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The Arabidopsis PIP1;1 Aquaporin Represses Lateral Root Development and Nitrate Uptake Under Low Nitrate Availability

Thayssa Rabelo Schley[1] | Ting Zhu[1] | Birgit Geist[1] | Amandine Crabos[2] | Daniela Dietrich[3] | Regina A. Alandes[3] | Malcolm Bennett[3] | Philippe Nacry[2] | Anton R. Schäffner[1]

[1]Department of Environmental Sciences, Institute of Biochemical Plant Pathology, Helmholtz Zentrum München, Neuherberg, Germany | [2]IPSiM, CNRS, INRAE, Institut Agro, University of Montpellier, Montpellier, France | [3]Plant & Crop Sciences, School of Biosciences, University of Nottingham, Nottingham, UK

Correspondence: Philippe Nacry (philippe.nacry@inrae.fr) | Anton R. Schäffner (schaeffner.lab@gmail.com)

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ABSTRACT

Nitrate (NO_3^-) deficiency decreases root water uptake and root hydraulic conductance. This adaptive response is correlated with reduced abundance and activity of plasma membrane intrinsic protein (PIP) aquaporins. We therefore screened changes in the root architecture of a complete set of Arabidopsis *pip* loss-of-function mutants grown under NO_3^- deficiency to systematically approach the impact of PIPs under these conditions. NO_3^- deprivation led to attenuated responses of specific *pip* single mutants compared to the strongly altered LR parameters of wild-type plants. In particular, *pip1;1* exhibited a lower relative reduction in LR length and LR density, revealing that PIP1;1 represses LR development when NO_3^- is scarce. Indeed, PIP1;1 compromises root and shoot NO_3^- accumulation during early developmental stages. A fluorescent VENUS-PIP1;1 fusion revealed that PIP1;1 is specifically repressed in the pericycle, endodermis and at the flanks of emerging LRs upon NO_3^- deficiency. Thus, LR plasticity and NO_3^- uptake are affected by an interactive mechanism involving aquaporins (PIP1;1) and nitrate accumulation during seedling development under NO_3^--deficient conditions.

1 | Introduction

Water is essential for plant growth, development and productivity. The root system is responsible for water absorption from the soil and transport up to the shoots. Water transport occurs throughout the roots along radial and axial paths. Axial water transport mainly relies on xylem vessels, and membrane barriers do not hamper it. The radial path is represented by water transport from the soil to the vasculature and can occur by three pathways: apoplastic (along cell walls, in extracellular space), symplastic (through plasmodesmata and cytoplasmic continuities) or transcellular (across membranes in and out). The transcellular and symplastic pathways are enhanced by the activity of aquaporins.

Aquaporins are abundantly expressed transmembrane proteins that enhance water permeability and are proposed to be responsible for more than 85% of water transport into plant cells (Chaumont and Tyerman 2014; Peret, Larrieu, and Bennett 2009; Maurel et al. 2008). In higher plants, aquaporins are classified into five subfamilies based on sequence similarity and related to their subcellular localization: the plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), small

basic intrinsic proteins (SIPs) and uncharacterized intrinsic proteins (XIPs) (Tyerman et al. 2021).

PIP aquaporins are abundant proteins that play a major role in the passive permeation of water across the cell membrane, which is driven by water potential gradients between the external and intracellular compartments (Robinson et al. 1996; Maurel et al. 2015). PIPs may mainly act and be activated when high water fluxes are required; thus, they are associated with tissue and cellular hydraulic conductance and involved in water uptake, growth and cell elongation (Javot et al. 2003; Kaldenhoff et al. 2008; Chaumont and Tyerman 2014; Maurel et al. 2015; Kelly et al. 2017; Ding et al. 2020; Wang et al. 2021). Arabidopsis *pip2;1*, *pip2;2* and *pip1;2* single knockout mutants were shown to reduce root and leaf hydraulic conductivity by 20% to 30% (Javot et al. 2003; Da Ines et al. 2010; Postaire et al. 2010; Prado et al. 2013). In contrast, Arabidopsis PIP1;4 and PIP2;5 overexpression lines show 40% higher root hydraulic conductivity than wild-type plants when submitted to a short cold treatment, although it is not clear whether this is an unspecific effect of an aquaporin or related to these specific isoforms (Lee et al. 2012).

PIP aquaporins are also involved in root development. The root elongation of *pip1;4* and *pip2;5* single and double mutants was retarded during recovery from freezing stress in comparison to the wild type (Rahman et al. 2020). Accordingly, a *PIP2;5* overexpression line grown in hydroponics has a 10% higher root growth rate than wild type when submitted to a long cold treatment (Lee et al. 2012). PIP aquaporins also play a role in lateral root (LR) development. Several *pip* mutants, in particular *pip2;1*, show a delayed LR emergence and cellular defects of the LR primordium (Péret et al. 2012). Taken together, these results suggest that PIP aquaporins may be involved in LR plasticity.

LR plasticity is essential for soil foraging (Muller et al. 2019), and it is highly responsive to changes in the surrounding environment, such as variations in water and nutrient concentration or soil structure and composition (Liu and von Wirén 2022). Under suboptimal conditions, LR plasticity is an important strategy required for plant survival (Sun, Yu, and Hu 2017). Low nutrient availability, including nitrogen, is among the abiotic stresses that modulate LR plasticity the most.

Nitrogen is a macronutrient essential for plant metabolism as a component of, for example, nucleic acids, proteins and chlorophyll. Nitrate (NO_3^-) is the primary nitrogen source for many plant species including Arabidopsis (*Arabidopsis thaliana*). NO_3^- is highly mobile in soil, and plants often encounter huge variations in concentration including deficiency. Therefore, they have a specialized and tightly regulated transport system that adjusts the NO_3^- uptake depending on its availability and plant demand (Noguero and Lacombe 2016). The NO_3^- transporters are classified into low-affinity, NRT1/NPF, and high-affinity, NRT2, transporter systems (Li et al. 2016). In addition to their role in NO_3^- uptake, the Arabidopsis NO_3^- transporters NRT1.1 (Krouk et al. 2010; Lay-Pruitt and Takahashi 2020; Remans et al. 2006a) and NRT2.1 (Little et al. 2005; Remans et al. 2006b; Pélissier, Motte, and Beeckman 2021) are also critical in regulating root system architecture in response to NO_3^- deficiency. The primary root is less responsive to NO_3^- availability than LR (Sun, Yu, and Hu 2017). In general, LR development is inhibited by a high (>10 mM) or very low (<1 mM) NO_3^- concentration, impairing LR elongation and, in some cases, LR initiation and emergence (Liu et al. 2020). In contrast, a moderate NO_3^- concentration stimulates LR development (Gruber et al. 2013; Jia et al. 2019).

In fluctuating environments, mechanisms modulating nutrient uptake and water transport may mutually influence each other or affect LR plasticity (Barzana et al. 2021). Indeed, NO_3^- does positively affect root water uptake, and deficiency decreases root hydraulic conductivity (Wang et al. 2016). This is correlated with reduced PIP aquaporin abundance or activity in root cells (Clarkson, 2000; Maurel et al. 2015; Li et al. 2016). The activity of PIP aquaporins, for example, controlled by their phosphorylation level, is positively correlated with root hydraulic conductivity under NO_3^- deficiency in Arabidopsis (Di Pietro et al. 2013). In addition, the abundance of PIP aquaporins correlates with NO_3^- availability (Hacke et al. 2010; Ishikawa-Sakurai, Hayashi, and Murai-Hatano 2014; Ren et al. 2015; Wignes 2017; Gao et al. 2018; Araus et al. 2020; Pou et al. 2022). Li et al. (2016) investigated the effects of NO_3^- availability, signalling and metabolism on root hydraulic conductivity using Arabidopsis mutants. They revealed a specific role of the high-affinity NO_3^- transporter NRT2.1 in enhancing root hydraulic conductivity, which was not linked to decreased NO_3^- accumulation and independent of reduced growth of *nrt2;1* plants. Interestingly, reduced root hydraulic conductivity of *nrt2.1* plants under control conditions and NO_3^- deficiency was linked to the reduction of highly expressed *PIP* genes with reduced transcript levels of *PIP1;1*, *PIP1;2*, *PIP2;1* and *PIP2;7*. Transcripts of *PIP1;1*, *PIP2;1*, *PIP2;3* and *PIP2;7* were significantly lowered in NO_3^--deficient versus NO_3^--supplied control *nrt2;1* plants independent from internal NO_3^- levels. PIP1 proteins of *nrt2;1* roots were also reduced as detected by an anti-PIP1;1/1;2/1;3/1;4 antiserum under NO_3^--deficient and control conditions, whereas PIP2;1/2;2/2;3 proteinswere only lowered in NO_3^--deficient *nrt2;1* (Li et al. 2016). Interestingly, NO_3^- deficiency as such did not suppress *PIP* transcripts or the investigated PIP protein levels of wild-type plants within the first 3 days of starvation, and reduced levels were only found at a later phase of nutrient stress (Di Pietro et al. 2013; Li et al. 2016).

