Coumarin enhances nitrate uptake in maize roots through modulation of plasma membrane H^+-ATPase activity

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ABSTRACT

•Coumarin is one of the simplest plant secondary metabolites, widely distributed in the plant kingdom, affecting root form and function, including anatomy, morphology and nutrient uptake. Although, some plant responses to coumarin have been described, comprehensive knowledge of the physiological and molecular mechanisms is lacking.

•Maize seedlings exposed to different coumarin concentrations, alone or in combination with 200 µM nitrate (NO_3^-), were analysed, through a physiological and molecular approach, to elucidate action of coumarin on net NO_3^- uptake rate (NNUR). In detail, the time course of NNUR, plasma membrane (PM) H^+-ATPase activity, proton pumping and related gene expression (*ZmNPF6.3*, *ZmNRT2.1*, *ZmNAR2.1*, *ZmHA3* and *ZmHA4*) were evaluated.

•Coumarin alone did not affect nitrate uptake, PM H^+-ATPase activity or transcript levels of *ZmNRT2.1* and *ZmHA3*. In contrast, coumarin alone increased *ZmNPF6.3*, *ZmNAR2.1* and *ZmHA4* expression in response to abiotic stress. When coumarin and NO_3^- were concurrently added to the nutrient solution, a significant increase in the NNUR, PM H^+-ATPase activity, together with *ZmNAR2.1:ZmNRT2*.1 and *ZmHA4* expression was observed, suggesting that coumarin affected the inducible component of the high affinity transport system (iHATS), and this effect appeared to be mediated by nitrate. Moreover, results with vanadate, an inhibitor of the PM H^+-ATPase, suggested that this enzyme could be the main target of coumarin.

•Surprisingly, coumarin did not affect PM H^+-ATPase activity by direct contact with plasma membrane vesicles isolated from maize roots, indicating its possible elicitor role in gene transcription

INTRODUCTION

Coumarin (1,2-benzopyrone), the simplest allelopathic com- pound among cinnamic acid derivatives, is widely distributed in many plant species and localised on the surface of leaves, seeds, flowers and fruits, where it is involved in plant–plant communications (Zobel & Brown 1995). Released into the environment, coumarin affects plant growth and development of many species (Rice 1984) and in particular, seed germination (Aliotta et al. 1993; Abenavoli et al. 2006), respiration and photosynthesis (Moreland & Novitzky 1987; Kupidlowska et al. 1994) and energy metabolism, causing oxidative stress (Pergo et al. 2008). However, plant response to coumarin depends on species, concentration and physiological processes, being stimulatory and inhibitory at lowest and highest concentrations, respectively (Abenavoli et al. 2006; Pergo et al. 2008; Lupini et al. 2016a). Negative effects of coumarin on cell division and root polarity in oat and *Phleum pratense* were previously reported (Goodwin & Avers 1950; Avers & Goodwin 1956). In contrast, Neumann (1959) demonstrated that coumarin markedly stimulated elongation of excised segments of hypocotyls in sunflower, suggesting an auxin-like behaviour of this compound. Coumarin effect on root morphology and histology was later reported, suggesting that the root system may be the main target of this allelochemical, affecting its form and function (Svensson 1971; Kupidlowska et al. 1994; Abenavoli et al. 2001). Later, a different degree of sensitivity to coumarin among root types, i.e. nodal>seminal>primary roots, was reported in maize (Abenavoli et al. 2004). Furthermore, the same authors demonstrated that, at low concentrations, coumarin increased the elongation of lateral roots but not of the primary root in *Arabidopsis* (Abenavoli et al. 2008). Using a morphological and electrophysiological approach, Lupini et al. (2010) indicated the root apex (within 20 mm of the tip) of the maize primary root as most sensitive to coumarin, suggesting that this effect could be mediated by auxin. Recently, the same authors (Lupini et al. 2014) evidenced a functional interaction between coumarin and auxin polar transport in driving root development, using *Arabidopsis* auxin mutants. In addition, coumarin was also able to modify root function. Indeed, 100 µM coumarin was able to increase net nitrate (NO_3^-) uptake rate (NNUR) and its translocation in shoot and root vessels in wheat seedlings (Abenavoli et al. 2001). In particular, coumarin stimulated the inducible high-affinity NO_3^- transport system (iHATS), leaving unchanged the constitutive transport (cHATS; Abenavoli et al. 2001), two components of HATS that operate at low (<500 µM) NO_3^- external concentrations (Glass & Siddiqi 1995). Similar results were also observed in alfalfa, where the lowest coumarin concentrations (25 and 50 µM) stimulated net NO_3^- uptake and N metabolism (Zhou et al. 2013). Beside the HATS, plant roots exhibit a low-affinity transport system (LATS), which operates at high NO_3^- concentrations (for review and references see Forde & Clarkson 1999; Forde 2000; Glass et al. 2002; Glass 2009). NPF6.3 (previously called NRT1.1) and NPF4.6 are two proteins involved in LATS (Kiba et al. 2012), whereas three NRT2 proteins (NRT2.1, NRT2.2 and NRT2.4) represent the main components of HATS (Li et al. 2007). In addition, recent studies pointed out that *NAR2.1*, an accessory protein, is required for functioning of NO_3^- uptake in *Poaceae* roots (Saia et al. 2015; Lupini et al. 2016b; Pii et al. 2016; Chen et al. 2017). These sophisticated and plastic NO_3^- transport systems needed to take up this mobile anion from the soil solution, which limits plant growth and development, thereby reducing plant productivity (Xu et al. 2012). Nevertheless, information concerning the effect of coumarin on genes encoding high affinity transporters is still lacking.

Nitrate transport across the plasma membrane (PM) depends on the cellular energy supply and is coupled to the proton electrochemical gradient ($2H^+$:NO_3^- symporter) generated by the PM H^+-ATPase (Miller & Smith 1996; Palmgren 2001; Miller et al. 2007). In maize roots, a relationship between nitrate transport and PM H^+-ATPase activity has been observed (Santi et al. 1995), especially under nitrate uptake induction (Santi et al. 2003; Sorgonà et al. 2011). Recent results suggested that some cinnamic acid derivatives, such as trans- cinnamic, ferulic and p-coumaric acids, affected PM H^+- ATPase activity independently of NO_3^- uptake (Abenavoli et al. 2010). However, the coumarin mechanism influencing the NO_3^- uptake system is not yet fully clarified, due to scarce knowledge of functional relationships among anion uptake and PM H^+-ATPase activity at protein and transcript levels. Thus, in the present work, using molecular and physiological approaches, this relationship was investigated

in maize roots. The results could provide more insight into the action of this allelopathic compound, and in general on the role of allelochemicals on plant nutrient uptake.

MATERIAL AND METHODS

Plant material and growth condition

Maize seeds (*Zea mays* L., cv. Cecilia; Pioneer, Italy) were sterilised for 20 min in 20% (v/v) sodium hypochlorite solution and then rinsed with deionised water. Seeds were then steeped in aerated deionised water for 48 h, germinated for 72 h in the dark at 24 °C in a plastic container filled with 0.5 mM $CaSO_4$. After 72 h, individual seedlings, selected for uniform size, were transferred into a glass growth unit (4.5 dm^3, 12 plants) containing 4.3 l of aerated one-quarter strength Hoagland hydroponic N-free solution (Hoagland & Arnon 1950). The pH was adjusted to 6.0 with 0.1 M KOH. The growing units were placed into a growth chamber at 25 °C with a 14-h photoperiod, a photosynthetic photon flux density (PPDF) of 350 µmol m^{-2} s^{-1} at plant height and 70% RH. The nutrient solution was renewed every 2 days (Lupini et al. 2016a,b). Coumarin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Net nitrate uptake assay

Nitrate uptake measurements were performed in maize seedlings (7 days old) grown in an N-free nutrient solution. Seed- lings (7 days old) were transferred into a single growing unit containing the same solution with 0, 25, 100 or 400 µM coumarin and/or 200 µM NO_3^-. Seedlings grown in the absence of NO_3^- and coumarin are referred to as the control (CK). After 0, 8, 24 and 48 h of contact, the Net Nitrate Uptake Rate (NNUR) was measured. In particular, for each time of expo- sure and coumarin treatment, three seedlings were collected and their roots immersed into 80 ml aerated uptake solution containing 0.5 mM CaSO4 and 200 lM NO_3^-. From the root contact, samples (1 ml) from the uptake solution were taken up at 5-min intervals over a 60-min period and NO_3^-concen- tration was measured at 210 nm with a UV–vis spectrophotometer (Lambda 35; Perkin Elmer, Waltham, MA, USA). The NNUR was calculated from the linear phase of the nitrate depletion curve and was expressed as µmol NO_3^- h^{-1} g^{-1} FW (Sorgonà et al. 2011; Lupini et al. 2016a). Fifteen biological replicated were adopted for the NNUR assay.

