Plant Soil (2016) 398:301–312
DOI 10.1007/s11104-015-2675-2

REGULAR ARTICLE

Transcriptional response of plasma membrane H^+-ATPase genes to ammonium nutrition and its functional link to the release of biological nitrification inhibitors from sorghum roots

Houqing Zeng · Tingjun Di · Yiyong Zhu · Guntur Venkata Subbarao

Received: 17 July 2015 / Accepted: 10 September 2015 / Published online: 15 September 2015

Abstract
Aims Sorghum (*Sorghum bicolor*) roots release biological nitrification inhibitors (BNIs) to suppress soil nitrification. Presence of NH_4^+ in the rhizosphere stimulates BNIs release and it is hypothesized to be functionally associated with plasma membrane (PM) H^+-ATPase activity. However, whether the H^+-ATPase is regulated at the transcriptional level, and if so, which isoforms of the H^+-ATPases are involved in BNIs release are not known. Also, it is not clear whether the stimulation on BNIs release from roots is due to NH_4^+ uptake or its assimilation, which are addressed in this study.
Methods Root exudates from intact sorghum plants were collected using aerated solutions of NH_4^+ or methyl-ammonium (MeA); and the BNI-activity release was determined. PM vesicles were isolated from fresh roots using a two-phase partitioning system; and the hydrolytic H^+-ATPase activity was determined. All genes encoding PM H^+-ATPases were searched in sorghum genome, and their expression in response to NH_4^+ or MeA were analyzed by quantitative RT-PCR in sorghum roots.
Results BNIs release and PM H^+-ATPase activity increased with NH_4^+ concentration (≤1.0 mM) in the root-exudate collection solutions, but at higher concentrations, it did not respond further or declined in case of the PM H^+-ATPase activity. Twelve PM H^+-ATPase genes were identified in sorghum genome; and these isoforms were designated *SbA1* to *SbA12*. Five H^+-ATPase genes were stimulated by NH_4^+ in the rhizosphere, and have similar expression pattern, which is consistent with the variation in H^+-ATPase activity. MeA, a non-metabolizable analogue of NH_4^+, had no significant effects on BNIs release, H^+-ATPase activity, or expression of the H^+-ATPase genes.
Conclusions Our results suggest that the functional link between PM H^+-ATPase activity and BNIs release is evident only at NH_4^+ levels of ≤1.0 mM in the rhizosphere. The variation in PM H^+-ATPase activity by NH_4^+ is due to transcriptional regulation of five isoforms of the H^+-ATPases. The stimulatory effect of NH_4^+ on BNIs release is functionally associated with NH_4^+ assimilation and not just with NH_4^+ uptake alone.

Keywords Plasma membrane H^+-ATPase · Biological nitrification inhibition (BNI) · Biological nitrification inhibitors (BNIs) · Ammonium · Transcriptional regulation · Sorghum (*Sorghum bicolor*)

Responsible Editor: Ad C. Borstlap.

H. Zeng · T. Di · G. V. Subbarao (✉)
Crop, Livestock & Environment Division, JIRCAS, 1-1 Ohwashi, Tsukuba, Ibaraki 305-8686, Japan
e-mail: subbarao@jircas.affrc.go.jp

H. Zeng
College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, People's Republic of China

Y. Zhu
College of Resource and Environmental Sciences, Nanjing Agricultural University, Nanjing 210095, People's Republic of China

Introduction

Plasma membrane (PM) H^+-ATPase is the universal electrogenic H^+ pump, a functional monomer and has N- and C-terminal segments protruding into cytoplasm (Palmgren and Harper 1999; Palmgren 2001). The C-terminal region of the H^+-ATPase contains a penultimate Thr known as autoinhibitory domain to keep the H^+-ATPase in a low-activity state (Palmgren 2001). The phosphorylation of the penultimate Thr and subsequent binding of the 14-3-3 protein to the phosphorylated penultimate Thr results in the activation of the H^+-ATPase (Olsson et al. 1998). Plant PM H^+-ATPases are encoded by a multi-gene family. There are 12 members in Arabidopsis (*Arabidopsis thaliana*) (AHA1-AHA12) and 10 members in rice (*Oryza sativa*) (OSA1-OSA10) classified into five sub-families (Arango et al. 2003). PM H^+-ATPases are involved in generating H^+ electrochemical gradient to provide driving force for the active influx, efflux of ions and metabolites across the PM (Portillo 2000; Palmgren 2001; Arango et al. 2003; Duby and Boutry 2009).

Ammonium (NH_4^+) is oxidized to nitrite (NO_2^-) and subsequently to nitrate (NO_3^-), by nitrifying bacteria (*Nitrosomonas* and *Nitrobacter*, respectively) in the soil. This biological process termed nitrification, however, is one of the major causes of nitrogen loss and associated environmental pollution due to NO_3^- leaching and denitrification. If the nitrification process is repressed, NO_3^- leaching and N_2O emission are reduced, then, nitrogen recovery in agricultural system can be improved (Subbarao et al. 2015). The existence of plant-derived nitrification inhibitors are known (Moore and Waid 1971; Lata et al. 1999, 2004). The phenomenon of nitrification inhibitors (BNIs) produced and released from plant roots is termed "biological nitrification inhibition" (BNI) (Subbarao et al. 2006, 2015). Several BNIs have been isolated and characterized; e.g., brachialactone is isolated from root exudates of pasture grass *Brachiaria humidicola*; methyl 3-(4-hydroxyphenyl) propionate (MHPP), sakuranetin and sorgoleone are isolated from root exudates of sorghum (*Sorghum bicolor*) (Subbarao et al. 2006, 2009, 2012a, b, 2013; Zakir et al. 2008; Tesfamariam et al. 2014). Presence of NH_4^+ in the growth media or root exudate collection solution is reported to stimulate BNIs release from sorghum roots (Subbarao et al. 2007b, 2009; Zakir et al. 2008).

The stimulation of BNIs release by NH_4^+ is reported to be related to the PM H^+-ATPase activity in sorghum (Zhu et al. 2012). The PM H^+-ATPase activity was stimulated by NH_4^+ nutrition, and the BNIs release was repressed by pharmacological inhibitor, vanadate or stimulated by fusicossin, a known stimulant of PM H^+-ATPase (Zhu et al. 2012). The release of BNIs is a tightly regulated physiological process where an interplay among NH_4^+ uptake, rhizosphere pH, and PM H^+-ATPase regulate BNIs release in sorghum roots (Zhu et al. 2012). Whether the PM H^+-ATPase genes respond to NH_4^+ presence in the rhizosphere and which isoforms are involved in BNIs release is not known; but such information is needed to understand the role of NH_4^+ nutrition on BNIs release. In this study, we investigated the effect of NH_4^+ nutrition on expression of PM H^+-ATPase genes from sorghum genome. Also, it is not known whether NH_4^+ uptake alone or its assimilation is responsible for stimulating BNIs release; this is addressed by comparing the influence of methyl-ammonium (MeA) (a non-metabolizable analogue of NH_4^+) (Ermilova et al. 2007; Kosola and Bloom 1994) and NH_4^+ on BNIs release, PM H^+-ATPase activities and expression of PM H^+-ATPase genes in sorghum roots.

