

Exposure to high nitrogen triggered a genotype-dependent modulation of cell and root hydraulics, which can involve aquaporin regulation

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ABSTRACT

Root nitrogen acquisition has been proposed to be regulated by mass flow, a process by which water flow brings nutrients to the root surface, depending on a concerted regulation of the root hydraulic properties and stomatal conductance. As aquaporins play an important role in regulating transcellular water flow, we aimed at evaluating the short-term effect of high nitrogen (HN) availability on the dynamics of hydraulic parameters at both the root and cell level and the regulation of aquaporins. The effect of short-term HN (8 mM NO₃⁻) treatment was investigated on 12 diverse 15-day-old maize genotypes. Root exposure to HN triggered a rapid (< 4 h) increase in the root hydraulic conductivity (L_{pr}) in seven genotypes while no L_{pr} variation was recorded for the others, allowing the separation of the genotypes into two groups (HN-responsive and HN-non-responsive). A remarkable correlation between L_{pr} and the cortex cell hydraulic conductivity (L_{pc}) was observed. However, while differences in gas exchange parameters were also observed, the variations were genotype-specific and not always correlated with the root hydraulic parameters. We then investigated whether HN-induced L_{pr} variations were linked to the activity and regulation of plasma membrane PIP aquaporins. While some changes in *PIP* mRNA levels were detected, this was not correlated with the protein levels. On the other hand, the rapid variation in L_{pr} observed in the B73 genotype was correlated with the PIP protein abundance in the plasma membrane, highlighting PIP posttranslational mechanisms in the short-term regulation of root hydraulic parameters in response to HN treatment.

Keywords: Aquaporins, maize, root and cell hydraulic conductivity, RNA and protein expression, plasma membrane

INTRODUCTION

Plant growth and development are highly dependent on nitrogen (N), making this element of pivotal importance for agriculture sustainability. The predominant form of N in most high-input agricultural soils is nitrate (NO_3^-), which is taken up by the roots using NO_3^- transporters (for review, Nacry et al. 2013). The delivery and maintenance of high N (HN) concentration at the root surface have been proposed to be regulated by an increase in mass flow that depends on a concerted regulation of the root hydraulic properties and stomatal conductance with nitrate availability (Cramer et al. 2009). Enhancing the plant water flow when NO_3^- is sensed may bring more NO_3^- at the root surface (Gorska et al. 2008a, Tyerman et al. 2017). The effectiveness of mass flow depends on factors that influence transpiration as well as nutrient type due to its dependence on nutrient concentration, solubility, and mobility in the soil (Barber 1995). Thus, it is expected that more water-use efficient crops would also be more efficient in plant nutrient acquisition. Nutrient regulation of plant water flux may occur through N-flux-linked signalling mechanisms (Desikan et al. 2002, Wilkinson et al. 2007, Gloser et al. 2007) that operate in both roots and shoots. Several studies have shown that the whole root hydraulic conductivity (L_{pr}) is sensitive to N form and concentration in a species-dependent manner (for review, see Tyerman *et al.* 2017). For many species, including maize, high nitrogen (HN) addition in the bathing medium, for several hours to days, enhances the L_{pr} (Carvajal et al. 1996, Hoarau et al. 1996, Gloser et al. 2007, Gorska et al. 2008a and b, Li et al. 2016). In addition, split-root experiments in maize showed that the water flow is only increased in portions of the root system exposed to HN, suggesting a root-specific response and NO_3^- signalling (Gorska et al. 2008b).

The movement of water through the root tissues is dependent on the presence and activity of aquaporins in the plasma membrane called PIPs (plasma membrane intrinsic proteins) (Chaumont and Tyerman 2014). Depending on the species, 35 to 80% of the total root radial water flow occurs through aquaporins (Amodeo et al. 1999, Maurel et al. 2008, Chaumont and Tyerman 2014), making these channels important actors in the modulation of root hydraulics. There are several ways to control the density and activity of aquaporins in the membranes, starting from the regulation of the gene expression and of the protein levels, as well as through the control of their trafficking in the secretory pathway and gating in the target membranes (Chaumont and Tyerman 2014). The rapid increase (min to h) in L_{pr} and the cell membrane hydraulic conductivity (L_{pc}) after a sudden exposure to HN (5 mM NO_3^-), together with the L_{pr} reduction under anoxia treatment, inducing an inhibition of aquaporin activity, indicate an important role for aquaporins in this process (Gorska et al. 2008a, 2008b). However, no significant change in the mRNA levels of the maize *PIP* genes was detected during this short-term treatment (Gorska et al. 2008b). On the other hand, a transcriptomic analysis of maize split-root system exposed or not to short term HN treatment (1 mM NO_3^- , 1 h) revealed that NO_3^- induces a transcriptional upregulation of genes

involved in NO_3^- uptake and assimilation, and water transport (*PIPs* and *TIPs*), together with genes encoding protein kinases and phosphatases, auxin response factors, ethylene receptors and cytokinin oxidases (Liu et al. 2008), compared to non-exposed control. In Arabidopsis, there is also a correlation between L_{pr} and NO_3^- concentration in the external medium, as well as in roots and shoots (Li et al. 2016). Conversely, N starvation induced L_{pr} inhibition, which is correlated to a decrease in abundance of PIP2 aquaporins and phosphorylated PIP peptides (di Pietro et al. 2013). Altogether, these studies strongly suggest that NO_3^- ions regulate maize and Arabidopsis root hydraulics through, in addition to hormonal and local NO_3^- signaling pathways, aquaporin abundance and/or activity involving posttranslational mechanisms such as trafficking from intracellular storage sites to the plasma membrane or gating of the pores (Chaumont and Tyerman 2014).

We hypothesized that short-term changes in hydraulic parameters in maize seedlings exposed to HN are mainly explained by aquaporin functioning. To test this hypothesis, we investigated the root and shoot hydraulic parameters in 12 contrasted inbred maize lines differing in their response to short-term HN treatment (8 mM NO_3^- during 3 to 4 h). In particular, we measured plant water-related physiological parameters (L_{pr} , L_{pc} , stomatal conductance (g_s), and leaf transpiration rate (E)) and then investigated whether NO_3^- induced variations of these parameters could be accounted for by the regulation of PIP aquaporins at the mRNA or protein levels. We showed that the increase in L_{pr} observed in the NO_3^- responsive genotypes upon short-term NO_3^- treatment is correlated with a higher L_{pc} . Although we did not observe any positive correlations between PIP protein or mRNA levels and L_{pr} and L_{pc} variations for the different genotypes upon HN treatment, an increased amount of PIP aquaporins in the plasma membrane of the B73 genotype was measured, suggesting that the response involved posttranslational mechanisms regulating PIP trafficking and activity.

MATERIALS AND METHODS

Plant material and growing conditions

Experiments were carried out with 12 maize inbred lines (*Zea mays* L.) with potentially contrasting root systems. The hybrids were crosses of the parent UH007 with the lines B73, UH250, Mo17, EC169, F98902, FV353, MS153, F7028, EZ47, EZ37, ABA(+) and ABA(-), supplied by Delley Seeds and Plants Ltd, Delley, Switzerland. Seeds were surface-sterilized using 50% sodium hypochlorite and germinated for two days in moist filter paper as described in Hachez et al. (2008). The seedlings were grown hydroponically for up to 15 days in 30 L containers (15 plants per box) in a 16/8 h light (25°C)/dark (19°C) photoperiod with photosynthetically active radiation (PAR) of 150 to 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Plants were grown in a modified Hoagland solution (3 mM CaSO_4 , 795 μM KNO_3 , 603 μM $\text{Ca}(\text{NO}_3)_2$, 190 μM

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KH₂PO₄, and 270 μM MgSO₄; micronutrients: 20 μM H₃BO₄, 0.085 μM ZnSO₄, 0.15 μM CuSO₄, 0.25 μM Na₂MoO₄, 40.5 μM Fe(III)-EDTA, pH 6.1) that was continuously aerated using aquarium diffusers and renewed once a week. On the day of measurements, three different treatments were applied: control (CTRL) consisting of the replacement of the medium by a new Hoagland solution and an incubation of 3 to 4 h, high nitrogen treatment (HN; 3-4 h) during which the medium was replaced with a high nitrate solution (3.6 mM Ca(NO₃)₂, 795 μM KNO₃, 190 μM KH₂PO₄, and 270 μM MgSO₄; micronutrients: 20 μM H₃BO₄, 0.085 μM ZnSO₄, 0.15 μM CuSO₄, 0.25 μM Na₂MoO₄, 40.5 μM Fe(III)-EDTA, pH 6.1) and the recovery treatment (REC; 3 h) during which HN treated plants were transferred to the former Hoagland solution. As no difference in L_{pr} measured at 10 a.m. and 2 p.m. was observed for the B73 genotype in control conditions (Fig. S1) we decided, for technical reasons, not to measure the different parameters in these conditions at the time of the recovery analysis for each genotype. The osmolality (Osm l⁻¹) of the solutions was measured using a freezing point osmometer (Model 3300, Advanced Instruments). Values were identical for both grow mediums (CTRL and HN).