Plasma membrane H+-ATPase

Isolation of plasma membrane vesicles

Plasma membrane vesicles were isolated from maize roots (7 days old) after exposure to 0 or 400 µM coumarin with or without 200 µM NO_3^- for 0, 8 and 24 h, using a small-scale procedure described in Giannini et al. (1988) and modified by Santi et al. (1995). Maize roots (about 2 g FW) were homogenised in extraction buffer (250 mM sucrose, 10% (v/v) glycerol, 10 mM glycerol-1-phosphate, 2 mM MgSO4, 2 mM EDTA, 2 mM EGTA, 2 mM ATP, 2 mM DTT, 5.7% (w/v) choline chloride, 25 mM BTP) buffered to pH 7.6 with MES, 1 mM PMSF and 20 mg ml^{-1} chymostatin freshly added before use. Then plant material was filtered and centrifuged twice at 12,700 g for 3 and 25 min at 4 °C. The suspension was layered over a 25/38% discontinuous sucrose gradient (10 mM DL-a-glycerol-1-phosphate, 2 mM $MgSO_4$, 2 mM EGTA, 2 mM ATP, 1 M PMSF, 2 mM DTT, 20 mg ml^{-1} chymostatin, 5.7% (w/v) choline chloride, 5 mM BTP buffered to pH 7.4 with MES) and centrifuged at 12,700 g for 60 min at 4 °C. The vesicles, banding at the 25/38% interface layers, were collected and centrifuged at 14,000 g for 45 min at 4 °C. The pellet was re-suspended in medium (20% glycerol (v/v), 2 mM EGTA, 2 mM EDTA,

0.5 mM ATP, 1 mM PMSF, 2 mM DTT, 20 mg $ml^{—1}$ chymostatin, 5.7% (w/v) choline chloride, 5 mM BTP buffered to pH 7 with MES), immediately frozen in liquid N2 and stored at —80 °C until use.

Plasma membrane H+-ATPase activity

The ATP-hydrolysing activity was determined by measuring the release of inorganic phosphate, as described previously (Forbush 1983). The assay medium (0.6 ml) contained 50 mM MES-BTP, pH 6.5, 5 mM $MgSO_4$, 5 mM ATP, 0.6 mM Na_2MoO_4, 100 mM KNO_3, 1.5 mM NaN_3, 0.01% (w/v) Brij58, with or without 100 mM vanadate (V_2O_5), an inhibitor of P-type H^+-ATPase (Sze 1985). Sodium azide (NaN_3, 1 mM) and potassium nitrate (KNO_3, 150 mM) were used as selective inhibitors of mitochondria and tonoplast H^+-ATPase, respectively (Santi et al. 1995). The reaction was started adding 0.5–1.5 g of membrane protein and stopped after 30 min using a solution containing 0.6 M HCl, 3% (w/v) SDS, 3% (w/v) ascorbic acid and 0.5% (w/v) ammonium molybdate at 2 °C. The PM H^+-ATPase activity was expressed as nmol Pi $\mu g^{—1}$ protein $h^{—1}$. Twelve independent experiments were repeated.

For the experiments on the *direct effect* of coumarin, plasma membrane vesicles, isolated as described above from roots of maize seedlings (7 days old), were exposed to 0 or 200 µM nitrate and treated with the allelochemical (from 0 to 400 lM) for 24 h. Fifteen independent experiments were repeated.

Total soluble protein was estimated according to Bradford (1976) using bovine serum albumin as standard.

Proton transport assay

The formation of a ΔpH gradient across membrane vesicles was monitored as absorbance changes at 492 nm of the pH probe acridine orange (A.O.) using a Perkin-Elmer UV-vis spectrophotometer (Lambda 35) in five independent experiments. The assay medium contained 50 mM BTP-MES (pH 6.5), 7.5 µM A.O, 100 mM KCl (or KNO_3) and 12 µg plasma membrane vesicle proteins from maize roots exposed to 0 or 400 µM coumarin for 24 h. The reaction was triggered by addition of 5 mM $MgSO_4$-ATP into the medium (1 ml) and carried out at 25 °C.

Proton extrusion assay

The proton extrusion rate (conversion of pH changes to H^+ fluxes) was calculated according to Glass et al. (1981). A total of 3 g (FW) maize roots (7 days old) were transferred into 80 ml of continuously aerated deionised water adjusted to pH 6.0 with KOH, containing 0 or 400 µM coumarin with or with- out 0.5 mM V_2O_5. The pH was monitored at 30-min intervals for 8 h. The proton extrusion rate was expressed as µmol H^+ $h^{—1}$ $g^{—1}$ FW, and the experiment was repeated ten times.

Western blot analysis

Vesicle membrane proteins of maize roots treated with 0 or 400 µM coumarin with or without 200 µM nitrate for 24 h were prepared and resolved on denaturing 8% SDS-PAGE as previously described in Santi et al. (1995). Resulting gels were stained with Coomassie brilliant blue or electro-transferred to PVDF membrane filters (Immobilon; Millipore, Bedford, MA, USA) overnight at 4 °C. Filters were blocked for 1 h in TBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% (w/v) skimmed milk powder and then incubated for 3 h with the antiserum (Santi et al. 2003). Afterwards, filters were blot- ted and incubated for 1 h with the secondary antibody (horse- radish peroxidase-conjugate antirabbit IgG; Sigma-Aldrich). The protein fragment size expected for PM H+-ATPase was detected by chemiluminescence (ECL Western Blotting Detection System; Amersham) on

autoradiography film (Kodak X-Omat AR). Polyclonal antibodies were previously raised against a synthetic peptide designed based on the sequence of the maize PM H^+-ATPase (Santi et al. 2003). One 18-mer was designed on the C-terminal domain of the *ZmHA3* and *ZmHA4* sequences, belonging to subfamily II: 273-AKRRAEIARL-RELHTLKG-290. The peptide was synthesised and conjugated to keyhole limpet haemocyanine by Sigma-Genosys (Sigma- Genosys, Cambridge, UK). The same manufacturer produced the antisera. Five independent replicates were conducted.

Gene expression analysis

RNA extraction

Total RNA from maize seedlings (7 days old) was extracted from 100 mg fresh tissue after 24 h of exposure to 0 or 400 µM coumarin with or without 200 µM NO3—. RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Milan, Italy), following the manufacturer's instructions. The RNA quality and quantification were assayed with a NanoDrop 2000 system (Thermo Scientific, Waltham, MA, USA).

Qualitative reverse transcript-PCR RT-PCR

Qualitative Reverse Transcript-PCR (RT-PCR) methods, starting from 100 ng total RNA in 25 µl reaction (Qiagen OneStep RT-PCR kit) for each treatment, were used to detect *ZmNPF6.3*, *ZmNRT2.1*, *ZmNAR2.1*, *ZmHA3* and *ZmHA4* genes. Specific primers for each gene were designed based on sequences available in the NCBI database. Ubiquitin (Bio-Fab research s.r.l., Rome, Italy) was used for housekeeping (Table S1). A set of different numbers of cycles was tested as reported in Lupini et al. (2016b). The PCR reactions were per- formed under the following conditions: 30 cycle of 30 s at 94 °C (denaturation), 45 s at 64 °C (annealing) and 60 s at 72 °C (extension); a 30 min reverse transcription at 55 °C; a 15 min hot start at 95 °C at the beginning of the reactions and, finally, a 10 min extension at 72 °C were performed. The PCR products were verified in agarose gel (1.5% w/v) using 123 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) as molecular standard. All PCR experiments were repeated five times to con- firm the repeatability of the banding patterns.

q-PCR

First-strand cDNA was synthesised from 1 µg total RNA after DNase treatment, using QuantiTect Reverse Transcription Kit (Qiagen), following the protocol provided by the manufacturer. Real-Time quantitative PCR (qPCR) was performed using the SYBR® Green RT-PCR master mix kit (Applied Biosystems, Branchburg, NJ, USA) adding 1 ml cDNA, 0.2 mM of the specific primer and 12.5 ml 2x SYBR Green PCR Master Mix, in 25 ml total volume, as suggested in the manufacturer's protocol. The conditions of amplifications were as previously described in Lupini et al. (2016b). According to the quantification method (Livak & Schmittgen 2001), specific primers were designed to amplify target fragments (Table S1). The qPCR results were analysed with the $2^{-\Delta CT}$ comparative method, as described in User Bulletin No 2 (Applied Biosystems) and reported in Livak & Schmittgen (2001). RT-PCR experiments were repeated five times to assess repeatability.