Therefore, we investigated whether PIP aquaporins may affect root development and nutrient acquisition efficiency by adjusting LR systems in response to NO_3^- deficiency. First, we screened the root architecture of a complete set of Arabidopsis *pip* loss-of-function mutants under NO_3^- deficiency on vertical agar plates. Deprivation of NO_3^- leads to altered root growth parameters and some mutants showed reduced changes under nitrate starvation; in particular, *pip1;1* was consistently less affected indicating that PIP1;1 impairs lateral root development in NO_3^--deficient conditions. In addition to this developmental phenotype, PIP1;1 has a negative impact on root and shoot nitrogen uptake when plants are grown with limited NO_3^- supply in an agar system, but that does not turn into altered shoot development of adult plants grown in pots.

2 | Materials and Methods

2.1 | Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) wild-type Columbia (Col) and *pip* single mutants, *pip1;1-1* (GABI_437B11, Rosso et al. 2003), *pip1;1-2* (CRISPR/Cas9 deletion, this work, Ordon et al. 2019), *pip1;2-2* (SALK_019794, Alonso et al. 2003), *pip1;3-1* (SALK_051107), *pip1;4-4* (GABI_412E06), *pip1;5-2* (CRISPR/Cas9 deletion, this work, Stuttmann et al. 2021), *pip2;1-2* (SM_3_35928, Tissier et al. 1999), *pip2;2-3* (SAIL_169A03), *pip2;3-1* (SALK_117876), *pip2;4-1* (SM_3_20853), *pip2;5-1* (SAIL_452H09), *pip2;6-3* (SALK_092140), *pip2;7-2* (CRISPR/Cas9 deletion, this work, Ordon et al. 2019) and *pip2;8-2* (SK16840, Robinson et al. 2009) were used. Insertion lines had been obtained from the Nottingham Arabidopsis Stock Centre (Scholl, May, and Ware 2000). All *pip* mutants are in Col background. All *pip* mutants are loss-of-function lines except *pip1;1-1*, which is a knockdown allele (Ceciliato et al. 2019).

pip1;1-2, *pip1;5-2* and *pip2;7-2* loss-of-function lines were obtained by CRISPR/Cas9-induced deletions. Two or four gene-specific gRNA sequences were selected according to target regions suggested at http://chopchop.cbu.uib.no (Labun et al. 2016, 2019) and assembled into binary pDGE-series vectors (Ordon et al. 2020, 2021) (Supporting Information: Table S1). These vectors targeting *PIP1;1*, *PIP1;5* and *PIP2;7* were used for *Agrobacterium*-mediated transformation of *A. thaliana* wild type (Col) (Clough and Bent 1998). Homozygous T2 plants harbouring deletions within the *PIP* genes were identified by PCR using flanking, gene-specific oligonucleotides and confirmed by DNA sequencing. *pip1;1-2* resulted in the deletion of 290 bp (nucleotides 1195–1484, with A of the start codon being nucleotide 1), *pip1;5-2* had a 184 bp deletion (nucleotides 596–779) and *pip2;7-2* showed a deletion of 220 bp (nucleotides 156–375) and two further CRISPR/Cas9-induced mutations (Supporting Information: Table).

PIP1;1$_{pro}$::*VENUS-PIP1;1* and *PIP2;1*$_{pro}$::*VENUS-PIP2;1* cassettes were compiled in pGGZ003 by GreenGate recombination (Lampropoulos et al. 2013) of (i) the relevant promoter sequences including the ATG start codon of the *PIP* genes cloned into pGGA000, (ii) a pGGB module harbouring the coding sequence of VENUS fluorescent protein without stop codon, (iii) a pGCC plasmid encompassing the relevant *PIP* genomic sequence starting (in frame with VENUS) at the ATG start codon and ending at the 3′-UTR sequences, (iv) pGGD002 dummy vector, (v) a pGGE vector with the same dummy insert as pGGD002 and finally, (vi) pGGF harbouring the At2-S$_{pro}$::GFP-NOS$_{ter}$ module (Bensmihen et al. 2004) as a selection marker for plant transformation (oligonucleotides used for amplifying the modules and thus defining the respective sequences, borders and junctions are compiled in the Supporting Information: Table S2). These vectors were used for *Agrobacterium tumefaciens* GV3101 pMP90-mediated transformation of *pip1;1-2* and *pip2;1-2* (Clough and Bent 1998). Homozygous, single-insertion lines were selected for experiments.

Surface-steriled seeds were prepared and sown on 12 × 12 cm plates containing modified Hoagland culture medium as described in Nacry et al. (2005). Plates were kept at 4°C for 48 h and transferred in a vertical position to a growth chamber with 65% humidity, 23°C, and a photoperiod of 16 h of light (165 µE m^{-2} s^{-1} of LED light in Montpellier and 160–190 µE m^{-2} s^{-1} of fluorescent lighting, colour 840, in Munich). On the fifth day after germination, seedlings were transferred to control (according to Nacry et al. 2005) or NO_3^- deficiency conditions. For NO_3^- deficiency, KNO_3^- was replaced by 1 mM K_2SO_4; thus, 0 mM nitrate was added with the media components. For NO_3^- dose-response analyses, KNO_3^- and K_2SO_4 concentrations were adjusted to reach a constant 30 mM K^+ concentration.

For phenotypic analyses of the growth of rosettes, as well as nitrogen and carbon contents of rosettes in control and NO_3^--deprived conditions, plants were grown for 1 week in 95-mL pots containing soil:quartz sand (4:1) mixture to allow proper germination and initial growth. Seven-day-old plants had their roots washed with distilled water before being transferred to 95-mL pots containing perlite:vermiculite (1:2) mixture. This substrate allowed controllable regular NO_3^- and low NO_3^- supply by watering them with a nutrient solution containing either control level (2 mM KNO_3^-) or low NO_3^- (0.2 mM KNO_3^-); in the latter case, the missing KNO_3^- was substituted by 0.9 mM K_2SO_4. The nutrient solution was otherwise composed as described in Nacry et al. (2005) without adding MES. These 7-day-old plants were irrigated three times a week, for the first two times with the control solution, then either the control or the NO_3^--deficient solution was used for a further 22 days. Plants were grown at 20°C (night 18°C), and 40 +/− 5% relative humidity with a 10 h light photoperiod. Plants were illuminated by LEDs comprising cool white (set to 35% intensity), blue (2%), FAR (15%) and deep red (0%); the average light intensity was 127 µE m^{-2} s^{-1}.

2.2 | Morphological Analysis

The root system of 11-day-old plants vertically grown on agar plates was scanned as described on The Root Phenotyping Platform in Montpellier (https://www1.montpellier.inra.fr/wp-inra/bpmp/en/platform/root-phenotyping-platform/, Fernandez et al. 2022) or images were taken by a Nikon D600 camera in Munich at a stand equipped with high-frequency illumination. Root morphological parameters were analysed on the part of root that grew during the 6 days of stress imposition. The primary root (PR) and LR lengths were measured using ImageJ software, LR number was counted, and LR density was calculated as LR number divided by the length of the PR.

RGB images of rosettes were taken daily for 27 days, 5 days before stress imposition and 22 days during stress imposition, using high-throughput, conveyor belt system equipped with an RGB camera (Photon Systems Instruments [PSI], Czech Republic). After 27 days of growth, rosettes were harvested, weighed to obtain their fresh weight, dried at 70°C for 5 days and weighed to obtain their final dry weight.

2.3 | Microscopy

Imaging of seedlings was performed on a Leica SP8 confocal microscope using a ×20 objective and zoom of ×1.5. Excitation of VENUS fluorescent protein was at 514 nm selected from a white laser, and emission was detected at 524–560 nm. The laser intensity was set at 7% for *PIP1;1*$_{pro}$::*VENUS-PIP1;1* and at 2% for *PIP2;1*$_{pro}$::*VENUS-PIP2;1*. All other settings were identical for both lines grown on vertically positioned plates under control and NO_3^- deficiency (0 mM KNO_3^-) conditions. At least 10 individuals per line per condition were analysed. Each individual showed at least one LR primordium at stages VI–VIII, which was analysed. Standard images were established based on a clear focus of the xylem poles together with an undistorted longitudinal view of the LR primordium. Single images were taken and the signal was quantified by ImageJ software.