Materials and methods

Plant cultivation

Sorghum seeds (*Sorghum bicolor* L. Moench var. hybrid sorgo) were germinated in trays containing vermiculite. After 1 week, the seedlings were transferred to aerated nutrient solution of 70 L tanks covered by a styrofoam board with 4 plants grown in each of the 45 holes. The seedlings were grown in a growth chamber under controlled conditions (photoperiod 14-h-light/10-h-dark at 30/28 °C, light intensity 300 μmol m^{-2} s^{-1}). The composition of the nutrient solution is as follows: $(NH_4)_2SO_4$ 0.5 mM; KH_2PO_4 0.3 mM; K_2SO_4 0.2 mM; $CaCl_2$ 0.1 mM; $MgSO_4$ 0.15 mM; Fe-EDTA 0.2 mM; H_3BO_3 20 μM; $CuSO_4$ 0.3 μM; $MnSO_4$ 9.0 μM; Na_2MoO_4 0.5 μM; $ZnSO_4$ 0.8 μM. The nutrient solutions were replaced every 3 days. At the end of every day, the pH of the nutrient solution was adjusted to 5.0 using 1 N NaOH or H_2SO_4.

Root exudates collection

Two weeks after transplanting, the intact roots of sorghum seedlings (a sample size of 12 plants with three replications) were removed from the nutrient solution and rinsed

with distilled water, then immersed for 4 h (from 10:00 AM to 14:00 PM) in 1 L aerated root exudates collection solution (200 μM $CaCl_2$) containing NH_4Cl or methyl-ammonium (tetra methyl-ammonium chloride); using a pH-stat system (NPH-660 NDE, Nissin, Japan), the pH of the root exudate solution was maintained at 5.0 during the 4 h collection period, as described earlier (Zhu et al. 2012). For vanadate experiment, 0.5 mM vanadate was used in the root exudates collection solution. After collection of root exudates, roots were separated from shoots, dried at 70 °C for 2 days in a forced air-circulating oven before determining the dry weight. For PM isolation, fresh roots were rinsed with distilled water and then ground immediately. For RNA isolation and gene expression analysis, fresh roots were frozen in liquid nitrogen for 4 h and then stored at −80 °C.

Nitrification inhibition determination

The inhibition of nitrification was determined following the methods described previously (Subbarao et al. 2006; Zhu et al. 2012). For the extraction of biological nitrification inhibition (BNI) compounds, root exudates were evaporated to dryness using a rotary evaporator (Buchi, V-850, Flawil, Switzerland) under vacuum at 45 °C, followed by extraction with 20 mL of methanol. The methanol extract was then further evaporated to dryness using a rotary evaporator at 40 °C; and the residue was extracted with 50 μL of dimethyl sulphoxide (DMSO). The DMSO extract was then used to determine the BNI activity using a modified bioassay that employs recombinant luminescent *N. europaea* (Iizumi et al. 1998; Subbarao et al. 2006). The BNI activity of samples is expressed in units defined in terms of the action of a standard inhibitor, allylthiourea (AT); the inhibitory effect of 0.22 μM AT in an assay containing 18.9 mM of NH_4^+ is defined as one ATU (AT unit) of activity (Subbarao et al. 2006).

PM isolation and H^+ ATPase activity analysis

PM isolation and H^+-ATPase activity analysis were conducted using the methods as described previously (Yan et al. 2002; Zhu et al. 2012; Zeng et al. 2013).

Identification of PM H^+-ATPase genes in sorghum

To identify novel PM H^+-ATPase genes of sorghum in a genome-wide level, the amino acid sequence of Arabidopsis AHA2 (a well-characterized PM H^+-ATPase member) was used to blast against the sorghum genome database (version 2.1) (http://www.phytozome.

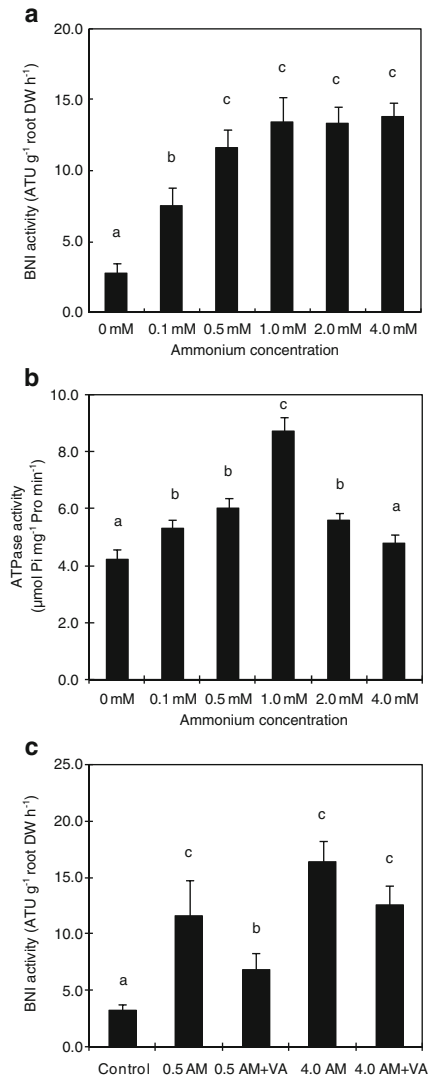

Fig. 1 Effects of ammonium (AM) on the BNI release and the PM H^+-ATPase activity. After growing in nutrient solution for 2 weeks, sorghum seedling roots were incubation with 1 L aerated root exudate collection solution containing NH_4Cl of different concentrations (0, 0.1, 0.5, 1.0, 2.0, 4.0 mM) for 4 h, and then root exudates were collected for BNI determination (**a**), and PM vesicles were isolated from fresh roots for analysis of the activity of PM H^+-ATPases (**b**). **c** To investigate the effect of vanadate (VA) on BNIs releases from sorghum roots, root exudates were collected after incubation for 4 h in 1 L aerated root exudate collection solution with 0.5 mM or 4.0 mM NH_4Cl, and with or without 0.5 mM VA. The data of the BNI activity and the PM H^+-ATPase activity at 0–1.0 mM NH_4^+ is adopted from Zhu et al. (2012). The *bars* represent means±SE of three replications. *Different letters* indicate that the values are significantly different at $P<0.05$ (based on one-way ANOVA)

Table 1 Characteristics of sorghum PM H^+-ATPase genes

Locus name (version 2.1)	Gene name	Chromosome	Gene length (bp)	Number of introns	Number of amino acid (aa)	Amino acid identity to AHA2 (%)
Sobic006g247100	*SbA1*	6	7024	13	951	83.9
Sobic008g190500	*SbA2*	8	7049	20	956	79.2
Sobic004g331500	*SbA3*	4	3705	1	951	79.2
Sobic010g207800	*SbA4*	10	4738	10	953	78.9
Sobic007g087700	*SbA5*	7	5269	10	953	77.5
Sobic001g132400	*SbA6*	1	9919	20	954	77.5
Sobic002g065000	*SbA7*	2	6193	20	956	77.4
Sobic001g519300	*SbA8*	1	4865	10	959	75.3
Sobic003g436400	*SbA9*	3	4367	11	952	74.9
Sobic001g543900	*SbA10*	1	8293	18	1004	71.8
Sobic001g480700	*SbA11*	1	5894	19	958	67.0
Sobic010g063700	*SbA12*	10	3184	2	874	78.0

net/search.php?org=Org_Sbicolor_v2.1). In addition, sorghum genes containing a functional annotation of KOG0205 (PM H^+-transporting ATPase) were also collected. The nucleotide and amino acid sequences, as well as other information of these putative PM H^+-ATPase genes were obtained.