Statistical analysis

A completely randomised design was adopted for all experiments. All data were evaluated for normality and homogeneity with Kolmogorov–Smirnov and Levene median tests, respectively. Significance was determined with Student's t-test at $P < 0.05$, except the PM H^+-ATPase and gene expression where ANOVA was performed

and means were separated by Tukey's HSD test at $P < 0.05$. Statistical analyses were performed using Systat version 8.0 software (Systat, Chicago, IL, USA).

RESULTS

Net nitrate uptake rate

The NNUR (about 6.0 μmol NO_3^- g^{-1} FW h^{-1}) of maize seedlings, not previously exposed to nitrate, was not affected by coumarin at all concentrations tested (Table S2). Conversely, exposure of maize roots to 200 μM NO3— alone caused a progressive increase of NNUR, which reached, after 8 h of contact with the anion, peak of activity of 9.10 μmol NO_3^- g^{-1} FW h^{-1} (full induction), thereafter, a subsequent decline was observed (Fig. 1). In addition, concurrent exposure to 25 μM coumarin and NO_3^- did not modify the pattern described above (Table S2), whereas the increase of coumarin concentration, at 100 and 400 μM, caused a shift of the full induction from 8 to 24 h, compared to NO_3^- alone (Table S2, Fig. 1). In addition, 100 and 400 μM coumarin significantly increased the maximum NNUR by 21% and 34%, respectively, compared to the control at 8 h (Table S2, Fig. 1). Finally, similarly to NO_3^- alone, in all treatments, a decay phase was observed after 48 h of exposure (Table S2, Fig. 1).

Plasma membrane H^+-ATPase activity

The ATP hydrolytic activity was about 60 nmol Pi μg^{-1} h^{-1} in the control (CK) and this value was maintained throughout the experimental period. Exposure to 200 μM NO_3^- brought about an increase in ATP hydrolytic activity, although not statistically significant, which reached a maximum value already after 8 h (31%) and 24 h (33%) compared to the control (Fig. 2). Similarly, plants exposed to 400 μM coumarin alone increased ATP hydrolytic activity by 31 and 42% compared to the control after 8 and 24 h, respectively (Fig. 2). Furthermore, concurrent exposure to coumarin and NO_3^- caused a marked and significant increase in PM H^+-ATPase activity already after 8 h of contact, reaching a maximum peak in enzyme activity after 24 h (Fig. 2). In addition, the effect of coumarin-induced PM H^+-ATPase activity was also confirmed by measuring ATP- dependent H^+ accumulation in vesicles isolated from maize roots exposed to 0 or 400 μM for 24 h, which was more marked in coumarin-treated plants than the control (Fig. 3), thereby indicating a higher rate of proton transport in coumarin-treated roots. However, despite this effect on PM H+-ATPase activity, coumarin did not modify the pH optimum of the enzyme, which was around 6.5 in both treatments (Fig. 4).

To further investigate the coumarin effect on H^+-ATPase activity, its direct contact with vesicles isolated from maize roots exposed to 0 or 200 μM NO_3^- for 24 h was studied (Fig. 5). Coumarin at all concentrations (from 0 to 400 μM) did not affect ATP hydrolytic activity in plants grown with or without NO_3^-, although significantly high activity in vesicles isolated from roots treated with NO_3^- was observed (Fig. 5).

Proton extrusion rate

Control and coumarin-treated plants exhibited an increase in proton extrusion already detectable after 2 h of exposure, but no significant differences between the two were observed (Fig. 6). After 3 h of exposure, net proton extrusion rate in plants exposed to coumarin increased significantly compared to the control, and this trend continued up to 8 h. In contrast, the addition of vanadate, an inhibitor of PM H^+-ATPase, dramatically reduced the net proton extrusion rate, regardless the coumarin addition (Fig. 6).

Western blot analysis

To compare the amount of H^+-ATPase in PM vesicles, Western blotting analysis using a polyclonal antibody raised against maize PM H^+-ATPase was carried out. After 24 h, a significantly higher increase in PM H^+-ATPase was observed in the presence of 400 µM coumarin alone or in combination with nitrate (99% and 161%, respectively), compared to the control. Conversely, nitrate alone determined a lower increase (73%) in the PM H^+-ATPase level (protein), compared to the control (Fig. 7).

Gene expression

Quantitative real-time PCR analyses of components related to NO_3^- uptake (*ZmNPF6.3*, *ZmNRT2.1* and *ZmNAR2.1*) and PM H^+-ATPase activity (*ZmHA3* and *ZmHA4*) were carried out to assess the relative abundance of transcripts after 24 h of treatment (Fig. 8). Coumarin alone induced a four-fold increase in *ZmNPF6.3* transcript level compared to the control (Fig. 8A), which was not induced by 200 µM NO_3^- alone or in combination with coumarin (Fig. 8A). The concurrent addition of coumarin and NO_3^- significantly induced (~ three-fold) *ZmNRT2.1* transcript abundance compared to all other treatments (Fig. 8B). A similar trend was observed for *ZmNAR2.1* expression, although coumarin alone was able to significantly increase its transcript abundance (Fig. 8C). In addition, the expression pattern of two PM H^+-ATPase isoforms was also analysed. *ZmHA3* transcript level was significantly induced by NO_3^- alone or in combination with coumarin, but not in the control (Fig. 8D) and coumarin did not have any effect (Fig. 8D). In contrast, *ZmHA4* was induced by coumarin and NO_3^- (2.1- and 1.78-fold compared to the control, respectively), whereas their simultaneous presence reduced this up-regulation, with transcript level similar to the control (Fig. 8E).

DISCUSSION

The results of the present study confirmed that coumarin increased NNUR in maize roots induced with nitrate, as reported in wheat and alfalfa (Abenavoli et al. 2001; Zhou et al. 2013). In particular, the data indicated that 100 and 400 µM coumarin together with NO_3^- significantly stimulated the inducible component of the high affinity transport system (iHATS), leaving unchanged the constitutive form (cHATS). Hence, coumarin was not able to modify the nitrate uptake process regardless of nitrate, which is the inducer of its own influx together with many transporters belonging to NPF6 and NRT2 families involved on its uptake (Aslam et al. 1993). In addition, the results confirmed the high sensitivity of iHATS to allelochemicals, such as coumarin (Abenavoli et al. 2001) and trans-cinnamic acid derivatives, which in contrast inhibited nitrate transport activity (Bergmark et al. 1992; Abenavoli et al. 2010). Coumarin-treated plants exhibited a shift in the full induction, which is usually under feedback regulation of N concentration within the roots. As suggested in previous studies, this shift could be correlated with increased xylem vessel diameter (Svensson 1971; Kupidlowska et al. 1994), which increases NO_3^- translocation from roots to shoots (Abenavoli et al. 2001), maintaining lower cytoplasmic and vacuolar NO_3^- concentrations in roots. Furthermore, coumarin delayed the decay phase, maintaining a high NNUR compared to nitrate- induced plants, likely due to an insufficient increase in N plant concentration to elicit negative regulation of the transport system.

Coumarin alone after 24 h did not affect *ZmNRT2.1* transcript abundance, a gene strictly involved in iHATS activity (Zhuo et al. 1999; Okamoto et al. 2003), but significantly increased expression of the accessory protein *ZmNAR2*. These results highlight the role of *ZmNAR2.1* as a signal for induction of iHATS, as reported by Pii et al. (2016), who showed earlier induction of *ZmNAR2.1* (namely *ZmNRT3.1*) compared to *ZmNRT2.1* in maize roots exposed to nitrate.

