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Overexpression of Rice Genes *OsNRT1.1A* and *OsNRT1.1B* Restores Chlorate Uptake and *NRT2.1/NAR2.1* Expression in *Arabidopsis thaliana chl1-5* Mutant

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Abstract

Nitrogen uptake by plants is a key step for efficient nitrogen use, which affects plant growth and yield. *Arabidopsis thaliana* gene *NRT1.1* was identified as a transporter related to nitrate (NO_3^-) signaling and uptake. In rice, three orthologs of *NRT1.1*, named *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C*, have been identified. This study evaluated the potential of *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* in NO_3^- signaling and uptake through overexpression in the *Arabidopsis chl1-5* mutant. The expression of *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* was evaluated in the roots and shoots of rice cultivated with NO_3^- or NH_4^+. *OsNRT1.1A* was expressed in the roots and shoots cultivated with NO_3^- and NH_4^+. *OsNRT1.1B* was expressed predominantly in roots of rice cultivated with NO_3^-, while the expression of *OsNRT1.1C* was low in roots and shoots. *Arabidopsis chl1-5* plants were transformed by the floral dip method using *Agrobacterium tumefaciens* to overexpress *OsNRT1.1A* and the alternative splicing product named *OsNRT1.1As*, *OsNRT1.1B*, and *OsNRT1.1C*. The chlorate test showed the ability of *OsNRT1.1A*, *OsNRT1.1B* or *OsNRT1.1C* to take up chlorate, as evidenced by the decrease in fresh weight. The *OsNRT1.1B* lineages presented higher toxicity to chlorate. Gene expression analyses showed that the insertion of *OsNRT1.1A* and *OsNRT1.1B* into *Arabidopsis chl1-5* induced the expression of *NRT2.1* and *NAR2.1*. *OsNRT1.1As* overexpression did not significantly affect the expression of *NRT2.1* and *NAR2.1*. The results show the differential ability of NRT1.1 orthologs in rice to take up chlorate and signal the expression of other nitrate transporters, which may affect the efficiency of nitrogen utilization and its uptake.

Keywords Nitrate signaling · Gene expression · *Oryza sativa* · Gateway cloning · Chlorate toxicity · Nitrate transport

Introduction

Rice is one of the most consumed cereals worldwide and is part of the daily and staple diet of more than half the world's population; it is one of the main crops for the maintenance of food security of various countries. The majority of crops remove significant amounts of N from the soil, and significant applications of nitrogen fertilizers are necessary to maintain the productivity of these crops (O'Brien et al. 2016). Nitrogen is usually the element required the most by the plants and often limits crop yield, and the preferentially absorbed forms are inorganic NO_3^- and NH_4^+ (Glass, 2003). NO_3^- is absorbed by transporters of the NRT (Nitrate Transporter) and NPF (Nitrate Peptide Transporter Family) gene families, which may belong to the group of HATS (High-Affinity transport system) or LATS (Low-Affinity transport system), allowing the uptake of NO_3^- and H^+ ions via symport ($2H^+/1NO_3^-$) over a wide range of concentration (Glass et al. 1992; Glass 2003).

Characterization of different NRT1 transporters indicated the transport of several molecules besides NO_3^-, for example, the AtNRT1.1 transporter of *Arabidopsis thaliana*,

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besides transporting NO_3^- can also facilitate auxin transport (Krouk et al. 2010). Thus, Leran et al. (2014) and von Wittgenstein et al. (2014) performed a new classification of the NRT1 family and renamed it NPF family (Nitrate Peptide Transporter Family). In *Arabidopsis*, the NRT1/NPF family genes encode for LATS transporters that operate at high concentrations of nitrate, usually above 1 mM (Glass 2003). The transporter AtNRT1.1 (AtNPF6.3) has the ability to absorb NO_3^- in the high-affinity and low-affinity range and is regulated by phosphorylation and dephosphorylation of threonine 101 (T101) (Ho et al. 2009). This dual affinity transporter needs the protein kinase CIPK23 when NO_3^- is present at low concentrations (Ho et al. 2009; Sun and Zheng 2015). This regulatory mechanism describes how the plants adapt to varying concentrations of nitrogen in the environment (Liu and Tsay 2003; Fredes et al.2019). Phosphorylation of T101 of NRT1.1 is essential for the regulation of the transporter in response to NO_3^-, and for controlling the growth of lateral roots mediating the flow of auxin (Bouguyon et al. 2015, 2016). The deletion of the *NRT1.1* gene gave rise to the *Arabidopsis* mutant *chl1-5*, which confers chlorate tolerance and impairs signaling for the *AtNRT2.1* gene expression (Ho et al.2009).

In rice, three potential orthologs of the *AtNRT1.1* gene were identified, named *OsNRT1.1A* (*OsNPF6.3*), *OsNRT1.1B* (*OsNPF6.5*) and *OsNRT1.1C* (*OsNPF6.4*) (Plett et al. 2010). OsNRT1.1B is associated with uptake of NO_3^- through the roots and transport to the shoot, demonstrated by *osnrt1.1b* mutant rice plants that also exhibited decreased expression of *OsNIA1* and *OsNIA2* but no change in *OsNRT2.1* expression (Hu et al.2015). *OsNRT2.1* is induced by NO_3^-, and *osnrt2.1* mutant rice plants have decreased uptake of NO_3^- (Feng et al. 2011). In *Arabidopsis*, the expression of *NRT2.1* is controlled by NRT1.1 and depends on the phosphorylation of T101 (Ho et al. 2009; Bouguyon et al. 2015). Interestingly, *OsNRT1.1B* shares a common ancestor with *NRT1.1* from *Arabidopsis* (Hu et al. 2015).

It is essential to properly understand the regulation of the expression of NO_3^- transporters (NRT) to increase the efficiency of NO_3^- uptake (Plett et al. 2018). The uptake capacity of NO_3^- promoted by NRT2.1 depends on the NAR2.1 in *Arabidopsis* and rice (Orsel et al. 2006; Yan et al. 2011). It is difficult to study the individual function of *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* in rice because of a possible countervailing effect by another isoform of *OsNRT1.1* (Plett et al. 2010). Therefore, we used *chl1-5* mutant *Arabidopsis* plants which do not have the *NRT1.1* gene and thus could express *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* individually.

In the present study, the rice genes *OsNRT1.1A*, *OsNRT1.1As* (*OsNRT1.1A* alternative splicing), *OsNRT1.1B* and *OsNRT1.1C* were overexpressed to verify which genes are capable of reversing the *Arabidopsis chl1-5* mutant phenotype. Thus, the objectives of this study were to: (i) study the expression of the *OsNRT1.1A*, *OsNRT1.1B,* and *OsNRT1.1C* genes in rice roots and shoots grown with NO_3^- or NH_4^+, (ii) evaluate the survival of *Arabidopsis chl1-5* mutant plants overexpressing the *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B,* and *OsNRT1.1C* genes in the presence of chlorate, and (iii) assess whether the *chl1-5* mutant plants with overexpressed *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B* and *OsNRT1.1C* had restored the lost nitrate transport and signaling capacity in the *chl1-5* mutant. This is the first report showing the capacity of these genes to reverse the *Arabidopsis chl1-5* mutant for the NRT1.1 transceptor.

