of current ideas and methodology. *In* Developments in Plants and Soil Sciences: Fundamental, Ecological and Agricultural Aspect of Nitrogen Metabolism in Higher Plants (eds H. Lambers, J.J. Neeteson & I.Stulen), Marttinus Nijhoff Publishers, Dordrecht, The Netherlands, 3-27.

Crawford NM, Glass ADM. 1998. Molecular and physiological aspects of nitrate uptake in plants. Trends in Plant Science **3**, 389-395.

Crete P, Caboche M, Meyer C. 1997. Nitrite reductase expression is regulated at the post-transcriptional level by the nitrogen source in Nicotiana plumbaginifolia and Arabidopsis thaliana. The Plant Journal **11**, 625-634.

Delhon P, Gojon A, Tillard P, Passama L. 1995. Diurnal regulation of NO_3^- uptake in soybean plants. I. Changes in NO_3^- influx, efflux, and N utilization in the plant during the day/night cycle. Journal of Experimental Botany **46**, 1585-1594.

Du L, Poovaiah BW. 2004. A novel family of Ca2+/calmodulin-binding proteins involved in transcriptional regulation: interaction with fsh/Ring3 class transcription activators. Plant Molecular Biology **54**, 549-569.

Figueroa P, Gusmaroli G, Serino G, Habashi J, Ma L, Shen Y, Feng S, Bostick M, Callis J, Hellmann H, Deng XW. 2005. Arabidopsis has two redundant Cullin3 proteins that are essential for embryo development and that interact with RBX1 and BTB proteins to form multisubunit E3 ubiquitin ligase complexes in vivo. The Plant Cell **17**, 1180-1195.

Filleur S, Daniel-Vedele F. 1999. Expression analysis of a high-affinity nitrate transporter isolated from Arabidopsis thaliana by differential display. Planta **207**, 461-469.

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Filleur S, Dorbe MF, Cerezo M, Orsel M, Granier F, Gojon A, Daniel-Vedele F. 2001. An arabidopsis T-DNA mutant affected in Nrt2 genes is impaired in nitrate uptake. FEBS Letters **489**, 220-224.

Girin T, El-Kafafi el S, Widiez T, Erban A, Hubberten HM, Kopka J, Hoefgen R, Gojon A, Lepetit M. 2010. Identification of Arabidopsis mutants impaired in the systemic regulation of root nitrate uptake by the nitrogen status of the plant. Plant Physiology **153**, 1250-1260.

Gojon A, Nacry P, Davidian JC. 2009. Root uptake regulation: a central process for NPS homeostasis in plants. Current Opinion in Plant Biology **12**, 328-338.

Gojon A, Krouk G, Perrine-Walker F, Laugier E. 2011. Nitrate transceptor(s) in plants. Journal of Experimental Botany **62**, 2299-2308.

Jacquot A, Chaput V, Mauries A, Li Z, Tillard P, Fizames C, Bonillo P, Bellegarde F, Laugier E, Santoni V, Hem S, Martin A, Gojon A, Schulze W, Lejay L. 2020. NRT2.1 C-terminus phosphorylation prevents root high affinity nitrate uptake activity in Arabidopsis thaliana. New Phytologist **228**, 1038-1054.

Kiba T, Feria-Bourrellier AB, Lafouge F, Lezhneva L, Boutet-Mercey S, Orsel M, Brehaut V, Miller A, Daniel-Vedele F, Sakakibara H, Krapp A. 2012b. The Arabidopsis nitrate transporter NRT2.4 plays a double role in roots and shoots of nitrogen-starved plants. The Plant Cell **24**, 245-258.

Kiba T, Inaba J, Kudo T, Ueda N, Konishi M, Mitsuda N, Takiguchi Y, Kondou Y, Yoshizumi T, Ohme-Takagi M, Matsui M, Yano K, Yanagisawa S, Sakakibara H. 2018. Repression of Nitrogen Starvation Responses by Members of the Arabidopsis GARP-Type Transcription Factor NIGT1/HRS1 Subfamily. The Plant Cell **30**, 925-945.

Kotur Z, Glass AD. 2015. A 150 kDa plasma membrane complex of AtNRT2.5 and AtNAR2.1 is the major contributor to constitutive high-affinity nitrate influx in Arabidopsis thaliana. Plant Cell and Environment **38**, 1490-1502.

Krouk G, Tillard P, Gojon A. 2006. Regulation of the high-affinity NO3- uptake system by NRT1.1-mediated NO3- demand signaling in Arabidopsis. Plant Physiology **142**, 1075-1086.

Laugier E, Bouguyon E, Mauries A, Tillard P, Gojon A, Lejay L. 2012. Regulation of high-affinity nitrate uptake in roots of Arabidopsis depends predominantly on posttranscriptional control of the NRT2.1/NAR2.1 transport system. Plant Physiology **158**, 1067-1078.

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Accepted Manuscript

Lejay L, Tillard P, Lepetit M, Olive F, Filleur S, Daniel-Vedele F, Gojon A. 1999. Molecular and functional regulation of two NO3- uptake systems by N- and C-status of Arabidopsis plants. The Plant Journal **18**, 509-519.

Lezhneva L, Kiba T, Feria-Bourrellier AB, Lafouge F, Boutet-Mercey S, Zoufan P, Sakakibara H, Daniel-Vedele F, Krapp A. 2014. The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants. The Plant Journal **80**, 230-241.