However, this higher expression was not sufficient to increase the NNUR. Recently it has been demonstrated, in nitrate-fed plants, that higher expression of *NAR2.1* driven by a modified native promoter determined higher expression of NRT2.1, nitrogen use efficiency and grain yield in transgenic rice (Chen et al. 2017). Conversely, the simultaneous presence of NO_3^- and coumarin enhanced expression of both genes, *ZmNRT2.1* and *ZmNAR2.1*, leading to a significant increase in NNUR. This result confirmed the close relationship between *NRT2.1* and *NAR2.1* for iHATS activity, as proposed by Zhou et al. (2013), who demonstrated that only a *CrNRT2.1* and *CrNAR2.1* co-expression induced high-affinity nitrate uptake in *Xenopus* oocytes, revealing that higher expression of both *NRT2.1* and *NAR2.1* could have a functional interaction for nitrate acquisition. Later, evidence for the role of *NAR2.1* protein as an essential partner for iHATS were also provided for barley (Tong et al. 2005), *Arabidopsis* (Okamoto et al. 2006; Orsel et al. 2006; Yong et al. 2010), rice (Feng et al. 2011; Yan et al. 2011) and more recently along the maize root axis (Lupini et al. 2016a,b). The location of a 150-kDa *AtNAR2.1/AtNRT2.1* complex in PM of *Arabidopsis* roots seemed to con- firm its functionality for high-affinity nitrate influx (Yong et al. 2010). Recently, Pii et al. (2016) pointed out that iHATS regulation is mainly based on transcriptional/translational modulation of the accessory protein *ZmNRT3.1A*.

Coumarin alone up-regulated *ZmNPF6.3* (previously called *ZmNRT1.1*), which exhibited a higher expression compared to the NO_3^--induced plants with or without the allelochemical, highlighting the role of this gene as sensor of many nitrate- independent pathways (Glass et al. 2001; Hachiya et al. 2011; Wang et al. 2012). However, the results also showed a non-significant increase of *ZmNPF6.3* expression when plants were exposed simultaneously to coumarin and NO_3^-, confirming its role as a nitrate-dependent sensor (Ho et al. 2009; Wang et al. 2009) involved in root NO_3^- uptake (Tsay et al. 1993) and in lateral root growth (Krouk et al. 2010). In particular, *ZmNPF6.3* expression is strongly induced by auxin (Guo et al. 2002), being able to repress lateral root growth at low NO3— level, by affecting basipetal auxin transport in oat (Krouk et al. 2010). A functional interaction between coumarin and auxin polar transport in root growth regulation, y using auxin *Arabidopsis* mutants, was recently demonstrated (Lupini et al. 2014), although further studies are needed to reveal the interaction mechanism among coumarin, *ZmNPF6.3* expression and auxin.

The present paper confirmed the stimulatory effect of coumarin on PM H^+-ATPase activity, as already observed in maize primary roots (Lupini et al. 2010). Indeed, coumarin alone slightly increased PM H+-ATPase activity and this effect became significant when nitrate was added to the nutrient solution. The increased proton pump activity was also accompanied by a higher steady state of the enzyme in the presence of the allelochemical, suggesting that coumarin could elicit an up-regulating mechanism at transcriptional level. This hypothesis was supported by the increase of MHA3 transcript abundance with coumarin alone, and of both *ZmHA3* and *ZmHA4* in the presence of nitrate. The increase in enzyme activity is essential to support nitrate uptake, which requires a proton-motive force (McClure et al. 1990; Sondergaard et al. 2004) to overcome the negative electrical potential across the PM of root cells (Miller & Smith 1996; Miller et al. 2001). This close relationship has been reported at biochemical and molecular levels in maize seedlings (Santi et al. 1995, 2003; Sorgonà et al. 2011; Pii et al. 2016). Conversely, although not comparable with the present experiments, Podbiekowska et al. (1996) recorded a coumarin-induced inhibitory effect of H^+-ATPase activity in onion. Furthermore, negative effects on PM H^+-ATPase activity caused by several allelochemicals, such as trans-cinnamic acid and some derivatives (Abenavoli et al. 2010; Lupini et al. 2016a), sorgoleone, juglone and 2-benzoxazolinone (BOA) (Friebe et al. 1997; Hejl & Koster 2004; Romero-Romero et al. 2005; Sanchez-Moreiras & Reigosa 2005) were also reported.

In contrast, the positive effect of coumarin on the PM H^+- ATPase was also confirmed with the results on proton extrusion and pumping, which were considered activities dependent on this enzyme (Kotyk et al. 1991). Indeed, coumarin stimulated active proton extrusion from maize roots after 4 h of exposure, and this effect appeared to also be related to proton pump activity of PM H+-ATP. This coumarin role was already demonstrated in tomato roots, where membrane potential was significantly stimulated by the allelochemical, displaying fast membrane hyperpolarisation (more negative electrical potential, 18 mV versus 3 mV; Princi et al. 2016). The addition of

vanadate, a specific inhibitor of PM H^+-ATPase blocked the proton efflux with or without coumarin. The coumarin-stimulated H^+ extrusion caused immediate acidification of the external medium (low external pH values), which could be associated with the increase of NNUR and *ZmNPF6.3* expression, as previously reported by McClure et al. (1990) and Wang et al. (1998), respectively. However, when plasma membrane vesicles were directly exposed to coumarin, PM H^+-ATPase activity was not affected, suggesting an indirect effect of the allelochemical on the regulation of PM H^+-ATPase activity. Hence, the coumarin effect is probably mediated by a signalling component, such as 14-3-3 regulatory proteins (Palmgren 2001; Duby & Boutry 2009), as reported for fusicoccin (Jahn et al. 1997), or regulated at translational and post-translational level (Portillo 2000) by involving protein kinases. A post-translational regulation mechanism involving the auto-inhibitory action of the C-terminal domain (ca. 100 amino acids) of the enzyme protein and the role of phosphorylation has recently been reviewed (Haruta et al. 2015). The PM H+-ATPase is regulated by several endogenous and exogenous factors, such as hormones, calcium, blue light, fungal elicitors, small bioactive molecules and stress stimuli that regulate plant growth and development (Johansson et al. 1993; Niu et al. 1993; Frias et al. 1996; Kinoshita & Shimazaki 1999; Janicka-Russak & Kłobus 2007; Hayashi et al. 2014; Spartz et al. 2014).

Most interestingly, gene expression of two different isoforms of PM H^+-ATPase, *ZmHA3* and *ZmHA4*, members of PM H+- ATPase subfamily II, appeared differently regulated by coumarin alone or in combination with nitrate. This is not surprisingly because different H^+-ATPase genes have been identified in maize (Santi et al. 2003; Pii et al. 2016), and their role in nutrient uptake (Sondergaard et al. 2004) and in responses to abiotic stresses (e.g. Janicka-Russak & Kłobus 2007) has been widely clarified. In particular, the involvement of some members of the PM H+-ATPase gene family in the NO_3^- induction phenomenon has been reported (Santi et al. 2003; Sorgonà et al. 2011; Pii et al. 2014, 2016; Lupini et al. 2016b). The results show that coumarin with or without NO_3^- significantly increased *ZmHA4* transcript abundance; conversely, it stimulated the *ZmHA3* isoform only in the presence of the anion, highlighting the specific *ZmHA4* transcription under coumarin abiotic stress.

In conclusion, the present study confirmed that coumarin did not directly affect NNUR, but it modulated PM H^+-ATPase activity and some nitrate transporters. Hence, as the energy conditions are favourable in terms of PM H^+-ATPase activity, the active proton pumping induced by coumarin could become essential for nutrient uptake. Indeed, proton pumping deter- mined H^+ fluxes across PM and consequently nitrate movement when present in the nutrient solution. Furthermore, the effect of coumarin on proton pumping could be related to the ability of this compound to modify membrane potential. Change in the PM induced by coumarin, together with modulation at transcriptional level of *ZmNAR2.1:ZmNRT2.1* and *ZmHA3* expression, regulated the functionality of the iHATS system in the short term. Finally, the correlation between gene expression and protein level did not appear to be accompanied by enzyme activity, suggesting a post-translational mechanism of regulation activated only by nitrate supply.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Specific forward and reverse primer sequences (50- 30 oriented) used in semi-quantitative PCR expression analysis of the genes under investigation.

