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ORIGINAL ARTICLE

Nitrate induction of root hydraulic conductivity in maize is not correlated with aquaporin expression

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Abstract Some plant species can increase the mass flow of water from the soil to the root surface in response to the appearance of nitrate in the rhizosphere by increasing root hydraulic conductivity. Such behavior can be seen as a powerful strategy to facilitate the uptake of nitrate in the patchy and dynamically changing soil environment. Despite the significance of such behavior, little is known about the dynamics and mechanism of this phenomenon. Here we examine root hydraulic response of nitrate starved *Zea mays* (L.) plants after a sudden exposure to 5 mM NO_3^- solution. In all cases the treatment resulted in a significant increase in pressure-induced (pressure gradient ~ 0.2 MPa) flow across the root system by ~50% within 4 h. Changes in osmotic gradient across the root were approximately 0.016 MPa (or 8.5%) and thus the results could only be explained by a true change in root hydraulic conductance. Anoxia treatment significantly reduced the effect of nitrate on xylem root hydraulic conductivity indicating an important role for aquaporins in this process. Despite a 1 h delay in the hydraulic response to nitrate treatment, we did not detect any change in the expression of six *ZmPIP1* and seven *ZmPIP2* genes, strongly suggesting that NO_3^- ions regulate root hydraulics at the protein level. Treatments with sodium tungstate (nitrate reductase inhibitor) aimed at resolving the information pathway regulating root hydraulic properties resulted in unexpected findings. Although this treatment blocked nitrate reductase activity and eliminated the nitrate-induced hydraulic response, it also produced changes in gene expression and nitrate uptake levels, precluding us from suggesting that nitrate acts on root hydraulic properties via the products of nitrate reductase.

Keywords Nitrate · Root hydraulic conductance · Tungstate · *ZmPIP* · *Zea mays*

Abbreviations

NR	Nitrate reductase
ZmPIP	*Zea mays* plasma membrane intrinsic protein gene
FAD	Flavin adenine dinucleotide
DTT	Dithiothreitol

Introduction

In well-aerated, non-acidic soils, NO_3^- ions are often the main source of nitrogen for plants. Because nitrate anions are not linked to negatively charged soil colloids, they move freely within the soil solution. This characteristic makes nitrate a highly variable commodity both spatially and temporally, with concentrations often varying several orders of magnitude even around the root system of a single plant (Crawford and Glass 1998). The adaptive mechanism that allows plants to respond to nitrate's variable distribution include both root proliferation in local high nitrate concentration volumes (Remans et al. 2006; Walch-Liu et al. 2006) and induction of NO_3^- ions transporters and nitrate assimilation enzymes by exogenous nitrate (Forde 2002; Stitt 1999). However, these mechanisms will lead to depletion of nitrate around the immobile roots and eventually to a self-inflicted reduction in nitrate uptake. Barber (1995) reports that 80% of nitrogen absorbed by maize roots moved to the

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root surface by mass flow and only 20% by diffusion (Barber 1995). Thus, to extend nitrate uptake activity, one can expect that plant should generate mass flow of water toward the root to overcome diffusive limitations.

There are two ways in which plants can increase the flow of water to roots. The first is to increase the mass flow of water toward roots by augmenting transpiration rates. In this scenario, the entire root system experiences higher water uptake, not just those roots exposed to increased nitrate levels. Although variation in transpiration rate and stomatal conductance in response to nitrate availability has been observed in a number of crop species (maize, tomato, cotton, barley), this response was mediated by adjustments in root hydraulic properties rather then via direct regulation of stomatal conductivity (Chapin et al. 1988; Radin 1990; Radin and Matthews 1989). The sensitivity of root hydraulic resistance to nitrate concentrations (Barthes et al. 1996; Carvajal et al. 1996; Ezeta and Jackson 1975; Gloser et al. 2007; Horau et al. 1996), provides the basis for an alternative scenario in which water flow is enhanced only to roots exposed to high nitrate levels (Gorska et al. 2008). An important outcome of such a local response is that it will magnify the positive effect of the altered root resistance on total nitrate uptake by prioritizing water uptake from roots exposed to nutrient rich patches.

The ability of roots to alter their hydraulic properties in response to nitrate availability provides a mechanism for increasing whole plant nutrient acquisition. However, the physiological mechanisms underlying the hydraulic response to nitrate availability are not fully known. Reverse genetics data and studies with mercury inhibition provide strong evidence that aquaporins facilitate water transport across the root (Javot et al. 2003; Maggio and Joly 1995; Martre et al. 2002; Siefritz et al. 2002), accounting for up to 70% of whole root radial water transport (Amodeo et al. 1999; Martre et al. 2001). Thus, fast changes of root hydraulic conductivity in response to nitrate most likely result from either the regulation of aquaporins activity (gating) or changes in their number due to altered transcription rate (expression). In this paper, we analyze the temporal dynamics of the influence of nitrate concentration on maize roots hydraulic conductivity and test if nitrate triggers an increase in water channel transcript levels. We further analyze the influence of tungstate salts on nitrate assimilation and its influence on nitrate-induced changes in root hydraulic conductivity.