Material and Methods

Expression of *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* Genes in Roots and Shoots of Rice Cultivated with NO_3^- and NH_4^+

Nipponbare rice cultivar seeds were disinfected in 2% sodium hypochlorite, followed by washes with distilled water, and germinated on gauze in containers containing distilled water. Six days after germination (DAG), four seedlings were transferred to 700 mL pots containing Hoagland's solution (Hoagland and Arnon 1950) modified to its 1/4 ionic strength (1.5 mM K, 0.25 mM P, 0.5 mM Mg, 1.0 mM Ca, 0.5 mM S, 2.72 µM Cl, 11.57 µM B, 2.25 µM Fe, 0.075 µM Cu, 0.025 µM Mo, 2.72 µM Mn, and 0.2 µM Zn) and containing 2 mM NO_3^--N or 2 mM NH_4^+-N and pH 5.8. After three days the two groups (NO_3^- and NH_4^+) of plants received a modified solution of 1/2 ionic strength, which was changed every three days and with 2 mM NO_3^--N or 2 mM NH_4^+-N. 18 DAG, 2/3 of the pots of each group received a modified solution without N while the rest of the pots continued receiving solution with 2 mM of NO_3^--N or 2 mM of NH_4^+-N. At 21 DAG plants with 2 mM N solution received the solution with the same earlier composition, while half of the plants grown in N-free solution for three days received 2 mM of N (NO_3^- or NH_4^+), and the other half received new solution without N (starvation). Sampling occurred on the 21 DAG three hours after the application of the solutions (Online Resource 1). Thus, the treatments were: (i) constant supply with NO_3^--N, (ii) resupply with 2.0 mM NO_3^--N after 72 h without NO_3^-, (iii) deficiency of NO_3^- for 72 h, (iv) constant supply with 2.0 mM of NH_4^+-N, (v) resupply with 2.0 mM of NH_4^+-N after 72 h without NH_4^+, and (vi) deficiency of NH_4^+ for 72 h.

Extraction of Total RNA

Total RNA was extracted as per the procedure described by Gao et al. (2001) with modifications cited in Sperandio et al. (2011). Samples of each plant were macerated in liquid nitrogen, and approximately 100 mg of macerated tissue was transferred to microtubes containing 500 µL of extraction buffer (200 mM Tris–HCl pH 7.5, 100 mM LiCl, 5 mM EDTA, and 1% SDS). After mixing samples and the extraction buffer, 250 µL of phenol and 250 µL of chloroform were added, mixed on a vortex mixer for one minute and then centrifuged for 10 min at 20000 × g. The aqueous phase was placed in fresh tubes containing 250 µL each of phenol and chloroform. This mixture was again mixed on a vortex mixer for one minute, centrifuged for 10 min at 20000 × g, and the aqueous phase transferred to a fresh tube. The RNA was precipitated with one volume of 6 M LiCl for 16 h at 4 °C.

The samples were centrifuged for 10 min at 20000 × g, the supernatant discarded and the precipitate was resuspended in 1 mL of 3 M LiCl for a second precipitation for 10 min at 20000 × g. The supernatant was discarded and the precipitate resuspended in 250 µL of ultrapure H_2O treated with Diethylpyrocarbonate (Milli-Q-DEPC). After complete dissolution of the precipitate, 0.1 volume of 3 M sodium acetate (25 µL) and two volumes of absolute ethanol (550 µL) were added. This mixture was maintained in the freezer −80 °C for 2 h and then centrifuged for 15 min at 20,000 × g. The supernatant was discarded and the precipitate was washed with 300 µL of 70% ethanol. After drying the precipitate, the RNA was resuspended in 50 µL of milliQ-DEPC water and stored in a freezer at −80 °C.

Treatment with DNase and Synthesis of cDNA

After quantification of total RNA using a Nanodrop 2000C (Thermo Scientific), 500 ng of total RNA was treated with DNase I using a kit (Thermo Scientific) according to the manufacturer's instructions. The synthesis of cDNA was performed using the GoScript™ Reverse Transcription System kit (Promega) according to the manufacturer's instructions.

Real-Time PCR

PCR reactions were performed in a StepOne Plus system (Applied Biosystems) using cDNA, primers (Online Resource 2) and EvaGreen (Solis Biodyne) according to the manufacturer's specifications. The gene sequences were obtained from the TAIR site (https://www.arabidopsis.org/) for *Arabidopsis* and TIGR (https://rice.plantbiology.msu.edu/) for rice. The specificity of the primers was verified using the primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). After the PCR reaction, the specificity of the reactions was verified by analyzing the dissociation curve. All primers used showed high specificity for the target gene. The expression was calculated according to Livak and Schmittgen (2001) using the Ct values (cycle threshold) obtained after real-time PCR. The sequences of the primers used for real-time PCR are listed in Online Resource 2.

Construction of Vectors Overexpressing *OsNRT1.1A*, *OsNRT1.1A* Splicing, *OsNRT1.1B* and *OsNRT1.1C* in *Arabidopsis chl1-5*

Total RNA from the Nipponbare rice variety was used to synthesize cDNA using the SuperScript™ III Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Genes were cloned using the Gateway cloning system (Thermo Scientific) according to the manufacturer's recommendations. The primers for *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1*.1B, and *OsNRT1.1C* were synthesized containing the Gateway *attB* recombination sites (Online Resource 3). Two PCR reactions were performed, where a part of the *attB* site was placed in the primer containing the annealing site in the target gene, and the remainder of the *attB* site was placed in a second PCR reaction. PCR reactions were performed using the Phusion kit (Finnzymes) according to the manufacturer's instructions. The *OsNRT1.1A* gene undergoes alternative splicing, one called *OsNRT1.1A* complete (or only *OsNRT1.1A*) containing all exons, while the second part, *OsNRT1.1As* does not contain the first exon (alternative splicing).

After isolating the genes with the *attB* sites, they were cloned in the vector pDONR™221 using the Gateway BP Clonase™ II Enzyme mix kit (Thermo Scientific) according to the manufacturer's recommendations to obtain the entry vectors containing the *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B* and *OsNRT1.1C* sequences. The reaction product was transferred in *Escherichia coli* strain *DH5α* by electroporation and plated on solid LB medium containing the antibiotic kanamycin (50 µg mL^{-1}). The vector was extracted using the PureYield™ Plasmid Miniprep system (Promega) according to the manufacturer's recommendations. The expression vectors were obtained by carrying out a triple LR reaction containing the pK7m34GW target vector and the entry vectors containing the 35S promoter (pENTR-35S), the genes of interest (*OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B*, and *OsNRT1.1C*) and a HA tag (pENTR-3xHA), using the Gateway™ LR Clonase™ Enzyme mix Kit (ThermoScientific) according to the manufacturer's

recommendations. The reaction product was electroporated into *E. coli* strain DH5α, plated on solid LB medium containing the antibiotics spectinomycin/streptomycin (Sm/Sp) (50 µg mL^{-1} and 20 µg mL^{-1}, respectively). Vectors were isolated with the PureYield™ Plasmid Miniprep System Kit (Promega) and were used to transform *Agrobacterium tumefaciens* strain LBA4404 by heat shock for further transformation of *Arabidopsis*. A schematic model of expression vector assembly is presented in Online Resource 4.

Transformation of *Arabidopsis chl1-5* Mediated by *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain LBA4404 was used to transform the *Arabidopsis chl1-5* mutant with the expression vectors containing the genes *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B*, and *OsNRT1.1C*. After the transformation of *Agrobacterium* by heat shock and confirmation by colony PCR, pre-inoculum was grown in 1 mL of YEB medium with Sm/Sp antibiotics (50 µg mL^{-1} and 20 µg mL^{-1}, respectively). Subsequently, the volume was raised to 10 mL with YEB medium and stirred overnight to reach an OD (optical density) close to 2. Forty milliliters of a solution containing 10% sucrose and 0.05% silwet was added to the 10 mL culture and floral dip was performed by dipping the inflorescence of *Arabidopsis chl1-5* mutant plants in the pre-flowering phenological phase (Clough and Bent 1998; Mara et al. 2010). Eight *chl1-5* plants, grown under the same previously reported conditions were used for each construct.