Root Hydraulic conductivity

Measurements of root hydraulic conductivity (L_{pr}) were carried out between 4 and 8 h after the light onset when L_{pr} was maximum (Hachez et al. 2012), using a high-conductance flow meter (HCFM, Dynamax Inc.), as described by Tyree et al. (1995). The primary root of each plant was cut under water and immediately connected to the HCFM with a water-tight seal to preclude any leakage that might interfere with final measurements. The flow rate was immediately measured using the transient method (Bogeat-Triboulot et al. 2002, Tyree et al. 1995) during which the HCFM system forces distilled and degassed water through the root under increasing pressure. Pressure (P; MPa) was ramped up to 0.5 MPa at a constant rate of 5-7 kPa s⁻¹ while simultaneously recording the flow (F, Kg s⁻¹) every 3 s. The root hydraulic conductance (k_{root}) was calculated as the slope of the plot of F versus P: $k_{root} = dF/dP$. The values were normalized by dividing k_{root} by the total root surface area (S_{root}) calculated from the scan of the root (Epson perfection V33 scanner) using ImageJ (National Institutes of Health; <https://imagej.nih.gov/ij/>) to finally obtain L_{pr} values.

Cell hydraulic conductivity

To determine the cell hydraulic conductivity (L_{pc}), cell pressure probe measurements were performed on intact primary roots (attached to the plant) in three contrasted genotypes (B73, EZ37 and F7028) as described in Hachez et al. 2012, Volkov et al. 2007. Root cortical cells were punctured at 5-8 cm from the root tip, a zone corresponding to the mature primary root where apoplastic barriers appear (Hachez et al., 2006), and hydrostatic pressure relaxations were induced to determine the $T_{1/2}$ across cell membranes, which is a measure of the water transport properties

of cells. Together with values of cell elastic modulus (ϵ) calculated from three successive hydrostatic pressure relaxations, the L_{pc} of cells was obtained (Hachez et al. 2012).

Leaf gas exchange parameters

Net CO₂ assimilation rate (A_N), stomatal conductance (g_s) and leaf transpiration (E) of the 4th fully expanded leaf were *in situ* determined during the late morning (3 to 4 h after HN) using an open-infrared gas exchange analyzer (IRGA) system equipped with a leaf chamber fluorometer (Li-6400XT, Li-Cor). At least four measurements per treatment were done under a light-saturating photosynthetic photon flux density (PPFD) of 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (provided by the light source of the Li-6400XT with 10% blue light). External CO₂ was provided by a cartridge plugged into the Li-6400XT to adjust and maintain the CO₂ concentration in the leaf chamber (C_a) during the measurements at 400 $\mu\text{mol CO}_2 \text{mol}^{-1}$ air. The temperature was set at 25°C and the relative humidity of the leaf chamber was 50 and 60%.

Detection of apoplastic barriers

Hand-cut cross-sections of primary roots (at 2 cm and 5 cm from the root tip) from seven selected genotypes (B73, Mo17, F7028, EZ47, EZ37, ABA+ and ABA-) grown in CTRL conditions, were stained in 0.1% (w/v) berberine hemisulfate for 1 h and 0.5% (w/v) toluidine blue O for 45 min, mounted in 0.1% (w/v) FeCl₃ in 50% glycerol, and immediately examined under a fluorescent microscope (DMR, Leica) with a CCD camera (ORCA-ER C4742-800) (excitation wavelength: 340-380 nm and emission wavelength: 425 nm) (Brundrett et al. 1988). Casparian strips and deposition of lignin were observed in the endo- and exodermis of the root tissue as a yellowish fluorescent signal. Three to four root systems were imaged and analyzed for each genotype.

RNA extraction and real-time quantitative PCR (RT-qPCR)

A pool of three plants per genotype was sampled for each treatment and immediately frozen in liquid N. The samples were stored at -80°C until analysis. The whole experiment was performed three times to obtain three independent biological replicates. Total RNA was extracted from ground frozen root tissue using Spectrum Plant Total RNA extraction Kit (Sigma-Aldrich). DNase I digestion was performed on a column during RNA extraction according to the manufacturer's recommendations. cDNA synthesis and qPCR were performed as described by Hachez et al. (2006, 2008, 2012). Results were normalized using three maize internal control genes: the polyubiquitin gene (gi: 248228), the actin-1 gene (gi: 103625937) and the elongation factor 1-alpha (*EF1- α*) gene (gi: 2282583). *PIP* primers were those used by Hachez et al. (2006). RT-qPCR products were verified by agarose gel electrophoresis and melt-curve analysis to confirm that a single PCR product was being amplified.

Protein extraction and analysis

Root microsomal fractions from three pooled plants per genotype and treatment were prepared as described in (Hachez et al. 2006, 2008 and 2012). The experiment was carried out twice. Fifteen micrograms of crude microsomal membranes were solubilized and separated by SDS-PAGE using 8-16% precast gel (BioRad).

Western-blot analysis was performed on the nitrocellulose membranes (Trans-Blot Turbo Transfer System, Bio-Rad) using antisera raised against the N-terminal peptides of PIP1;2, PIP2;1/2;2, PIP2;5, or PIP2;6 (Hachez et al. 2006, Heinen et al. 2014), at dilution of 1:1000 (PIP1;2, PIP2;5, and PIP2;6) or 1:3500 (PIP2;1/ 2;2). Quantification was carried out using a Kodak Image Station 4000R (Carestream Health, Inc.) as described in Hachez et al. (2012).

Confocal microscopy

Transgenic maize plants expressing cyan fluorescent protein (CFP) -tagged PIP2.1 (Mohanty *et al.*, 2009) were grown in a hydroponic medium as explained above. Root cells were imaged using a confocal laser-scanning microscope (LSM 710, Zeiss, Jena, Germany) and a Plan-Apochromat 63/1.40 oil immersion objective. The CFP was excited with the 445 nm laser line and the fluorescence was detected through a dichroic mirror from 450 to 510 nm. First, detection of the CFP was performed on root samples from non-treated plants under a glass coverslip. Then, these plants were transferred into a nutrient solution complemented or not with NO₃⁻ for 3.5 h and the CFP signals were again examined and compared in both conditions. The same parameters were used for all acquired images, allowing a subsequent calculation and comparison of the plasma membrane fluorescence intensity by means of the Zen 2009 software (Zeiss) as described in Chevalier et al. (2014). The experiment was performed three times on 4 to 6 plants per experiment and treatment.