Methods

Plant material

Seeds of *Zea mays* (L.) "Hybrid Corn—White Sweet" by Jonny's Selected Seeds (955 Benton Avenue Winslow, Maine 04901, USA) were germinated on wet filter paper (Whatman Quantitative Circles, 90 mm Ø, Cat No 1001 090, Whatman®, Schleicher & Schuell) in covered Petri dishes at room temperature. Three days after germination, the seedlings were moved to an aerated hydroponic solution (6.5 L containers). We used a modified Hoagland solution (pH ~ 6.1; 795 μM KNO_3, 603 μM $Ca(NO_3)_2$, 270 μM $MgSO_4$ and 109 μM KH_2PO_4; micronutrients: 405 μM Fe(III)-EDTA, 20 μM H_3BO_4, 2 μM $MnSO_4$, 0.085 μM $ZnSO_4$, 0.15 μM $CuSO_4$ and 0.25 μM Na_2MoO_4). Plants were grown in growth chamber (14 h light : 10 h dark cycle, 25:20°C, 60% humidity and a photon flux density of 500 μmol m^{-2} s^{-1}). After 1 week, the plants were transferred to 42 L containers (15 plants per box) and allowed to grow for an additional week. For plants assigned to the low nitrate treatment, the medium was then replaced with a low nitrate solution (pH ~ 6.1; 79.5 μM KNO_3, 60.3 μM $Ca(NO_3)_2$, 270 μM $MgSO_4$ and 109 μM KH_2PO_4, 795 μM K_2SO_4, 603 μM $CaCl_2$; micronutrients: 405 μM Fe(III)-EDTA, 20 μM H_3BO_4, 2 μM $MnSO_4$, 0.085 μM $ZnSO_4$, 0.15 μM $CuSO_4$ and 0.25 μM Na_2MoO_4. Plants were grown in the low nitrate medium for a minimum of 7 days, but no longer than 12 days, before being measured. In all cases, hydroponic solutions were exchanged two times per week.

Hydraulic measurements

The hydraulic properties of maize root systems were determined by measuring flow rates resulting from the application of a constant pressure gradient of 0.2 MPa. Briefly, a de-topped root system was fitted with a plastic tube filled with DI water and connected to a beaker located on a balance (±0.01 mg; Sartorius BP230, Germany; Fig. 1). The root system was then sealed in a pressure chamber filled with solution from the hydroponic container to avoid any shock related to change in solution quality. To avoid pH changes associated with the addition of nitrate later in the procedure, the solution was kept at ~6.1 pH using MES (2-(*N*-morpholino)ethanesulfonic acid) buffer (1 g per 1 L). The pressure in the chamber was regulated using a needle valve, set to produce a small "leak" leak such that the air used to pressurize the chamber also served to aerate and mix the medium (Fig. 1). Water flow through the root system was automatically recorded at 30 s intervals. In most cases flow stabilized within 10 to 20 min after the plant was exposed to pressure. Plants were maintained under these conditions for an additional 2 h, after which we added either a mixture of 3.2 M KNO_3 and 2.4 M $Ca(NO_3)_2$ in solution to reach a final concentration of 5 mM NO_3^- or the same volume of low nitrate solution from the growing medium as control. We also assayed the impact of supplying nitrogen in the form of ammonium or urea; in these

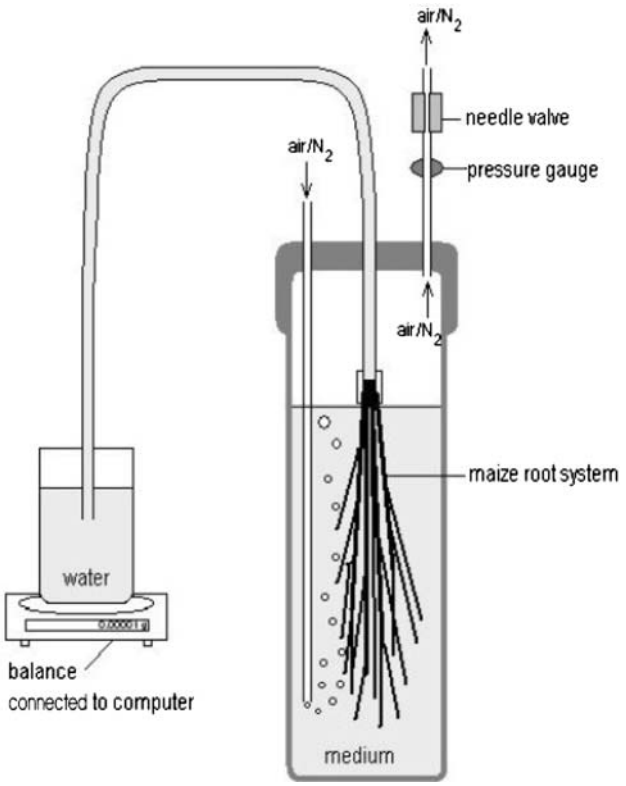

Fig. 1 Schematic of experimental system used to generate pressure-induced root exudation

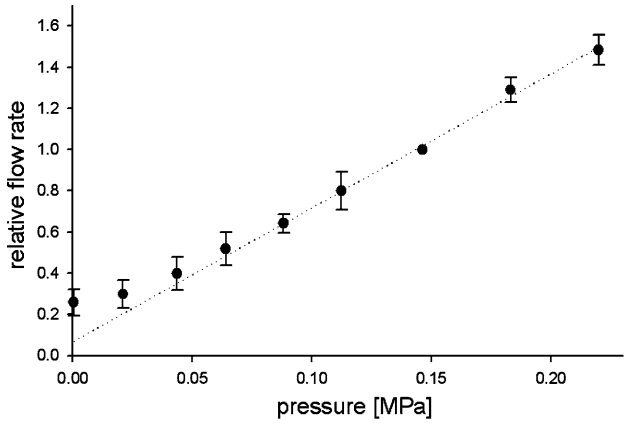

Fig. 2 Relation between applied pressure and water flow through maize root systems. Data are means of four independent experiments (*error bars* denote SE). To account for differences in root size we relate changes in flow rate to flow at 0.15 MPa