Table S2. Time course of nitrate uptake rate in maize root (7 days old) exposed to 0, 25, 100 and 400 μM coumarin with or without 200 μM nitrate.

REFERENCES

Abenavoli M.R., De Santis C., Sidari M., Sorgonà A., Badiani M., Cacco G. (2001) Influence of coumarin on the net nitrate uptake in durum wheat. New Phytologist, 150, 619–627.

Abenavoli M.R., Sorgonà A., Albano S., Cacco G. (2004) Coumarin differentially affects the morphology of different root types of maize seedlings. Journal Chemical Ecology, 30, 1871–1883.

Abenavoli M.R., Cacco G., Sorgonà A., Marabottini R., Paolacci A.R., Ciaffi M., Badiani M. (2006) The inhibitory effects of coumarin on the germination of durum wheat (Triticum turgidum ssp. durum, CV. Simeto) seeds. Journal Chemical Ecology, 32, 489– 506.

Abenavoli M.R., Nicolo` A., Lupini A., Oliva S., Sorgonà A. (2008) Effects of different allelochemicals on root morphology of *Arabidopsis thaliana*. Allelopathy Journal, 22, 245–252.

Abenavoli M.R., Lupini A., Oliva S., Sorgonà A. (2010) Allelochemical effects on net nitrate uptake and plasma membrane H+-ATPase activity in maize seedlings. Biologia Plantarum, 54, 149– 153.

Aliotta G., Cafiero G., Fiorentino A., Strumia S. (1993) Inhibition of radish germination and root growth by coumarin and phenylpropanoids. Journal of Chemical Ecology, 19, 175–183.

Aslam M., Travis R.L., Huffaker R.C. (1993) Comparative induction of nitrate and nitrite uptake and reduction systems by ambient nitrate and nitrite in intact roots of barley (*Hordeum vulgare* L.) seedlings. Plant Physiology, 102, 811–819.

Avers C.J., Goodwin R.H. (1956) Studies on root. IV. Effects of coumarin and scopoletin on the standard root growth pattern of *Phleum pratense*. American Journal of Botany, 43, 612–620.

Bergmark C.L., Jackson W.A., Volk R.J., Blum U. (1992) Differential inhibition by ferulic acid of nitrate and ammonium uptake on *Zea mays* L. Plant Physiology, 98, 639–645.

Bradford M.M. (1976) A rapid and sensitive method of quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry, 72, 248–254.

Chen J., Fan X., Qian K., Zhang Y., Song M., Liu Y., Xu G., Fan X. (2017) pOsNAR2.1:OsNAR2.1 expression enhances nitrogen uptake efficiency and grain yield in transgenic rice plants. Plant Biotechnology Journal, 15, 1273–1283.

Duby G., Boutry M. (2009) The plant plasma mem- brane proton pump ATPase: a highly regulated P- type ATPase with multiple physiological roles. European Journal of Physiology, 457, 645–655.

Feng H., Yan M., Fan X., Li B., Shen Q., Miller A.J., Xu G. (2011) Spatial expression and regulation of rice high-affinity nitrate transporters by nitrogen and carbon status. Journal Experimental Botany, 62, 2319–2332.

Forbush B. (1983) Assay of the Na^+ K^+-ATPase in plasma membrane preparations: increasing the permeability of membrane vesicles using sodium dodecyl sulfate buffered with bovine serum albumin. Analytical Biochemistry, 128, 159–163.

Forde B.G. (2000) Nitrate transporters in plants: structure, function and regulation. Biochimica et Biophysica Acta, 1465, 219–235.

Forde B.G., Clarkson D.T. (1999) Nitrate and ammonium nutrition of plants: physiological and molecular perspectives. Advances in Botanical Research, 30, 1–90.

Frias I., Caldeira M.T., Perez C.J.R., Navarro A.J.P., Culianez M.F.A., Kuppinger O., Stransky H., Pages M., Hager A., Serrano R. (1996) A major isoform of the maize plasma membrane H+-ATPase: characterization and induction by auxin in coleoptiles. The Plant Cell, 8, 1533–1544.

Friebe A., Roth U., Kuck P., Schnabl H., Schulz M. (1997) Effects of 2,4-dihydroxy-1,4-benzoxazin-3- ones on the activity of plasma membrane H^+- ATPase. Phytochemistry, 44, 979–983.

Giannini J.L., Ruiz-Cristin J., Briskin D.P. (1988) A small-scale procedure for the isolation of transport competent vesicles from plants tissues. Analytical Biochemistry, 174, 561–567.

Glass A.D.M. (2009) Nitrate uptake by plant roots. Botany-Botanique, 87, 659–667.

Glass A.D.M., Siddiqi M.Y. (1995) Nitrogen absorption in higher plants. In: Srivastava H. S., Singh R. P. (Eds), Nitrogen Nutrition in Higher Plants. Associated Publishing, New Delhi, India, pp 21–55.

Glass A.D.M., Siddiqi M.Y., Giles K.I. (1981) Correlations between potassium uptake and hydrogen efflux in barley varieties. A potential screening method for the isolation of nutrient efficient lines. Plant Physiology, 68, 457–459.

Glass A.D.M., Brito D.T., Kaiser B.N., Kronzucker H.J., Kumar A., Okamoto M., Rawat S.R., Siddiqi M.Y., Silim S.M., Vidmar J.J., Zhou D. (2001) Nitrogen transport in plants, with an emphasis on the regulation of fluxes to match plant demand. Journal of Plant Nutrition and Soil Science, 164, 199–207.

Glass A.D., Britto D.T., Kaiser B.N., Kinghorn J.R., Kronzucker H.J., Kumar A., Okamoto M., Rawat S., Siddiqi M.Y., Unkles S.E., Vidmar J.J. (2002) The regulation of nitrate and ammonium transport systems in plants. Journal Experimental Botany, 53, 855–864.

Goodwin R.H., Avers C.J. (1950) The effect of coumarin derivatives on growth of *Avena* root. American Journal of Botany, 37, 224–227.

Guo F.-Q., Wang R., Crawford N.M. (2002) The *Arabidopsis* dual-affinity nitrate transporter gene AtNRT1.1 (CHL1) is regulated by auxin in both shoots and roots. Journal Experimental Botany, 53, 835–844.

Hachiya T., Mizokami Y., Miyata K., Tholen D., Watanabe C.K., Noguchi K. (2011) Evidence for a nitrate-independent function of the nitrate sensor NRT1.1 in *Arabidopsis thaliana*. Journal of Plant Research, 124, 425–430.

Haruta M., Gray W.M., Sussman M.R. (2015) Regulation of the plasma membrane proton pump (H^+-ATPase) by phosphorylation. Current Opinion Plant Biology, 28, 68–75.

Hayashi Y., Takahashi K., Inoue S., Kinoshita T. (2014) Abscisic acid suppresses hypocotyl elongation by dephosphorylating plasma membrane H+-ATPase in *Arabidopsis thaliana*. Plant and Cell Physiology, 55, 845–853.

Hejl A.M., Koster K.L. (2004) The allelochemical sorgoleone inhibits root H^+-ATPase and water uptake. Journal of Chemical Ecology, 30, 2181–2191.

Ho C.H., Lin S.H., Hu H.C., Tsay Y.F. (2009) CHL1 functions as a nitrate sensor in plants. Cell, 138, 1184–1194.

Hoagland D.R., Arnon D.I. (1950) The water-culture method for plants without soil. California Agricultural Experiment Station Circular, 347, 32.

Jahn T., Fuglsang A.T., Olsson A., Bruntrup I.M., Collinge D.B., Volkmann D., Sommarin M., Palmgren M.G., Larsson C. (1997) The 14-3-3 protein interacts directly with the C-terminal region of the plant plasma membrane H^+-ATPase. The Plant Cell, 9, 1805–1814.