Statistical analysis

Statistical differences between means were assessed by an analysis of variance (ANOVA). When differences were significant, a multiple comparison of means post hoc Duncan ($P < 0.05$) was performed with SPSS 19.0 (IBM Corp.). PCA analyses were performed in R (R Development Core Team, 2016, Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Root and cell hydraulic conductivity upon short term high nitrogen treatment

The effect of a short-term (3.5 h) HN treatment and the recovery thereof on the root hydraulic conductivity (L_{pr}) was first investigated in 12 maize genotypes (15-day-old), using the HCFM method. As this method involves a large application of external hydrostatic pressure and change

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in root medium water potential, it does drive water uptake along the cell-to-cell path (involving aquaporins) and the apoplastic path. The results revealed highly variable and genotype-dependent responses (Fig. 1). Based on the L_{pr} response after HN, two groups were observed. Seven genotypes (B73, Mo17, EC169, F98902, FV353, EZ37 and ABA+) showed a significant L_{pr} increase in presence of HN (group 1 or HN-responsive), whereas five genotypes (UH250, MS153, F7028, EZ47 and ABA-) did not respond to HN (group 2 or HN-non-responsive). When the plants were transferred from HN to control NO_3^- concentration (recovery), L_{pr} of the HN-responsive genotypes decreased except for FV353 and EC169, which remained at high levels. Among the HN-non-responsive genotypes, UH250, MS153 and F7028 stayed unresponsive to the recovery conditions, whereas EZ47 and ABA- showed an increased L_{pr} . In none of the genotypes, HN treatment induced a decrease in L_{pr} . In addition, we observed that the control absolute L_{pr} values varied according to the genotypes, whatever the group they belong to, with significantly higher values for F7028, EZ47, EZ37 and ABA+, compared to the other genotype ($P < 0.05$; Duncan test).

L_{pc} and L_{pr} were measured in an independent assay using 10-day-old plants, in three contrasting genotypes (B73, F7028 and EZ37) displaying different L_{pr} behaviors (Fig. 2). We first measured the L_{pc} of cortex cells using a cell pressure probe with intact plants and, then, the L_{pr} from the same plants by HCFM. L_{pr} and L_{pc} variations in these three genotypes showed similar trends in HN and recovery treatments (Fig. 2). Under HN, L_{pr} and L_{pc} increased for B73 and for EZ37, while, after recovery, L_{pr} and L_{pc} fully recovered within a 3.5 h to control levels. In contrast, F7028 maintained constant L_{pr} and L_{pc} values both for HN treated plants and after recovery. Interestingly, L_{pr} and L_{pc} absolute values differed in B73 and EZ37 upon HN treatment. For B73, L_{pr} was higher than L_{pc} while the opposite happened for EZ37. We also noted that the absolute L_{pr} control values were similar for the three genotypes in contrast to the data obtained in Fig. 1. This difference could be due to the plant age.

Gas exchange rates under control, high nitrogen and recovery conditions

Seven genotypes behaving differently in presence of HN were selected in terms of L_{pr} variability for further experiments (group 1: B73, Mo17, EZ37, ABA+; group 2: F7028, EZ47 and ABA-). Changes between CTRL and HN treatments produced a different range of gas exchange responses in all genotypes whatever the group they belong (Fig. 3). Striking differences between genotypes were observed during the transition from CTRL to HN. g_s of B73 and EZ47 decreased (closed their stomata) whereas EZ37 and ABA- experienced an increase in g_s . The stomata of Mo17, F7028 and ABA+ were found to be much less dynamic in their responses to HN getting similar g_s values before and after being resupplied with HN (Fig. 3A). Interestingly, two genotypes of group 2 (F7028 and EZ47) that did not show any L_{pr} modification upon HN, had large L_{pr} and g_s values (Fig. S2), suggesting that the plant water flow might be sufficient for N acquisition in these genotypes. Among the genotypes that showed a L_{pr} increase in HN conditions (group 1), different

g_s behaviors were observed, suggesting different response mechanisms. The most dynamic genotype was, however, EZ37, which exhibited the largest increase in both *L_{pr}* and *g_s* in response to HN (Figs 1 and 3; Fig. S2). Upon recovery, *g_s* also responded differently according to the genotype. In B73, Mo17, F7028 and EZ47, constant *g_s* values were maintained, while for EZ37, ABA⁺ and ABA⁻, a significant drop in *g_s* was observed (Fig. 3A). Differences in *g_s* between genotypes were also accompanied by similar differences in *A_N* and *E* (Fig. 3B and 3C, respectively), suggesting that there was a feedback control between assimilation rate and stomatal conductance that enables leaves to maintain a constant high ratio of CO₂:H₂O exchange under changing conditions.

Development of root apoplastic barriers in control conditions

Because apoplastic water movement plays an important role in the total root water flow, we checked the deposition of apoplastic barriers in the seven selected maize genotypes to elucidate if the presence/absence of these barriers might influence the *L_{pr}* behavior in each of these genotypes. Because we did not expect any modification of these apoplastic barriers during the 4 h HN treatment, we only visualized them in control conditions. In all genotypes, we observed Casparian bands and lignin deposits in the endodermis and exodermis cell layers as a yellowish fluorescent signal (Fig S3). Arrowheads show marked lipid deposition in the cell walls of F7028 and ABA⁺ (Fig. S3C and S3G). The formation of Casparian bands increased from the immature tip toward the mature, halfway region along the main axis of seminal roots, so as the root tissue matures (at 5 cm from the root tip) and creates apoplastic barriers. The same pattern occurred in all genotypes, although F7028 and ABA⁺ genotypes developed stronger lipid deposition in the cell walls.

***PIP* mRNA and protein levels in roots upon short term high nitrogen treatment,**

We hypothesized that NO₃⁻ affects the *L_{pr}* through regulation of the root aquaporin activity as the conductance of the cell-to-cell pathway is largely determined by the aquaporin activity. Thus, to investigate if the effects of short term NO₃⁻ responses in maize plants in terms of water flux adaptations were aquaporin-mediated, the seven selected inbred maize lines were grown in hydroponics and equally treated with HN and subsequently recovered, and RT-qPCR was used to measure the mRNA levels of *PIP1;1*, *PIP1;2*, *PIP1;3*, *PIP1;4*, *PIP1;5*, *PIP1;6*, *PIP2;1*, *PIP2;2*, *PIP2;3*, *PIP2;4*, *PIP2;5* and *PIP2;6* genes in roots. *PIP1;1*, *PIP2;1*, *PIP2;5* and *PIP2;6* were the most abundantly expressed PIPs in all the genotypes, as previously reported (Hachez et al. 2006). However, variations in *PIP* mRNA levels for each treatment were genotype-dependent (Fig. 4). Indeed, we noted that each isoform had a different response pattern against each treatment depending on the genotype, obtaining, for example, a significant decrease in expression of *PIP2;1* after 3.5 h HN in B73 and F7028, though in other genotypes this significant reduction was not

observed (Fig. 4). In addition, upon HN treatment, *PIP2;5* mRNA levels decreased in five genotypes (B73, EZ37, ABA+, ABA- and F7028) while it was stable in Mo17 and EZ47 (Fig. 4). Quantification of the total *PIP* mRNA levels (of all *PIP* genes) for each treatment and genotype showed a significant decline in *PIP* expression for B73 and ABA+ during HN while no significant change was observed for all other genotypes (one-way ANOVA, $P < 0.05$), even if a trend in decline was also observed for F7028, EZ37 and ABA- (Fig. S4).

Our main goal was to detect if the induced NO_3^- increase in L_{pr} was aquaporin mediated. Under control conditions, *PIP* transcript levels did not correlate with any of the obtained physiological variables (Table S2). However, significant negative correlations between *PIP1;2* and *PIP2;4* transcript levels and L_{pc} were observed under HN conditions (Table S3). We, therefore, quantified the protein abundance for *PIP1;2*, *PIP2;1/2;2*, *PIP2;5* and *PIP2;6*, for which we had specific antibodies (Fig. 5) (Hachez et al. 2006, Heinen et al. 2014). The protein levels of *PIP1;2*, *PIP2;1/2;2*, *PIP2;5* and *PIP2;6* were generally similar for CTRL, HN and recovery conditions for all the genotypes, with the exception of Mo17 and EZ47, for which a decrease in *PIP1;2*, and *PIP2;5* levels were measured upon HN and recovery treatments, compared with control conditions. Moreover, Mo17 showed a similar trend for *PIP2;1/2;2*. Additionally, a higher abundance of *PIP1;2* and lower abundance of *PIP2;5* were also observed in F7028 and ABA+, respectively, upon HN treatments (Fig. 5). Finally, correlation analyses (Tables S2 and S3) revealed that neither L_{pr} nor L_{pc} were positively correlated with the overall *PIP* protein abundance, suggesting that the role of *PIPs* in the variation of the L_{pr} and L_{pc} in response to HN is due to the regulation of their subcellular localization and/or activity.