Maeda Y, Konishi M, Kiba T, Sakuraba Y, Sawaki N, Kurai T, Ueda Y, Sakakibara H, Yanagisawa S. 2018. A NIGT1-centred transcriptional cascade regulates nitrate signalling and incorporates phosphorus starvation signals in Arabidopsis. Nature Communications **9**, 1376.

Maghiaoui A, Gojon A, Bach L. 2020. NRT1.1-centered nitrate signaling in plants. Journal of Experimental Botany **71**, 6226-6237.

Malagoli P, Laine P, Le Deunff E, Rossato L, Ney B, Ourry A. 2004. Modeling nitrogen uptake in oilseed rape cv Capitol during a growth cycle using influx kinetics of root nitrate transport systems and field experimental data. Plant Physiology **134**, 388-400.

Medici A, Marshall-Colon A, Ronzier E, Szponarski W, Wang R, Gojon A, Crawford NM, Ruffel S, Coruzzi GM, Krouk G. 2015. AtNIGT1/HRS1 integrates nitrate and phosphate signals at the Arabidopsis root tip. Nature Communications **6**, 6274.

Munos S, Cazettes C, Fizames C, Gaymard F, Tillard P, Lepetit M, Lejay L, Gojon A. 2004. Transcript profiling in the chl1-5 mutant of Arabidopsis reveals a role of the nitrate transporter NRT1.1 in the regulation of another nitrate transporter, NRT2.1. The Plant Cell **16**, 2433-2447.

Nacry P, Bouguyon E, Gojon A. 2013. Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. Plant Soil **370**, 1-29.

Nazoa P, Vidmar JJ, Tranbarger TJ, Mouline K, Damiani I, Tillard P, Zhuo D, Glass AD, Touraine B. 2003. Regulation of the nitrate transporter gene AtNRT2.1 in Arabidopsis thaliana: responses to nitrate, amino acids and developmental stage. Plant Molecular Biology **52**, 689-703.

O'Brien JA, Vega A, Bouguyon E, Krouk G, Gojon A, Coruzzi G, Gutierrez RA. 2016. Nitrate Transport, Sensing, and Responses in Plants. Molecular Plant **9**, 837-856.

Ohkubo Y, Kuwata K, Matsubayashi Y. 2021. A type 2C protein phosphatase activates high-affinity nitrate uptake by dephosphorylating NRT2.1. Nature Plants **7**, 310-316.

Accepted Manuscript

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Ohkubo Y, Tanaka M, Tabata R, Ogawa-Ohnishi M, Matsubayashi Y. 2017. Shoot-to-root mobile polypeptides involved in systemic regulation of nitrogen acquisition. Nature Plants **3**, 17029.

Okamoto M, Vidmar JJ, Glass AD. 2003. Regulation of NRT1 and NRT2 gene families of Arabidopsis thaliana: responses to nitrate provision. Plant & Cell Physiology **44**, 304-317.

Oliveira IC, Coruzzi GM. 1999. Carbon and amino acids reciprocally modulate the expression of glutamine synthetase in Arabidopsis. Plant Physiology **121**, 301-310.

Ota R, Ohkubo Y, Yamashita Y, Ogawa-Ohnishi M, Matsubayashi Y. 2020. Shoot-to-root mobile CEPD-like 2 integrates shoot nitrogen status to systemically regulate nitrate uptake in Arabidopsis. Nature Communications **11**, 641.

Rubin G, Tohge T, Matsuda F, Saito K, Scheible WR. 2009. Members of the LBD family of transcription factors repress anthocyanin synthesis and affect additional nitrogen responses in Arabidopsis. The Plant Cell **21**, 3567-3584.

Safi A, Medici A, Szponarski W, Martin F, Clement-Vidal A, Marshall-Colon A, Ruffel S, Gaymard F, Rouached H, Leclercq J, Coruzzi G, Lacombe B, Krouk G. 2021. GARP transcription factors repress Arabidopsis nitrogen starvation response via ROS-dependent and -independent pathways. Journal of Experimental Botany **72**, 3881-3901.

Sato T, Maekawa S, Konishi M, Yoshioka N, Sasaki Y, Maeda H, Ishida T, Kato Y, Yamaguchi J, Yanagisawa S. 2017. Direct transcriptional activation of BT genes by NLP transcription factors is a key component of the nitrate response in Arabidopsis. Biochemical and Biophysical Research Communications **483**, 380-386.

Tsay YF, Schroeder JI, Feldmann KA, Crawford NM. 1993. The herbicide sensitivity gene *CHL1* of *Arabidopsis* encodes a nitrate-inducible nitrate transporter. Cell **72**, 705-713.