Phylogenetic tree construction and sequence analysis

Protein sequences of PM H^+-ATPases of Arabidopsis, rice and sorghum were obtained from TAIR (http://www.arabidopsis.org/), NCBI (http://www.ncbi.nlm.nih.gov/) and Phytozome (http://www.phytozome.net/sorghum.php), respectively. The amino acid sequences of PM H^+-ATPase proteins were aligned using ClustalW (BLOSUM series was used for Protein Weight Matrix), and a phylogenetic tree was constructed by the neighbor-joining method using the software MEGA4.0 (Tamura et al. 2007). Gene structure feature was analyzed by Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/index.php).

RNA extraction and gene expression analysis

Total RNAs were extracted with the RNeasy Plant Mini Kit (QIAGEN) and digested with DNase I (QIAGEN) to eliminate DNA contamination, and then 1 μg RNA was used for reverse transcription in a 20 μL reaction system with PrimeScript RT reagent kit (TAKARA). Quantitative real-time RT-PCR (qRT-PCR) was performed with SYBR Premix EX Taq (TAKARA), and amplification was real-time monitored on a miniOpticon

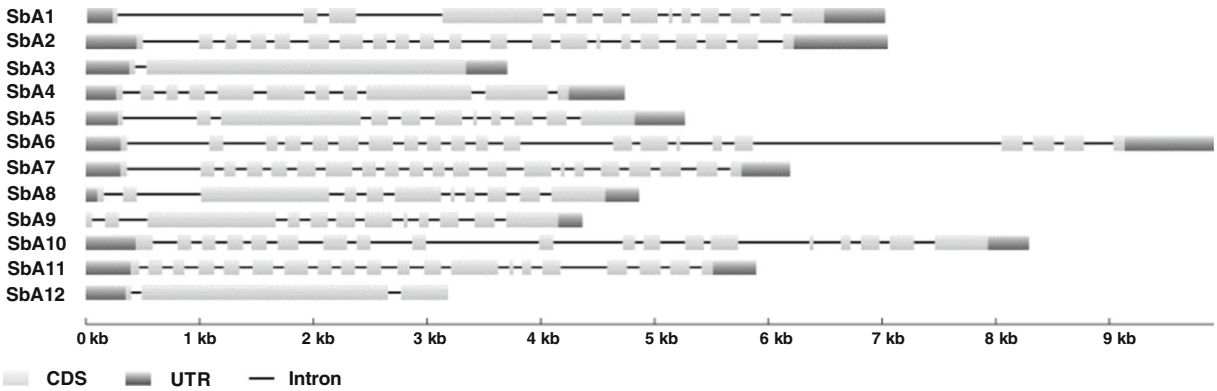

Fig. 2 Gene structures of 12 sorghum PM H^+-ATPase genes, *SbA1-12*. A *dark gray box* indicates 5′- and 3′- untranslated regions; a *light gray box* indicates exons; a *black line* indicates introns

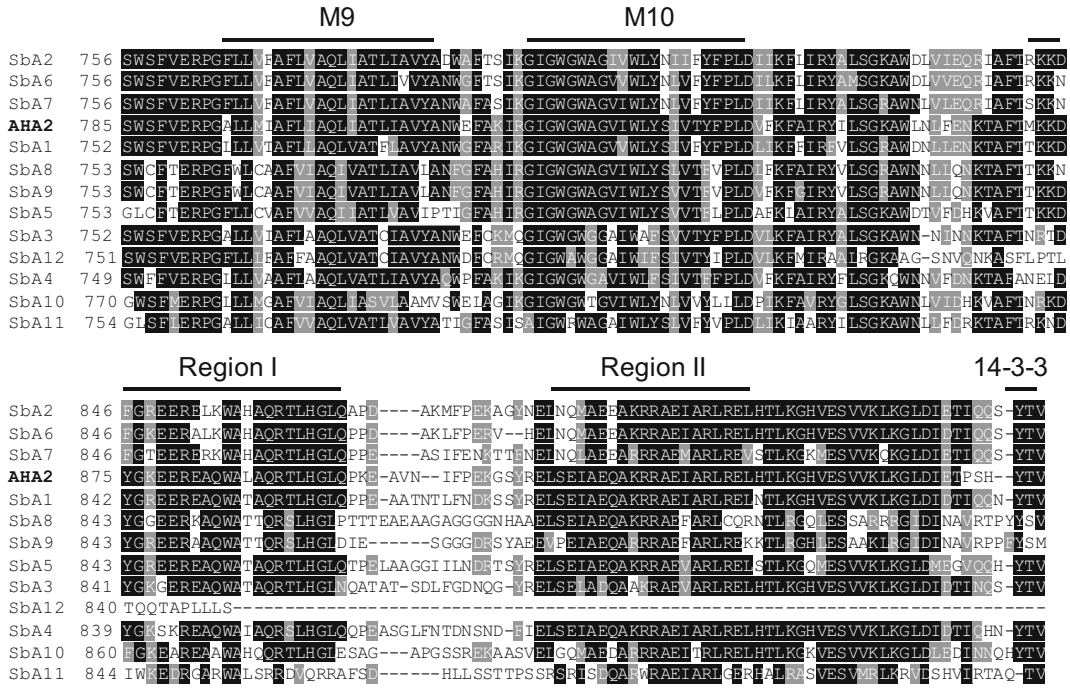

Fig. 3 Alignment of the C-terminal regions of AHA2 and 12 sorghum PM H^+-ATPases with ClustalW (http://www.genome.jp/tools/clustalw/) and BoxShade (http://www.ch.embnet.org/software/BOX_form.html). *Black blocks* indicate highly conserved residues. *Dashes* indicate gaps introduced to allow for optimal alignment of the sequences. The 9th and 10th transmembrane segments (M9 and M10), the region I, the region II and the 14-3-3 protein binding site within the C-terminal regions are indicated by *lines*. *SbA12* is a probable pseudogene which lacks C-terminal auto-inhibitory domain

real-time PCR system (Bio-Rad). Briefly, 2 μL of a 1/10 dilution of cDNA, 10 μL of the 2×SYBR Premix Ex Taq (TaKaRa), 1 μL each primer of 5, and 6 μL water was mixed in a final volume of 20 μL. The reaction was amplified for 30s at 95 °C, followed by for 5 s at 95 °C and 30 s at 60 °C for 40 cycles. All reactions were run in triplicate and included no template or reverse transcription controls for each gene. PCR efficiency was determined by a series of 2-fold dilutions of cDNAs, and the calculated efficiency of all primers varied from 0.8 to 1.0. The primers used for amplification of sorghum H^+-ATPase genes were as follows: 5′ CGCCTTATTGC GACGGA 3′ and 5′ CGCTTTACAACTAGGGCT GCT 3′ for *SbA1*; 5′ CCACATTCACCACCGAGC 3′ and 5′ CCCTTGCAGTTCCAGATTTATA 3′ for *SbA2*; 5′ TCAGGTTTCTTTTGGATTAGACA 3′ and 5′ AACTTACAAGGAGGGAGGAGG 3′ for *SbA3*;; 5′ TCACCACCAAGAAGGACTACG 3′ and 5′ GGTGCGGTCGTTGAGGAT 3′ for *SbA5*; 5′ GCCTCCGACCCTTCTTCT 3′ and 5′ GACGGTTTCGTTGGTGATG 3′ for *SbA10*; 5′ AGCAGCACAACACCATCTTC 3′ and 5′ CGCTTCAGCCTCATCACA 3′ for *SbA11*. Gene relative expression levels were normalized to one *ACTIN* gene (*Sobic005g047100*) and presented as $2^{-\Delta\Delta CT}$ to simply the presentation of data. The specific primers for *ACTIN* were 5′CATCCCCA CTTCGGCTCC 3′ and 5′ CCTCAATAGG CTGGCAATCTC 3′.