Selection of Homozygous *Arabidopsis* Plants

Seeds of *Arabidopsis chl1-5* submitted to the floral dip transformation process were transferred to Petri dishes of 12 mm diameter containing MS culture medium (Murashige and Skoog, 1962) at half of the ionic strength with 1% sucrose, 0.5 g L^{-1} of MES, 50 mg L^{-1} of kanamycin, solidified with 0.7% bacto-agar, pH 5.7. The dishes were closed and sealed with microporous tape, and placed in the refrigerator at 4 °C for three days. The dishes were transferred to a BOD at 25 °C with approximately 80 µmol photons m^{-2} s^{-1} and 14 h photoperiod. The plants with the kanamycin-resistance phenotype were selected for propagation on the substrate to obtain homozygous inbred lineages (T3) (Clough and Bent 1998; Zhang et al.2006; Mara et al. 2010). For the subsequent analytical steps, two homozygous inbred lineages (T3) were chosen for each transformation: *OsNRT1.1A* lineages *1.2* and *3.1, OsNRT1.1As* lineages *2.2* and *1.1, OsNRT1.1B* lineages *1.8* and *2.2*, and *OsNRT1.1C* lineages *1.1* and *2.1*. The confirmation of the insertion and expression levels of *Arabidopsis chl1-5* overexpressing *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B* and *OsNRT1.1C* genes was done by Real-Time PCR as described above with the primers listed in Online Resource five. The expression of NRT1.1 in *Arabidopsis* WT was used as control.

Analysis of Gene Expression, Nitrate and Amino Acid Content in *Arabidopsis chl1-5* Containing the Isoforms *OsNRT1.1A, OsNRT1.1As, OsNRT1.1B*, and *OsNRT1.1C*

Seeds of WT *Arabidopsis* Columbia ecotype (Col 0), *chl1-5* mutant and homozygous plants transformed with the *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B*, and *OsNRT1.1C* genes were propagated on substrate mixed with vermiculite (2:1) in 150 mL pots and placed in plastic trays covered with PVC film. The trays were placed in a plant growth chamber with an average luminous flux of 140 µmol photons m^{-2} s^{-1}, an average temperature of 23 °C and 65% humidity. Twenty-three days after planting, eight applications of a 15 mL solution containing 12 mM sodium chlorate, 3.75 mM ammonium sulfate and 8.25 mM potassium nitrate per pot (Wang et al. 1988) were initiated. On the 39th day after planting, the plants were photographed whit a camera Canon model SX400, after they harvested to obtain comparative parameters of the effect of chlorate on their metabolism. The control treatment consisted of seeds of all transformed, WT and *chl1-5* mutant treated with the same nutrient concentrations without chlorate.

The plants cultivated without chlorate were used for the analysis of gene expression by real-time PCR. The extraction of total RNA, treatment with DNase, synthesis of cDNA and real-time PCR were performed as previously described. The expression of the *NRT2.1* and *NAR2.1* (Santos et al.2012) were assessed. The primers used are listed in Online Resource 6. The content of NO_3^--N and amino acid were performed according to the methods of Miranda et al. (2001) and Yemm and Cocking (1955), respectively.

Alignment of Rice OsNRT1.1 Isoforms and *Arabidopsis* NRT1.1

Multiple sequence alignment of the amino acids sequences between rice OsNRT1.1 isoforms (OsNRT1.1A, OsNRT1.1As, OsNRT1.1B and OsNRT1.1C) and *Arabidopsis* NRT1.1 was performed using Clustalw2 with default parameters (https://www.genome.jp/tools-bin/clustalw).

Statistical Analysis

The experiments were conducted using a completely randomized design. The results were subjected to analysis of variance using the program Sisvar (Ferreira 2014). Comparisons of the means were made by the LSD test ($p < 0.05$).

Results

Expression of *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C* Genes in Roots and Shoots of Rice

The expression profile of the isoforms *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C* varied with treatments in NO_3^- and NH_4^+ (Fig. 1). The isoform *OsNRT1.1A* displayed higher expression in the roots of plants grown with NO_3^- and NH_4^+, in particular with a constant supply of 2 mM N (Fig. 1). The expression of *OsNRT1.1B* was higher in the roots with a constant supply of 2 mM of NO_3^--N, while it was downregulated in plants grown with NH_4^+ (Fig. 1a, b). Resupply of 2 mM of NO_3^--N did not induce the expression of *OsNRT1.1A* and *OsNRT1.1B* in roots (Fig. 1a, b). Expression of *OsNRT1.1A* and *OsNRT1.1B* are lower in shoots, meanwhile *OsNRT1.1C* displayed low expression in roots and shoots in plants grown with NO_3^- or NH_4^+ (Fig. 1). Thus, the expression of *OsNRT1.1A* in roots occurred regardless of the form of N applied, while *OsNRT1.1B* was expressed mainly in the roots of plants grown in a constant supply of NO_3^- and downregulated by NH_4^+ provision. The gene expression analysis suggests the higher importance of *OsNRT1.1A* and *OsNRT1.1B* in rice compared to *OsNRT1.1C*.

Effect of Overexpression of *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B*, and *OsNRT1.1C* in *Arabidopsis chl1-5*

The *chl1-5* lineages overexpressing *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B*, and *OsNRT1.1C* presented different expression levels (Fig. 2). The lineages *OsNRT1.1A L3.1*, *OsNRT1.1B L2.2* and *OsNRT1.1C L2.1* presented higher expression levels compared to *OsNRT1.1As L2.2*, *OsNRT1.1B L1.8* and *OsNRT1.1C L1.1* (Fig. 2).

As expected, the application of chlorate to WT plants resulted in a strong reduction in growth, while no toxic effect of chlorate was observed in the *chl1-5* mutant (Fig. 3). On comparing the lineages overexpressing *OsNRT1.1A* and *OsNRT1.1As*, a variation in growth compared to *chl1-5* was observed (Fig. 3). The chlorate-sensitive phenotype by the *OsNRT1.1B* transporter overexpression for the *OsNRT1.1B L1.8* and *L2.2* was more characteristic and approached the WT plant (Fig. 4), indicating that *OsNRT1.1B* may be the predominant isoform in low-affinity nitrate absorption in rice, as its expression was able to revert the mutant *chl1-5* phenotype as that of WT. The overexpression of *OsNRT1.1C L1.1* and *L2.1* also showed evidence of damage due to the chlorate treatment (Fig. 4).

When 12 mM of chlorate was applied there was no significant reduction of fresh mass in the mutant *chl1-5* (Fig. 5). However, in the case of the WT plant and all the transformed lineages, a significant reduction of fresh weight was observed with chlorate application (Fig. 5).

The importance of the chlorate test and the function of *NRT1.1* were characterized and the effect of comparison of the effect of chlorate on plants with and without chlorate treatment, are shown in Fig. 6. *Arabidopsis* plants overexpressing *OsNRT1.1B* showed growth reduction similar to that of WT plants when treated with chlorate (90% fresh mass reduction), while plants overexpressing *OsNRT1.1A* (complete and splicing) and *OsNRT1.1C* plants displayed more than 40% fresh mass reduction when treated with chlorate (Fig. 6).

The overexpression of *OsNRT1.1A* and *OsNRT1.1B* increased the expression of transporter *NRT2.1,* although it was not affected by the overexpression of *OsNRT1.1C* when compared with *chl1-5* plants (Fig. 7a). Curiously, the overexpression of the alternative splicing product of *OsNRT1.1A (OsNRT1.1As)* did not increase the expression of *NRT2.1* when compared with *chl1-5* plants, and unlike plants overexpressing the complete sequence of *OsNRT1.1A* (Fig. 7a). The lineage *OsNRT1.1A L3.1* displayed higher expression of *OsNRT1.1A* (Fig. 2) and *NRT2.1* (Fig. 7a), suggesting the differences of *NRT2.1* expression was partially caused by different levels of *OsNRT1.1A* expression in *Arabidopsis chl1-5*.