CFP-PIP2;1 abundance in the plasma membrane in high nitrogen condition

Regulation of the trafficking and the subcellular localization of *PIP* aquaporins allows for rapid modification of their abundance in the plasma membrane, and therefore the membrane water permeability (for review, see Hachez et al. 2013 and Chevalier and Chaumont 2015). Transgenic maize plants (B73 background) expressing *PIP2;1* fused to the cyan fluorescent protein (CFP) (Mohanty et al. 2009), were observed by laser-scanning confocal microscopy before and after treatment with HN for 3.5 h in comparison with control. A consistent expression pattern of *PIP2;1* in the plasma membrane in the three conducted experiments was recorded (Fig. 6). The CFP-*PIP2;1* fluorescence signal intensity increased in the plasma membrane of cortex cells after HN treatment, while no change was observed in CFP-*PIP2;1*- plants maintained in the control solution (Fig. 6A). The plasma membrane fluorescence intensity in the root cells was quantified as described in Chevalier et al. (2014) and showed a significant increase upon HN treatment (Fig. 6B), suggesting that the observed re-localization of CFP-*PIP2;1*- at the subcellular level could be a valuable molecular mechanism to regulate L_{pc} and L_{pr} after a short term NO_3^- application (3.5 h).

DISCUSSION

Plants facing changes in nutrient or water content in their environment can adapt the root development, growth and branching to optimize the resource uptake (Dinneny, 2019). However, in addition to these long-term responses, short-term mechanisms allow roots to rapidly (h) respond to changes in the root environment. In general, L_{pr} decreases when NO_3^- availability is low and increases when NO_3^- supply is high, and the response can occur over a couple of hours as observed in maize (Carvajal et al. 1996, Clarkson et al. 2000, Gloser et al. 2007, Gorska et al. 2008a). The mechanism by which sudden changes in NO_3^- availability lead to rapid changes in root hydraulic properties is not entirely understood, but a modification of the number or activity of plasma membrane aquaporins has been suggested (Carvajal et al. 1996, Henzler et al. 1999, Gorska et al. 2008b). In this work, we identified maize lines with different L_{pr} responses to a 3 to 4 h treatment with high nitrogen (8 mM NO_3^-), and developed a comprehensive analysis of the contribution of root aquaporins.

Short term high nitrogen treatment induces genotype-dependent changes in root and cell hydraulic conductivity

In seven out of the twelve studied maize genotypes (B73, Mo17, F9802, EZ47, EC169, FV353 and ABA+), L_{pr} increased upon HN treatment. This process was reversible, except in EC169, FV353, after NO_3^- withdrawal, indicating that the dynamics of the response to NO_3^- addition versus withdrawal was not necessarily symmetric and that the underlying mechanisms may differ between genotypes. In addition, HN-induced changes in L_{pr} varied from non-existent (UH250, MS153, F7028, EZ47 and ABA-) to more than 50% increase (B73 and EZ37), suggesting the presence of some distinctive physiological traits that link the soil NO_3^- availability with the plant NO_3^- needs and their root hydraulic properties. This finding was already reported by Gorska et al. (2010), who correlated the magnitude of the hydraulic response to NO_3^- presence with the NO_3^- uptake rate in different species. The distinct short-term behaviour of the studied maize genotypes facing HN allowed us to define two groups, the first one rapidly increasing their L_{pr} contrary to the second group that maintains constant L_{pr} .

In maize and other cultivated crops, improving nitrogen use efficiency (NUE) is an important trait for further exploring the intra- and inter-cultivar genetic diversity and its use for genetic improvement. In our work, we investigated the major QTL (named *root-ABAI*) backcross-derived lines homozygous either for the presence or absence of the allele (here referred to as ABA+ and ABA-, respectively), increasing or decreasing, respectively, root diameter and dry weight, and leaf ABA concentration (Giuliani et al. 2005). When grown hydroponically, the ABA+ line showed significantly higher root surface (data not shown) in comparison with ABA-,

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as previously shown by Giuliani et al. (2005). Interestingly, ABA+ line exhibited higher L_{pr} when the plants were grown under control conditions, seemingly due to altered cell-scale hydraulic properties, as from the geometrical point of view alone larger maize roots are expected to have lower L_{pr} (Heymans et al., 2021). Moreover, after HN treatment, L_{pr} largely increased in ABA+ line and tended to decrease in the ABA- line, suggesting that the magnitude of hydraulic response to NO_3^- is ABA-dependent. An effect of ABA on maize root and cell hydraulic conductivity was also previously reported (Hose et al. 2000, Wan et al. 2004) and was suggested to be dependent on aquaporins as the expression levels of the latter were positively regulated by ABA (Zhu et al. 2005, Parent et al. 2009).

In maize, the largest resistance to root water flow is associated with radial transport from root surface to xylem vessels (70–90% of the resistance (Steudle and Peterson, 1998)) and the remaining part is attributed to axial transport along xylem vessels. To understand the factors that control root radial hydraulic conductance, Zhu and Steudle (1991) simultaneously measured hydraulic parameters at the scale of individual cells and of excised roots in young maize seedlings. They concluded that besides a dominant apoplastic transport across the rhizodermis and cortex, the contribution of the hydraulic conductance of the cell-to-cell path to the overall conductance increased significantly from the first layer of the cortex toward the inner layers from 2 to 23%. At the endodermal diffusion barrier, there is increasing evidence that purely apoplastic water transport is excluded (Knipfer et al., 2010; Pascut et al., 2021) while endodermal cell membrane aquaporins largely mediate cell-to-cell water flow (Maurel 1997, Tyerman et al. 1999) and would control up to 70% of the radial conductance prior to suberisation (Couvreur et al., 2018). However, the analysis of cell-scale factors controlling root radial conductance is complicated by the fact that the conductance of the multiple composite paths vary radially (Ding et al., 2020) and longitudinally with the maturation of diffusion barriers (Wang et al., 2019; Hachez et al., 2006), and depend on the root direct environment and anatomy (Bramley et al., 2009; Heymans et al., 2020). In this work, cell pressure probe measurements allowed us to investigate how HN modified the cortex cell L_{pc} and make a link between L_{pr} and aquaporins. Interestingly, when comparing L_{pr} and L_{pc} data in three different genotypes after HN treatment and NO_3^- withdrawal, similar behaviours were observed, suggesting that changes in cell and root hydraulic properties are related to the assimilation of NO_3^- by root cells. A parallel decrease of both L_{pr} and L_{pc} were reported in Armand et al. (2019) and Knipfer et al. (2021) for barley roots exposed to long-term (several days) low-N and high salt conditions, respectively, indicating that multiple treatments may reduce the conductance of the cell-to-cell radial path.

From this point it would be tempting to conclude that for these genotypes in these conditions, root radial water transport was dominantly transmembrane. However, given the similar absolute values of L_{pr} and L_{pc} , this argument does not resist a simple mathematical analysis: crossing two membranes in series whose surfaces equal the root outer surface would

already make L_{pr} twice smaller than L_{pc} . Adding more membranes in series in the inner part of the root (6-8 cortex cell layers have to be crossed radially (Zhu and Steudle 1991)) would further reduce L_{pr} , particularly after correction by their even smaller surfaces, as in Bramley et al. (2009). Yet, scenarios with L_{pr} equal to or even larger than L_{pc} are possible. In Appendix S1, we identify five of them. Considering that cells are cylindrical and that diffusion barriers (Casparian strip and suberin lamellae) at both the exodermis and epidermis offer a substantial impedance to radial water flow, the most likely scenario would be that radial water flow is cell-to-cell across diffusion barriers (with flow through plasmodesmata required to bypass suberin lamellae when present) and water flow is apoplastic with low resistance across the cortex. These specific pathways concur with recent estimations combining a hydraulic anatomy model to multiscale maize root hydraulic data in wild-type and aquaporin mutants (Ding et al., 2020). This approach also suggests that the contribution of average cortex L_{pc} to L_{pr} is particularly low (Couvreur et al., 2018), and that the latter is essentially controlled by the conductance of “gatekeeper” cells nearby diffusion barriers (e.g., at the endodermis and neighbouring cell layers before and after its suberisation, respectively), whose cell L_p is likely larger than that of the cortex (Hachez et al., 2006; Ding et al., 2020). With that in mind, our results of similar behavior of L_{pr} and L_{pc} in response to HN and recovery treatments would imply that L_{pc} did not just change in the cortex but must also have varied accordingly at gatekeeper cells, critical locations that determine L_{pr} values.