2.4 | Total RNA Extraction and RT-qPCR

Roots were harvested and homogenized as described by Remans et al. (2006b). Total RNA was extracted using the Tri-Reagent protocol (Sigma, Deisenhofen, Germany) or acccording to Logemann et al. (1987). cDNA was prepared according to the manufacturer's instructions from 500 ng of total RNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). qPCR was performed using 2× Sensimix SYBR (Meridian Bioscience Inc., TN, USA) on a 7500 fast real-time PCR system (Applied Biosystems, Thermo Fisher Scientific, Germany). PCR was performed in 96-well optical reaction plates heated for 10 min to 95°C, followed by 40 cycles with 15 s at 95°C, 35 s at 55°C (for *UBQ5*, *CLATHRIN*, *RHIP1*) or 58°C (for *NRT* genes), 45 s at 72°C and dissociation stage (15 s at 95°C, 1 min at 60°C, 15 s at 95°C). Gene expression level was analysed using the gene-specific primers listed in the Supporting Information: Table S3. Data were analysed and normalized by LinReg and geNorm (Ruijter et al. 2009; Vandesompele et al. 2002) using *UBQ5* (At3g62250), *CLATHRIN* (At5g46630) and *RHIP1* (At4g26410) as reference genes.

2.5 | Root and Shoot Nitrate Uptake Activity Assay

Root NO_3^- uptake was determined by ^{15}N labelling as described by Remans et al. (2006b). Briefly, 11-day-old in vitro grown seedlings were transferred to a 5-cm-diameter petri dish containing 0.1 mM $CaSO_4$, with the roots in the solution and the aerial parts outside. This solution was replaced after 1 min with the 0.2 mM $^{15}NO_3^-$ solution for 5 min. Plant roots were then rinsed again for 1 min in 0.1 mM $CaSO_4$ before being harvested, roots and shoots were separated, dried at 70° C for 48 h, and weighed. Influx was calculated as μmol $^{15}NO_3^-$ h^{-1} g^{-1} root dry weight after the determination of total ^{15}N in roots and shoots. The ^{15}N analyses were performed using an integrated system for continuous flow isotope ratio mass spectrometry (Euro-EA elemental analyzer; EuroVector S.P.A., France; Isoprime Mass Spectrometer; GV Instruments, France).

2.6 | Rosette C/N Content and Substrate Nitrate Content Measuring

Plant rosettes from the pot experiment in the PSI facility were directly harvested after the last round of phenotype recording. After drying for 5 days, two rosettes with the same genotype and treatment were grouped as one sample and were put into a 2 mL polypropylene tube (Sarstedt, Nümbrecht, Germany) with tissue lyser beads. Samples were homogenized and grounded into fine powder using a mixing mill (Retsch MM400, Retsch, Hann, Germany) with a frequency of 30 s^{-1} for 1 min. Around 2 mg of each sample was then weighed by using a microbalance (Sartorius CP2P microbalance, Sartorius AG, Göttingen, Germany) with three technical replicates. Samples were filled and capsuled into tin cans (4 × 6 mm, IVA Analysentechnik, Meerbusch, Germany) and loaded to an isotope ratio mass spectrometer (Delta V Advantage, Thermo Fisher, Dreieich, Germany) coupled to an elemental analyzer (EuroEA, Eurovector, Pavia, Italy) to measure the carbon and nitrogen content and ratio.

After removing the plant materials from the pots, two pots grown with the same genotype and treatment were grouped as one sample, each with three biological replicates. Sample weights were recorded and 200 mL ddH_2O was added to extract the NO_3^-. After soaking the sample for 3 days, 40 mL of the supernatant was collected as the measuring sample. Dimatoc 2000 (Dimatec Analysetechnik, Essen, Germany) together with continuous flow analysis with a photometric autoanalyser (CFA-SAN Plus; Skalar Analytik, Germany) were used for determining the NO_3^- concentration.

2.7 | Statistical Analysis

For all data, normality was tested by Shapiro–Wilk. For the root morphology analysis performed in Montpellier and in Munich, the analysis of variance Kruskal–Wallis was performed, followed by Dunn's Method. *t*-test or Mann–Whitney was performed for the PIP expression pattern and the NO_3^- uptake assay. Two-way ANOVA analysis followed by Holm–Šidák Method was performed for the expression levels of NRT genes, the data on fresh and dry weights, as well as nitrogen and carbon contents of the rosettes.

3 | Results

3.1 | Lateral Root Morphology of *pip* Single Mutants in Control and Nutrient Deficiencies

To investigate whether PIP aquaporins alter the root development under nutrient deficiency, the root morphology of the wild-type Columbia (Col) and a complete collection of *A. thaliana* plasma membrane aquaporin *pip* single mutants, *pip1;1–2*, *pip1;2-2*, *pip1;3-1*, *pip1;4-4*, *pip1;5-2*, *pip2;1-2*, *pip2;2-3*, *pip2;3-1*, *pip2;4-1*, *pip2;5-1*, *pip2;6-3*, *pip2;7-2* and *pip2;8-2*, was analysed at the root phenotyping facility of IPSIM-INRAE (Montpellier, France) in control (C: 2 mM KNO_3^-) and nitrate deficiency (–N: 0 mM KNO_3^-) conditions. We measured

primary root (PR) length, as well as number, length and density of lateral roots (LR) of 11-day-old plants grown on vertical agar plates 6 days after stress imposition. Therefore, only the part of roots that had grown during these 6 days was assessed.

In the C condition, Col plants showed 5.40 ± 0.31 cm growth of PR, the initiation of 8.00 ± 1.80 LRs with an average LR length of 2.14 ± 0.71 cm, and an LR density of 1.48 ± 0.28 LR cm^{-1}. Nitrate deficiency (–N condition) severely inhibited root growth of wild-type roots (Figure 1; Supporting Information: Figure S1), reducing PR growth to 3.59 ± 0.27 cm, LR number to 1.00 ± 0.50, LR length to 0.05 ± 0.08 cm and LR density to 0.14 ± 1.29. Many *pip* mutants showed better LR growth in the –N condition compared to Col plants. In contrast to the severe, 99% reduction in LR length of the wild type under –N condition, *pip1;1-2*, *pip2;2-3* and *pip2;4-1* had their LR length reduced by only 83%, 86% and 87%, respectively (Figure 1A). In addition, the LR density of these *pip* mutants was less severely affected, showing reductions from 38% to 66% instead of the 91% reduction of wild-type plants (Figure 1B). Other *pip* mutants also showed a less pronounced reduction of LR density, but only *pip1;1-2*, *pip2;2-3* and *pip2;4-1* had an attenuated response for both LR length and density compared to Col (Figure 1). Consequently, we selected these *pip* mutant lines for further investigation and phenotype validation. The differential NO_3^--deficiency response of *pip1;1-2*, *pip2;2-3* and *pip2;4-1* was independently assessed at a different location (Helmholtz Zentrum Munich, Germany). All mutants showed the same trends for the LR phenotypic parameters, but the differences were statistically significant only for *pip1;1-2* for both LR length and density (Figure 2). Thus, *pip1;1-2* showed a robust amelioration of the NO_3^- deficiency-dependent suppression of LR growth. The *pip1;1-2* resistant phenotype was confirmed by