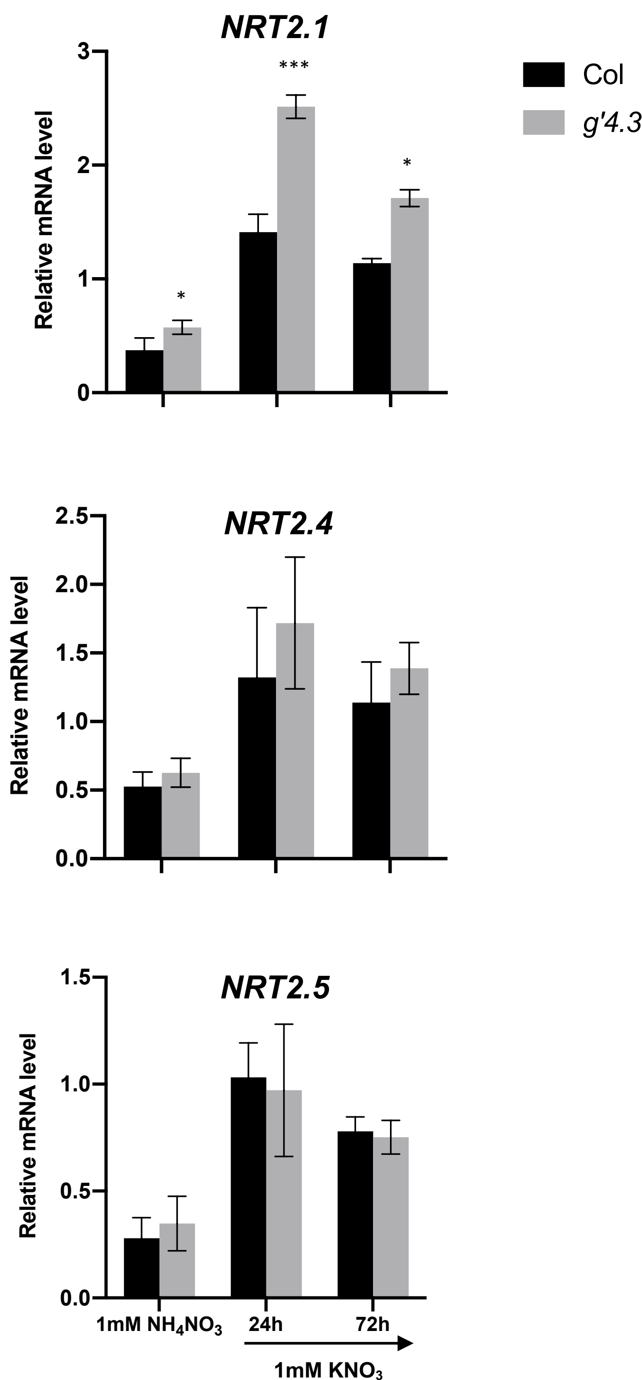
Wilkinson JQ, Crawford NM. 1993. Identification and characterization of a chlorate-resistant mutant of *Arabidopsis thaliana* with mutations in both nitrate reductase structural genes *NIA1* and *NIA2*. Molecular & General Genetics **239**, 289-297.

Wirth J, Chopin F, Santoni V, Viennois G, Tillard P, Krapp A, Lejay L, Daniel-Vedele F, Gojon A. 2007. Regulation of root nitrate uptake at the NRT2.1 protein level in Arabidopsis thaliana. Journal of Biological Chemistry **282**, 23541-23552.

Zhuo D, Okamoto M, Vidmar JJ, Glass AD. 1999. Regulation of a putative high-affinity nitrate transporter (*Nrt2;1At*) in roots of *Arabidopsis thaliana*. The Plant Journal **17**, 563-568.