Janicka-Russak M., Kłobus G. (2007) Modification of plasma membrane and vacuolar H^+-ATPases in response to NaCl and ABA. Journal of Plant Physiology, 164, 295–302.

Johansson F., Sommarin M., Larsson C. (1993) Fusicoccin activates the plasma membrane H^+-ATPase by a mechanism involving the C-terminal inhibitory domain. The Plant Cell, 5, 321–327.

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

Kinoshita T., Shimazaki K.I. (1999) Blue light activates the plasma membrane H+-ATPase by phosphorylation of the C-terminus in stomatal guard cells. EMBO Journal, 18, 5548–5558.

Kotyk A., Fischer-Schliebs E., Lu€ttge U. (1991) Medium acidification by maize root tips and its inhibition by heavy water. Botanica Acta, 104, 433–438.

Krouk G., Lacombe B., Bielach A., Perrine-Walker F., Malinska K., Mounier E., Hoyerova K., Tillard P., Leon S., Ljung K., Zazimalova E., Benkova E., Nacry P., Gojon A. (2010) Nitrate-regulated auxin trans- port by NRT1.1 defines a mechanism for nutrient sensing in plants. Developmental Cell, 18, 927–937.

Kupidlowska E., Kowalec M., Sulkowski G., Zobel A.M. (1994) The effect of coumarin on root elongation and ultrastructure of meristematic cell protoplast. Annals of Botany, 73, 25–530.

Li W., Wang Y., Okamoto M., Crawford N.M., Siddiqi M.Y., Glass A.D.M. (2007) Dissection of the AtNRT2.1, AtNRT2.2 inducible high-affinity nitrate transporter gene cluster. Plant Physiology, 143, 425–433.

Livak K.J., Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods, 25, 402–408.

Lupini A., Sorgonà A., Miller A.J., Abenavoli M.R. (2010) Short-time effects of coumarin along the maize primary root axis. Plant Signaling and Behavior, 5, 1395–1400.

Lupini A., Araniti F., Sunseri F., Abenavoli M.R. (2014) Coumarin interacts with auxin polar trans- port to modify root system architecture in *Arabidopsis* thaliana. Plant Growth Regulation, 74, 23–31.

Lupini A., Sorgonà A., Princi M.P., Sunseri F., Abenavoli M.R. (2016a) Morphological and physiological effects of trans-cinnamic acid and its hydroxylated derivatives on maize root types. Plant Growth Regulation, 78, 263–273.

Lupini A., Mercati F., Araniti F., Miller A.J., Sunseri F., Abenavoli M.R. (2016b) NAR2.1/NRT2.1 functional interaction with NO3— and H+ fluxes in high-affinity nitrate transport in maize root regions. Plant Physiology and Biochemistry, 102, 107–114.

McClure P.R., Kochian L.V., Spanswick R.M., Shaff I.E. (1990) Evidence for cotransport of nitrate and protons in maize roots. Effect of nitrate io the membrane potential. Plant Physiology, 93, 281–289.

Miller A.J., Smith S.J. (1996) Nitrate transport and compartmentation in cereal root cells. Journal of Experimental Botany, 47, 843–854.

Miller A.J., Cookson S.J., Smith S.J., Wells D.M. (2001) The use of microelectrodes to investigate compart- mentation and the transport of metabolized inorganic ions in plants. Journal of Experimental Botany, 52, 541–549.

Miller A.J., Fan X., Orsel M., Smith S.J., Wells D.M. (2007) Nitrate transport and signalling. Journal Experimental Botany, 58, 2297–2306.

Moreland E.D., Novitzky W.P. (1987) Effects of acids, coumarins and flavonoids on isolated chloroplast and mitochondria. In: Waller G. R. (Ed), Allelochemicals: Role in Agriculture and Forestry. American Chemical Society, Washington, DC, USA, pp 247–261.

Neumann J. (1959) An auxin-like action of coumarin. Science, 129, 1675–1676.

Niu X., Narasimhan M.L., Salzman R.A., Bressan R.A., Hasegawa P.M. (1993) NaCl regulation of plasma membrane H^+-ATPase gene expression in a glycophyte and a halophyte. Plant Physiology, 103, 713–718.

Okamoto M., Vidmar J.J., Glass A.D.M. (2003) Regulation of NRT1 and NRT2 gene families of *Arabidopsis thaliana*: responses to nitrate provision. Plant and Cell Physiology, 44, 304–317.

Okamoto M., Kumar A., Li W., Wang Y., Siddiqi M.Y., Crawford N.M., Glass A.D.M. (2006) High-affinity nitrate transport in roots of *Arabidopsis* depends on expression of the NAR2-like gene AtNRT3.1. Plant Physiology, 140, 1036–1046.

Orsel M., Chopin F., Leleu O., Smith S.J., Krapp A., Daniel-Vedele F., Miller A.J. (2006) Characterization of a two-component high-affinity nitrate uptake sys- tem in *Arabidopsis*. Physiology and protein-protein interaction. Plant Physiology, 142, 1304–1317.

Palmgren M.G. (2001) Plant plasma membrane H+- ATPases: powerhouses for nutrient uptake. Annual Review of Plant Physiology and Plant Molecular Biology, 52, 817–845.

Pergo E.M., Abrahim D., Dasilva P.C.S., Kern K.A., Dasilva L.J., Voll E., IshiiIwamoto E.L. (2008) *Bidens pilosa* L. exhibits high sensitivity to coumarin in comparison with three other weed species. Journal of Chemical Ecology, 34, 499–507.

Pii Y., Alessandrini M., Guardini K., Zamboni A., Varanini Z. (2014) Induction of high-affinity NO_3^- uptake in grapevine roots is an active process correlated to the expression of specific members of the NRT2 and plasma membrane H^+-ATPase gene families. Functional Plant Biology, 41, 353–365.

Pii Y., Alessandrini M., Dall'Osto L., Guardini K., Princi B., Espen L., Zamboni A., Varanini Z. (2016) Time-resolved investigation of molecular compo- nents involved in the induction of NO_3^- high affinity transport system in maize root. Frontiers in Plant Science, 7, 1657.

Podbiekowska M., Waleza M., Dobrzynska K., Zobel A.M. (1996) Effect of two furanocoumarins and three other coumarins on ultrastructure, ATPases and acid phosphatases in meristematic cells of *Allium cepa* root tips. International Journal of Pharmacognosy, 34, 96–104.

Portillo F. (2000) Regulation of plasma membrane H^+-ATPase in fungi and plants. Biochimica et Biophysica Acta, 1469, 31–42.

Princi M.P., Lupini A., Longo C., Miller A.J., Sunseri F., Abenavoli M.R. (2016) Long- and short-term effect of boron excess to root form and function in two tomato genotypes. Plant Physiology and Biochemistry, 109, 9–19.

Rice E.L. (1984) Allelopathy. Academic Press, Orlando, FL, USA.

Romero-Romero T., Sanchez-Nieto S., Sanjuan Badillo A., Anaya A.L., Cruz-Ortega R. (2005) Comparative effects of allelochemical and water stress in roots of *Lycopersicon esculentum* Mill Plant (Solanaceae). Plant Science, 168, 1059–1066.

Saia S., Rappa V., Ruisi P., Abenavoli M.R., Sunseri F., Giambalvo D., Frenda A.S., Martinelli F. (2015) Soil inoculation with symbiotic microorganisms promotes plant growth and nutrient transporter gene expression in durum wheat. Frontiers in Plant Science, 6, 815.

Sanchez-Moreiras A.M., Reigosa M.J. (2005) Whole plant response of lettuce after root exposure to BOA (2(3H)-benzoxazolinone). Journal of Chemical Ecology, 31, 2689–2703.

Santi S., Locci G., Pinton R., Cesco S., Varanini Z. (1995) Plasma membrane H^+-ATPase in maize roots induced for NO_3^- uptake. Plant Physiology, 109, 1277–1283.

Santi S., Locci G., Monte R., Pinton R., Varanini Z. (2003) Induction of nitrate uptake in maize roots: expression of a putative high affinity nitrate transporter and plasma membrane H+- ATPase isoforms. Journal of Experimental Botany, 54, 1851–1864.

Sondergaard T.E., Schulz A., Palmgren M.G. (2004) Energization of transport processes in plants. Roles of the plasma membrane H^+-ATPase. Plant Physiology, 136, 2475–2482.