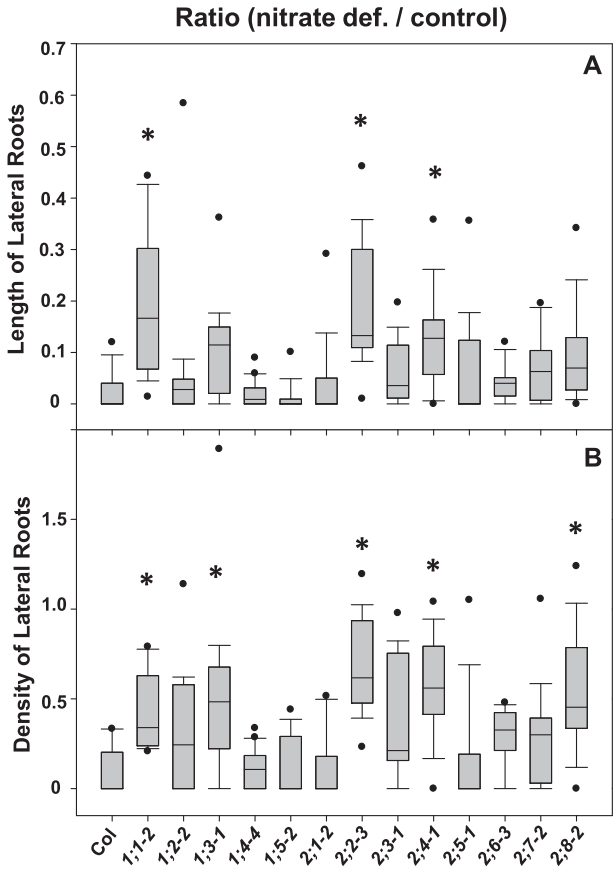

FIGURE 1 | Relative length (A) and density (B) of lateral roots of *Arabidopsis thaliana* wild type (Col) and all aquaporin single loss-of-function mutants, *pip1;1-2*, *pip1;2-2*, *pip1;3-1*, *pip1;4-4*, *pip1;5-2*, *pip2;1-2*, *pip2;2-3*, *pip2;3-1*, *pip2;4-1*, *pip2;5-1*, *pip2;6-3*, *pip2;7-2*, *pip2;8-2*, grown in presence of control nitrogen level (2 mM NO_3^-) and under nitrate deficiency (0 mM NO_3^- added). Experiments were performed in the Montpellier Laboratory. Relative values (absolute value on 0 mM nitrate/average of control) measured 6 days after stress imposition (11-day-old plants) are displayed. The absolute values of the control condition data are provided in the Supporting Information: Table S4. $n > 11$ plants per genotype per treatment. *Statistical differences (Kruskal–Wallis followed by Dunn's Method, $p < 0.05$) between the mutant and Col. Only the gene and allele numbers are displayed to indicate the *pip* loss-of-function mutants.

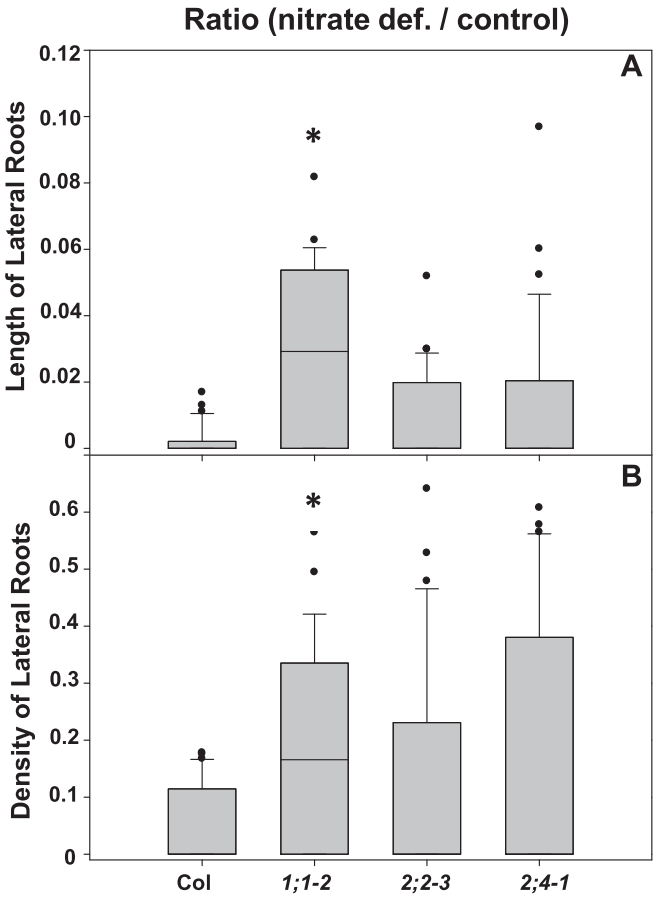

FIGURE 2 | Relative length (A) and density (B) of lateral roots of *Arabidopsis thaliana* wild type (Col) and selected aquaporin single mutants, *pip1;1-2*, *pip1;4-4*, *pip2;2-3*, *pip2;4-1*, submitted to control (2 mM NO_3^-) and nitrate deficiency (0 mM NO_3^- added). The experiment was independently performed in the Munich Laboratory. The presented data are relative values (absolute value of 0 mM nitrate/average of control) measured 6 days after stress imposition (11-day-old plants). The absolute values of the control condition data are provided in the Supporting Information: Table S4. Data were pooled from two independent rounds of experiment, totalling $n > 23$ plants per genotype per treatment. *Statistical differences (Kruskal–Wallis followed by Dunn's Method, $p < 0.05$) between the mutant and Col. Only the gene and allele numbers are displayed to indicate the *pip* loss-of-function mutants.

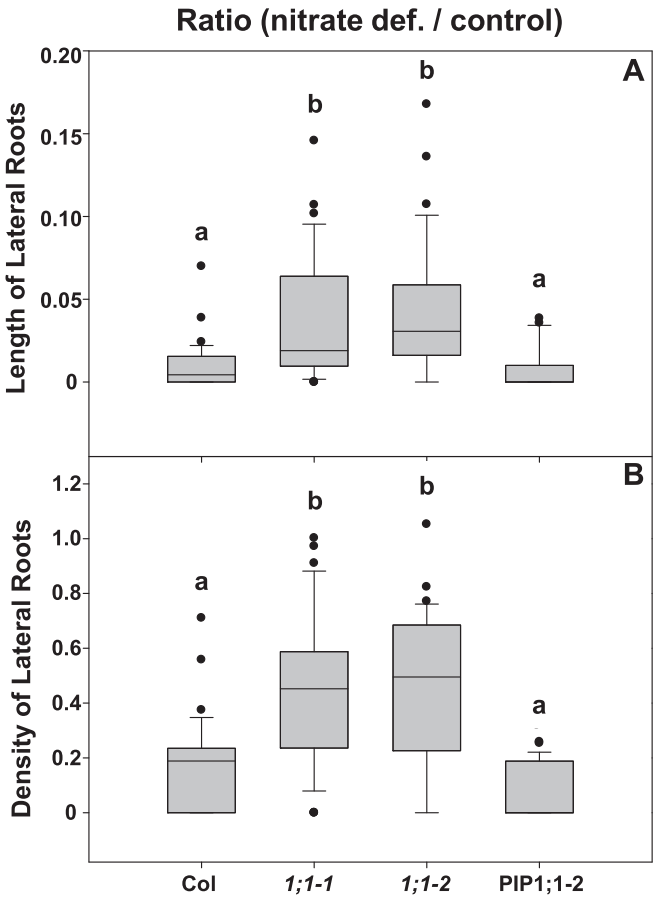

FIGURE 3 | Relative length (A) and density (B) of lateral roots of *Arabidopsis thaliana* wild type (Col), *pip1;1-1* and *pip1;1-2* single mutants, and the complementation line *PIP1;1*$_{pro}$::*VENUS-PIP1;1* submitted to control (2 mM NO_3^-) and nitrate deficiency (0 mM NO_3^- added). Data are relative values (absolute value of 0 mM nitrate/average of control) measured after 6 days of the stress imposition (11-day-old plants). The absolute values of the control condition data are provided in Supporting Information: Table S4. Data were pooled from two independent rounds of experiment, totalling $n > 25$ plants per genotype per treatment. Different letters indicate statistical differences (Kruskal–Wallis followed by Dunn's Method, $p < 0.05$). Only the gene and allele numbers are displayed to indicate the *pip* loss-of-function mutants.

pip1;1-1, an independent knock-down mutant allele (Figure 3; Supporting Information: Figure S2). In addition, the wild-type phenotype was restored by the *PIP1;1*$_{pro}$::*VENUS-PIP1;1* complementation line expressing a translational fusion of PIP1;1 driven by its native promoter in a *pip1;1-2* mutant background (Figure 3; Supporting Information: Figure S2). Taken together, these results showed that PIP1;1 plays a crucial role in repressing LR development when NO_3^- is scarce.

3.2 | Nitrate Deficiency Decreases the PIP1;1 Transcript and Protein Abundance

To further investigate the role of PIP1;1 in modulating the LR adaptive response to low NO_3^- availability, we analysed its expression pattern under the –N condition. In parallel, the expression pattern of PIP2;1 was assessed, as it is, together with PIP1;1, among the critical PIP aquaporins involved in root adaptive responses to NO_3^- deficiency (Li et al. 2016). In addition, PIP2;1 has a crucial function in root water transport (Boursiac et al. 2008; Da Ines et al. 2010) and, importantly, its involvement in LR development (Péret et al. 2012).