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

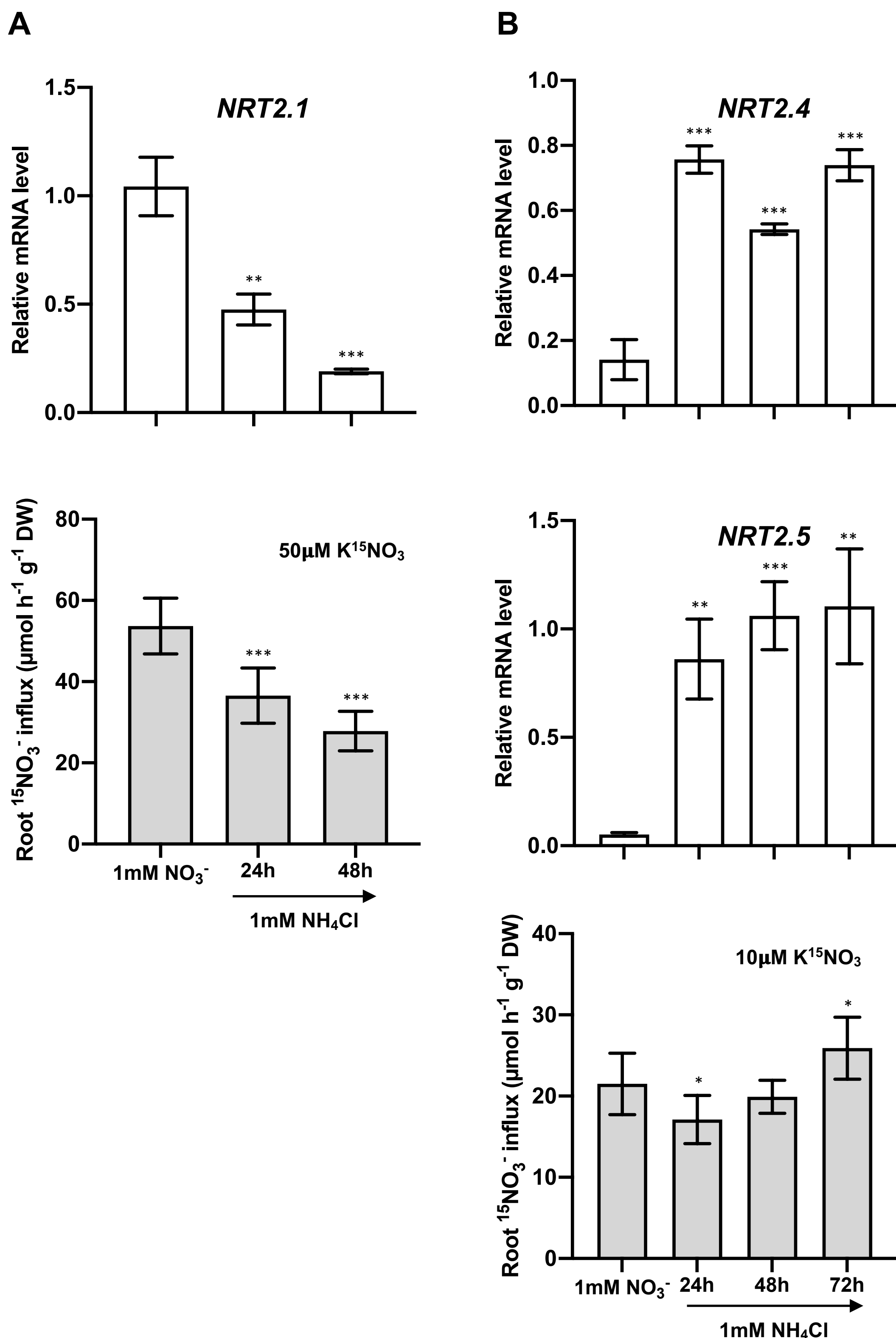
Accepted Manuscript



Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Figure 1. Impact of nitrate reductase mutation (*g'4.3*) on *NRT2.1*, *2.4* and *2.5* regulation by the N status.

Plants were grown on 1mM NH_4NO_3 for 5 weeks, before being transferred on 1mM KNO_3 during 24h and 72h. Roots have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col) and the mutant *g'4.3* are significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test).



Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Figure 2. Regulation of *NRT2.1*, *2.4, 2.5* expression and of root NO_3^- influx after transfer of the plants from NO_3^- to NH_4^+.

Plants were grown on 1mM NO_3^- for 5 weeks before being transferred on 1mM KNO_3 during 24h, 48h or 72h. Roots have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Root NO_3^- influx was measured at the external concentration of **(A)** 50 µM $^{15}NO_3^-$ and **(B)** 10 µM $^{15}NO_3^-$. Plants were treated in the same conditions as for *NRT2s* mRNA level measurements. Values are means of 12 replicates ± SD.
Differences between plants on 1mM NO_3^- and 1mM NH_4Cl are significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test).