Sorgonà A., Lupini A., Mercati F., Di Dio L., Sunseri F., Abenavoli M.R. (2011) Nitrate uptake along the maize primary root: an integrated physiological and molecular approach. Plant, Cell and Environment, 34, 1127–1140.

Spartz A.K., Ren H., Park M.Y., Grandt K.N., Lee S.H., Murphy A.S., Sussman M.R., Overvoorde P.J., Gray W.M. (2014) SAUR inhibition of PP2C-D phosphatases activates plasma membrane H^+-ATPases to promote cell expansion in *Arabidopsis*. The Plant Cell, 26, 2129–2142.

Svensson S.B. (1971) The effect of coumarin on root growth and root histology. Physiologia Plantarum, 24, 446–470.

Sze H. (1985) H^+-translocating ATPases: advances using membrane vesicles. Annual Review of Plant Physiology, 36, 175–208.

Tong Y., Zhou J.J., Li Z., Miller A.J. (2005) A two- component high-affinity nitrate uptake system in barley. The Plant Journal, 41, 442–450.

Tsay Y.F., Schroeder J.I., Feldmann K.A., Crawford N.M. (1993) The herbicide sensitivity gene CHL1 of *Arabidopsis* encodes a nitrate-inducible nitrate trans- porter. Cell, 72, 705–713.

Wang R., Liu D., Crawford N.M. (1998) The *Arabidopsis* CHL1 protein plays a major role in high affinity nitrate uptake. Proceedings of the National Academy of Science USA, 95, 15134– 15139.

Wang R., Xing X., Wang Y., Tran A., Crawford N.M. (2009) A genetic screen for nitrate regulatory mutants captures the nitrate transporter gene NRT1.1. Plant Physiology, 151, 472–478.

Wang Y.-Y., Hsu P.-K., Tsay Y.-F. (2012) Uptake, allocation and signaling of nitrate. Trends in Plant Science, 17, 458–467.

Xu G., Fan X., Miller A.J. (2012) Plant nitrogen assimilation and use efficiency. Annual Review of Plant Biology, 63, 153–182.

Yan M., Fan X., Feng H., Miller A.J., Sheng Q., Xu G. (2011) Rice OsNAR2.1 interacts with OsNRT2.1, OsNRT2.2 and OsNRT2.3a nitrate transporters to provide uptake over high and low concentration ranges. Plant, Cell and Environment, 34, 1360– 1372.

Yong Z., Kotur Z., Glass A.D.M. (2010) Characterization of an intact two component high-affinity nitrate transporter from *Arabidopsis* roots. The Plant Journal, 63, 739–748.

Zhou X., Song H., Wang J. (2013) Effects of coumarin on net nitrate uptake and nitrogen metabolism in roots of alfalfa (*Medicago sativa*). Allelopathy Journal, 31, 377–386.

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

Zobel A.M., Brown S.A. (1995) Coumarins in the interactions between the plant and its environment. Allelopathy Journal, 2, 9–20.

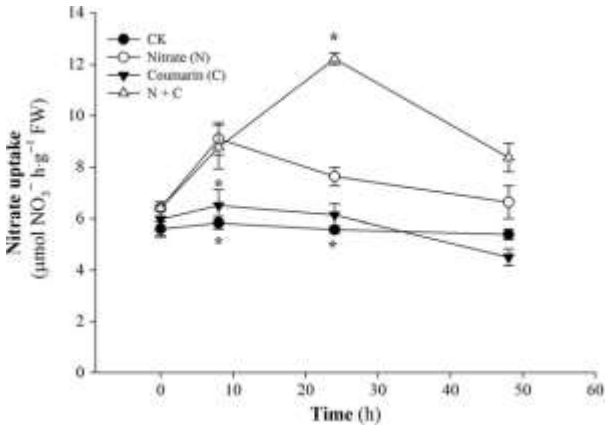

Fig. 1. Time course of the nitrate uptake rate in maize roots (7 days old) exposed to different coumarin and nitrate concentrations (CK, 0 µM NO_3^- + 0 µM coumarin; N, 200 µM NO_3^- + 0 µM coumarin; C, 0 µM NO_3^- + 400 µM coumarin; CN, 200 µM NO_3^- + 400 µM coumarin). The values are mean SE (n = 15). Asterisk indicates a significant difference compared to N (○) (Student's t-test at $P < 0.05$).

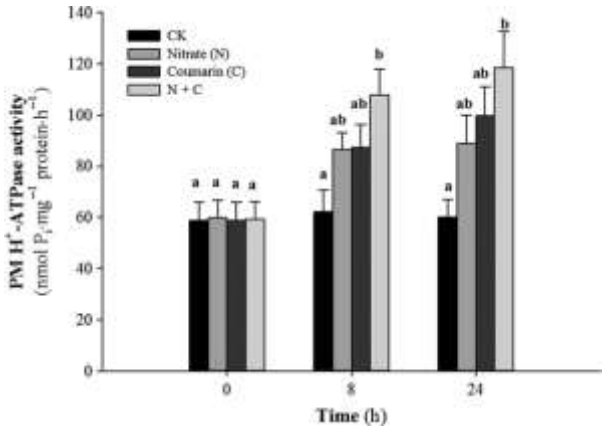

Fig. 2. Time course of PM H^+-ATPase activity (nmol Pi μg^{-1} protein h^{-1}) of plasma membrane vesicles isolated from maize roots (7 day old) exposed to different treatments (CK, 0 µM NO_3^- + 0 µM coumarin; N, 200 µM NO_3^- + 0 µM coumarin; C, 0 µM NO_3^- + 400 µM coumarin; CN, 200 µM NO_3^- + 400 µM coumarin). The values are mean SE (n = 12). Different letters within the time indicate means that differ significantly, according to Tukey's HSD test at $P < 0.05$.

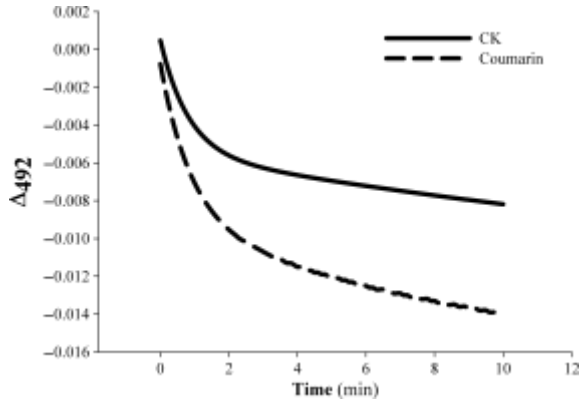

Fig. 3. Effect of coumarin on stimulation of the initial rate of ATP-dependent H^+ transport in plasma membrane vesicles isolated from maize roots exposed to 0 (CK) or 400 μM coumarin for 24 h. The figure is representative of five independent experiments.

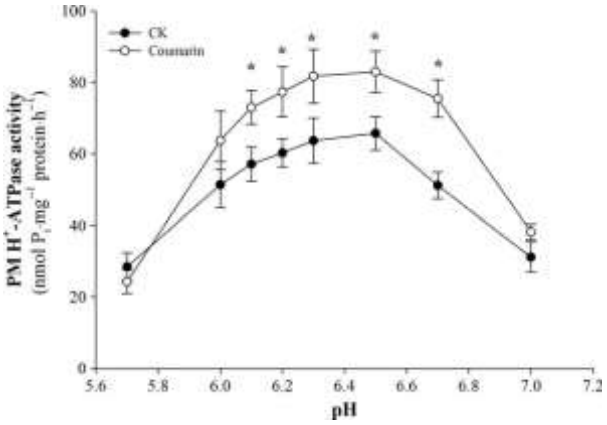

Fig. 4. Plasma membrane H^+-ATPase activity at different pH of plasma membrane vesicles isolated from maize roots exposed to 0 (CK) or 400 µM coumarin for 24 h. The values are mean SE (n = 15). Asterisk indicates a significant difference compared to CK (Student's t-test at $P < 0.05$).