Therefore, we first investigated the transcript level of *PIP1;1* and *PIP2;1* in Col roots submitted to C and –N conditions. *PIP1;1* and *PIP2;1* were similarly downregulated by 70% in –N condition (Supporting Information: Figure S3). We then analysed and quantified the PIP1;1 and PIP2;1 expression pattern using the complementation lines *PIP1;1*$_{pro}$::*VENUS-PIP1;1* and *PIP2;1*$_{pro}$::*VENUS-PIP2;1* expressing a translational fusion of the VENUS fluorescent protein and PIP driven by the native *PIP* promoter in a *pip1;1-2* and *pip2;1-2* mutant background, respectively. Under control conditions, PIP1;1 and PIP2;1 were similarly expressed in the stele, pericycle, endodermis, cortex and at the flanks of LR primordia (Figure 4A,E). In addition, PIP1;1 was also expressed in the epidermis (Figure 4A,B). The –N condition reduced the accumulation of both PIP proteins in all root tissues (Figure 4A–H). A quantitative analysis of the fluorescence indicated repression of PIP1;1 by 70%, whereas the PIP2;1 level was reduced by 37% (Figure 4I). Moreover, the reduction of PIP1;1 expression was particularly prominent in the pericycle, endodermis and at the flanks of LR primordia (Figure 4A,C).

3.3 | PIP1;1 Negatively Impacts Nitrate Uptake in Nitrate-Deficient Conditions

To further explore the interaction between PIP1;1 and NO_3^- nutrition, we investigated the transcript abundance of three NO_3^- transporter genes (*NRT1.5*, *NRT1.1* and *NRT2.1*) in roots of Col and *pip1;1-2* plants grown under C and –N conditions. We selected these three genes, since *NRT1.5* is an efflux carrier involved in N translocation (Lin et al. 2008), whereas *NRT1.1* and *NRT2.1* are the prevalent representatives of the low-affinity and high-affinity transport systems, respectively, and potential NO_3^- signalling components (Remans et al. 2006a, 2006b; Krouk et al. 2010; Gojon et al. 2011; Zheng et al. 2013). Only minor differences in transcript accumulation were observed when comparing Col and *pip1;1-2* roots (Figure 5). However, quantification of the root NO_3^- uptake capacity and content measured by the short-term $^{15}NO_3^-$ influx showed that *pip1;1-2* mutant roots accumulated 50% more NO_3^- than Col roots (Figure 6A) leading to a 4.25 times higher NO_3^- level of *pip1;1-2* shoots (Figure 6B).

3.4 | Loss-of-Function of PIP1;1 Has an Attenuated Effect During Long-Term Vegetative Growth Under Nitrate-Deficient Conditions

To investigate the suppressive role of PIP1;1 in NO_3^- uptake observed for young seedlings during long-term vegetative growth, we observed the effects of NO_3^- deficiency implemented on plants cultivated in vermiculite–perlite-filled pots. Here, NO_3^- was not completely withheld; instead, nitrate deficiency was initiated at Day 12 by irrigation with 0.2 mM NO_3^- containing nutrient solution compared to 2.0 mM NO_3^- for controls. A low NO_3^--containing nutrient solution was used to ensure plant

survival and long-term growth for another 22 days (Figure 8). At the end of the experiment, nitrate was depleted in an aqueous extract of the pot substrate under NO_3^--deficient conditions, showing a severe reduction compared to the C pots (Supporting Information: Figure S4). Col and *pip1;1-2* plants were similarly affected by the NO_3^- deficiency, which led to a 42% decrease in nitrogen content and a 7% increase in carbon content compared to control plants (Figure 7). NO_3^- deficiency also reduced the rosettes' surface area of both wild-type and *pip1;1-2* plants, which was apparent about 17 days after the switch to stress conditions. This reduction reached about 20% compared to the plants grown under C condition after 3 weeks (Figure 8A). Nevertheless, there was a notable difference between wild type and *pip1;1-2* showing a tendency of a suppressive role of PIP1;1 also on vegetative growth when NO_3^- is scarce. NO_3^- deficiency led to a 40% reduction in fresh weight of wild type, whereas *pip1;1-2* plants experienced a 30% reduction (Figure 8B). This led to a significant increase in the ratio of the fresh weight of controls versus NO_3^- deficiency of *pip1;1-2* plants in comparison to wild type. Thus, *pip1;1-2* may be more tolerant to low nitrate also when grown towards adult plants on a soil substrate, although this effect is at least partly attributed to the numerically lower fresh weight of *pip1;1-2* plants in well-nurtured controls (Figure 8B,C). Dry weight and dry weight ratios showed a similar tendency (Supporting Information: Figure S5).

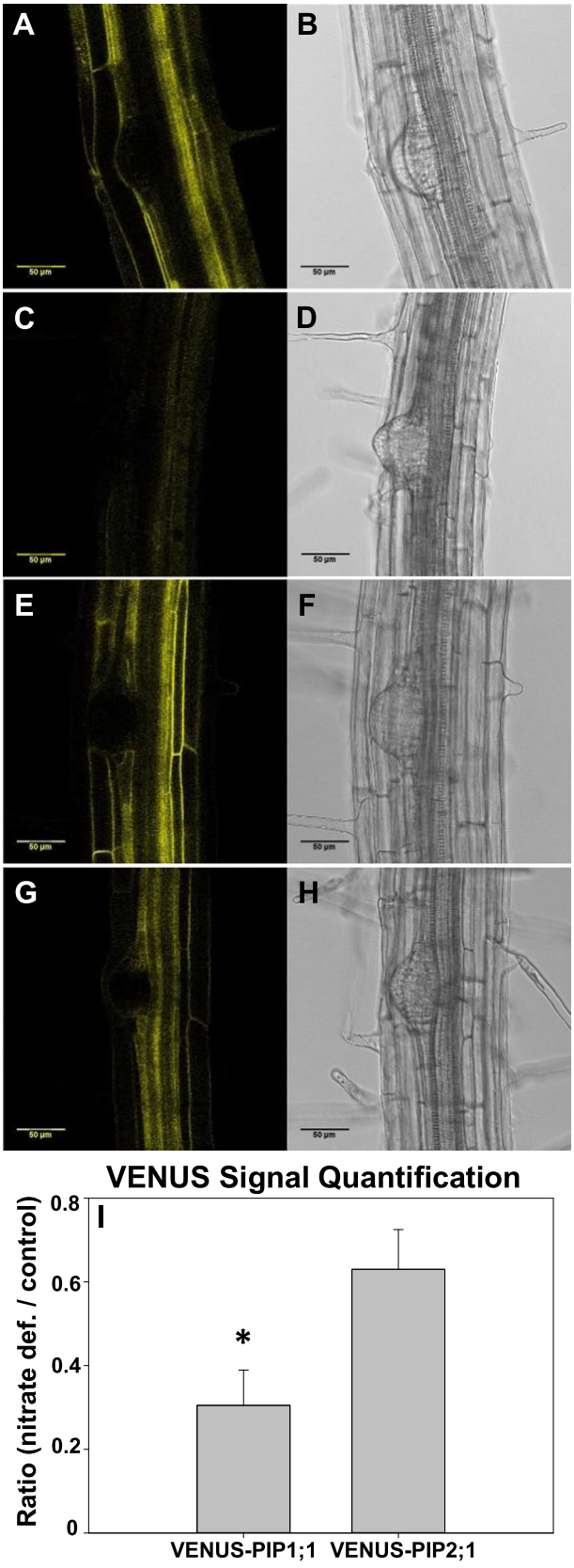

FIGURE 4 | Effects of external nitrate in PIP1;1 (A–D) and PIP2;1 (E–H) expression (yellow signal) at the primary root and lateral root primordia of *Arabidopsis* $PIP1;1_{pro}$::VENUS-PIP1;1 and $PIP2;1_{pro}$::VENUS-PIP2;1 lines. Plants were grown on agar plates for 5 days in C conditions and 6 days in the presence (C condition: 2 mM KNO_3^-) or deficiency of nitrogen (–N condition: 0 mM KNO_3^-) before microscope analysis. Eleven-day-old plants were used, and 2D single images were taken by a confocal microscope. (A, B, E, F) C conditions. (C, D, G, H) –N conditions. (I) Quantification of VENUS signal measured by ImageJ. $n = 10$ plants per genotype per treatment. *Statistical differences (*t*-test, $p < 0.001$).

4 | Discussion

LRs represent a major part of the root system and are essential for foraging for water and nutrients. Consequently, their development is responsive to environmental signals such as nutrient deficiency, and many molecular cues are integrated (Liu and von Wirén 2022). PIP aquaporins are also involved in LR development and root responses to stress (Péret et al. 2012; Maurel et al. 2015). The involvement of aquaporins in root responses to nutrient deficiency has been suggested based on gene expression or protein activity analyses. Common regulatory pathways between nutrient transporters and aquaporins have been proposed (Wang et al. 2016; Barzana et al. 2021). Therefore, we reversed the perspective and analysed the response of a complete collection of all 13 *pip* single mutants to the deficiency of one major macronutrient, NO_3^-, mainly focusing on LR development.