Results

Presence of NH_4^+ in the root exudate solution on BNIs release and PM H^+-ATPase activity in sorghum roots

The release of BNI activity from sorghum roots increased by the presence of NH_4^+ in the concentration range of 0 to 1.0 mM, but no further increase was observed at concentration >1.0 mM (1.0 to 4.0 mM) (Fig. 1a). Root PM H^+-ATPase activity also increased in the concentration range of 0 to 1.0 mM NH_4^+ in the root exudate solutions, but it declined subsequently at concentration >1.0 (1.0 to 4.0 mM) (Fig. 1b). Vanadate is a known inhibitor of PM H^+-ATPases. After incubation with 0.5 mM vanadate, BNI release from intact sorghum plants was significantly

suppressed at 0.5 mM and 4.0 mM NH_4^+ (Fig. 1c). These results suggest that PM H^+-ATPase is functionally involved in the stimulation of BNIs release by NH_4^+ in the concentration range of ≤1.0 mM.

Genome-wide identification of sorghum PM H^+-ATPase genes

To investigate whether the transcriptional levels of the H^+-ATPase genes are affected by NH_4^+, we first identified novel PM H^+-ATPase genes by blasting against sorghum genome database using the amino acid sequences of Arabidopsis AHA2 as query. A total of 12 homologous genes encoding putative PM H^+-ATPases were found in sorghum genome, and they showed high sequence identity with AHA2, arranged between 67 and 84 %. These genes were sequentially named SbA1-SbA12, based on their amino acid identity to AHA2 (Table 1). Similar to their counterparts in other plants, sorghum H^+-ATPase are relatively big proteins with lengths ranging from 874 to 1004 aa. The coding region of sorghum H^+-ATPase genes is interrupted by 1–20 introns (Table 1, Fig. 2), which is similar to that in Arabidopsis and rice (Arango et al. 2003). With the exception of SbA12, all the other 11 isoforms possess a penultimate Thr or Ser, and conserved region I and region II in the C-terminal regions (Fig. 3). Because of the lack of C-terminal auto-inhibitory domain, SbA12 was considered to be a probable pseudogene. Region I and region II in the C-terminal region have been identified to be important for the auto-inhibitory effects on the H^+-ATPase (Palmgren et al. 1991; Axelsen et al. 1999). The phosphorylation of penultimate Thr or Ser is required for 14-3-3 protein binding, which results in the activation of H^+-ATPases (Olsson et al. 1998; Maudoux et al. 2000; Kinoshita and Shimazaki 2002). Phylogenetic analysis using full-length amino acid sequences indicated that sorghum PM H^+-ATPase genes are localized in each of the five subfamilies (Fig. 4), similar to PM H^+-ATPase genes in Arabidopsis and rice (Arango et al. 2003).

Expression of PM H^+-ATPase genes in response to NH_4^+ in sorghum roots

Expression levels of sorghum H^+-ATPase genes in response to NH_4^+ were analyzed by qRT-PCR using specific primers. At least six of these 12 H^+-ATPase genes were successfully detected in sorghum roots, but no expression was detected for the other eight isoforms, which could be expressed in specific tissue or under specific conditions. Among the six genes, *SbA3* was only marginally affected by NH_4^+, whereas the other

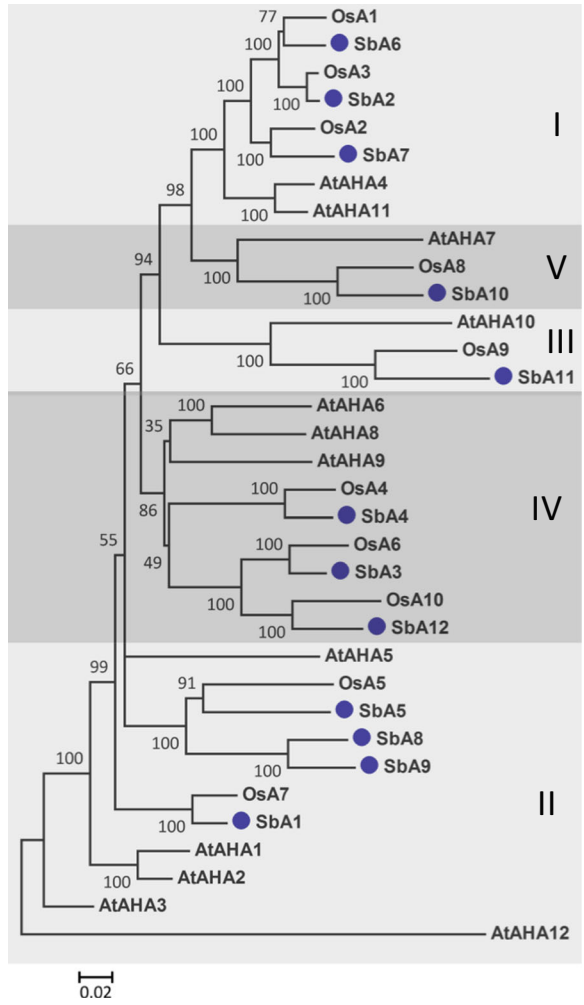

Fig. 4 Phylogenetic tree of PM H^+-ATPase proteins from Arabidopsis, rice and sorghum. The full-length amino acid sequences of the H^+-ATPase proteins were aligned using ClustalW, and a phylogenetic tree was constructed by the neighbor-joining method using the software MEGA4.0. Sorghum PM H^+-ATPase proteins are marked with a *blue circle* before the protein names. The *bar* indicates the relative divergence of the sequences examined and bootstrap values are displayed next to the branch. Roman numerals designate the subfamilies. The locus names of SbA1-SbA12 are listed in Table 1. The AGI (Arabidopsis Genome Initiative) number of AtAHA1-AtAHA12 is At2g18960, At4g30190, At5g57350, At3g47950, At2g24520, At2g07560, At3g60330, At3g42640, At1g80660, At1g17260, At5g62670, and At4g11730, respectively. The NCBI gene accession numbers of OsA1-OsA10 are AJ439999, AJ440000, AJ440001, AJ440002, AJ440216, AJ440217, AJ440218, AJ440219, AJ440220, and AJ440221, respectively. *AHA12*, *OsA10* and *SbA12* are probable pseudogenes which lack C-terminal auto-inhibitory domains (Axelsen and Palmgren 2001; Arango et al. 2003)

five genes (*SbA1*, *SbA2*, *SbA5*, *SbA10* and *SbA11*) were found to respond to NH_4^+ nutrition and their expression patterns were similar (Fig. 5). The expression of the five H^+-ATPase genes was increased by NH_4^+, and the highest expression occurred at concentration of 0.5 mM or 1.0 mM. But their expression levels were decreased by high NH_4^+ concentration (4.0 mM). The expression patterns of H^+-ATPase genes were largely consistent with the response of PM H^+-ATPase activities (Fig. 1b), although post-translational regulation could not be excluded.