WT *Arabidopsis* plants displayed a higher expression of *NAR2.1* compared to *chl1-5*, as well as *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* lineages, while *OsNRT1.1As* overexpression did not affect the expression of *NAR2.1* (Fig. 7b). WT *Arabidopsis* induced *NAR2.1* by twofold compared to *chl1-5*, meanwhile overexpression of *OsNRT1.1A L1.2* and *L3.1* induced *NAR2.1* by 77 and 26 times compared to *chl1-5*, respectively. Overexpression of *OsNRT1.1B L1.8* and *L2.2* induced *NAR2.1* by 53 and 14 times compared to *chl1-5*, respectively (Fig. 7b). Analysis of the amino acid sequences of OsNRT1.1A, OsNRT1.1As, OsNRT1.1B, OsNRT1.1C, and *Arabidopsis* NRT1.1 shows that OsNRT1.1A, OsNRT1.1B, and OsNRT1.1C have a threonine (T) residue in a position similar to the T101 on the *Arabidopsis* NRT1.1 transporter (positions T107, T104 and T118 in OsNRT1.1A, OsNRT1.1B and OsNRT1.1C, respectively), while OsNRT1.1As does not present the threonine residue due to loss of the first exon

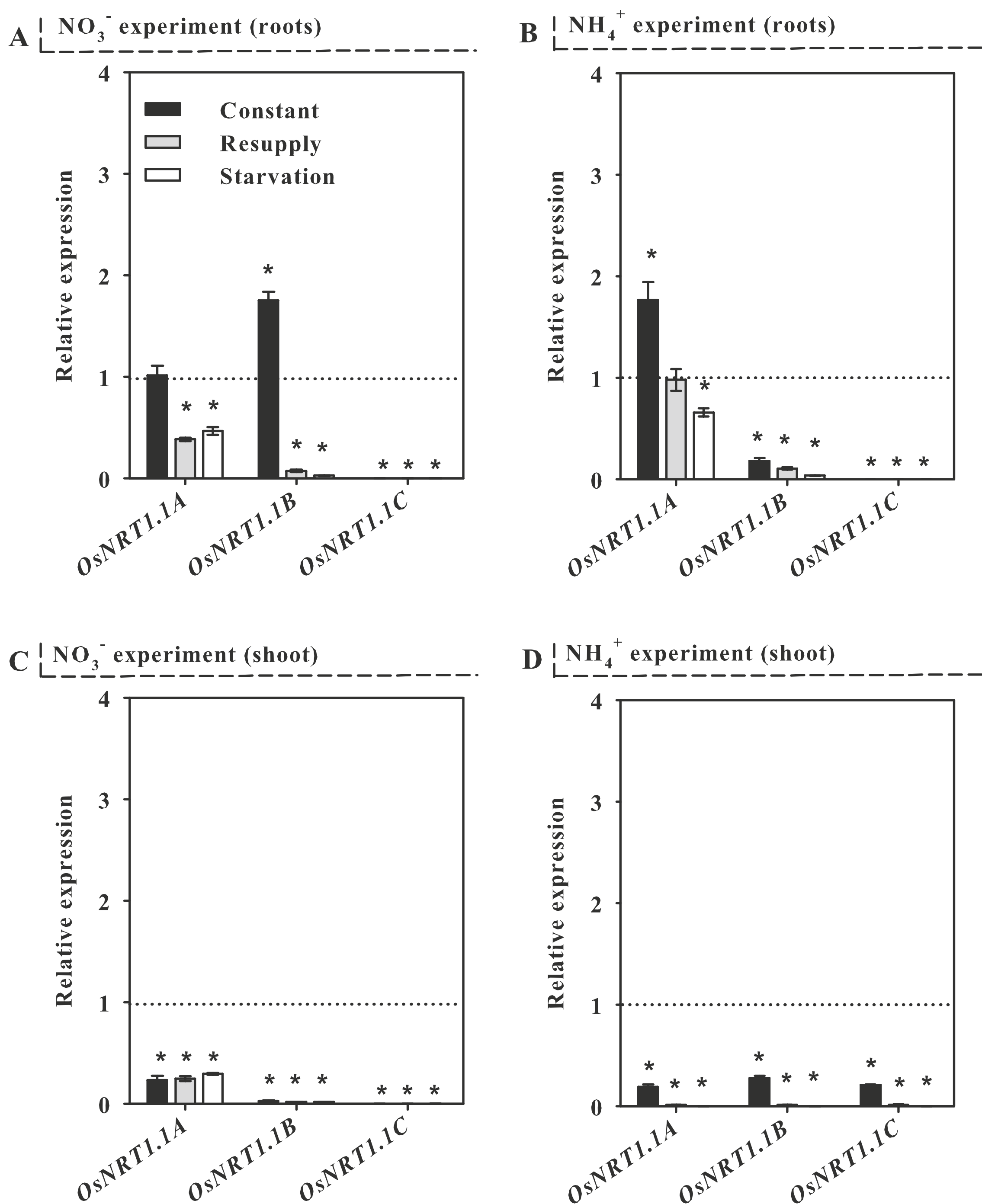

Fig. 1 Expression of *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C* in the roots **a** and **b** and shoots **c** and **d** of rice cultivated with NO_3^- **a** and **c** and NH_4^+ **b** and **d**. The treatments used were constant supply with 2 mM of NO_3^--N or NH_4^+-N, resupply with 2 mM of NO_3^--N or NH_4^+-N after 72 h of N-deprivation and NO_3^- and NH_4^+ deficit for 72 h. *Significantly different from OsNRT1.1A with constant NO_3^- treatment in the roots according to a Least Significant Difference test ($p < 0.05$)

with alternative splicing (Fig. 8). Altogether, the results indicate the first exon in OsNRT1.1A containing the T107 is essential to *NRT2.1* and *NAR2.1* induction.

WT *Arabidopsis* presented increased NO_3^--N and amino acid content compared to *chl1-5* (Fig. 9a, b). According to WT plants, *Arabidopsis chl1-5* overexpressing *OsNRT1.1A* and *OsNRT1.1B* increased NO_3^--N and amino acid content compared to *chl1-5* as well, however, *chl1-5* overexpressing *OsNRT1.1As* and *OsNRT1.1C* did not increase NO_3^--N and amino acid content (Fig. 9a, b).

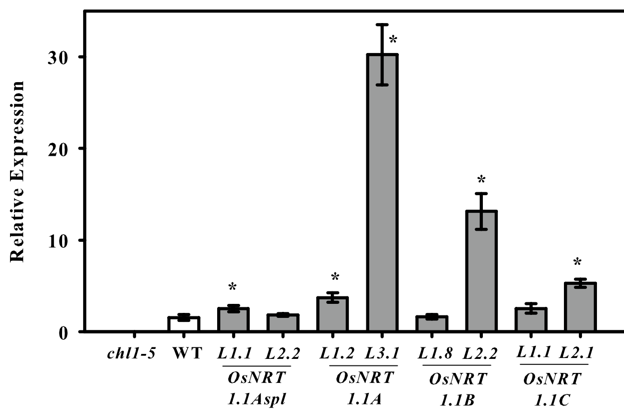

Fig. 2 Expression analysis of *OsNRT1.1* in lineages of *Arabidopsis chl1-5* overexpressing the rice genes *OsNRT1.1A* splicing (*OsNRT1.1As*), *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C*. Two lineages were used for each mutant overexpressing *OsNRT1.1* family. The WT expression of *NRT1.1* was assigned value 1. *Significantly different from WT according to a Least Significant Difference test ($p < 0.05$)

Discussion

Plants need to regulate the expression of NO_3^- transporters to grow in environments with varying levels of N. The expression of transporters essential for the absorption of NO_3^- in *Arabidopsis* is regulated by the *NRT1.1* transporter (Ho et al. 2009; Bouguyon et al. 2015, 2016). Rice has three *NRT1.1* orthologs, namely *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* (Plett et al. 2010). The importance of *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* in N uptake and expression of *NRT2.1* and *NAR2.1* promoted by NO_3^- was assessed by evaluating their expression in rice plants and subsequently by overexpressing *OsNRT1.1A*, *OsNRT1.1As*, *OsNRT1.1B*, and *OsNRT1.1C* in *Arabidopsis chl1-5*.