4.1 | PIP1;1 Impairs Lateral Root Growth Under Nitrate Deficiency

Aquaporins have been suggested to play a role in root responses to NO_3^- availability, since NO_3^- deficiency down-regulates and NO_3^- application upregulates aquaporin

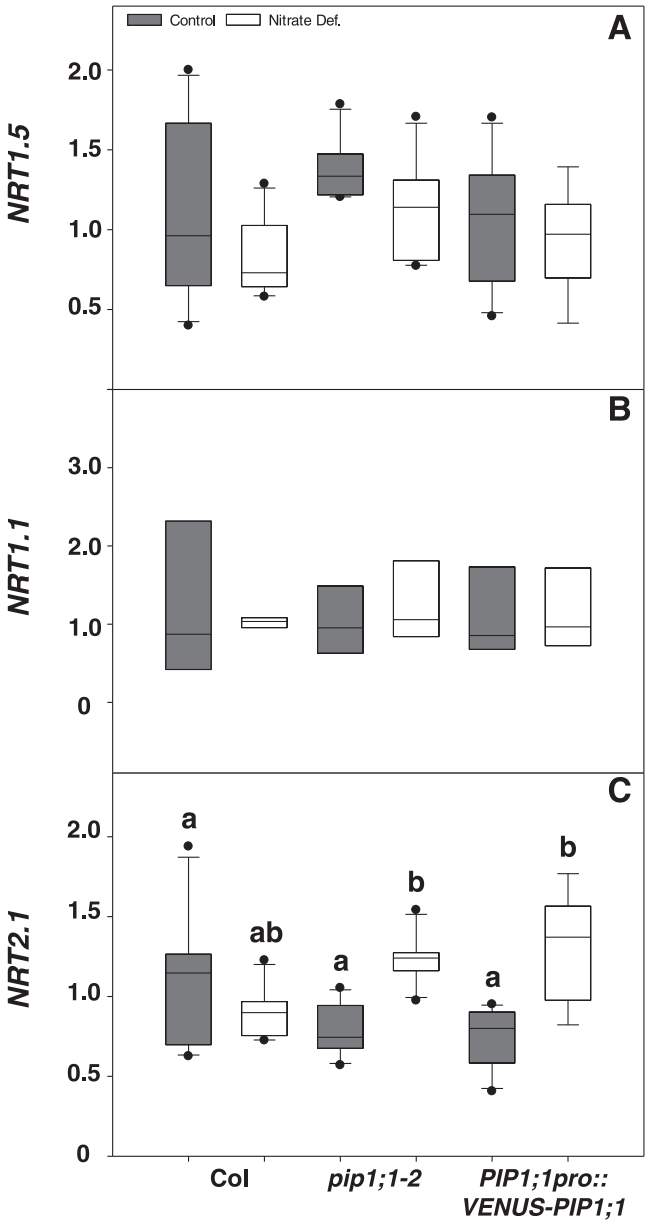

FIGURE 5 | Effects of external nitrate on *NRT1.5* (A), *NRT1.1* (B) and *NRT2.1* (C) transcript abundance in roots of *Arabidopsis thaliana* wild type (Col), *pip1;1-2* single mutant and the complementation line *PIP1;1*$_{pro}$::*VENUS-PIP1;1*. Plants were grown on agar plates for 5 days in C conditions and 6 days in the presence (C condition: 2 mM NO_3^-) or deficiency of nitrogen (–N condition: 0 mM NO_3^- added) before characterization of *NRT* expression using RT-qPCR. Data are presented as absolute values of the relative gene expression. $n > 4$ biological pools of roots per genotype per treatment. The absence of letters in (A) and (B) represents the absence of statistical differences. Different letters in (C) indicate statistical differences (two-way ANOVA followed by Holm–Šidák Method, all pairwise analysis, $p < 0.05$).

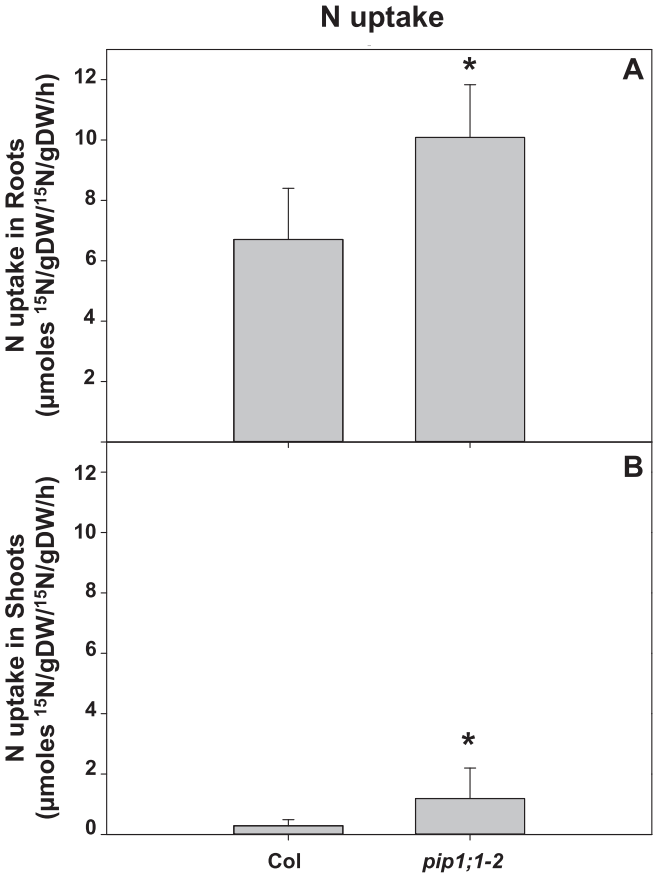

FIGURE 6 | Effects of external nitrate in the NO_3^- content in roots (A) and shoots (B) of *Arabidopsis thaliana* wild type (Col) and *pip1;1-2* single mutant. Plants were grown on agar plates for 5 days in C conditions and 6 days in the presence (C condition: 2 mM KNO_3^-) or deficiency of nitrogen (–N condition: 0 mM KNO_3^- added) before measurements of NO_3^- content. $n > 5$ plants per genotype per treatment. *Significant differences (*t*-test, $p < 0.05$) between the mutant and Col.

expression in roots of different plant species, including Arabidopsis (Gifford et al. 2008; Li et al. 2016), tomato (Wang, Garvin, and Kochian 2001), rice (Ishikawa-Sakurai et al. 2014; Ren et al. 2015), and maize (Gaspar 2003; Wignes 2017; Pou et al. 2022). In addition, aquaporins have been linked to facilitated LR emergence (Péret et al. 2012; Reinhardt et al. 2016). Therefore, we hypothesized that PIP aquaporins could also modulate root system architecture responses to NO_3^- deficiency. The –N condition drastically inhibited root growth and LR development of wild-type plants grown on vertical agar plates, which agrees with previous studies (Figure 1; Supporting Information: Figure S1; Gruber et al. 2013; Lay-Pruitt and Takahashi 2020). Several *pip1* and *pip2* mutants showed a less altered lateral root phenotype but only *pip1;1* showed a persistent reduction of the relative inhibition of LR growth compared to wild-type plants under –N condition (Figures 1–3). Thus, PIP1;1 is specifically involved in modulating LR plasticity under NO_3^- deficiency, exerting a suppressive role on LR length and density relative to control conditions.