Table 1 Osmotic pressure (MPa) of maize xylem sap

Time (min)	Osmotic pressure of xylem sap	
−30	0.033	±0.011
0	0.035	±0.010
30	0.037	±0.008
60	0.037	±0.007
90	0.036	±0.009
120	0.039	±0.008
150	0.043	±0.005
180	0.045	±0.004
210	0.048	±0.008
240	0.049	±0.007

Time 0 is the moment of 5 mM NO_3^- addition into the medium in root pressure chamber. Data are means (MPa) ± SE from four independent experiments

experiments a final concentration of 1 mM was used. These additional liquids were injected though the aeration system, such that the pressure in the chamber remained unchanged and the added nutrients became quickly mixed throughout the existing medium.

Examination of the relation between applied pressure and flow rate indicated that the linear phase of the pressure:flow curve starts around 0.1 MPa, i.e. the influence of the osmotic pressure of xylem sap is undetectable above this threshold pressure (Fig. 2). Thus, we selected 0.2 MPa as our driving force to ensure that all measurements were conducted in the linear phase. At the end of each measurement, we applied an anoxia treatment induced by application of nitrogen gas instead of the air in the pressurization system. This treatment was introduced for two reasons (1) to determine the influence of protonation on aquaporin gating (Tournaire-Roux et al. 2003) and (2) to check for the presence of leaks or root damage.

Determination of xylem sap osmotic pressure

Although our measurements were made within the linear portion of the pressure:flow relation for maize roots, we also determined xylem sap osmotic potentials so that we could be sure that the influence of the osmotic pressure of xylem sap during the experiments on the measured flow rates was negligible. De-topped root systems of maize were treated in the same way as for the hydraulic measurements, but instead of directing the out-flow to the balance, exudate was collected and its osmotic potential measured using a vapor pressure osmometer (5520 Varro, Wescor). Xylem sap samples were collected every 30 min starting half an hour before the nitrate addition. Observed changes in xylem sap osmotic pressure are shown in Table 1.

Plant material harvests

Material for gene expression analysis and enzyme activity determination was harvested from plants exposed to the same nutrient levels as used in the physiological measurements described above. An important difference, however, is that these analyses were made on plant material harvested from directly from intact plants to avoid problems associated with sugar depletion or changes in hormonal

balance. The assumption here is that the response of intact plants to nitrate is similar to that of de-topped plants, as shown in former studies in vivo (Gloser et al. 2007). Plants were removed from the hydroponic container and the roots were immediately excised, washed in DI water and then dried with paper towels. Dry roots were wrapped in aluminium foil and placed in liquid N_2. The roots were subsequently ground to a fine powder in liquid nitrogen and stored at −80°C for further analyses that included nitrate tissue concentration, nitrate reductase activity, and quantitative real-time PCR of 13 *ZmPIP* encoding genes. There were three treatments: (1) control (no nitrate addition), (2) nitrate treatment (addition of nitrate to 5 mM NO_3^- concentration) and (3) nitrate treatment on plants pre-treated with tungstate (1 mM sodium tungstate, 48 h). Five collections were scheduled for each treatment that included time zero (beginning of the experiment) and 0.5, 1, 2, and 4 h after nitrate addition for a total of 36 plants in three separate treatments.

Nitrate tissue concentration

For determination of tissue nitrate content, hot water extracts from frozen, pulverized maize roots were prepared in a weight ratio 1:2 (100°C, 20 min). After centrifugation (18,000*g*, 10 min) the NO_3^- concentration of the supernatant was determined using the salicylic acid nitration method (Cataldo et al. 1975).

Nitrate reductase activity

Frozen powder of root tissue was added to an extraction buffer (50 mM Hepes-KOH (pH 7.6), 1 mM DTT, 10 µM FAD, 10 mM $MgCl_2$ and 50 µM cantharidine) in a 1:2 ratio and ground until thawed using a glass tissue grinder. After centrifugation (18,000*g*, 10 min, 4°C) a part of the supernatant was removed for nitrite determination (to establish baseline nitrite levels). The remaining aliquot was desalted at 4°C on home-made Sephadex G-25 columns (1 ml gel volume, 600 µl extract). Enzyme activity was assayed in the presence of Mg^{2+} ions (actual activity) (Kaiser et al. 2000). Aliquots (500 µl) were added to the reaction mix (500 µl, 50 mM Hepes-KOH (pH 7.6), 20 µM FAD, 2 mM DTT, 20 mM $MgCl_2$, 10 mM KNO_3, 0.4 mM NADH) and incubated for 4 min at 23°C. The reaction was stopped by the addition of 30 µl of 2 M zinc acetate. After centrifugation the supernatant was used for colorimetric determination of nitrite production (Wray and Filner 1970).