The expression results indicate a complex regulation of the *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* genes in rice (Fig. 1). While *OsNRT1.1A* expression in roots and shoots displayed less variation, *OsNRT1.1B* seems to be expressed mainly in the roots and with a constant supply of NO_3^-, and *OsNRT1.1C* exhibited lower expression between *OsNRT1.1* orthologs. These results indicate a greater involvement of *OsNRT1.1A* and *OsNRT1.1B* orthologs in rice, and the participation of *OsNRT1.1A* in the movement of NO_3^- in the shoots. NH_4^+ and its assimilation products, especially glutamine, are powerful inhibitors of several NO_3^- transporters (Glass et al. 2003). Here, NH_4^+ was used as a source of N in the nutrient solution to verify the expression of *OsNRT1.1A* , *OsNRT1.1B*, and *OsNRT1.1C* in repressive conditions. The expression of *OsNRT1.1A* presented little inhibitory effect on the roots of plants grown in the presence of NH_4^+, indicating low sensitivity to variations in the N status in the plant. On the other hand, the expression of *OsNRT1.1B* was only induced by NO_3^- in the roots of rice and repressed by NH_4^+, suggesting that it can contribute to the uptake of NO_3^- in the roots and was less significant in the flow of NO_3^- in the shoots. A higher expression of *OsNRT1.1B* is related to higher NO_3^- uptake and transport to the shoots in rice (Hu et al. 2015), while, high expression of *OsNRT1.1A* in the shoots (Fig. 1) suggests that this transporter can also participate in the internal flow of NO_3^-.

Rice plants overexpressing *OsNRT1.1A* reveal its regulation by NH_4^+, suggesting that this transporter is important in flooded areas for rice production (Wang et al. 2018). The results reported by Wang et al. (2018) are similar to the results of our study (Fig. 1), where NH_4^+ is responsible for inducing the expression of *OsNRT1.1A*. Wang et al. (2018) also demonstrated that under field conditions the mutant *osnrt1.1a* has decreased plant size, panicle size, and weight of 1000 seeds, on the other hand, the overexpression of *OsNRT1.1A* in *Arabidopsis* resulted in an increase of the size of the plant and shorter flowering with a reduction of 6 to 15 days (Wang et al. 2018).

The *Arabidopsis chl1-5* plants overexpressing *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C* and *OsNRT1.1As* were assessed by applying chlorate. Fig. 3, 4 show the toxicity promoted by the chlorate (molecule analogous to nitrate). After being absorbed, chlorate is reduced within the cells to chlorite by nitrate reductase, which is toxic to the plant, resulting in the reduction of fresh mass for all transformed isoforms as well as the WT plant. The decrease of fresh weights with the overexpression of *OsNRT1.1A*, *OsNRT1.1B*, *OsNRT1.1C*, and *OsNRT1.1As* indicates the individually isoform function in the transport of nitrate, however, overexpression of *OsNRT1.1B* led to a greater reduction of fresh weight between the lineages and similar to that exhibited by WT after the application of chlorate (Fig. 6), which could be confirmed visually (Fig. 4). The fresh weight reduction of *Arabidopsis* overexpressing *OsNRT1.1A* and *OsNRT1.1As* treated with chlorate clearly indicates that the first exon is not necessary for the absorption function of NO_3^- (Fig. 3). On the other hand, even a low expression of *OsNRT1.1C* in rice (Fig. 1) may enable the transport of NO_3^- (Fig. 4).

The NRT1.1 transporter is essential for the regulation of *NRT2.1* expression in *Arabidopsis* (Ho et al. 2009; Bouguyon et al. 2015, 2016). The analysis of expression in *Arabidopsis chl1-5* plants overexpressing *OsNRT1.1A*, *OsNRT1.1B*, *OsNRT1.1C*, and *OsNRT1.1As* revealed that only *OsNRT1.1A* and *OsNRT1.1B* could increase the expression of *NRT2.1* compared to the *Arabidopsis chl1-5* (Fig. 7a). The nitrate-induced high-affinity transport system requires NAR2.1 for its functionality (Orsel et al. 2006; Yan et al. 2011), although the NAR2.1 does not transport NO_3^- (Orsel et al. 2006). *Arabidopsis chl1-5* lineages overexpressing *OsNRT1.1A*, *OsNRT1.1B,* and *OsNRT1.1C*