PIP1;1 is highly expressed in roots and is among the few PIP isoforms that are downregulated under NO_3^- deficiency along with the observed reduced hydraulic conductivity (Monneuse et al. 2011; Di Pietro et al. 2013; Li et al. 2016). Conversely, *PIP1;1* is upregulated at the transcriptional level, especially at the pericycle and endodermis, when NO_3^- is applied for 2 h to wild-type roots (Gifford et al. 2008). We showed that *PIP1;1* was downregulated in wild-type roots submitted to the –N

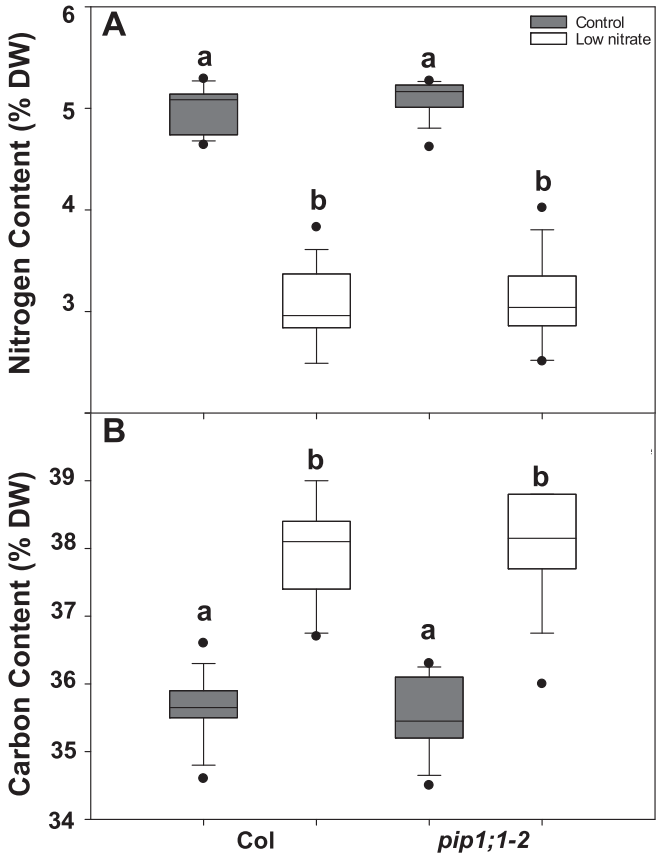

FIGURE 7 | Effects of external nitrate in the nitrogen (A) and carbon (B) contents of rosettes of *Arabidopsis thaliana* wild type (Col) and *pip1;1-2* single mutant. Seven-day-old plants were transferred to pots containing perlite:vermiculite (1:2) mixture and were irrigated at a frequency of three times a week for 5 days with control solution, then either with control (C condition: 2 mM KNO_3^-; grey) or a nitrate-deficient (–N: 0.2 mM KNO_3^-; white) solution for further 22 days. At the end of the experiment, the N and C content of the rosettes of 34-day-old plants was analysed. Different letters indicate significant differences (two-way ANOVA followed by Holm–Šidák Method, all pairwise analysis, $p < 0.05$).

condition (Supporting Information: Figure S3). Thus, NO_3^- availability strongly regulates *PIP1;1* transcript abundance. In addition, NO_3^- starvation decreases the abundance of PIP1;1 along with PIP2;1, PIP2;2, PIP2;4, PIP1;2 and PIP1;3 in Arabidopsis roots at the protein level (Di Pietro et al. 2013). The downregulated PIP1;1 peptides included a phosphorylated peptide, which is a predominant posttranslational modulation positively correlating with the hydraulic conductivity of roots. In accordance with Di Pietro et al. (2013), the lines expressing a translational fusion of PIP with the VENUS fluorescent protein showed a reduced abundance of both PIP1;1 and PIP2;1 protein under –N conditions (Figure 4C,G). The reduction of PIP1;1 was stronger than the repression of PIP2;1 (Figure 4I). This suggests that PIP aquaporins are not subjected to a global, but rather a more isoform-specific regulation in response to NO_3^- deficiency.

To further explore the involvement of PIP1;1 in the root adaptive response to NO_3^-, *pip1;1-2* mutant and complemented lines were cultivated under a wide range (0–30 mM) of NO_3^- concentrations. Low and high NO_3^- concentrations have a negative impact on both primary and lateral root elongation of wild-type seedlings (Supporting Information: Figure S6). Interestingly, primary and lateral root elongation of *pip1;1-2* mutants is significantly reduced under high (over 1 mM) NO_3^- concentrations (Supporting Information: Figure S6). Thus, under high nitrate levels, the loss of PIP1;1 has an opposite impact than under NO_3^- deficiency, suggesting a complex interaction of PIP 1;1 and nitrogen nutrition.

4.2 | PIP1;1 Suppresses NO_3^- Uptake Under Nitrate Deficiency

The positive impact of *pip1;1* on LR development under nitrate-deficient conditions was paralleled by a higher NO_3^- accumulation than wild-type plants in both roots and shoots (Figure 6). LRs are responsible for the highest uptake rate of NO_3^- when NO_3^- is scarce (York, Silberbush, and Lynch 2016). Among several NO_3^- transporters mutants tested in response to NO_3^- starvation, only the *nrt2.1* mutant showed reduced root hydraulic conductivity (Li et al. 2016). This reduced hydraulic conductivity was correlated with a downregulation of *PIP* aquaporins, including *PIP1;1*. Thereby, the downregulation of *PIP1;1*, and the parallel downregulation of *PIP2;1* and *PIP2;3*, showed an additional NRT2.1-dependent component: while nitrate deficiency did not affect *PIP1;1* (and *PIP2;1* and *PIP2;3*) transcription of wild type, the *nrt2.1* loss-of-function mutant showed reduced *PIP* transcripts in control condition in comparison to wild type, which were further downregulated by –N conditions (Li et al. 2016). In contrast, *NRT* transcripts were not significantly affected by the loss of PIP1;1 (Figure 5). Thus, the observed enhanced NO_3^- uptake of *pip1;1* is regulated at different levels. Indeed, for example, phosphorylation of NRT2.1 was related to promoted NO_3^- accumulation (Jacquot et al. 2020; Ohkubo, Kuwata, and Matsubayashi 2021). Phosphorylation of NRT1.1 leads to a switch from low to high nitrate affinity (Liu and Tsay 2003).

NRT2.1 and other NRTs have been proposed to be involved in nitrate sensing and/or signalling apart from their nitrate transporter activities. This dual function connects the fundamentally important nitrate acquisition and the control of carbon/nitrogen balance to root development, to the hormonal control of development involving cytokinin, ethylene and auxin, and to light sensing and photosynthetic activity (Little et al. 2005; Remans et al. 2006a, 2006b; Zheng et al. 2013; Ruffel et al. 2021; Wang et al. 2023).

In conclusion, *PIP1;1* and NO_3^- uptake are subject to a coordinated and mutually interacting regulation under NO_3^- deficiency. The less severe relative reduction in LR length and LR density of *pip1;1* acted synergistically together with the upregulation of NO_3^- uptake under –N conditions (Figure 9). A feedback regulation may exist, in which NRT2.1 positively regulates PIP1;1 to maintain hydraulic conductivity and/or to repress LR development under nitrate deficiency (Figure 9). A deeper understanding of the underlying regulation will offer interesting breeding targets to improve both root development and NO_3^- uptake under