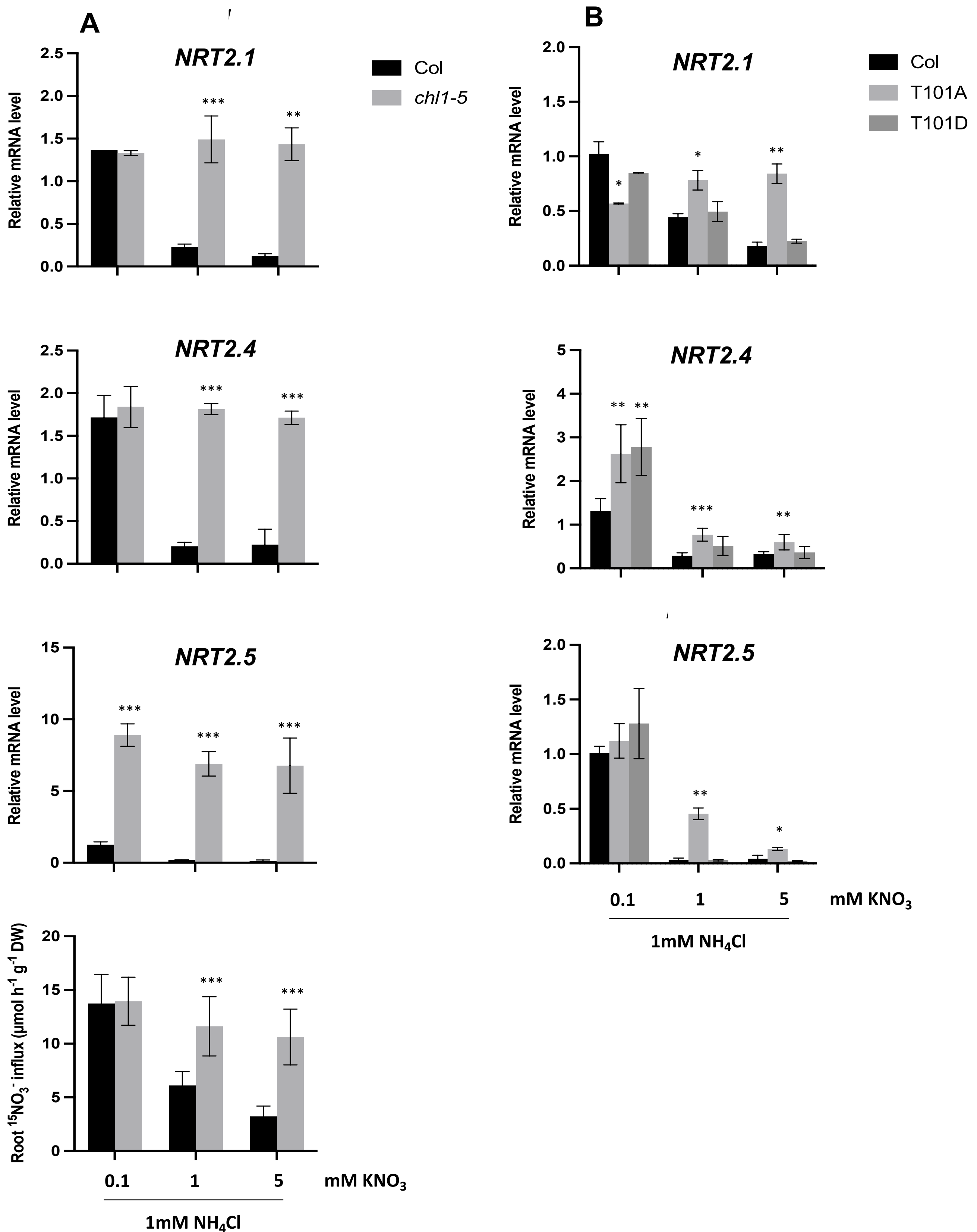
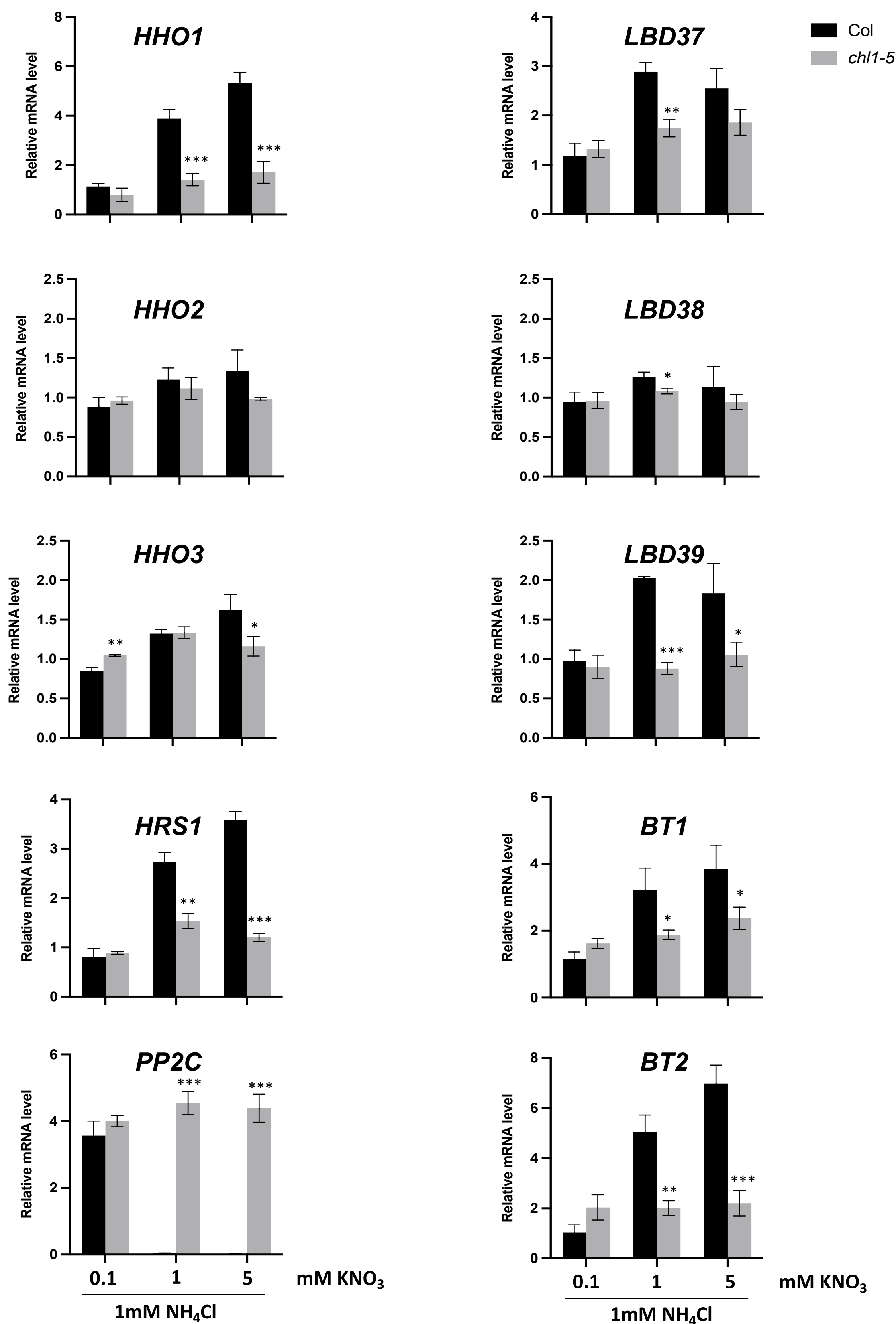


Figure 3. Impact of *NRT1.1* mutations on *NRT2.1*, *2.4, 2.5* and root NO_3^- influx repression by NO_3^-.

Plants were grown on 1mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1mM NH_4Cl with 0.1, 1 or 5mM KNO_3. Roots of **(A)** wild type and *chl1-5* knock-out mutant and **(B)** wild type and T101A and T101D mutants have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Root NO_3^- influx was measured at the external concentration of 10 µM $^{15}NO_3^-$. Plants were treated in the same conditions as for *NRT2s* mRNA level measurements. Values are means of 12 replicates ± SD.
Differences between WT (Col) and the mutants are significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t* test).