Influence of methyl ammonium (MeA) on BNIs release and PM H^+-ATPase activity in sorghum roots

To investigate whether the effect of NH_4^+ nutrition on PM H^+-ATPase activity of sorghum roots is related to NH_4^+ uptake or assimilation, we applied MeA, a non-metabolizable analogue of NH_4^+ to the root exudate collection solution (Kosola and Bloom 1994; Ermilova et al. 2007), and examined the influence of MeA on BNIs release and PM H^+-ATPase activity in sorghum roots. No significant change was observed for either of these processes following MeA treatment (Fig. 6a, b). But the H^+-ATPase activity decreased slightly at 4.0 mM of MeA, which may be caused by its toxicity at higher concentration. Consistent with the H^+-ATPase activity, the expression of most PM H^+-ATPase genes were not activated by MeA treatment (Fig. 6b). But the expression of some *SbA5* and *SbA10* were repressed by MeA at higher concentration and *SbA11 was marginally* increased by MeA. Thus, unlike NH_4^+, MeA has little or no stimulatory effect on PM H^+-ATPase activity and BNIs release.

Discussion

Root exudates from sorghum plants were reported to inhibit nitrification (Alsaadawi et al. 1986), and their direct suppressive effect on *Nitrosomonas bacteria* has been validated recently (Subbarao et al. 2007a, 2013, 2015; Zakir et al. 2008; Tesfamariam et al. 2014). The presence of NH_4^+ in the rhizosphere has been shown to stimulate BNIs release in sorghum roots (Subbarao et al. 2007b, 2009, 2012a). It is hypothesized that PM H^+-ATPase is functionally linked to BNIs release and is stimulated by the presence of NH_4^+ (≤1.0 mM in the rhizosphere; the concentration range where high affinity AMT1-type ammonium-transporters operate in PM) (Yuan et al. 2007). At concentrations of >1.0 mM, an apparent disconnect was observed between the BNIs release and the PM H^+-ATPase activity, indicating alternative BNIs release mechanisms associated with mass-flow of NH_4^+, which is open to future research.

Vanadate, a known inhibitor of PM H^+-ATPase (O'Neill and Spanswick 1984), suppressed the release of BNIs in the presence of NH_4^+ in rhizosphere (Fig. 1c), providing further evidence to the functional link of PM H^+-ATPase to BNIs release. Such functional link between PM H^+-ATPase and BNI release was

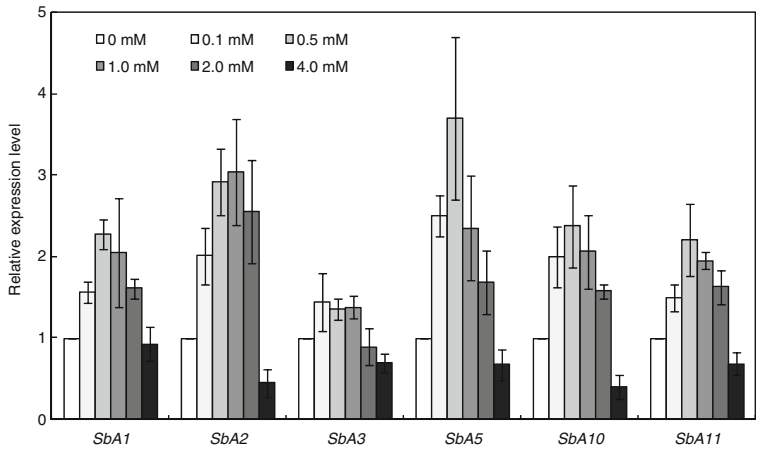

Fig. 5 Expression of six sorghum PM H^+-ATPase genes in response to NH_4^+ nutrition. After growing in nutrient solution for 2 weeks, sorghum seedling roots were incubated with 1 L aerated root exudate collection solution containing NH_4Cl of different concentrations (0, 0.1, 0.5, 1.0, 2.0, 4.0 mM) for 4 h, and then total RNA was isolated from roots for expression analysis. Relative expression level of each individual PM H^+-ATPase gene was normalized to that of *SbACTIN* (*Sobic005g047100*). The relative expression levels were normalized to 1 in control (without ammonium). The bars represent means±SE of three replicates

however disrupted at >2.0 mM NH_4^+ concentration, suggesting that other BNIs release mechanisms that do not require PM H^+-ATPase activity may be operational. The BNI compounds are suggested to be anionic substances (Subbarao et al. 2007b). It is thus assumed that BNIs release mechanisms may be akin to mechanisms associated with the release of other organic anions, such as citrate and malate. For example, the aluminum-activated root citrate exudation is mediated by a PM-localized citrate transporter belonging to the multi-drug and toxic compound extrusion (MATE) family (Furukawa et al. 2007); and this physiological process is linked to the activity of PM H^+-ATPase (Shen et al. 2005). During the release of organic compounds, the PM H^+-ATPase activity seem crucial for providing the driving force and maintaining the charge balance in plant cells.

In the present study, we identified 12 PM H^+-ATPase genes from sorghum genome (Table 1, Fig. 1); and most of the encoded H^+-ATPases possess a conserved C-terminal region, with the exception of SbA12 (Fig. 3). Arabidopsis AHA12 and rice OsA10 also lack such C-terminal regions, and they are considered to be probable pseudogenes (Axelsen and Palmgren 2001; Arango et al. 2003). All the 12 sorghum H^+-ATPases show high identity to AHA2, which is a well-characterized PM H^+-ATPase member in Arabidopsis (Palmgren 2001); and they are closely related to the H^+-ATPases from Arabidopsis and rice based on the phylogenetic analysis (Fig. 4), suggesting their functions are similar in plant species.

NH_4^+ nutrition acidifies the rhizosphere in higher plants (Schubert and Yan 1997; Zhu et al. 2009, 2012); the induction of PM H^+-ATPase activity by NH_4^+ nutrition could partly be due to acidification of rhizosphere (Yan et al. 1998; Zhu et al. 2009). In the present study, the pH of root exudate collection solutions (containing varying levels of NH_4^+) were kept at 5.0 using pH-stat system, thereby removing the secondary effects of rhizosphere-acidification from ammonium uptake and assimilation on BNI release. The stimulatory effect from NH_4^+ on BNI release thus seems the direct effect from its assimilation and not from the secondary effects (i.e., rhizosphere-acidification) associated with its uptake.