Quantitative real-time PCR

Total RNAs were isolated from powdered root tissue using the RNeasy Plant Mini Kit (Qiagen). Quantification of RNA samples was done by spectrophotometrical measurement at $\lambda = 260$ nm. cDNA was synthesized from 1 µg of total RNAs using QuantiTect Reverse Transcription Kit (Qiagen) including a special step for genomic DNA degradation. Q-PCR reaction was conducted with QuantiTect SYBR Green PCR Kit (Qiagen) and maize aquaporins gene-specific primers designed by Hachez et al. (2006) according to the manufacturer's instructions. The reaction mix had a final volume of 25 µl and contained 12.5 µl 2 × QuantiTect SYBR Green PCR Master Mix, 0.3 µM of each primer and 12.5 ng of cDNA. Thermocycling conditions were as follows: 15 min at 95°C; 40 cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 72°C, with a final melt gradient starting from 50°C and heating to 95°C at a rate of 0.5°C s1. The signals were detected on an MJ Opticon 2 (Bio-Rad). The real-time PCR reaction was normalized by glyceraldehyde phosphate dehydrogenase, GAPDH (gi:22237) (Hachez et al. 2006), which showed a constant transcript level in all performed treatments. The results were expressed as the average ± SE of at least three independent RNA samples.

Results

Nitrate addition to hydroponic solution resulted in increased water flux across the root system only in plants that had been exposed to the low nitrate solution for a minimum of 4 days. Temporal dynamics of the flow response to nitrate addition showed a significant delay (∼1 h) in the start of a gradual increase of flow rate across the root, which reached a new steady state flow not earlier than 4 h later (Fig. 3a). The average increase in flow rate was ∼50% of the initial value. The osmotic gradient between medium and xylem sap rose from ∼0.033 to ∼0.049 MPa (i.e. by ∼0.016 MPa) and could account only for ∼7% of the flow rate increase (Table 1). Thus, the majority of the observed increase in flow rate resulted directly from changes in root hydraulic conductivity.

The anoxia treatment reduced the flow rate within a few minutes of replacing air with nitrogen gas and resulted in a significant reduction of flow rate over the one hour of application, reaching levels 45 and 36% of initial flow rate, respectively, for nitrate treated and control roots (Fig. 3b). The difference in flow rates between nitrate treated and control roots was almost entirely eliminated by anoxia, indicating that the gain in root hydraulic conductivity in response to nitrate was related to physiological processes associated with cell respiration. Because anoxia was previously shown to specifically gate aquaporins by protonation (changes in cellular pH) (Tournaire-Roux et al. 2003), we propose that most of the nitrate induced increase in root conductivity was aquaporin mediated.

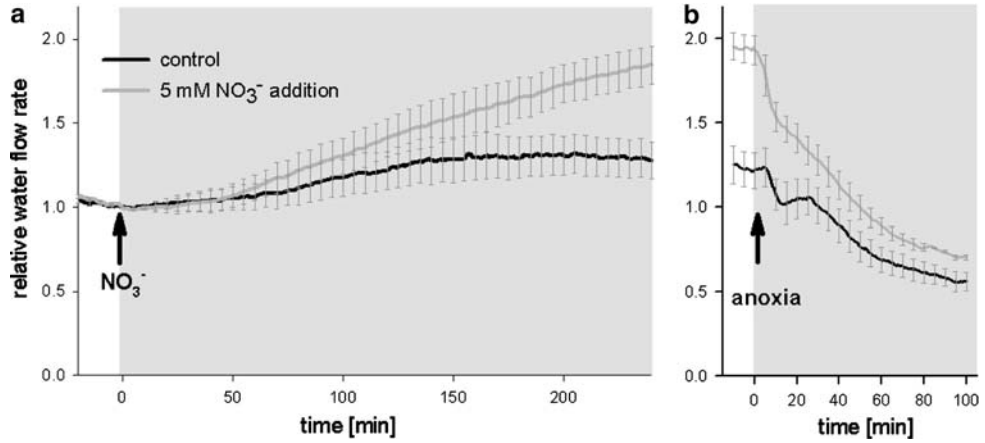

Fig. 3 Pressure driven exudation from whole root systems of *Zea mays*. Due to size differences between plants, data were standardized by the flow at the moment of nitrate application. Time of nitrate application (**a**) is indicated by *downward pointing arrow* and *gray shading*. Each *line* is the average of four plants; *vertical bars* indicate SE and are, for clarity, are shown only for every tenth time point. *Upward pointing arrow* and *light gray shadowing* denotes application of anoxia treatment (**b**), 4–5 h after the nitrate addition

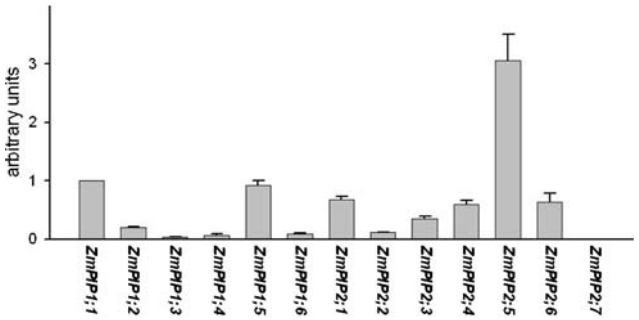

Fig. 4 The relative levels of maize aquaporin gene expression in roots of 1-week nitrogen starved maize plants. Expression was adjusted and compared to PIP1:1 level. The data represent means of six plants from three independent experiments. *Error bars* denote SE