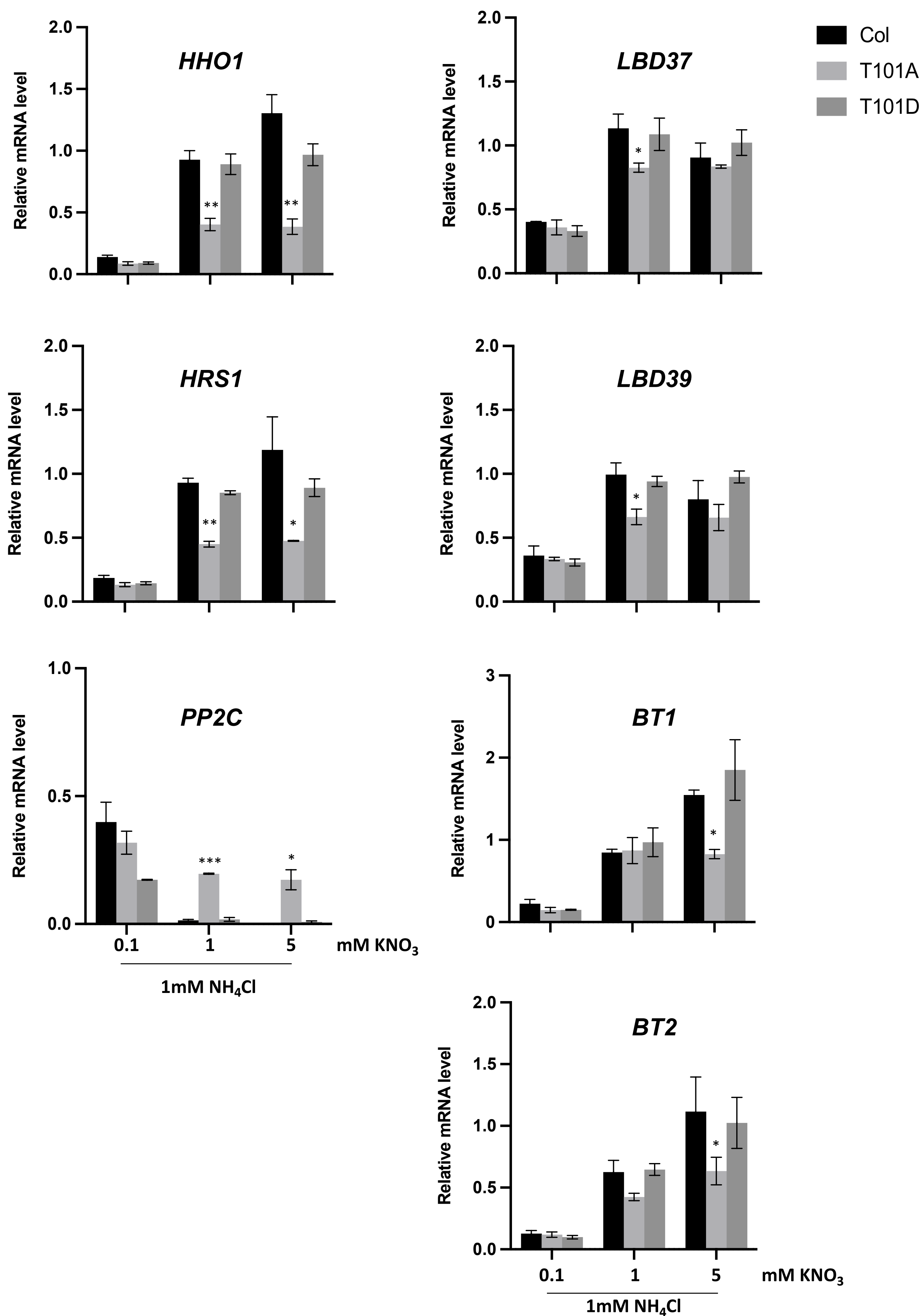
Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023



Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Figure 4. Impact of *nrt1.1* mutation (*chl1.5*) on *HHO1, HHO2, HHO3, HRS1, LBD37, LBD38, LBD39, BT1* and *BT2* regulation by NO_3^-.