Consistent with the activation of the PM H^+-ATPase activity in sorghum roots, at least five PM H^+-ATPase genes responded to moderate concentration of NH_4^+ similarly in expression pattern (Figs. 1b and 5). Thus, variation in the H^+-ATPase activity with NH_4^+ nutrition is at least partly due to transcriptional regulation of H^+-ATPase genes. The H^+-ATPase activity was also activated by NH_4^+ nutrition in the roots of barley and rice (Yamashita et al. 1995; Zhu et al. 2009; Zeng et al. 2012). Recently, it was shown that the H^+-ATPase activity is stimulated by NH_4^+ treatment in potassium (K)-

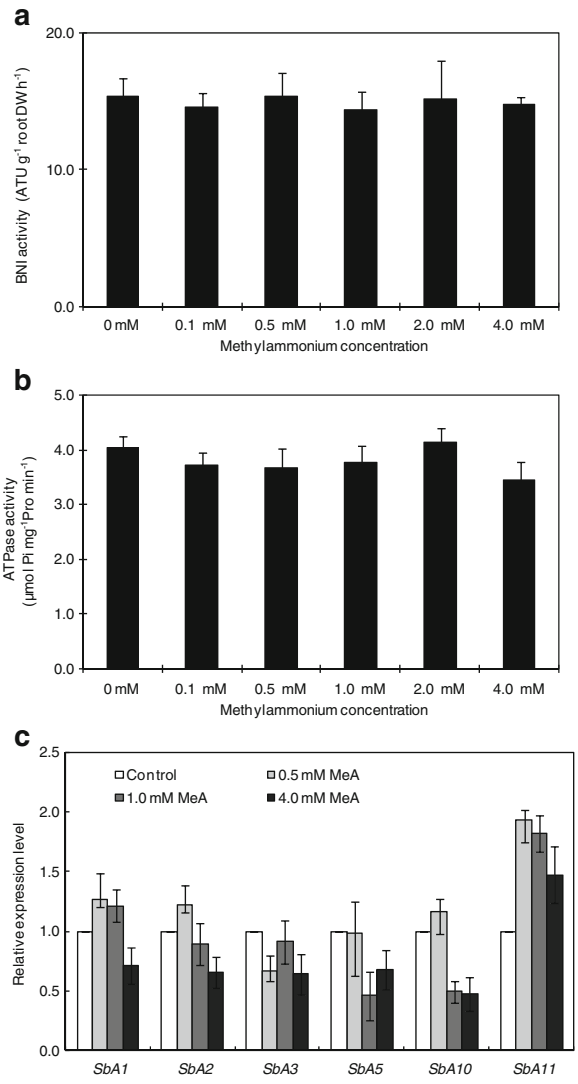

Fig. 6 Effect of methyl-ammonium (MeA) on the BNIs release, the H^+-ATPase activity and the expression of the H^+-ATPase genes in sorghum roots. After growing in nutrient solution for 2 weeks, sorghum seedling roots were incubated with 1 L aerated root exudate collection solution containing MeA of different concentrations (0, 0.5, 1.0, 4.0 mM) for 4 h, root exudates were collected for BNI determination (**a**), and PM vesicles were isolated from fresh roots for analysis of the activity of H^+-ATPases (**b**), and then total RNA was isolated from roots for expression analysis (**c**). Relative expression level of each individual PM H^+-ATPase gene was normalized to that of *SbACTIN* (*Sobic005g047100*). The relative expression levels were normalized to 1 in control (without MeA). The *bars* represent means±SE of three replicates

deficient sorghum roots, where two H^+-ATPase genes *SbA1* and *SbA2* were induced by NH_4^+ compared to NO_3^- (Alvarez-Pizarro et al. 2014). In the present study, we have extended the members of NH_4^+-responsive H^+-ATPase genes of sorghum to five (*SbA1*, *SbA2*, *SbA5*, *SbA10* and *SbA11*) (Fig. 5). In addition to transcriptional regulation, the rapid activation of the H^+-ATPase activity in sorghum roots byNH_4^+ nutrition could be related to post-transcriptional regulation, such as phosphorylation of penultimate Thr and subsequent binding of 14-3-3 regulatory protein. Most sorghum isoforms of H^+-ATPase possess penultimate Thr or Ser in the C-terminal region (Fig. 3). The phosphorylation of penultimate Thr or Ser enables the binding of 14-3-3 protein, which is needed for the activation of PM H^+-ATPase in plants (Olsson et al. 1998; Maudoux et al. 2000; Kinoshita and Shimazaki 2002). The H^+-ATPase activity can be modulated at transcriptional- and post-translational level under aluminum stress in soybean roots (Shen et al. 2005). Further research is thus needed to determine whether H^+-ATPase activity is also modulated at the post-translational level when sorghum roots are fed with NH_4^+.

Methyl ammonium (MeA) is a non-metabolizable analogue to NH_4^+ (Kosola and Bloom 1994; Ermilova et al. 2007) and is taken up by plant roots through NH_4^+ transporters (Kosola and Bloom 1994; Ninnemann et al. 1994). However, unlike in NH_4^+ treatment, there was no significant variation in PM H^+-ATPase activity and in BNIs release following MeA treatment (Fig. 6a). In addition, a lack of transcriptional response for the NH_4^+-responsive H^+-ATPase genes under MeA (Fig. 6b) suggests that variation in PM H^+-ATPase and BNIs release from NH_4^+ nutrition is not just due to NH_4^+ uptake, but from NH_4^+ assimilation (Fig. 7).

Although the uptake of NH_4^+ or MeA can lead to depolarization of cell membrane potential because of positive inward currents across PM (Walker et al. 1979; Ullrich et al. 1984), the membrane potential change or the depolarization of cell membrane potential caused by NH_4^+ or MeA uptake is not sufficient to activate PM H^+-ATPase activity. It has been suggested that the uptake and assimilation of NH_4^+ are closely synchronized in plant roots (Ishiyama et al. 2003; Loqué and von Wirén 2004); and the assimilation of NH_4^+ is a proton-generating process (Van Beusichem et al. 1988; Xu et al. 2012). In order to pump additional H^+ out of cytoplasm and maintain the electrochemical potential gradient necessary for ion uptake, the H^+-ATPase activity is thus stimulated to further BNIs release. With the results presented in this study, we propose a hypothesis that NH_4^+ assimilation and not just uptake alone sustains H^+ supply for the continued functioning of PM-H^+-pumps,

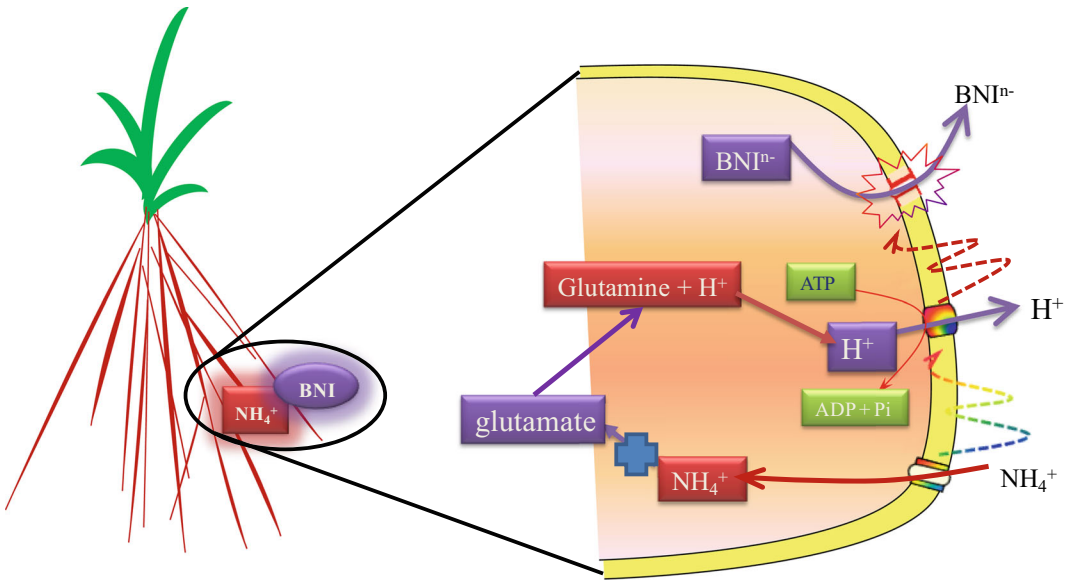

Fig. 7 A hypothesis involving schematic description of ammonium (NH_4^+)-induced BNIs release in relation to the PM H^+-ATPase in sorghum roots. After NH_4^+ is taken up by high affinity ammonium transporters located on PM of the cell (in the concentration range of ≤1.0 mM), NH_4^+ is incorporated into glutamate to produce glutamine and other amino acids, which facilitates the generation of H^+ in cytoplasm; to pump these additional H^+ out of the cell, the PM H^+-ATPase activity is activated and this facilitates the release of anionic BNIs possibly through anionic channels on the cell PM

which is critical for BNIs release; and that is probably mediated through anion-channels when rhizosphere NH_4^+ concentration is ≤1.0 mM (Fig. 7).