The ~1-hour time delay in the response of root hydraulic conductivity to nitrate treatment raises the possibility that the observed increase in hydraulic conductivity resulted from a change in the number of aquaporins and thus was related to their expression. The level of *ZmPIP1* and *ZmPIP2* gene transcripts was measured on roots of 1-week nitrogen starved plants (Fig. 4). In general, there were significant differences between expression levels of different genes. *ZmPIP2;5* was the gene of highest expression level *ZmPIP1;1*, *ZmPIP1;5*, *ZmPIP2;1*, *ZmPIP2;4* and *ZmPIP2;6* had expression levels about 3–4 times lower than *ZmPIP2;5*. The lowest level of transcript was detected for *ZmPIP1;3* ~100 times lower than *ZmPIP2;5*. We failed to find any transcript amplification of gene *ZmPIP2;7* (Fig. 4). Relative level of expression between analyzed genes was almost identical to formerly reported levels (Hachez et al. 2006).

Control plants showed a significant variation in gene expression over the time of experiment (Fig. 5). However, since there was no change in the experimental treatment of these plants from during the harvest day in comparison to preceding days we can only assume that this variation reflects diurnal trend associated with growing environment as has been reported in previous studies (Henzler and Steudle 1994). Addition of nitrate was not followed by any significant change in the expression level of all tested *ZmPIP* genes when compared to control plants within 4 h (Fig. 5). Surprisingly, addition of sodium tungstate a pretreatment known to inhibit NR activity had a generally negative effect on expression of *ZmPIP,* particularly: *ZmPIP2;5*, *ZmPIP2;3*, *ZmPIP1;1*, *ZmPIP1;5* and *ZmPIP1;6* (Fig. 5).

As expected, addition of NO_3^- to nitrate starved plants resulted in a significant increase in nitrate reductase activity (Fig. 6). Incubation of plants for two days in a hydroponic solution with 1 mM sodium tungstate (nitrate reductase inhibitor) and no molybdenum eliminated NO_3^- induction of nitrate reductase activity (Fig. 6). Maize roots treated with nitrate showed a steady increase in root nitrate concentration (Fig. 7) despite the strong increase in NR activity (Fig. 6). In contrast, roots pretreated with tungsten did not exhibit an increase in nitrate concentrations despite elimination of NR activity (Fig. 7). This suggests that either nitrate uptake was affected by tungstate or that root cells unloaded nitrate to the xylem as fast as it was absorbed.

Incubation in tungstate salt had a marked effect on root hydraulic conductivity, causing a significant reduction over the control plants: 2.9 ± 0.3 and 5.4 ± 0.7 [ng H_2O s^{-1} Pa^{-1} g^{-1} root DW], respectively (Fig. 8), which coincided with the fact that tungstate treatment also reduced the expression level of many *ZmPIP* genes. In addition, tungstate incubation eliminated the response of root hydraulic conductivity to the addition of nitrate (Fig. 9a), while the effect of anoxia on the tungstate treated roots was smaller to that of control plants ~27 and ~55%, respectively, most