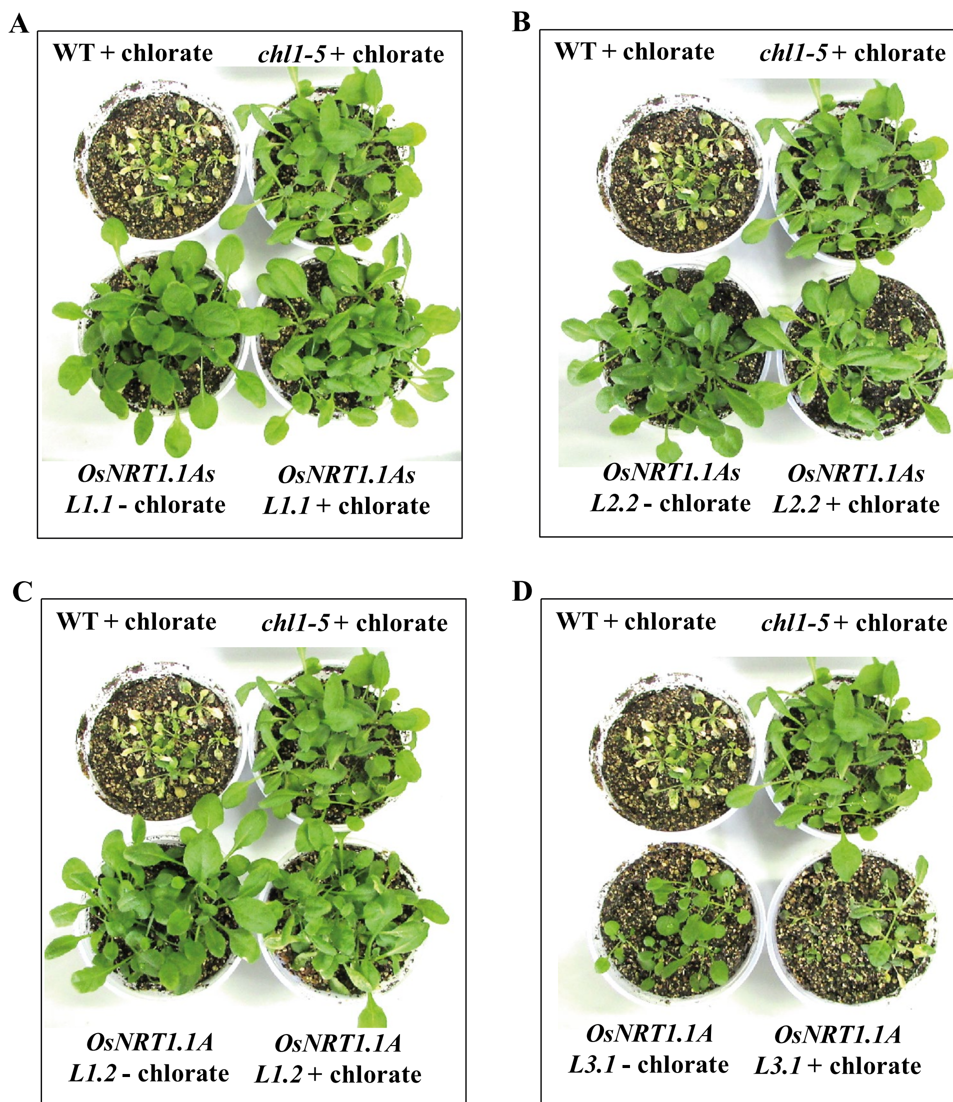

Fig. 3 *Arabidopsis thaliana* wild-type (WT) plants, mutant *chl1-5,* and lineages of *Arabidopsis chl1-5* overexpressing *OsNRT1.1As L1.1* **a**, *OsNRT1.1As L2.2* **b**, *OsNRT1.1A L1.2* **c** and *OsNRT1.1A L3.1* **d**, with and without sodium chlorate ($NaClO_3$)

exhibited high *NAR2.1* induction compared to *chl1-5*, while *Arabidopsis* WT displayed low induction (Fig. 7b).

The results obtained show the ability of OsNRT1.1A and OsNRT1.1B to induce the expression of *NRT2.1* and *NAR2.1* in *Arabidopsis chl1-5* and may also have signaling functions in rice, as the expression of *OsNRT2.1* and *OsNAR2.1* is induced by NO_3^- in a manner similar to that in *Arabidopsis* (Araki and Hasegawa 2006). The T101 residue in the NRT1.1 transporter of *Arabidopsis* is essential for signaling activity (Ho et al. 2009; Bouguyon et al. 2015, 2016). The overexpression of *OsNRT1.1As* in *Arabidopsis chl1-5* was not able to induce the expression *NRT2.1* and *NAR2.1* as observed in plants overexpressing *OsNRT1.1A* and *OsNRT1.1B* which possess the threonine residue in a position similar to the T101 of *Arabidopsis* NRT1.1 (Fig. 8). The gene expression analysis indicates that the first exon present in *OsNRT1.1A*, *OsNRT1.1B*, and *OsNRT1.1C* containing the T107, T104, and T118 residue, respectively (Fig. 8), may present an essential function for the signaling promoted by NO_3^- in rice. However, the first exon in *OsNRT1.1A* does not seem to be essential for the transport capacity of NO_3^-, given that *Arabidopsis chl1-5* overexpressing *OsNRT1.1As* displayed effects of toxicity in the presence of chlorate (Fig. 6). The importance of *OsNRT1.1A* and *OsNRT1.1B* to induce the expression of *NRT2.1* and *NAR2.1* was also supported by the increased NO_3^--N and amino acid content in *chl1-5* lineages overexpressing *OsNRT1.1A* and *OsNRT1.1B* compared to *chl1-5* plants, similar to those presented in WT plants (Fig. 9). The protein CPSF30-L regulates *NRT1.1* expression in *Arabidopsis* and the *cpsf30* mutant decreases *NRT1.1* expression and NO_3^- accumulation compared to WT (Li et al. 2017). Compared to *chl1-5* mutants, *chl1-5* overexpressing *OsNRT1.1As* and *OsNRT1.1C* did not increase *NRT2.1* expression, NO_3^- content and amino acid, indicating the importance of *OsNRT1.1A* and *OsNRT1.1B* in NO_3^- signaling.

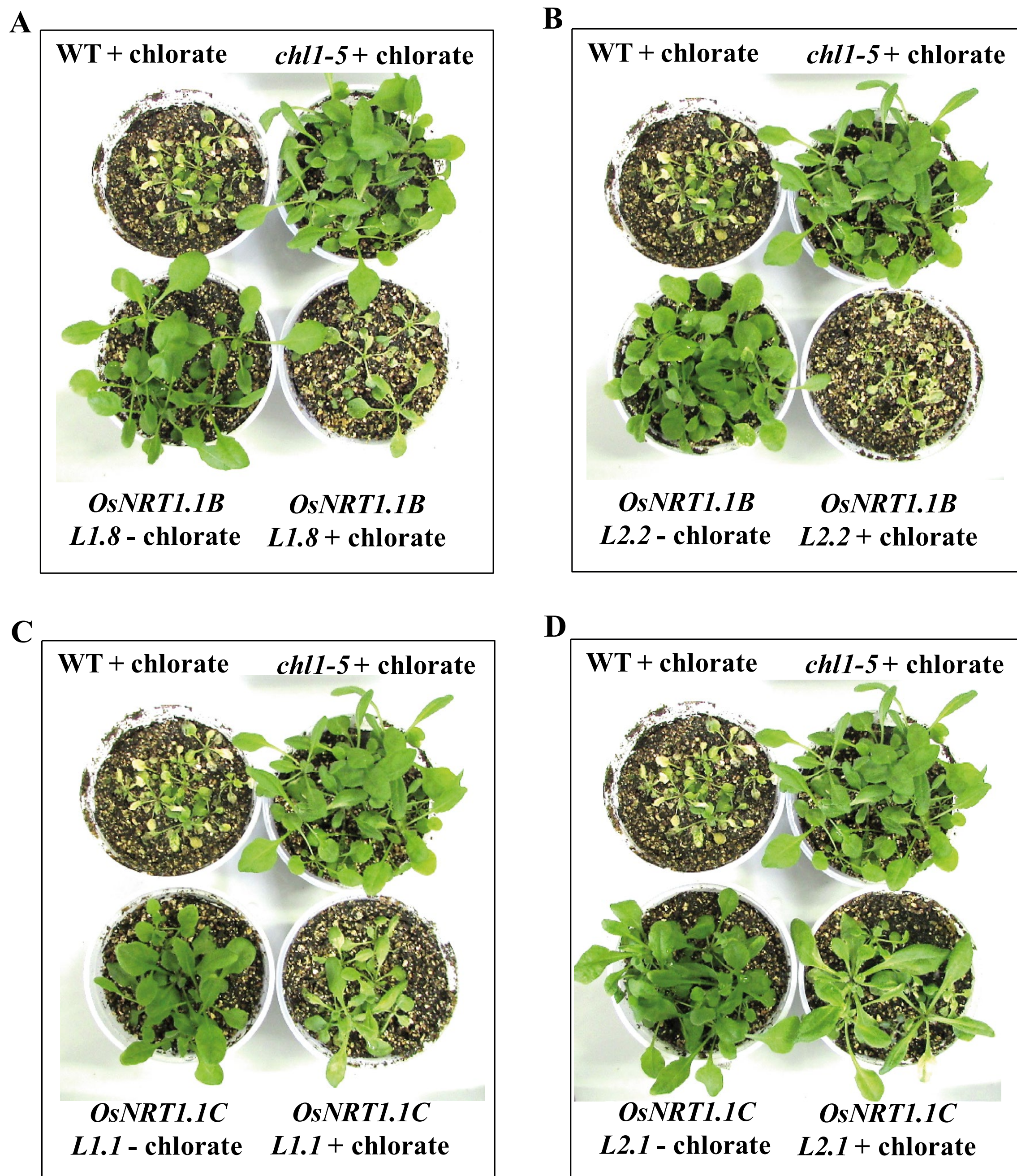

Fig. 4 *Arabidopsis thaliana* wild-type (WT) plants, mutant *chl1-5,* and lineages of *Arabidopsis chl1-5* overexpressing *OsNRT1.1B L1.8* **a**, *OsNRT1.1B L2.2* **b**, *OsNRT1.1C L1.1* **c** and *OsNRT1.1C L2.1* **d**, with and without sodium chlorate ($NaClO_3$)