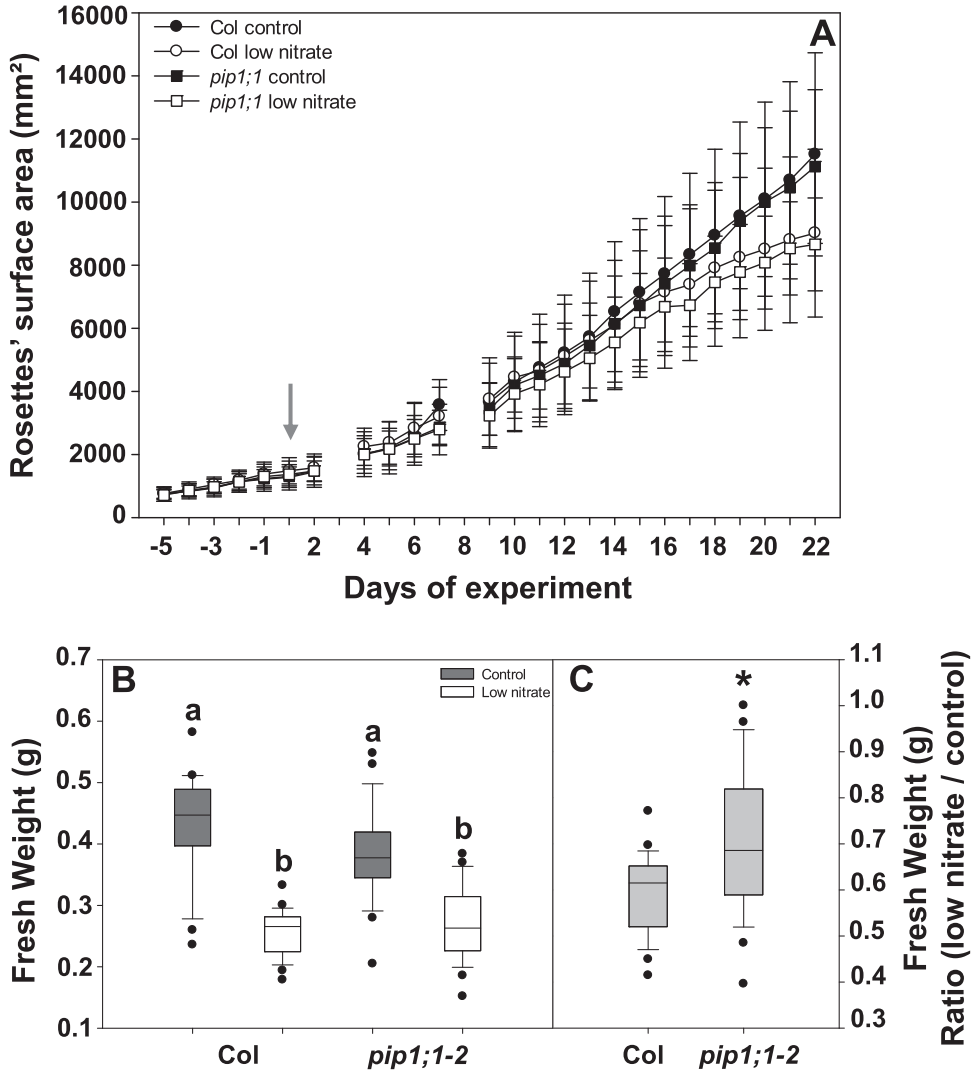

FIGURE 8 | Effects of external nitrate in the rosettes of *Arabidopsis thaliana* wild type (Col) and *pip1;1-2* single mutant. (A) Rosette surface area. (B) Fresh weight of rosettes. (C) Relative values of fresh weight of rosettes (absolute values on 0.2 mM nitrate/average of control). Seven-day-old plants were transferred to pots containing perlite:vermiculite (1:2) mixture and were irrigated at a frequency of three times a week for 5 days with control solution, then either with control (C condition – 2 mM NO_3^-) or the nitrate-deficient (0.2 mM NO_3^-) solution for further 22 days. The grey arrow in (A) represents the start of stress imposition. Data shown in (B) and (C) were measured for 34-day-old plants, that is, at the last time point of (A). $n > 15$ plants per genotype per treatment. Different letters in (B) indicate significant differences (two-way ANOVA followed by Holm–Šidák Method, all pairwise analysis, $p < 0.05$). In (C), *Significant differences (Mann–Whitney, $p < 0.05$) between the mutant and Col.

suboptimal NO_3^- availability. The detailed physiological function of PIP1;1 in this interactive regulation is not yet clear. Nevertheless, a regulation of root development through a sole or parallel impact on water permeation can be hypothesized. This is supported by the correlation of root hydraulic conductivity and PIP1;1 expression under –N conditions (Li et al. 2016) or due to the localization and nitrate deficiency-induced downregulation of PIP1;1 in the pericycle, endodermis and flanks of LR primordia which potentially may affect LR development (Figure 4C,G) (Péret et al. 2012). Since the loss of PIP1;1 improves LR emergence during NO_3^- deficiency, the downregulation of wild-type PIP1;1 may be a measure to counteract the impairment of LR emergence (Figure 9). Interestingly PIP1;1 also regulates primary and lateral root elongation under high NO_3^- concentration (Supporting Information: Figure S6). This suggests a wide function for this aquaporin and further research will be necessary to fully characterize the complex interactions between aquaporins and nitrate nutrition.

4.3 | Vegetative Development Under Low NO_3^- May Be Less Improved by the Loss of PIP1;1

NO_3^- deficiency similarly reduced shoot development and biomass production of both wild-type and *pip1;1* plants, when nitrate was reduced at later stages (day 12 after germination) and long-term cultivation of the plants (Figures 7 and 8). However, *pip1;1* was less severely affected than Col, since the relative drop in fresh weight was significantly attenuated (Figure 8B,C). Thus, the loss-of-function of PIP1;1 still conferred a less sensitive phenotype under NO_3^- deficiency, although the pronounced phenotypic differences of *pip1;1* seedlings on agar plates

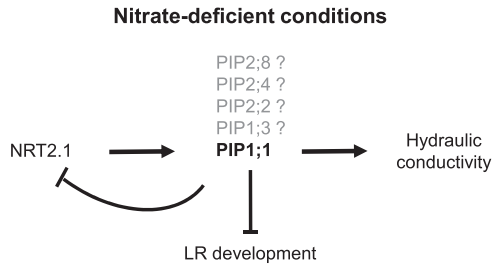

FIGURE 9 | Interacting regulation of *Arabidopsis thaliana* PIP1;1 and NRT2.1 affecting hydraulic conductivity and nitrate uptake under nitrate deficiency. Nitrate deficiency is involved in sensing low nitrate and reduces root hydraulic conductivity, but it has no impact on *PIP1;1* (and grey-labelled *PIP1;2*, *PIP2;1*, *PIP2;2*, *PIP2;3* and *PIP2;7*) transcription (Li et al. 2016). However, *nrt2.1* loss-of-function mutants display lowered transcript levels of these *PIP* genes already under control nitrate conditions. Importantly, nitrate deficiency further amplifies this repression of *PIP1;1* (and *PIP2;1* and *PIP2;3*) transcription and further reduces root hydraulic conductivity (Li et al. 2016). In turn, PIP1;1 is suppressing nitrate uptake, and it has a negative impact on LR development when nitrate is low. Other PIP members, such as PIP1;3, PIP2;2, PIP2;4 and PIP2;8, may be also involved in this regulation dependent on nitrate deficiency, since they also affected relative responses of LR parameters, although in a more variable manner.

(Figures 1–6) were less evident in the pot system (Figures 7 and 8). This attenuated phenotype of *pip1;1* during vegetative growth may be attributed to several cues, since two different culture systems, different NO_3^- regimes and distinct timing were employed, that is, young seedlings on agar plates (5- to 11-day-old plantlets transferred to N-free medium) versus adult plants in soil-filled pots with a later implementation of nitrate deficiency (12- to 34-day-old plants irrigated with 0.2 mM NO_3^-). First, the stress applied in the pot system was less severe and started considerably later than on agar plates (Figures 1–3 and 8A). Second, agar plates generally provide a more uniform distribution of nutrients compared to pots (Chapman et al. 2012; Hanlon et al. 2018). The physical structure of the perlite–vermiculite mixture may affect root penetration and development and trap NO_3^- during initial control irrigation, thereby influencing substrate exploration and the response to NO_3^- deficiency (Chapman et al. 2012). Finally, the developmental stage of the plants influences strategies to cope with nutrient deficiencies (Takehisa and Sato 2019; Ganther et al. 2022; Guo et al. 2022). Thus, the altered response of *pip1;1* in early developmental stages on agar plates may be attenuated or compensated in later stages when grown in pots. Nonetheless, the observed impacts of PIP1;1 during early developmental stages are critically important, as this phase can influence the entire life cycle of the plant especially under stress conditions (Khalid et al. 2019). Thus, the suppressive role of PIP1;1 in affecting LR development and NO_3^- uptake during early stages plays a crucial effect during seedling establishment and may influence subsequent growth under a NO_3^--deficient scenario.

4.4 | Final Remarks and Future Perspectives

Even though nitrogen and water availabilities have been reported to impact essential plant traits (Araus et al. 2020), studies that investigate the effects of combining nitrogen and water stresses in plant responses, especially at the molecular level, are scarce. Most of the studies about multifactorial abiotic stresses combined drought, heat and salinity (Rizhsky et al. 2004; Mittler and Blumwald 2010; Prasch and Sonnewald 2013; Suzuki et al. 2014; Choudhury et al. 2017; Shaar-Moshe et al. 2019; Zandalinas et al. 2021, 2022). In a study of combined nitrogen and water deficiency stresses in tomatoes, Babazadeh et al. (2021) focussed only on yield effects. Therefore, future studies to investigate the effects of NO_3^- and other major nutrients with parallel water stress on root development and other plant traits are needed. At the molecular level, there are still many open questions, such as how aquaporins interact with NO_3^- transporters and regulators, which specific, or perhaps even dual functions of the proteins are involved, whether this mutual regulation is also effective in leaves, and how combined NO_3^- and water stress would affect this regulation. Such a regulatory network may also include other PIP isoforms including PIP–PIP interactions, for example, by PIP1-PIP2 heterotetramer formation (Chaumont and Tyerman 2014; Maurel et al. 2015), since *pip1;3*, *pip2;2*, *pip2;4* and *pip2;8*, also displayed differential, although variable responses of LR root development to –N conditions (Figures 1, 2 and 9).

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

The data that support the findings of this study are available in the Supporting Information of this article. Additional data are available on request from the corresponding authors.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.