Significance

Physiological understanding of the mechanisms governing BNIs release has implications to practical agriculture, as this helps in determining suitable niches in agro-ecological regions where BNI expression is likely favored. For example, light-soils that have low-buffering capacity such as Alfisols of the SAT (Semi-arid Tropics, where sorghum is predominantly grown as a rainy-season crop) India or sandy-loams of West Africa, where sorghum is grown as a major staple crop, seem better suited to develop acidic-rhizosphere (pH <6.0) compared to Vertisols, which are of heavy-clay type and have an alkaline pH of >7.5 (Subbarao et al. 2013). Alfisols with a natural soil pH of <6.5, are better suited for the expression of BNI function in sorghum as NH_4^+ uptake and assimilation (results from the present study) further reduces the rhizosphere pH in the acidic range (i.e., pH <6.0), critical to sustain operations of PM H^+-pumps for BNIs release (Zhu et al. 2012; Subbarao et al. 2013). In contrast, Vertisols, where sorghum is grown as a post-rainy season crop in parts of Asia may not allow BNI expression due to resistance to changes in rhizosphere pH; thus NH_4^+ uptake and assimilation will not result in rhizosphere pH reaching <6.0, a level that is conducive for BNIs release in sorghum (Zhu et al. 2012; Subbarao et al. 2013, 2015). Knowledge of the underlying physiological mechanisms governing BNIs release thus helps in choosing suitable agro-ecological niche production systems where BNI function is expressed to its genetic potential for controlling nitrification.

The results from the present study improve our understanding of the physiological processes operating in sorghum roots for the BNIs release, thus a step forward to deploy sorghum BNI function in practical agriculture for the benefit of small-holder farmers in dry areas of tropical regions. In addition, the use of slow-release fertilizers can facilitate keeping soil ammonium levels <1.0 mM, this coupled with the development of genetically modified crops with high PM H^+-ATPase activity can accelerate BNI release to make production systems low-nitrifying that is beneficial to the environment.

Acknowledgments The research presented here is supported through JIRCAS invitation fellowship program to co-authors (Drs. Houqing Zeng, Tingjun Di and Prof. Yiyong Zhu), and is funded by grant-in-Aid for scientific research from Ministry of Agriculture, Forestry and Fisheries of Japan (MAFF) to JIRCAS under BNI project. Funding support also came from Natural Science Foundation of China (NSFC 31172035) and Program of New Century Excellent Talent in Universities (NCET-11-0672).

References

Alsaadawi I, Al-Uqaili J, Alrubeaa A, Al-Hadithy S (1986) Allelopathic suppression of weed and nitrification by selected cultivars of *Sorghum bicolor* (L.) moench. J Chem Ecol 12: 209–219

Alvarez-Pizarro JC, Gomes-Filho E, Prisco JT, Grossi-De-Sá MF, De Oliveira-Neto OB, Da Rocha Fragoso R (2014) Plasma membrane H^+-ATPase in sorghum roots as affected by potassium deficiency and nitrogen sources. Biol Plant 58:507–514

Arango M, Gevaudant F, Oufattole M, Boutry M (2003) The plasma membrane proton pump ATPase: the significance of gene subfamilies. Planta 216:355–365

Axelsen KB, Palmgren MG (2001) Inventory of the superfamily of P-type ion pumps in *Arabidopsis*. Plant Physiol 126:696–706

Axelsen K, Venema K, Jahn T, Baunsgaard L, Palmgren M (1999) Molecular dissection of the C-terminal regulatory domain of the plant plasma membrane H^+-ATPase AHA2: mapping of residues that when altered give rise to an activated enzyme. Biochemistry 38:7227–7234

Duby G, Boutry M (2009) The plant plasma membrane proton pump ATPase: a highly regulated P-type ATPase with multiple physiological roles. Pflugers Arch Eur J Physiol 457: 645–655

Ermilova EV, Nikitin MM, Fernández E (2007) Chemotaxis to ammonium/methylammonium in *Chlamydomonas reinhardtii*: the role of transport systems for ammonium/methylammonium. Planta 226:1323–1332

Furukawa J, Yamaji N, Wang H, Mitani N, Murata Y, Sato K, Katsuhara M, Takeda K, Ma JF (2007) An aluminum-activated citrate transporter in barley. Plant Cell Physiol 48: 1081–1091

Iizumi T, Mizumoto M, Nakamura K (1998) A bioluminescence assay using *Nitrosomonas europaea* for rapid and sensitive detection of nitrification inhibitors. Appl Environ Microbiol 64:3656–3662

Ishiyama K, Kojima S, Takahashi H, Hayakawa T, Yamaya T (2003) Cell type distinct accumulations of mRNA and protein for NADH-dependent glutamate synthase in rice roots in response to the supply of $NH4^+$. Plant Physiol Biochem 41: 643–647

Kinoshita T, Shimazaki K (2002) Biochemical evidence for the requirement of 14-3-3 protein binding in activation of the guard-cell plasma membrane H^+-ATPase by blue light. Plant Cell Physiol 43:1359–1365

Kosola KR, Bloom AJ (1994) Methylammonium as a transport analog for ammonium in tomato (*Lycopersicon esculentum* L.). Plant Physiol 105:435–442

Lata JC, Durand J, Lensi R, Abbadie L (1999) Stable coexistence of contrasted nitrification statuses in a wet tropical savanna ecosystem. Funct Ecol 13:762–768

Lata JC, Degrange V, Raynaud X, Maron PA, Lensi R, Abbadie L (2004) Grass populations control nitrification in savanna soils. Funct Ecol 18:605–611

Loqué D, von Wirén N (2004) Regulatory levels for the transport of ammonium in plant roots. J Exp Bot 55:1293–1305

Maudoux O, Batoko H, Oecking C, Gevaert K, Vandekerckhove J, Boutry M, Morsomme P (2000) A plant plasma membrane H^+-ATPase expressed in yeast is activated by phosphorylation at its penultimate residue and binding of 14-3-3 regulatory proteins in the absence of fusicoccin. J Biol Chem 275: 17762–17770

Moore DRE, Waid JS (1971) The influence of washings of living roots on nitrification. Soil Biol Biochem 3:69–83

Ninnemann O, Jauniaux JC, Frommer WB (1994) Identification of a high affinity $NH4^+$ transporter from plants. EMBO J 13: 3464–3471

Olsson A, Svennelid F, Ek B, Sommarin M, Larsson C (1998) A phosphothreonine residue at the C-terminal end of the plasma membrane H^+-ATPase is protected by fusicoccin-induced 14-3-3 binding. Plant Physiol 118:551–555