In rice, the *OsNRT1.1A* and *OsNRT1.1B* transporters are highly expressed and can signal for the expression of *OsNAR2.1*, since *OsNRT2.1* depends also on *OsNAR2.1* to transport NO_3^- (Yan et al. 2011). The failure of *OsNRT1.1A* splicing overexpression in inducing the expression of *NAR2.1* and *NRT2.1* (Fig. 7) clearly indicates the importance of the first exon for signaling mediated by *OsNRT1.1A*. The *osnrt1.1b* mutant rice plant did not present a lower expression of *OsNRT2.1* (Hu et al. 2015), however, the results obtained in this study suggest that other homologs to *Arabidopsis NRT1.1*, as *OsNRT1.1A* can control the expression of NO_3^- transporters. In rice, *OsNRT1.1B* promotes the ubiquitination and degradation of SPX4, by activating NO_3^- and phosphorus (P) response genes (Hu et al. 2019). The results showed differences in intensity of chlorate sensitivity and the ability to induce *NRT2.1/NAR2.1* expression in *Arabidopsis chl1-5* plants overexpressing

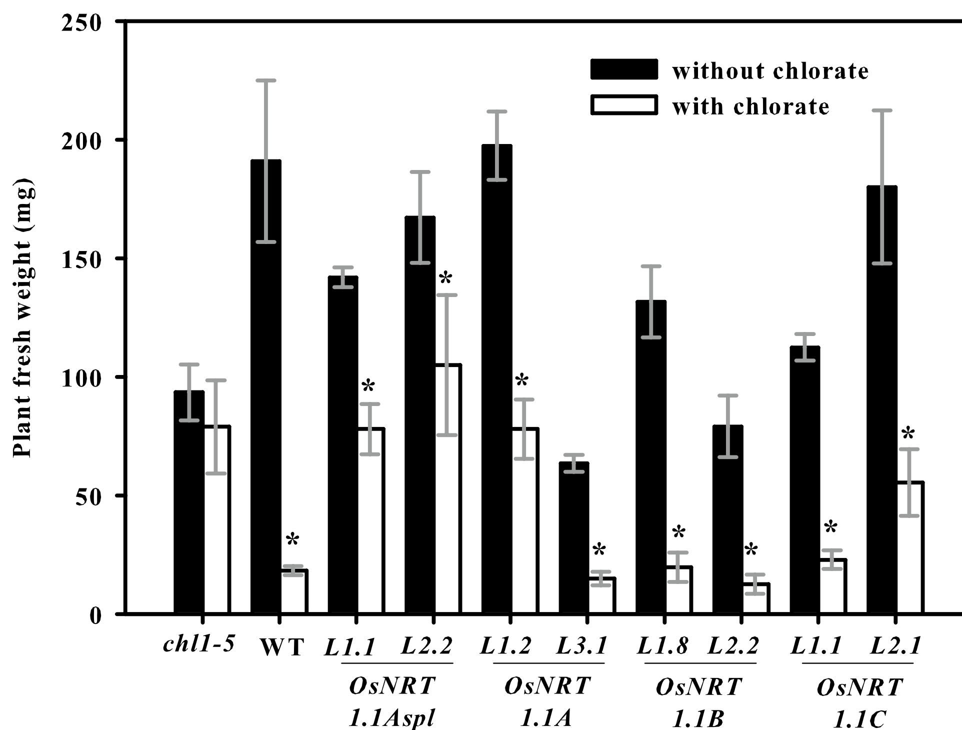

Fig. 5 Plant fresh weight of mutant *Arabidopsis chl1-5,* WT and lineages of *Arabidopsis chl1-5* with overexpressed rice genes *OsNRT1.1A splicing* (*OsNRT1.1As*), *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C*, with and without chlorate. Two lineages were used for each mutant overexpressing *OsNRT1.1*. *Significantly different from control (without chlorate) according to a Least Significant Difference test ($p < 0.05$)

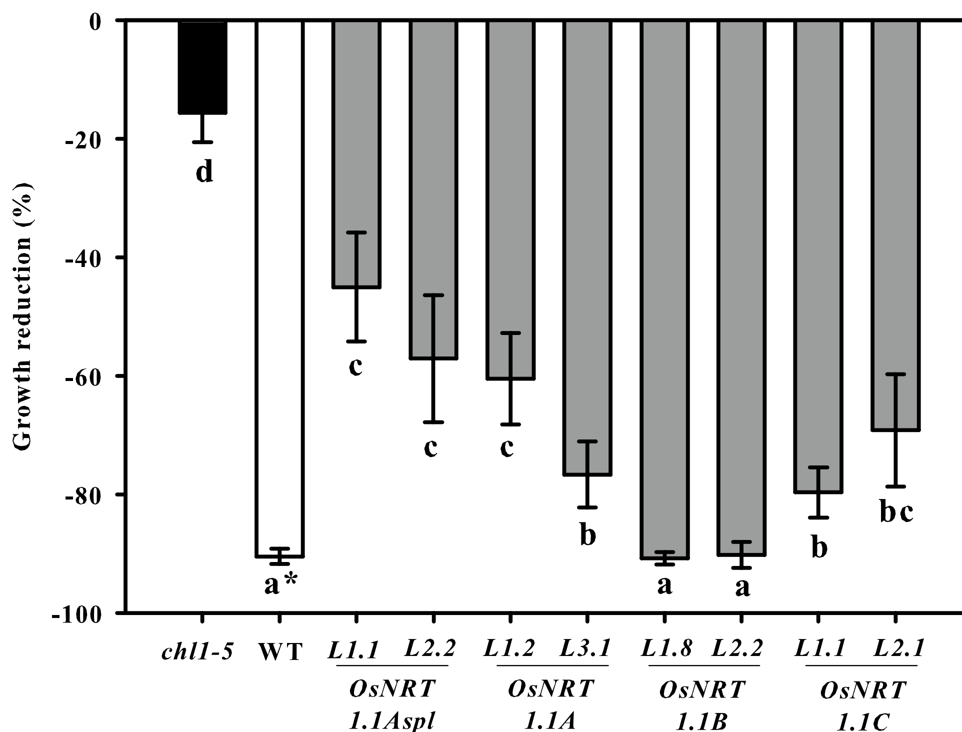

Fig. 6 Effect of chlorate on the reduction of fresh weight (%) in *Arabidopsis* mutant *chl1-5,* WT and lineages of *Arabidopsis chl1-5* overexpressing the rice genes *OsNRT1.1A splicing* (*OsNRT1.1As*), *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C*. Two lineages were used for each mutant overexpressing *OsNRT1.1*. *Different letter represents statistically significantly different according to a Least Significant Difference test ($p < 0.05$)

OsNRT1.1A and *OsNRT1.1B*, indicating that they may have distinct functions.