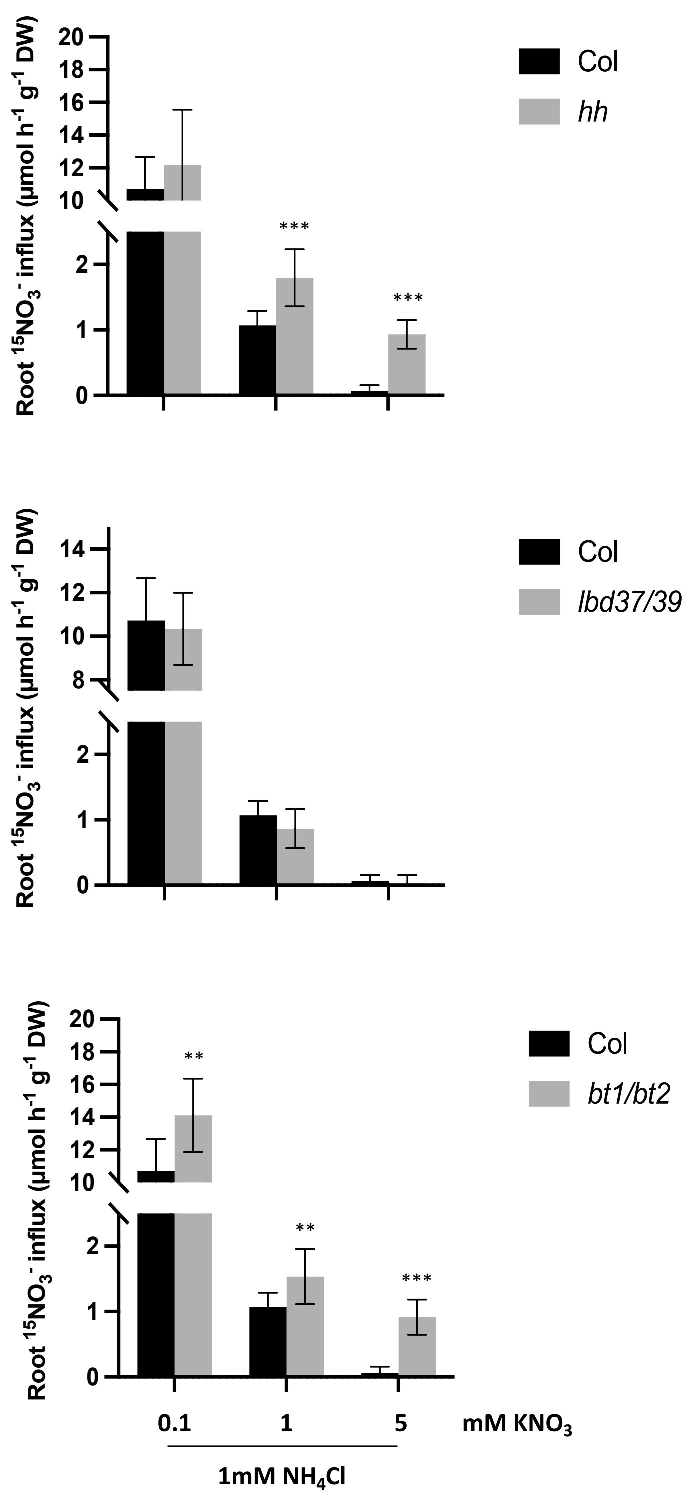
Plants were grown on 1mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1mM NH_4Cl with 0.1, 1 or 5mM KNO_3. Roots have been collected to assess mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col) and the mutant *chl1.5* are significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t* test).



Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Figure 5. Impact of *NRT1.1* mutations (T101A, T101D*)* on *HHO1, HRS1, LBD37, LBD39, BT1, BT2* and PP2C regulation by NO_3^-.

Plants were grown on 1mM NH_4NO_3 for 5 weeks, before being transferred during 72h on 1mM NH_4Cl with 0.1, 1 or 5mM KNO_3. Roots have been collected to assess mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col) and the mutant *chl1.5* are significant at *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Student's *t* test).



Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

Figure 6. Impact of *hh* (*hho1*/*hrs1*), *lbd37*/*39* and *bt1*/*bt2* mutations on root NO_3^- influx regulation by NO_3^-.

Plants were grown on 1mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1mM NH_4Cl with 0.1, 1 or 5mM KNO_3. Root NO_3^- influx was measured at the external concentration of 10 µM $^{15}NO_3^-$. Values are means of 12 replicates ± SD. Differences between WT (Col) and the mutants are significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (Student's *t* test)..