Interestingly, neither L_{pr} nor L_{pc} of the F7028 genotype varied during HN and recovery treatments suggesting that this genotype is non-responsive to short term HN or, more probably, that it responds differently, using different mechanisms to cope with variations in NO_3^- availability as observed for plants facing drought stress (anisohtyric versus isohydric behaviors, Chaumont and Tyerman 2014). It is interesting to observe that the L_{pr} of this genotype was already high in the control condition, suggesting that an increase in L_{pr} was maybe not necessary to ensure efficient NO_3^- uptake and distribution.

Impact effects of high nitrogen and recovery on gas exchange did not coincide with changes of L_{pr}

N availability induces interacting effects on gas exchange parameters and, thus, there is crosstalk between the regulation of N and water relations. It is considered that the supply of N to the plant increases its photosynthetic rate and its total growth since all the dry matter produced by the plant depends entirely on this process (Peil and Galvez 2012). Indeed, it was demonstrated in wheat (*Triticum aestivum* L.), sunflower (*Helianthus annuus* L.) and maize that high N supply induces an increase in CO_2 assimilation (Lindquist 2001, Cechin and de Fatima 2004). Similarly, in many studies, N deprivation or N supply regulates stomatal aperture and transpiration (Matimati et al. 2014, Ren et al. 2015). However, these behaviours were not occurring here for all the studied maize genotypes, for which HN was not always correlated with higher g_s , E and A_N , thus

providing a rather complex feedback regulation in relation to N availability. Similar behaviour was reported in *Lotus japonicus*, where changes in shoot transpiration were not reflected by changes in L_{pr} (Henzler et al. 1999). This could be explained through a longer-term shift in the distribution of ABA between roots, where ABA increases aquaporin activity and the hydraulic conductivity, and shoots, where ABA causes stomatal closure. However, it could also be due to the contrasted measurement condition of the leaf gas exchange rates ($1500 \mu\text{mol m}^{-2} \text{s}^{-1}$), different from that used during growth ($200 \mu\text{mol m}^{-2} \text{s}^{-1}$). Indeed, it is known that leaves that have developed in low irradiance have a lower rate of photosynthesis when they are exposed to saturating irradiance, and that also contain increased chlorophyll relative to electron transport proteins and relative to Rubisco protein (Leong and Anderson, 1986; Terashima and Evans, 1988) than leaves that have developed under high irradiance (Bjorkman, 1981). Thus, because Rubisco exerted little or no control at low measuring light, but exerted an increasing degree of control when the measuring light intensity increased, we may also suggest that these indirect effects of the measuring light conditions influence the gas exchange of the plants, which can be genotype dependent.

In contrast, EZ37 and F7028 genotypes belonging to group 1 and 2, respectively, showed a positive correlation between gas exchange rates and L_{pr} responses to HN and recovery conditions. In the former one, NO_3^- addition stimulated L_{pr} whereas NO_3^- starvation during recovery suppressed it, allowing transpiration to presumably drive convection of mobile N sources in the solution to the root surface. However, F7028 dynamics of the response to NO_3^- addition was non-existent for either L_{pr} or gas exchange parameters, raising the possibility that, in this case, an adjustment of these parameters was not necessary due to their already large values.

Rapid changes in root hydraulic properties are possibly due to a higher abundance of PIP aquaporins in the plasma membrane

The aquaporin mRNA levels were generally unchanged or decreased in HN treated plants, but did not increase as one would have predicted based on the L_{pc} data. No variation in maize aquaporin transcript levels was neither observed by Gorska et al. (2008b) for a period of up to 4 h post- NO_3^- treatment using the “Hybrid Corn-White Sweet” genotype, while observing a higher root water flow. Surprisingly, no positive correlation between PIP protein and mRNA levels and L_{pr} or L_{pc} for the different genotypes was observed, suggesting that the variation of L_{pr} and L_{pc} in response to short-term HN is due to post-translational PIP regulation mechanisms (Li et al. 2014). Moreover, under HN conditions, L_{pc} was negatively correlated with *PIP* transcript levels, concretely with *PIP1;2* and *PIP2;4*. A similar trend was also observed when comparing L_{pr} with the transcript abundance of several aquaporins in Arabidopsis and rice plants (Sutka et al. 2011 and Grondin et al. 2016) which could be due to the diurnal/circadian variation of *PIP* mRNA levels previously observed in maize (Hachez et al, 2012; Caldeira et al., 2014) and the delay

between transcription, protein synthesis and activity at the membrane level. However, further investigation of cell-specific expression data or PIP expression over the course of the day is needed.

Here, we also quantified PIP1;2, PIP2;1/2;2, PIP2;5 and PIP2;6 protein abundance and no significant differences were recorded for most genotypes in the tested conditions with the exception of Mo17 and EZ47, in which a decrease in abundance of two or three PIP proteins, was recorded after HN and recovery. Actually, this data was not so surprising considering the short-term HN treatment (3.5 h) and the aquaporin feature and regulation. Firstly, discrepancies between aquaporin mRNA and protein levels were previously reported in maize and other plant species after different stresses (i.e. Hachez et al. 2012, Muries et al. 2011, Qian et al. 2015). Secondly, while there was a good correlation between L_{pc} and the aquaporin abundance during the day or night when maize plants were growing in well-watered conditions (Hachez et al. 2012) or in maize PIP2;5 overexpressing or knockout plants (Ding et al. 2020), modification of the growing medium can affect at short term the L_{pc} without leading to a rapid change in protein abundance due to regulation of aquaporin subcellular localization or activity (Hachez et al. 2014, Pou et al. 2016, Chaumont and Tyerman 2014). Quantification of CFP-PIP2;1 abundance in B73 root plasma membrane after 4 h HN treatment showed an accumulation of the proteins in the membrane suggesting that trafficking regulation might be responsible for the L_{pc} increase. The molecular mechanism leading to an accumulation of PIP2;1 in the plasma membrane is still unknown but, could be related to a decrease in its internalization/degradation. In addition, we cannot exclude that HN also increases the activity of PIPs already present in the plasma membrane. Deciphering how NO_3^- signalling leads to regulation of PIP trafficking and activity is the next challenge. The Arabidopsis high-affinity NO_3^- transporter NRT2.1 was previously shown to act as a NO_3^- sensor/transducer to regulate long-term plant development, but also *PIP* expression (Little et al. 2005, Remans et al. 2006, Li et al. 2016). Determining whether the maize NRT2;1 ortholog is an actor contributing to the rapid regulation of PIP subcellular trafficking or activity will have to be investigated.

DATA AVAILABILITY

Data available on request from the authors.

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AUTHOR CONTRIBUTIONS

AP and FC designed the experiments. AP, CH and AI performed the experiments. AP, CH, VC, LM, AI and FC analyzed the data. AP, VC and FC wrote the manuscript. All the authors contributed to the discussion and revision of the manuscript.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article:

Table S1. Cell pressure probe measurements of maize root cortex cells of B73, EZ37 and F7028 genotypes measured after 3.5 h incubation in control (CTRL; black) or high nitrogen (HN; white), and after 3 h recovery (RECOV; dark gray) solutions.

Table S2. Pearson's coefficient of correlation between root hydraulic properties (L_{pr} , L_{pc}), leaf gas exchange parameters (photosynthesis, stomatal conductance and transpiration rates) and root PIP transcript abundance under control conditions (CTRL).

Table S3. Pearson's coefficient of correlation between root hydraulic properties (L_{pr} , L_{pc}), leaf gas exchange parameters (photosynthesis, stomatal conductance and transpiration rates), root PIP transcript abundance and root PIP abundance under high nitrogen conditions (HN).