Altogether, using *chl1-5* mutant overexpression system we were able to study the individual function of *OsNRT1.1A*, OsNRT1.1As, *OsNRT1.1B* and *OsNRT1.1C*, avoiding potential redundancy of functions among the OsNRT1.1 in rice. In this study, *OsNRT1.1A* and *OsNRT1.1B* genes were overexpressed in *Arabidopsis chl1-5* mutant and their NO_3^- transport function was revealed, as well as the *NRT2.1/NAR2.1* induction in the *Arabidopsis chl1-5*

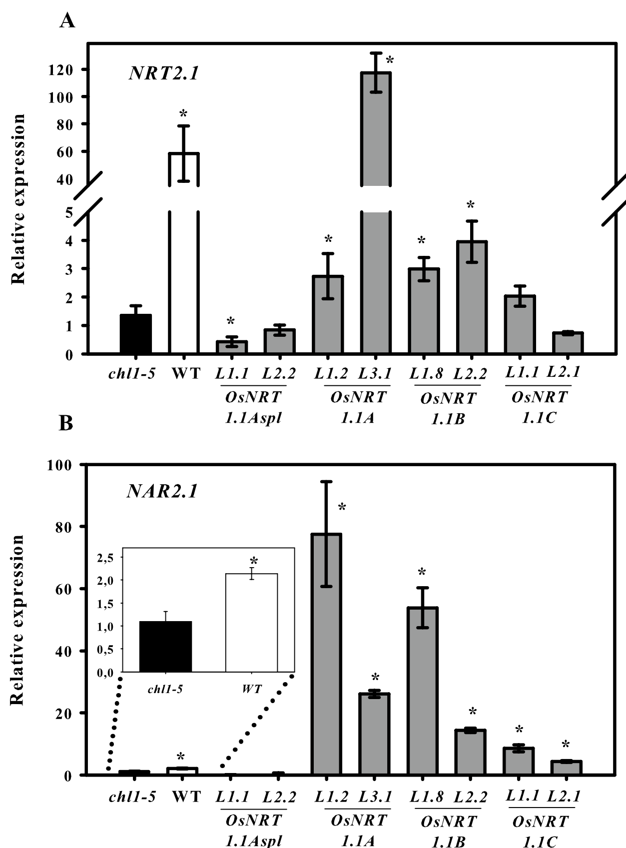

Fig. 7 Expression of the NO_3^- transporter *NRT2.1* **a** and *NAR2.1* **b** in *Arabidopsis* mutant *chl1-5,* WT and lineages of *Arabidopsis chl1-5* overexpressing the rice genes *OsNRT1.1A splicing* (*OsNRT1.1As*), *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C*. Two lineages were used for each mutant overexpressing *OsNRT1.1*. The expression in the mutant *chl1-5* was assigned value 1. *Significantly different from *chl1-5* according to a least significant difference test ($p < 0.05$)

```
OsNRT1.1B     ERMTTLGIAVNLVPYMTGTMHLGNAAAANTVTNFIGTSFMLCLLGGFVADTYLGRYLTIA  106
OsNRT1.1C     ERLTTLGVAVNLVTYLTGTMHLGSAASANAVTNFLGTSFMLCLLGGFLADTYLGRYLTIA  120
OsNRT1.1A     ERLTTLGIAVNLVTYLTATMHAGNAEAANVVTNFMGTSFMLCLLGGFVADSFLGRYLTIA  109
OsNRT1.1As    ------------------------------------------------------------
NRT1.1        ERLTTLGIGVNLVTYLTGTMHLGNATAANTVTNFLGTSFMLCLLGGFIADTFLGRYLTIA  103
```

Fig. 8 Alignment of partial amino acid sequences of OsNRT1.1A, OsNRT1.1As (alternative splicing of OsNRT1.1A without the first exon), OsNRT1.1B, OsNRT1.1C and NRT1.1 (*Arabidopsis*) in the region where residue T101 is found in *Arabidopsis* NRT1.1. Threonine residues in a similar position to T101 of *Arabidopsis* NRT1.1 characterized by Ho et al. (2009) are in red and underlined. The position of threonine residues in rice are T107, T104 and T118 in OsNRT1.1A, OsNRT1.1B and OsNRT1.1C, respectively (Color figure online)

overexpression system. The overexpression of *OsNRT1.1A* transporter showed a strong effect on the expression of *NRT2.1*/*NAR2.1* promoted by NO_3^-, while the overexpression of *OsNRT1.1B* displayed a strong sensitivity towards chlorate, showing its likely role in the absorption of NO_3. Although *OsNRT1.1A* has higher protein sequence identity to *Arabidopsis* NRT1.1 (Wang et al. 2018), the present study indicates *OsNRT1.1A* and *OsNRT1.1B* contribute to NO_3^- uptake and *NRT2.1* and *NAR2.1* expression. Additionally, *OsNRT1.1A* and *OsNRT1.1B* display different expression among rice root and shoot as well as N source (NO_3^- or NH_4^+), indicating different functions of NRT1.1 family in rice. Rice is NH_4^+ tolerant and responsive to NO_3^- and NH_4^+ provision (Kronzucker et al. 2000). Although *chl1-5* lineages overexpressing *OsNRT1.1C* presented chlorate sensitivity, those lineages did not induce *NRT2.1* expression compared to *chl1-5* mutants. Further, the low expression of *OsNRT1.1C* in rice indicates the importance of the expression of *OsNRT1.1A* and *OsNRT1.1B* in rice. The T101 residue in NRT1.1 is essential to mediate NO_3^- signaling in *Arabidopsis* (Ho et al. 2009). The analysis of alternative splicing of *OsNRT1.1A* revealed the importance of the first exon in signaling and the likely importance of T107 of *OsNRT1.1A* in signaling promoted by NO_3^-.

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Author Contributions VJS: conceptualization, plant transformation, performing the experiments, experiment analysis, data analysis, and writing the manuscript. SMVL: conceptualization, gene expression analysis, vectors construction, data analysis, and writing the manuscript. FMS: conceptualization, data analysis, and writing the manuscript. SLA: conceptualization, supervision, project administration, vectors construction, and writing the manuscript.

Compliance with Ethical Standards

Conflict of Interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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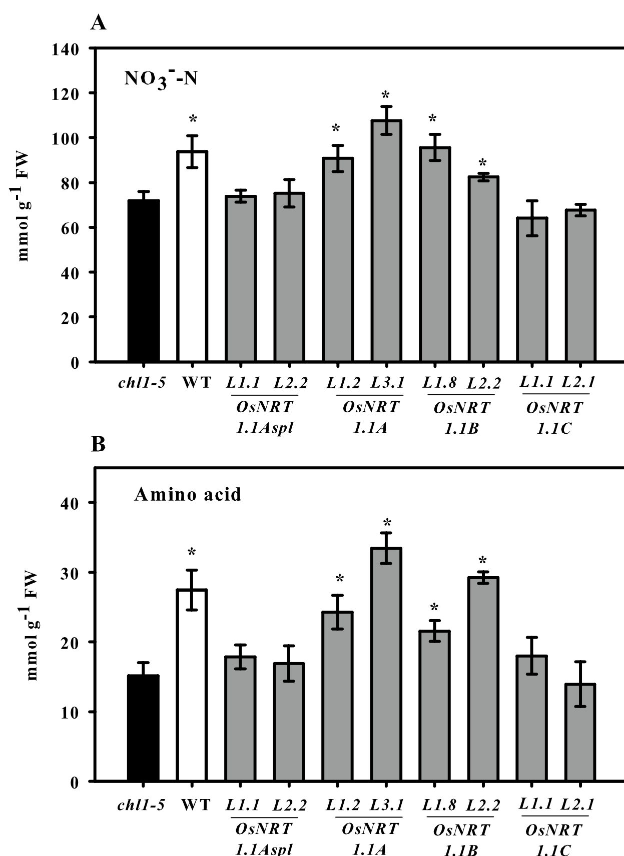

Fig. 9 Plant NO_3^--N content **a** and amino acid content **b** of mutant *Arabidopsis chl1-5,* WT and lineages of *Arabidopsis chl1-5* with overexpressed rice genes *OsNRT1.1A splicing* (*OsNRT1.1As*), *OsNRT1.1A*, *OsNRT1.1B* and *OsNRT1.1C*. Two lineages were used for each mutant overexpressing *OsNRT1.1*. *Significantly different from *chl1-5* according to a Least Significant Difference test ($p < 0.05$)

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