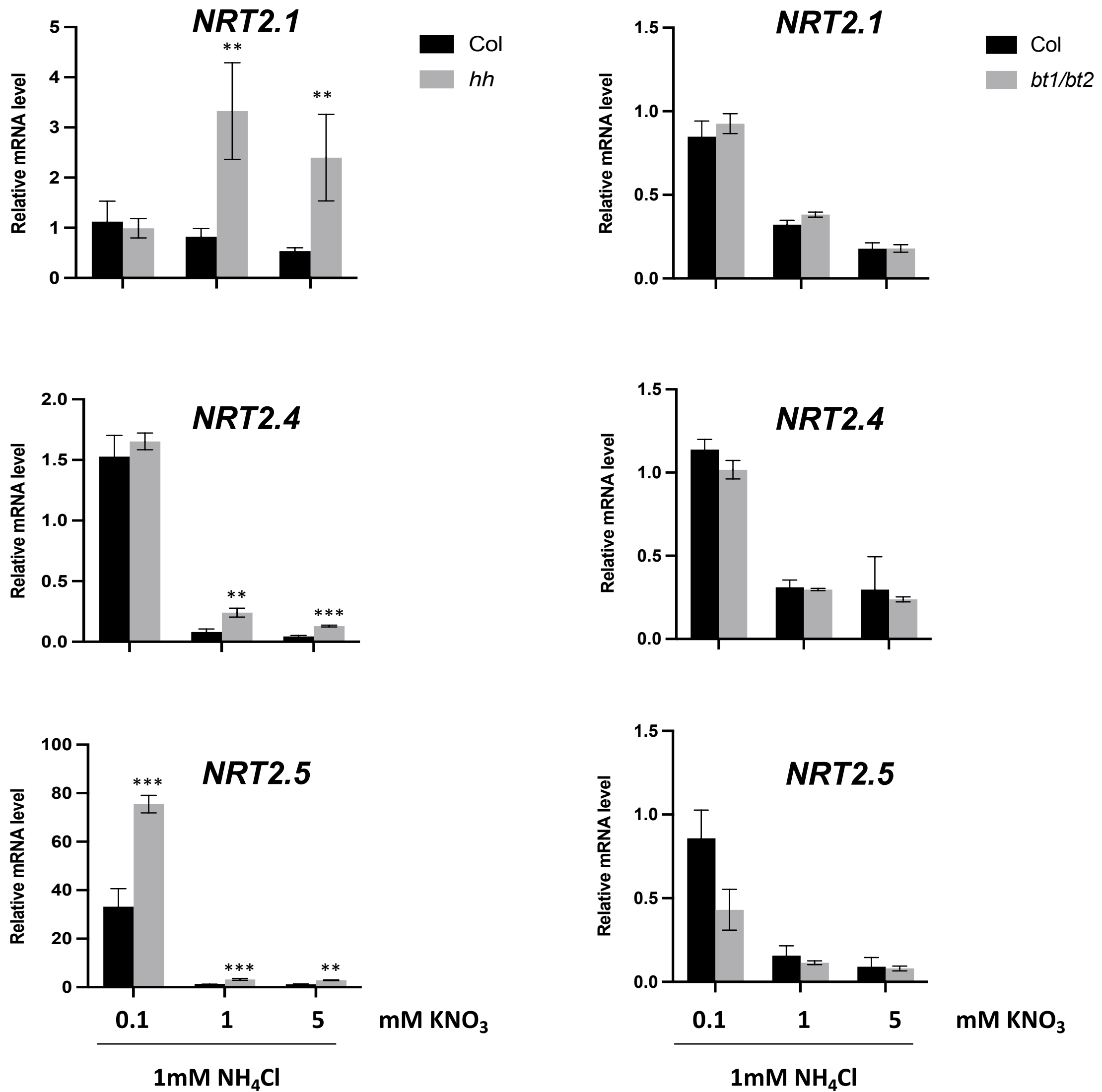


Figure 7. Impact of the double mutation *hho1*/*hrs1* (*hh*) and *bt1*/*bt2* on *NRT2.1*, *2.4, 2.5* repression by NO_3^-.

Plants were grown on 1mM NH_4NO_3 for 5 weeks before being transferred during 72h on 1mM NH_4Cl with 0.1, 1 or 5mM KNO_3. Roots have been collected to assess *NRT2.1*, *NRT2.4* and *NRT2.5* mRNA accumulation by RT-QPCR (relative accumulation to *Clathrin* housekeeping gene). Values are means of three biological replicates ± SD. Differences between WT (Col) and the mutant *chl1.5* are significant at **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t* test).

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023

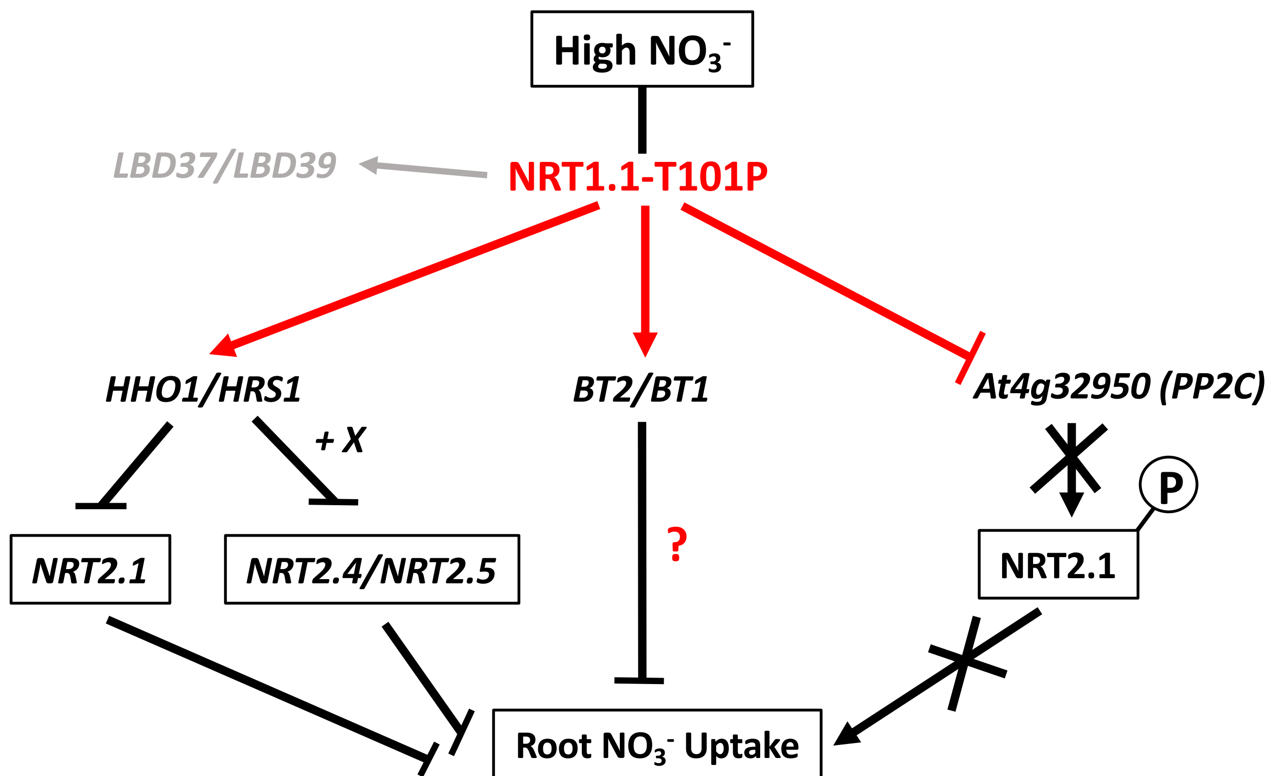


Figure 8. Model for the regulation of root NO_3^- uptake by high NO_3^- concentration

Downloaded from https://academic.oup.com/jxb/advance-article/doi/10.1093/jxb/erad149/7142713 by INIST-CNRS INSU user on 28 April 2023