Table S4. Pearson's coefficient of correlation between root hydraulic properties (L_{pr} , L_{pc}), leaf gas exchange parameters (photosynthesis, stomatal conductance and transpiration rates), root PIP transcript abundance and root PIP abundance under recovery conditions (REC).

Fig. S1. Root hydraulic conductivity (L_{pr}) of B73 maize genotype at different measuring times (10 AM and 2 PM).

Fig. S2. The relationship between stomatal conductance (g_s) (Y1 axis) and transpiration rate (E) (Y2 axis) and root hydraulic conductivity (L_{pr}) of 7 genotypes.

Fig. S3. Development of apoplastic barriers in primary roots of selected two-week-old genotypes growing in hydroponic conditions.

Fig. S4. Contribution of each isoform to total PIP transcript levels in roots of 7 different genotypes.

Appendix S1. Analysis of possible hydraulic scenarios under the constraint of equal root L_{pr} and cell L_p .

Figure legends

Figure 1. Root hydraulic conductivity (L_{pr}) of 12 maize genotypes. Two-week-old plants were incubated in hydroponic conditions in modified Hoagland medium (2 mM NO_3^- ; CTRL, black bars) or medium containing 8 mM NO_3^- (HN; white bars) for 3.5 h. In the recovery treatment, HN treated plants were transferred to the original modified Hoagland solution for 3 h (RECOV; gray bars). These data allowed us to define seven HN responsive maize genotypes (group 1) (A) and four HN non-responsive maize genotypes (group 2) (B). Values are means $\pm$ SE of 10 to 15 plants. For each genotype, different letters denote statistically significant differences between treatments determined using Anova with Tukey's test ($P < 0.05$).

Figure 2. Membrane water permeability of root hydraulic conductivity (L_{pr}) and cortex cells (L_{pc}) of B73, EZ37 and F7028 genotypes measured after 3.5 h incubation in control (CTRL; black) or high nitrogen (HN; white), and after 3 h recovery (RECOV; dark gray) solutions. Values are

means $\pm$ SE of 3 plants. Different letters denote statistically significant differences between treatments determined using Anova with Tukey's test ($P < 0.05$).

Figure 3. (A, B) Stomatal conductance (gs), (C, D) Net Photosynthesis (AN) and (E, F) transpiration rate (E) of 7 genotypes in CTRL (black), HN (white) and RECOV (dark grey) conditions. gs, AN and E were measured by using the Li-cor 6400 (Li-6400-40; Li-Cor Inc.). The values are means $\pm$ SE of 4 to 5 plants after 3.5 h acclimation in each treatment. Different letters denote statistically significant differences between treatments within each line by Anova with Duncan's test ($P < 0.05$).

Figure 4. The relative levels of PIP aquaporin RNA levels in roots of seven selected two-week-old genotypes measured after 3.5 h incubation in control (CTRL; black) or high nitrogen (HN; white), and after 3 h recovery (RECOV; dark gray) solutions. The geometric mean of the mRNA level of three control genes (actin, growth factor EF-1a and ubiquitin) was used to normalize the data. Error bars denote levels of PIP transcript in maize roots. The results are means $\pm$ SE of three independent RNA samples extracted from pooled root material collected from a minimum of three plants per treatment. Asterisks denote statistically significant differences between treatments within each line determined using Anova with Tukey's test ($P < 0.05$).

Figure 5. PIP aquaporin mRNA levels in roots. (A) PIP1;2, PIP2;1/2;2, PIP2;5 and PIP2;6 protein levels in roots of seven selected genotypes. Microsomes were prepared from control (CTRL), high nitrogen (HN) and recovered (RECOV) roots and subjected to Western blotting using antibodies against PIP1;2, PIP2;1/2;2, PIP2;5 and PIP2;6. Anti-PMA antibody recognizing plasma membrane H^+ -ATPases was used as a loading control. The position of the molecular markers is shown. (B) For the sake of signal comparison between treatments, the signal intensity for CTRL roots was set to 1 (arbitrary units). The mean $\pm$ SE of two independent experiments is shown. For each genotype, asterisks denote statistically significant differences between CTRL and treated plants (either HN or RECOV) by Student's t test ($P < 0.05$).

Figure 6. CFP-PIP2;1 fluorescence intensity in the plasma membrane (PM) in root cells. (A) Representative laser-scanning confocal images of the CFP-PIP2;1 fluorescence in root epidermal cells at 5 cm from the apex from CFP-PIP2;1 transgenic plants growing in control (CTRL; black) or high nitrogen (HN; white) conditions for 3.5 h. Acquisitions were performed with the same settings for all samples. Scale bar, 10 μ m. (B) For the sake of signal comparison between treatments within the graph, the signal intensity for control (CTRL) grown roots was set to 1 (arbitrary units). Each bar represents the mean of three independent experiments (4-6 plants per experiment). Error bars show the 95% confidence interval. Asterisks denote statistically significant differences between treatments by Student's t test ($P < 0.05$).

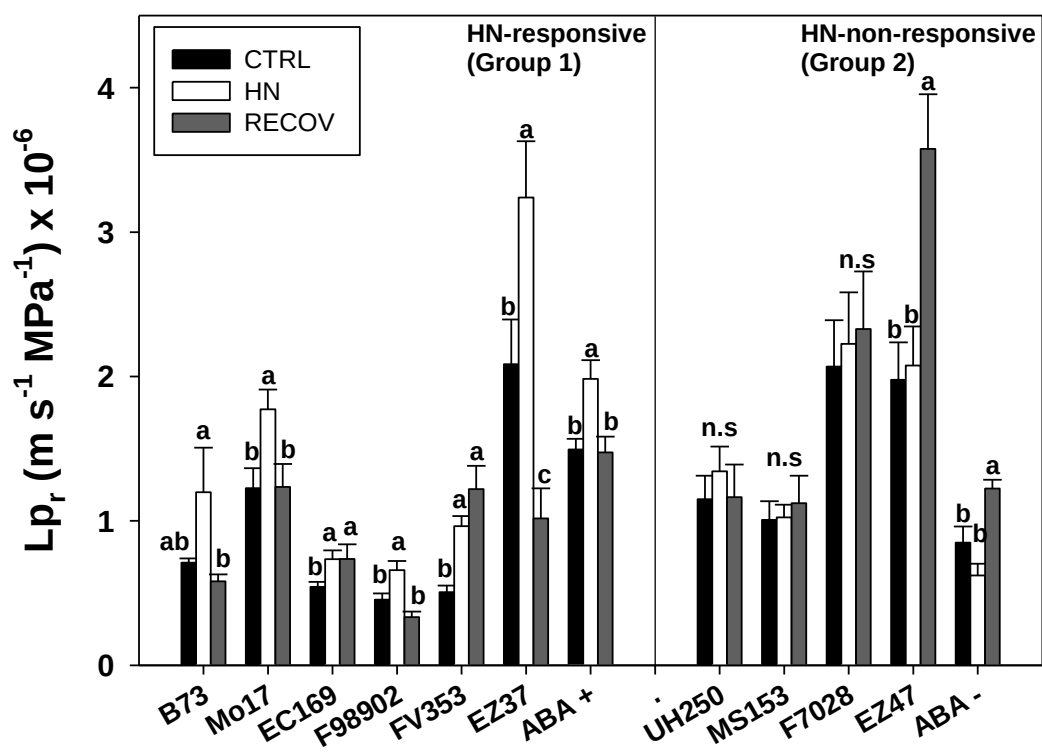


Figure 1.