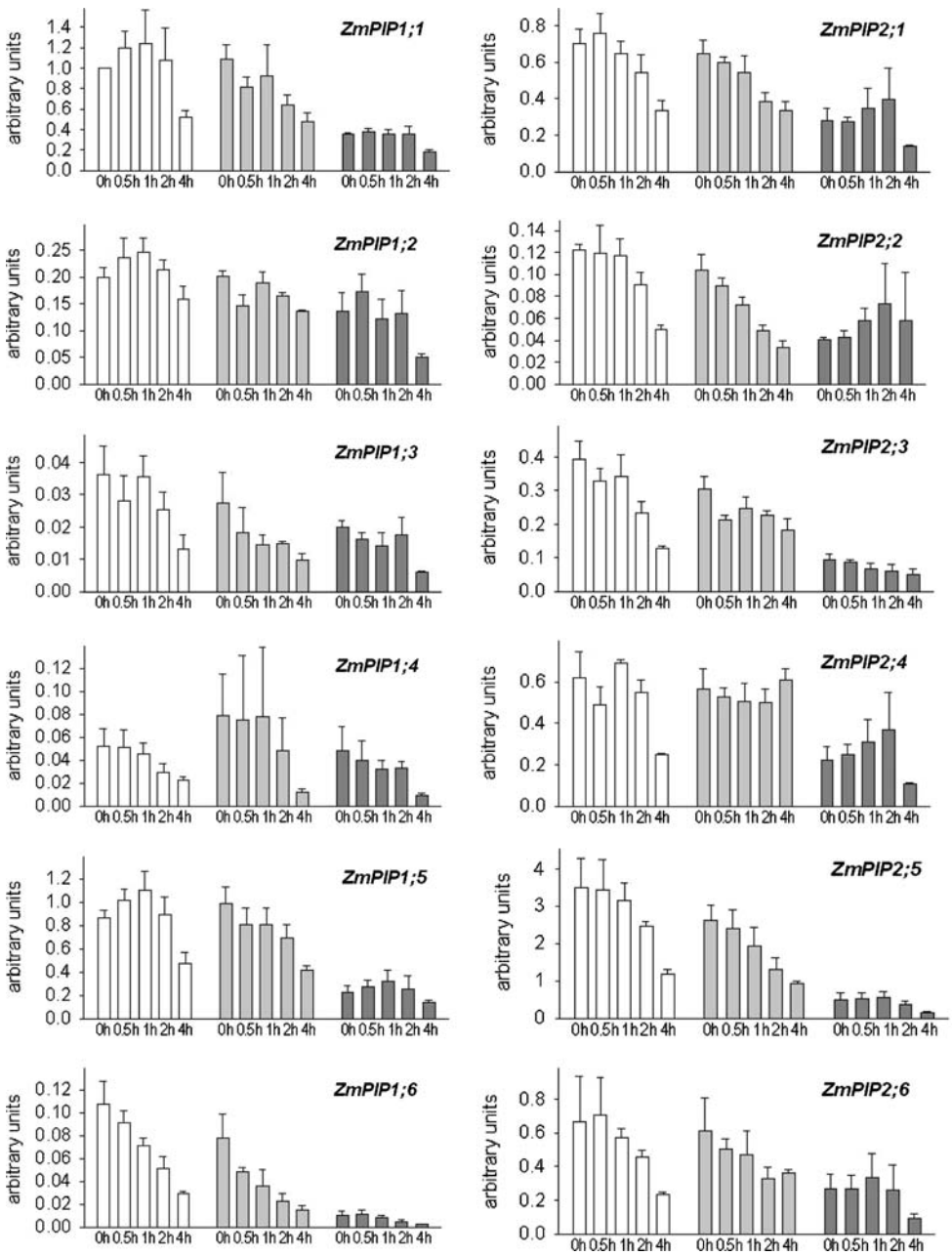

Fig. 5 Levels of *ZmPIP* transcript in maize roots. *Bars* with no shadings—control, *light grey shading*—5 mM NO_3^- addition, *dark grey columns*—5 mM NO_3^- addition to plants pretreated with tungstate for 48 h. The results are the averages of three independent RNA samples extracted from pooled root material collected from a minimum of three plants per treatments per time samples (*error bars* denote SE)

likely reflecting lower level of aquaporin expression (Figs. 3b, 9b) Because the tungstate treated roots did not exhibit the increase in tissue nitrate levels which characterized the effect of nitrate addition to plants that were not pretreated with tungstate, the failure of the tungstate treated roots to respond to nitrate addition in terms of their root hydraulic properties may be associated with the unchanged tissue nitrate concentrations, rather than a reduction in NR activity.

These findings are further reinforced by the failure of nitrogen-starved plants to respond to nitrogen supplied solely in the form of NR activity products: ammonium or urea (Fig. 10). Application of both chemicals initially caused a small decrease in the rate of water uptake, after which no stimulating affect was observed over a period of 4 h.

Discussion

The response of maize root hydraulic properties to nitrate addition was slightly delayed when compared to sunflower (Gloser et al. 2007). Within ~60 min from the time of nitrate addition, water flux across the root started to increase. This increased flow rate resulted from a true change in root hydraulic conductivity and is not due to

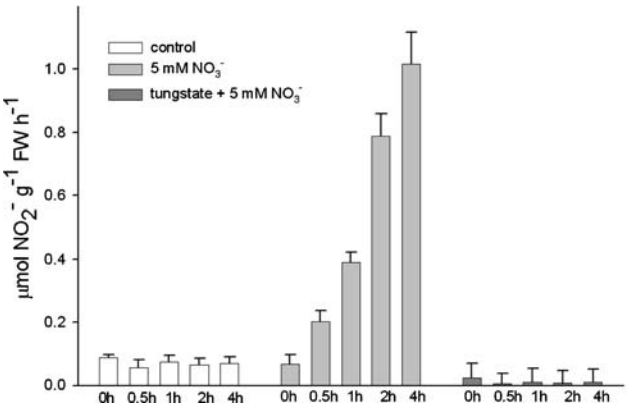

Fig. 6 Nitrate reductase activity in maize roots. Averages represent data collected from three independent experiments with pooled root samples from a minimum of three plants (*error bars* denote SE)

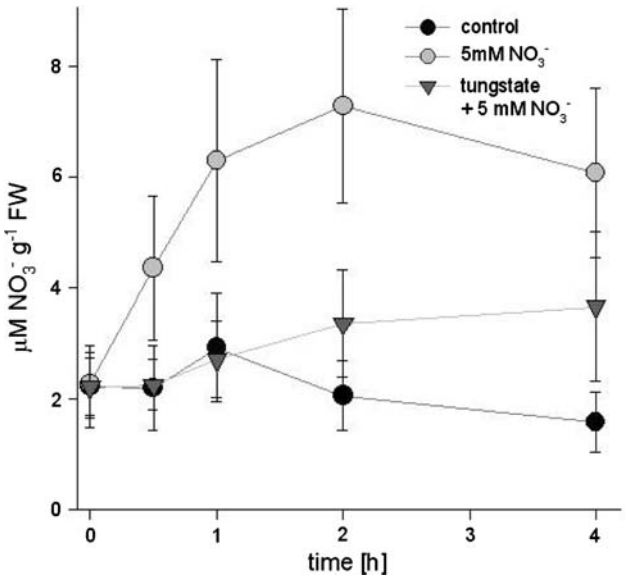

Fig. 7 Changes in nitrate tissue concentrations in maize roots. Data represent the average of three independent experiments one plant per experiment per time collection (*error bars* denote SE)

changes in osmotic driving force. It has been previously shown that nitrate addition to nitrogen starved maize plant can increase xylem sap exudation within over the period of 15 h (Barthes et al. 1996). In that study flow was generated by root pressure only (i.e. osmotic potential difference). Thus the evidence linking the observed flow increase to changes in root hydraulic conductivity was limited to the symplastic pathway (Steudle et al. 1993) and it was not clear if this response would be significant in hydrostatic pressure driven flow. Our results confirm that changes in the nutrient solution in the root medium affect root hydraulic conductivity at flow rates similar to those expected for transpiring plants and thus can be of importance in nitrate acquisition under field conditions.