O'Neill SD, Spanswick RM (1984) Effects of vanadate on the plasma membrane ATPase of red beet and corn. Plant Physiol 75:586–591

Palmgren MG (2001) Plant plasma membrane H^+-ATPases: powerhouses for nutrient uptake. Annu Rev Plant Biol 52:817–845

Palmgren MG, Harper JF (1999) Pumping with plant P-type ATPases. J Exp Bot 50:883–893

Palmgren M, Sommarin M, Serrano R, Larsson C (1991) Identification of an autoinhibitory domain in the C-terminal region of the plant plasma membrane H^+-ATPase. J Biol Chem 266:20470–20475

Portillo F (2000) Regulation of plasma membrane H^+-ATPase in fungi and plants. Biochim Biophys Acta 1469:31–42

Schubert S, Yan F (1997) Nitrate and ammonium nutrition of plants: effects on acid/base balance and adaptation of root cell plasmalemma H^+-ATPase. Z Panzenphysiol Bodenkd 160:275–281

Shen H, He LF, Sasaki T, Yamamoto Y, Zheng SJ, Ligaba A, Yan XL, Ahn SJ, Yamaguchi M, Sasakawa H (2005) Citrate secretion coupled with the modulation of soybean root tip under aluminum stress. Up-regulation of transcription, translation, and threonine-oriented phosphorylation of plasma membrane H^+-ATPase. Plant Physiol 138:287–296

Subbarao GV, Ishikawa T, Ito O, Nakahara K, Wang HY, Berry WL (2006) A bioluminescence assay to detect nitrification inhibitors released from plant roots: a case study with *Brachiaria humidicola*. Plant Soil 288:101–112

Subbarao GV, Rondon M, Ito O, Ishikawa T, Rao IM, Nakahara K, Lascano C, Berry WL (2007a) Biological nitrification inhibition (BNI)—is it a widespread phenomenon? Plant Soil 294:5–18

Subbarao GV, Wang HY, Ito O, Nakahara K, Berry WL (2007b) NH_4^+ triggers the synthesis and release of biological nitrification inhibition compounds in *Brachiaria humidicola* roots. Plant Soil 290:245–257

Subbarao GV, Nakahara K, Hurtado MP, Ono H, Moreta DE, Salcedo AF, Yoshihashi AT, Ishikawa T, Ishitani M, Ohnishi-Kameyama M, Yoshida M, Rondon M, Rao IM, Lascano CE, Berry WL, Ito O (2009) Evidence for biological nitrification inhibition in *Brachiaria pastures*. Proc Natl Acad Sci U S A 106:17302–17307

Subbarao GV, Sahrawat K, Nakahara K, Ishikawa T, Kishii M, Rao I, Hash C, George T, Srinivasa Rao P, Nardi P (2012a) Biological nitrification inhibition—a novel strategy to regulate nitrification in agricultural systems. Adv Agron 114:249

Subbarao GV, Sahrawat K, Nakahara K, Rao I, Ishitani M, Hash C, Kishii M, Bonnett D, Berry W, Lata J (2012b) A paradigm shift towards low-nitrifying production systems: the role of biological nitrification inhibition (BNI). Ann Bot 112:297–316

Subbarao GV, Nakahara K, Ishikawa T, Ono H, Yoshida M, Yoshihashi T, Zhu Y, Zakir H, Deshpande S, Hash C (2013) Biological nitrification inhibition (BNI) activity in sorghum and its characterization. Plant Soil 366:243–259

Subbarao GV, Yoshihashi T, Worthington M, Nakahara K, Ando Y, Sahrawat KL, Rao IM, Lata J, Kishii M, Braun H (2015) Suppression of soil nitrification by plants. Plant Sci 233:155–164

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tesfamariam T, Yoshinaga H, Deshpande SP, Srinivasa Rao P, Sahrawat KL, Ando Y, Nakahara K, Hash CT, Subbarao GV (2014) Biological nitrification inhibition in sorghum: the role of sorgoleone production. Plant Soil 379:325–335

Ullrich WR, Larsson M, Larsson CM, Lesch S, Novacky A (1984) Ammonium uptake in *Lemna gibba* G 1, related membrane potential changes, and inhibition of anion uptake. Physiol Plant 61:369–376

Van Beusichem ML, Kirkby EA, Baas R (1988) Influence of nitrate and ammonium nutrition on the uptake, assimilation, and distribution of nutrients in *Ricinus communis*. Plant Physiol 86:914–921

Walker N, Beilby M, Smith F (1979) Amine uniport at the plasmalemma of charophyte cells: I. Current–voltage curves, saturation kinetics, and effects of unstirred layers. J Membr Biol 49:21–55

Xu GH, Fan XR, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63:153–182

Yamashita K, Kasai M, Ezaki B, Shibasaka M, Yamamoto Y, Matsumoto H, Sasakawa H (1995) Stimulation of H^+ extrusion and plasma membrane H^+-ATPase activity of barley roots by ammonium-treatment. Soil Sci Plant Nutr 41:133–140

Yan F, Feuerle R, Schaffer S, Fortmeier H, Schubert S (1998) Adaptation of active proton pumping and plasmalemma H^+-ATPase activity of corn roots to low root medium pH. Plant Physiol 117:311–319

Yan F, Zhu Y, Muller C, Zorb C, Schubert S (2002) Adaptation of H^+-pumping and plasma membrane H^+ ATPase activity in proteoid roots of white lupin under phosphate deficiency. Plant Physiol 129:50–63

Yuan L, Loque D, Kojima S, Rauch S, Ishiyama K, Inoue E, Takahashi H, von Wiren N (2007) The organization of high-affinity ammonium uptake in *Arabidopsis* roots depends on the spatial arrangement and biochemical properties of AMT1-type transporters. Plant Cell 19:2636–2652

Zakir HAKM, Subbarao GV, Pearse SJ, Gopalakrishnan S, Ito O, Ishikawa T, Kawano N, Nakahara K, Yoshihashi T, Ono H,

Yoshida M (2008) Detection, isolation and characterization of a root-exuded compound, methyl 3-(4-hydroxyphenyl) propionate, responsible for biological nitrification inhibition by sorghum (*Sorghum bicolor*). New Phytol 180:442–451

Zeng HQ, Liu G, Kinoshita T, Zhang RP, Zhu YY, Shen QR, Xu GH (2012) Stimulation of phosphorus uptake by ammonium nutrition involves plasma membrane H^+ ATPase in rice roots. Plant Soil 357:205–214

Zeng H, Feng X, Wang B, Zhu Y, Shen Q, Xu G (2013) Citrate exudation induced by aluminum is independent of plasma membrane H^+-ATPase activity and coupled with potassium efflux from cluster roots of phosphorus-deficient white lupin. Plant Soil 366:389–400

Zhu YY, Di TJ, Xu GH, Chen X, Zeng HQ, Yan F, Shen QR (2009) Adaptation of plasma membrane H^+-ATPase of rice roots to low pH as related to ammonium nutrition. Plant Cell Environ 32:1428–1440

Zhu YY, Zeng HQ, Shen QR, Ishikawa T, Subbarao GV (2012) Interplay among NH_4^+ uptake, rhizosphere pH and plasma membrane H^+-ATPase determine the release of BNIs in sorghum roots—possible mechanisms and underlying hypothesis. Plant Soil 358:131–141