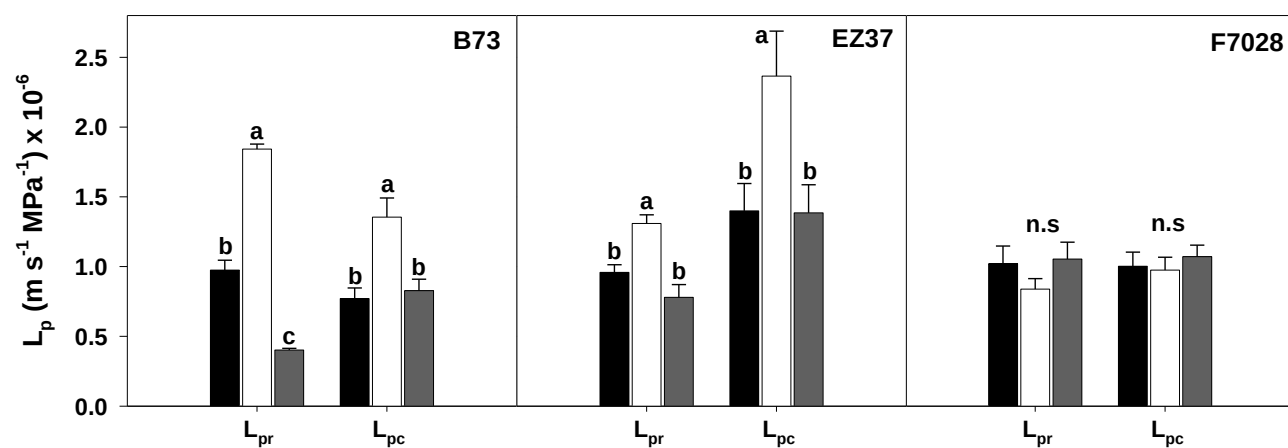


Figure 2.

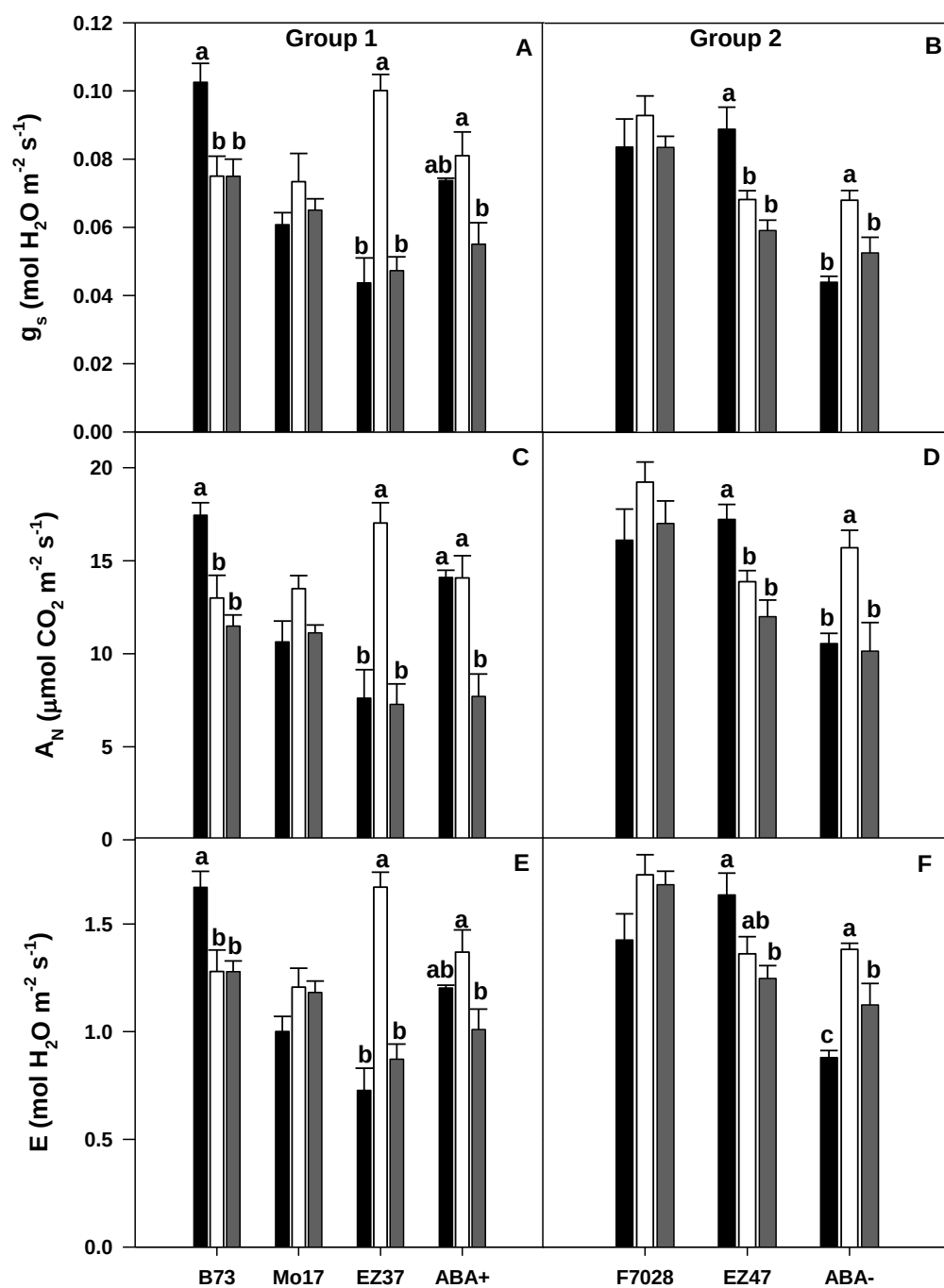


Figure 3.

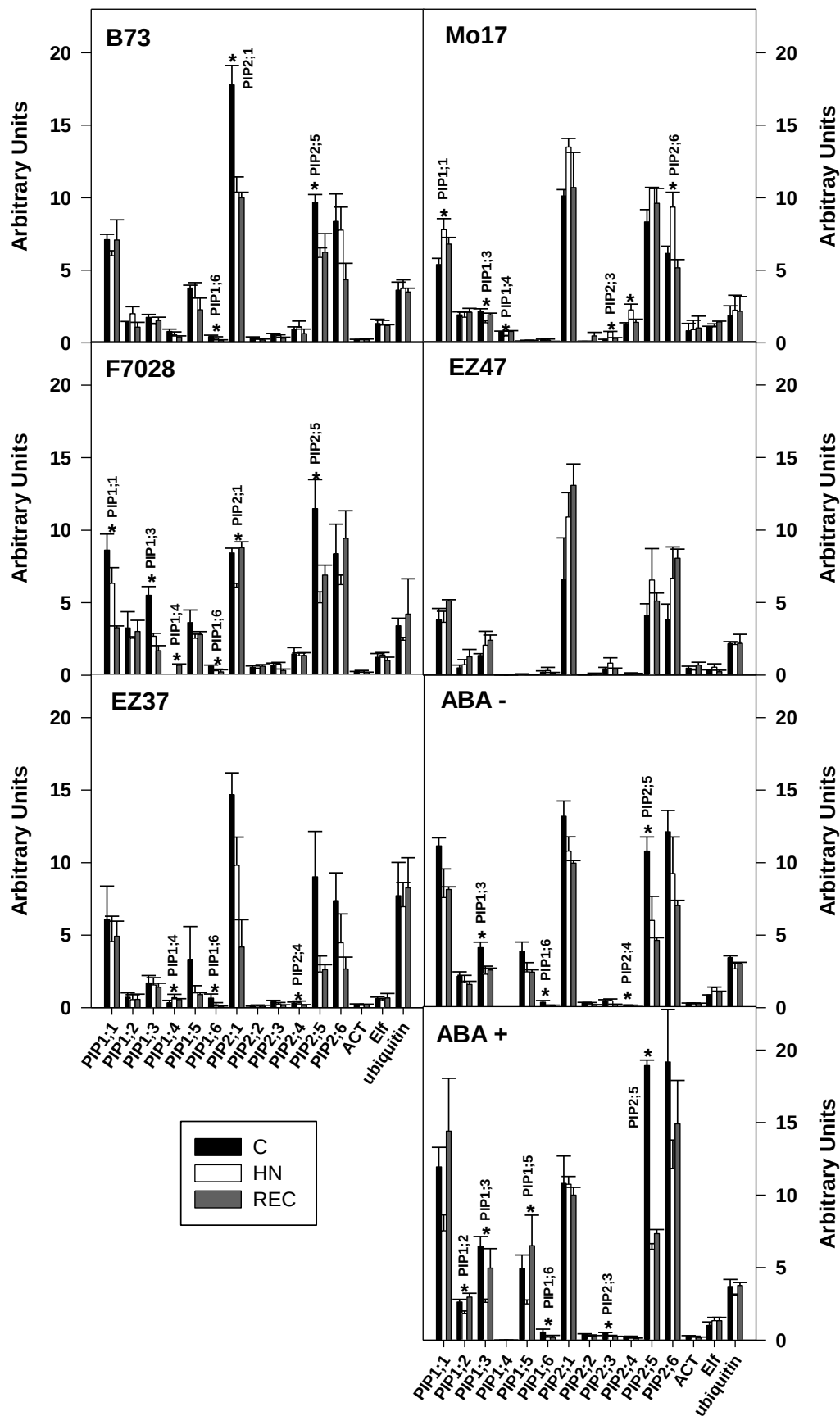


Figure 4.