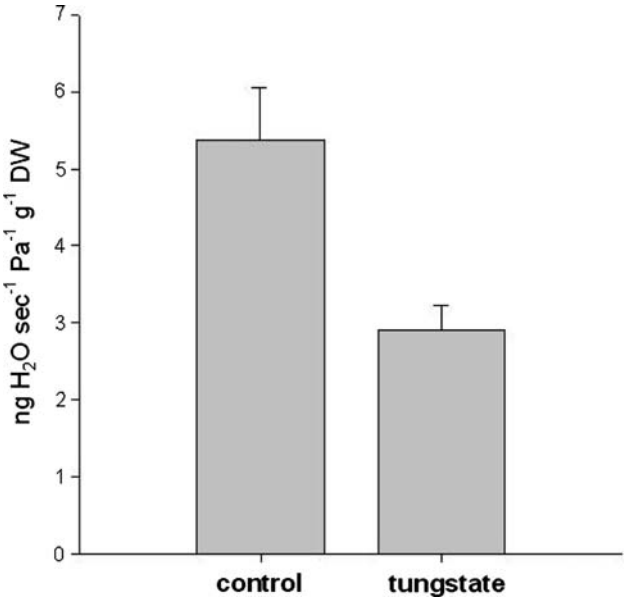

Fig. 8 Hydraulic conductivity of maize roots without (control) and with 48 h exposure to 1 mM sodium tungstate. *Each bar* is the average of six plants; *vertical bars* indicate SE

Rapid changes in root hydraulic properties could result from changes in membrane fluidity (Schaller 2003) or changes in water channel activity (Johansson et al. 2000; Tyerman et al. 1999, 2002). Plasma membrane fluidity has been shown to be lower in nitrogen deprived wheat plants than in nitrate treated ones, which could be responsible for lower root hydraulic conductance (Carvajal et al. 1996). However, there are also data suggesting that nitrate can regulate transcript levels of some water channel genes (Wang et al. 2001). Because root oxygen deprivation is a powerful and reversible (i.e. not damaging) process gating aquaporins (Tournaire-Roux et al. 2003) we used it to determine to what degree the observed changes in maize root hydraulic conductivity is mediated by aquaporins. Anoxia reduced root hydraulic conductivity for both nitrate starved and nitrate treated roots (Fig. 3b), suggesting that NO_3^- ions influence maize root hydraulic properties mainly by aquaporin regulation. Nevertheless, the major impact of NO_3^- ions on root hydraulic is likely to be via an influence on aquaporin-mediated water transport. Such changes could be the result of direct regulation (gating) of existing aquaporins, changes in gene expression levels, or modification of the protein synthesis/degradation balance. Analysis of temporal dynamics showed a significant delay (~1 h) between nitrate application and the initial increase in root hydraulic conductivity. This is in contrast to studies on sunflower that showed an immediate (within a few minutes) response to nitrate (Gloser et al. 2007). Such a delay suggests that, in maize, the response could be related to changes in expression levels. However, we found that nitrate did not alter

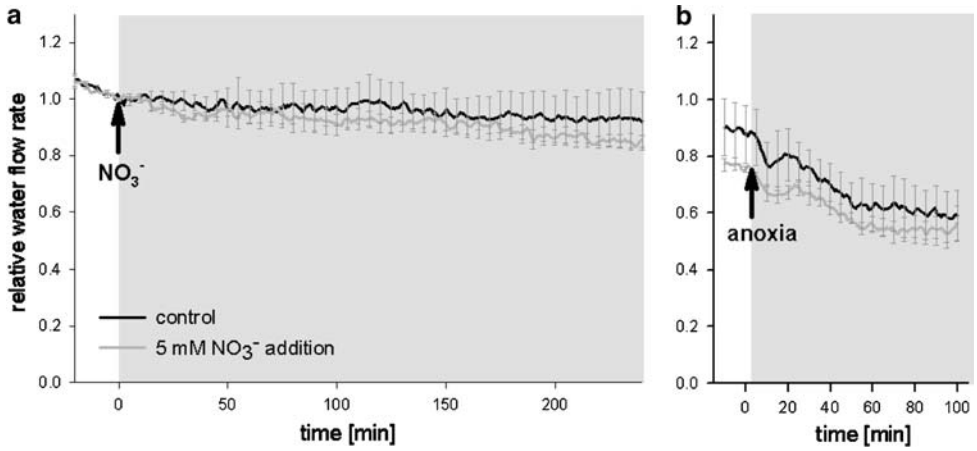

Fig. 9 Pressure driven exudation from whole root systems of *Zea mays* after 48 h exposure to tungstate. Due to the size difference between roots data were standardized by the flow at the moment of nitrate application. Application of nitrate (**a**) is indicated by *downward pointing arrow* and *gray shading*. Each *line* is the average of four plants; *vertical bars* indicate SE and, for clarity, are shown only for every tenth time point. *Upward pointing arrow* and *light gray shading* denotes application of anoxia (**b**), 4–5 h after the nitrate addition (note the change in *vertical scale*)