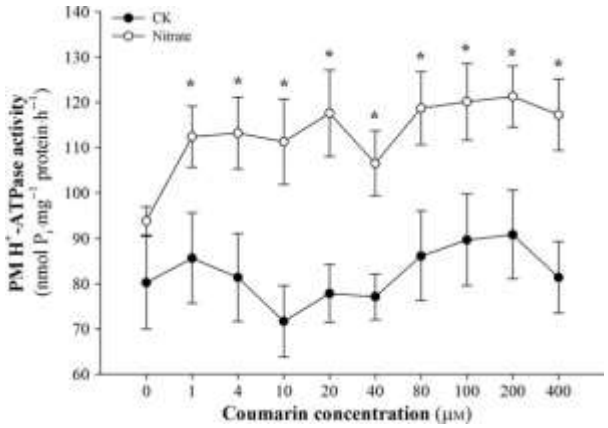

Fig. 5. Plasma membrane H^+-ATPase activity at different coumarin concentrations (from 0 to 400 μ) of plasma membrane vesicles isolated from maize roots exposed to 0 (CK) or 200 μM nitrate for 24 h. The values are mean SE (n = 15). Asterisk indicates a significant difference compared to CK (Student's t-test at $P < 0.05$).

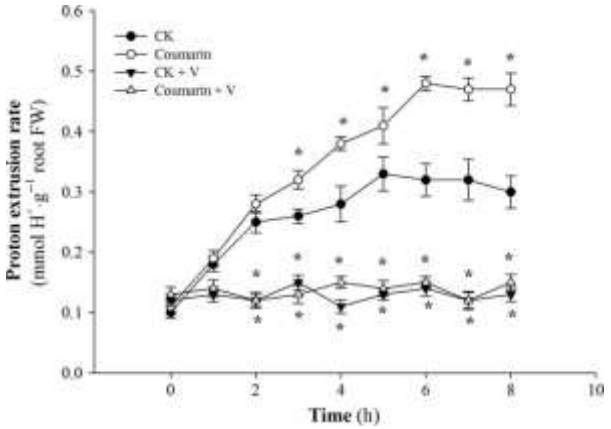

Fig. 6. Proton extrusion rate of maize root exposed to different coumarin and vanadate concentrations (CK, 0 μM coumarin + μ lM vanadate; Coumarin, 400 μM coumarin + 0 μM vanadate; CK+V, 0 μM coumarin + 0.5 mM vanadate; Coumarin+V, 400 μM coumarin + 0.5 mM vanadate) for 8 h. The values are mean SE (n = 10). Asterisks indicate a significant difference with respect to CK (Student's t-test at $P < 0.05$).

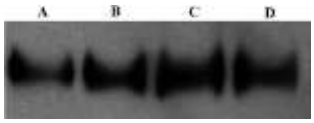

Fig. 7. Western blot analysis of plasma membrane vesicle protein in maize roots exposed to 0 µM nitrate (A), 200 µM nitrate (B), 200 µM nitrate + 400 µM coumarin (C) or 400 µM coumarin (D) for 24 h. The figure is representative of five independent replicates.

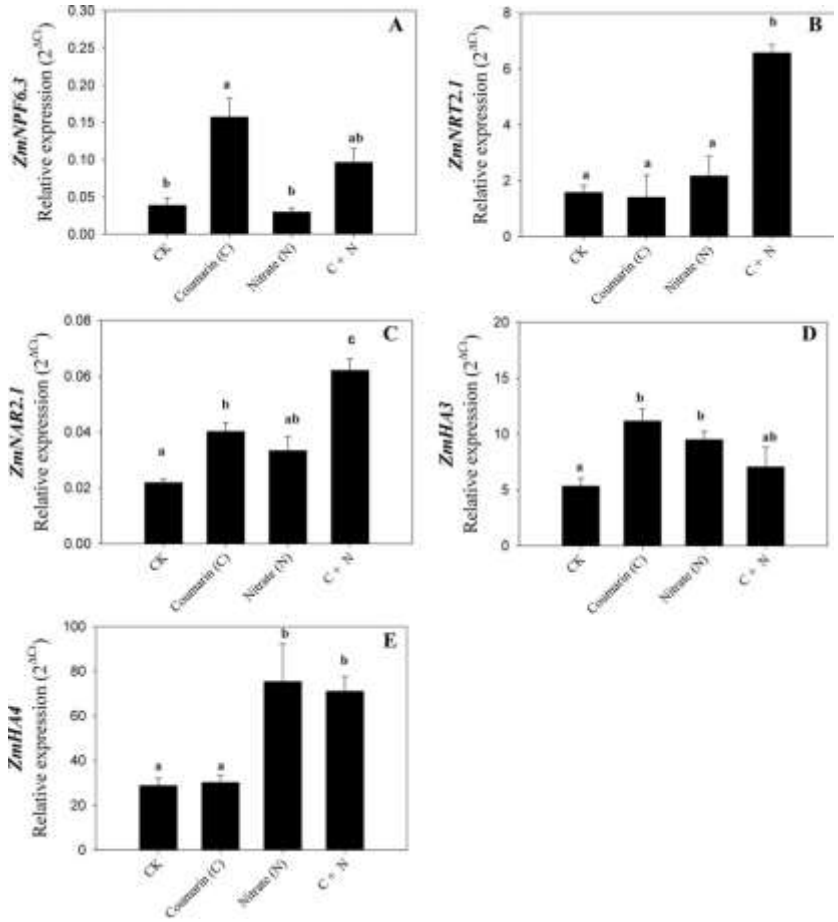

Fig. 8. Relative expression analysis of ZmNPF6.3 (A), ZmNRT2.1 (B), ZmNAR2.1 (C), ZmHA3 (D) and ZmHA4 (E) in maize roots exposed to 0 μM (CK), 400 μM coumarin (Coum), 200 μM nitrate (N) or Coum + N for 24 h. The values are mean SE (n = 5). Different letters indicate means that differ significantly, according to Tukey's HSD test at $P < 0.05$.

Table S1. Specific forward and reverse primer sequences (5'-3' oriented) used in semi-quantitative PCR expression analysis of the genes under investigation.

Gene	accession no.	Forward	Reverse
ZmNAR2.1	NM_001112459.1	CTCGCCTTCTTCTTCGTCAT	ATCAGCAACGACAGCCACT
ZmNrt1.1	AY187878.1	CCGCCTATGAAATCGTCCTA	GACCGTGTTGAGGTACGACCC
ZmNrt2.1	AY129953	ATCTTCGGGGTCATCCCCTTTGTCT	CAGCGTGCACGCCATGATCAT
ZmHA3	AJ441084.1	GAGAACAAGACCGCCTTCAC	AAGACGGGTACCCAACCATA
ZmHA4	AJ539534.1	GAGAACAAGACCGCCTTCAC	CTTGTTGTTCTTGCGACGAC
Ubiquitin	U29162	CCACTTGGTGCTGCGTCTTAG	CCTTCTGAATGTTGTAATCCGCA

Table S2. Time-course of the nitrate uptake rate in maize root (7 days old) exposed to 0, 25, 100 and 400 μM coumarin with and without 200 μM nitrate

		Net Nitrate Uptake Rate, NNUR (μmol NO_3^- h^{-1} FW)			
		***Time** (h)*			
Nitrate (μM)	***Coumarin (μM)***	***0***	***8***	***24***	***48***
0	0	5.60 (0.26)	5.83 (0.23)	5.58 (0.13)	5.39 (0.20)
0	25	5.56 (0.42)	6.27 (0.27)	5.53 (0.35)	5.19 (0.30)
0	100	6.24 (0.31)	6.20 (0.42)	5.53 (0.35)	5.19 (030)
0	400	5.96 (0.68)	6.50 (0.63)	6.13 (0.45)	4.51 (0.33)
200	0	6.40 (0.10)	9.10 (0.6)	7.64 (0.36)	6.63 (0.64)
200	25	6.31 (0.14)	9.97 (0.51)	8.62 (0.22)	7.57 (0.6)
200	100	6.01 (0.14)	8.58 (0.48)	10.99 (0.55)*	8.44 (0.55)
200	400	5.98 (0.13)	8.77 (0.84)	12.21 (0.22)*	8.37 (.055)

The value in the brackets indicates standard error (n = 15). The asterisk indicates significant level at $p < 0.05$ respect to 0 μM coumarin (gray rows) in both treatments (Student's test).