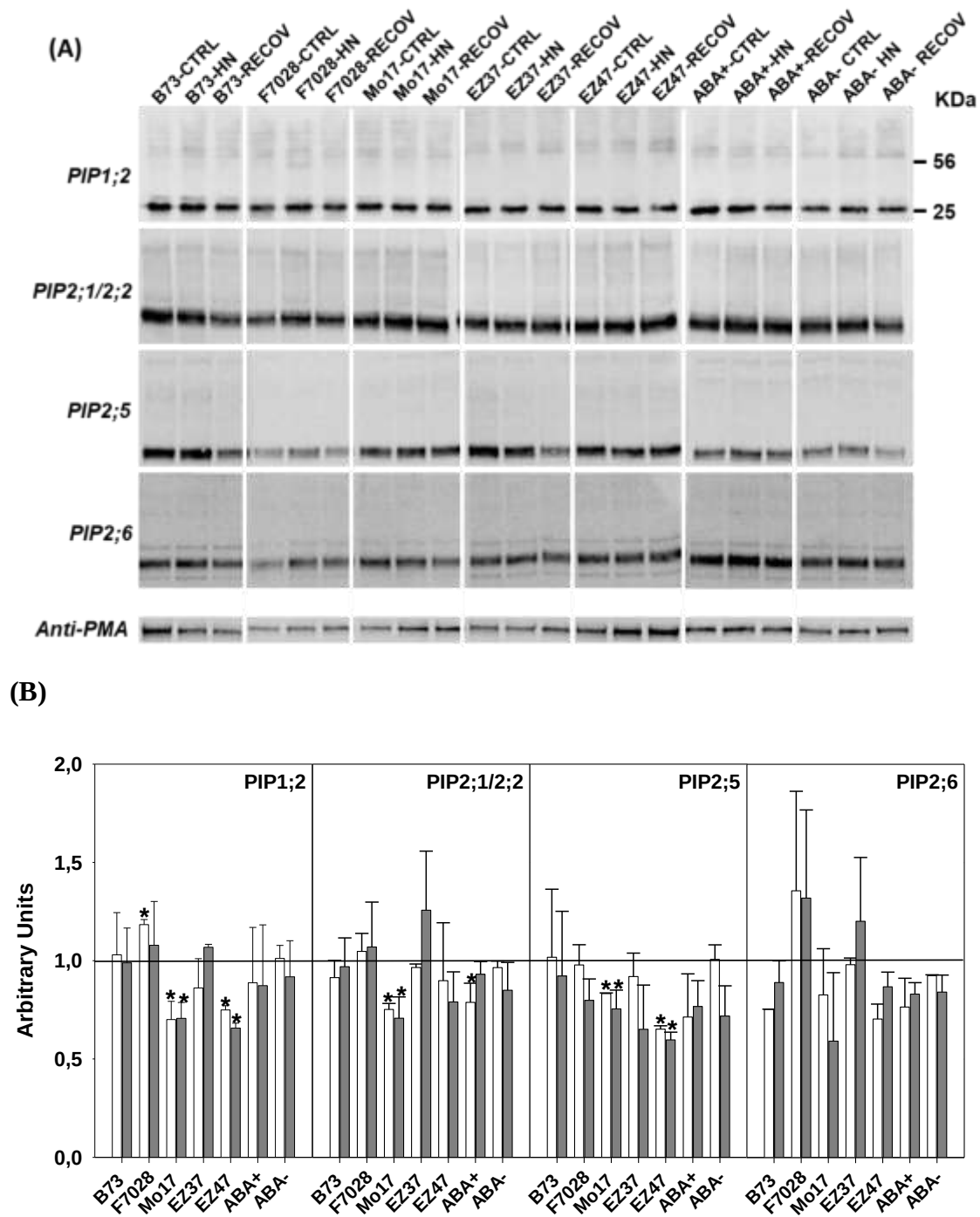


Figure 5.

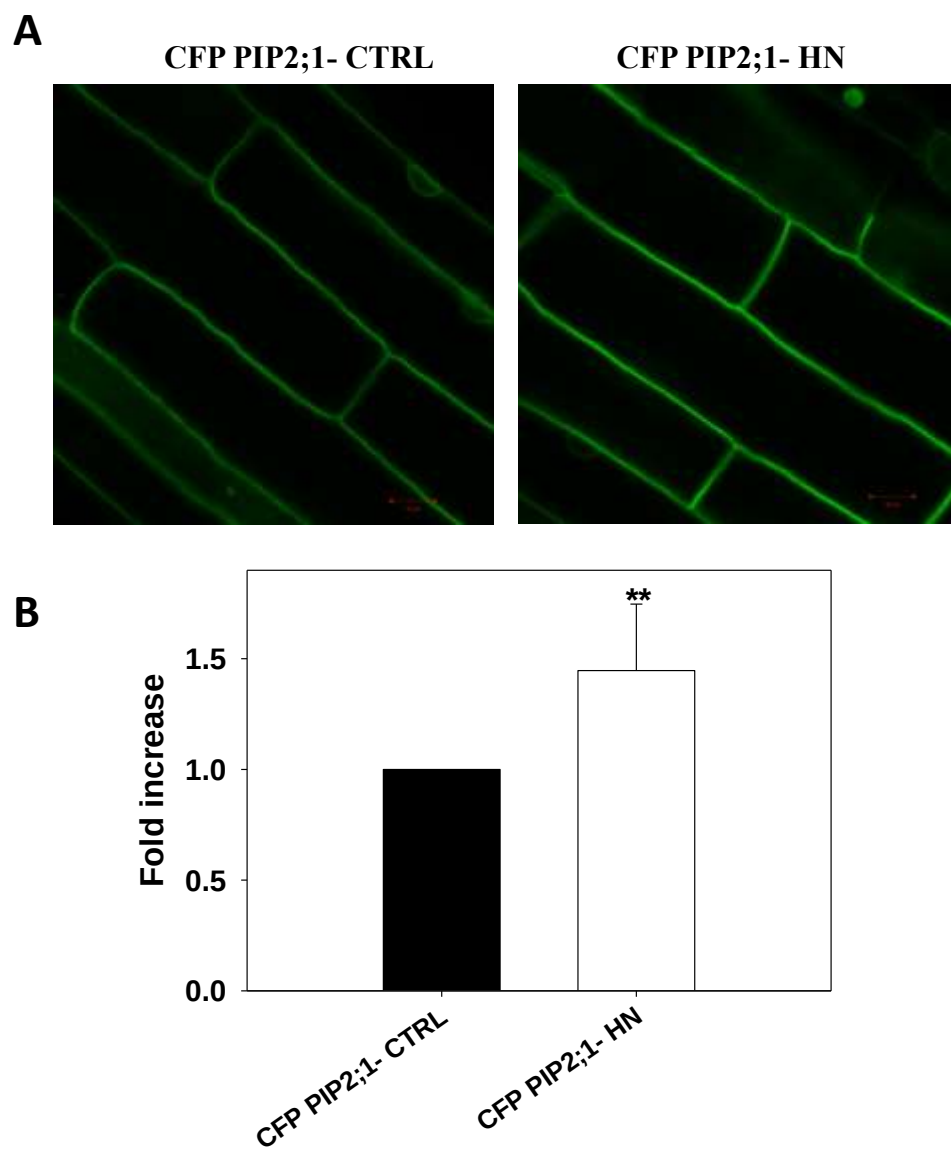


Figure 6.