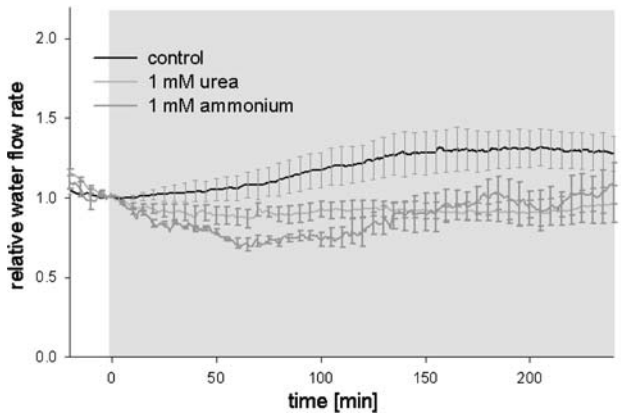

Fig. 10 Pressure driven exudation from whole root systems of *Zea mays*. Due to size differences between plants, data were standardized by the flow at the moment of ammonium or urea application. Time of ammonium or urea application is indicated by *gray shading*. Each *line* is the average of four plants; *vertical bars* indicate SE and are shown only for every tenth time point

maize aquaporin transcript levels relative to control plants for a period of up to 4 h post nitrate treatment, thus pointing toward protein regulation being the major source of the response. Our findings are in line with results reported for *Arabidopsis* and tomato roots. Nitrate treatment of nitrogen starved plant of *Arabidopsis thaliana* did not significantly change mRNA level of plasma membrane aquaporin genes within 3 h (Scheible et al. 2004) or in tomato roots for a period up to 12 h following nitrate addition (Wang et al. 2001).

Our findings add to the idea that the hydraulic response to nitrate is under strong physiological control acting over multiple temporal scales. In long term studies (multiple days) increased availability of nitrate leads to significant changes in root morphology and proliferation that increases the ability of roots to acquire nitrate from patchy environment (Barthes et al. 1996). A temporal response of hours to days was associated with up regulation of aquaporin expression in tomato plants (Wang et al. 2001). While our analysis of fast temporal response (minutes to hours) showed no changes in ZmPIP expression level, thus pointing towards posttranscriptional regulation i.e. changing the balance between open and closed aquaporins. The results presented in this study suggest that aquaporins in maize roots are only partially utilized and under favorable conditions root hydraulic conductivity can be rapidly up regulated by opening already present membrane proteins or that a rapid sub-cellular relocalization of aquaporins can occur. A fast and reversible response would allow for much more plastic response to changing soil environment. The nature of the mechanism responsible for maintaining a balance between open and closed aquaporins is not well known. There are many potential processes that could be involved: phosphorylation, protonation, methylation, divalent cation binding, hereromerization, high osmotic pressure, or mechanical tension (Chaumont et al. 2005; Maurel 2007). We did not try to resolve which of these processes is responsible for the observed hydraulic dynamics. However, because nitrate is a signaling molecule that induces many regulatory factors including protein kinases (Scheible et al. 2004), it is possible that nitrate changes aquaporin activity by altering their posphorylation/dephosphorylation status.

We were also interested in whether the products of nitrate assimilation are responsible for changes in root hydraulic dynamics as suggested previously (Forde 2002; Gloser et al. 2007). Application of NR activity products as a nitrogen source did not enhance water uptake in nitrogen

starved plants, suggesting that it is nitrate itself and particularly its in-plant concentration that is involved in the signaling pathway responsible for rapid changes in maize root hydraulic properties. A similar conclusion was drawn from cellular studies of cucumber roots in which tungstate treatment was shown to block nitrate uptake and NR activity but direct injection of nitrate to root cortical cells caused an increase in membrane hydraulic conductivity (Gorska et al. 2008).

In further tests of NR's role in observed changes in root hydraulic properties, we were successful in blocking nitrate assimilation path with tungstate, but unfortunately we are unable to make any meaningful conclusion regarding this subject. As we found out and what was not reported earlier, tungstate treatment resulted in a strong modification of several processes associated directly with root water relations. We observed that root hydraulic conductivity was about two times lower in tungstate treated plants than in control roots. This drop was associated with a significant reduction in the expression level of highly expressed aquaporins: *ZmPIP2;5*, *ZmPIP1;1* and *ZmPIP1;5* (Fig. 5) and a reduction in the response to anoxia that pointed toward a greater dependence of water transport on non aquaporin mediated paths (Fig. 9b). Tungstate is often used as a specific blocker of NR activity, but it has also been found to interact with other molybdoenzymes as sulfite oxidase, xanthine dehydrogenase, indole-3-acetaldehyde oxidase and abscisic aldehyde oxidase (Jiang et al. 2004; Porch et al. 2006). Because reaction catalyses of these enzymes are involved in auxin and abscisic acid synthesis (Mendel and Hansch 2002), hormones known to be involved in plant response to water stress, it is possible that *ZmPIP* expression pattern was influenced by this additional tungstate effect. Abscisic acid has been reported to regulate the promoter of PIP1-like gene in tobacco (Siefritz et al. 2004) and there are data suggesting that auxin can regulate aquaporin expression (Lin et al. 2007). The fact that tungstate addition can disturb *ZmPIP* expression and nitrate accumulation in the cell, as found in this study, and evidence from other studies where tungstate influenced hormonal balance indicates that extreme caution that has to be taken when using tungstate as a NR blocking treatment in the study of plant water relations.

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