Plasma membrane H^+-ATPase in sorghum roots as affected by potassium deficiency and nitrogen sources

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Abstract

We studied the influence of inorganic nitrogen sources (NO_3^- or NH_4^+) and potassium deficiency on expression and activity of plasma membrane (PM) H^+-ATPase in sorghum roots. After 15 d of cultivation at 0.2 mM K^+, the plants were transferred to solutions lacking K^+ for 2 d. Then, K^+ depletion assays were performed in the presence or absence of vanadate. Further, PMs from K^+-starved roots were extracted and used for the kinetic characterization of ATP hydrolytic activity and the immunodetection of PM H^+-ATPase. Two major genes coding PM H^+-ATPase (*SBA1* and *SBA2*) were analyzed by real-time PCR. PM H^+-ATPase exhibited a higher V_{max} and K_m in NH_4^+-fed roots compared with NO_3^--fed roots. The optimum pH of the enzyme was slightly lower in NO_3^--fed roots than in NH_4^+-fed roots. The vanadate sensitivity was similar. The expressions of *SBA1* and *SBA2* increased in roots grown under NH_4^+. Concomitantly, an increased content of the enzyme in PM was observed. The initial rate of K^+ uptake did not differ between plants grown with NO_3^- or NH_4^+, but it was significantly reduced by vanadate in NH_4^+-grown plants.

Additional key words: ammonium, immunodetection, K^+ uptake, nitrate, *Sorghum bicolor*, vanadate.

Introduction

In many plant species, potassium acquisition by the roots is impaired by a high concentration of ammonium in the root medium, but not by nitrate (Marschner 1995). Reduction in the K^+ influx rate is attributed to an NH_4^+-induced inhibition of the expression and activity of high-affinity K^+ carriers belonging to the KUP/HAK/KT family (Santa-María *et al*. 2000, Martínez-Cordero *et al*. 2005). In an NH_4^+-rich environment, K^+ uptake occurs through K^+ channels of the AKT family which are insensitive to NH_4^+. However, the ability of these channels to completely deplete external K^+ is lower than that exhibited by H^+-coupled K^+ carriers (Hirsch *et al*. 1998, Spalding *et al*. 1999, Rubio *et al*. 2008). Therefore, the plasma membrane (PM) H^+-ATPase is a key component involved in the adaptation to K^+ deficiency in the presence of external NH_4^+ since more negative membrane potentials developed by an increased activity of this enzyme may drive passive K^+ uptake through K^+ channels.

PM H^+-ATPases are encoded by members of a large gene family having at least 10 genes (Palmgren 2001, Arango *et al.* 2003). Some of them are required for ion homeostasis and are highly expressed in all plant tissues, whereas others have more specialized functions and display restricted expression patterns (Gaxiola *et al*. 2007). Accordingly, the question arises whether an increase in root PM H^+-ATPase activity in response to nutrient deficiency can be attributed to an enhanced transcription of housekeeping genes constitutively expressed even under normal conditions, or it should be attributed to nutritional stress-induced genes. In *Arabidopsis thaliana* roots, *AHA2* and *AHA7* are the

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Abbreviations: K_i - inhibition constant; K_m - Michaelis-Menten constant; PM - plasma membrane; V_{max} - maximum enzymatic activity.
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genes that are most responsive to Fe deficiency, whereas *AHA1*, one of the major H^+-ATPase isoforms in the PM, is not affected (Santi and Schmidt 2009). Concomitant with an increase in transcription, an increase in the amount of PM H^+-ATPase protein in roots under nutrient deficiency have also been observed (Dell'Orto *et al*. 2000, Santi and Schmidt 2009, Alvarez-Pizarro *et al*. 2011) indicating that this enzyme may be transcriptionally regulated. However, based on the lack of alteration in the protein content in PM H^+-ATPase, post-translational modifications of this enzyme or changes in membrane lipids are also suggested to be determining factors for an increase in the enzyme activity in response to nutrient stress (Palmgren and Harper 1999, Dell'Orto *et al*. 2000, Shen *et al*. 2006, Sperandio *et al*. 2011). To date, the mechanism by which PM H^+-ATPase is regulated under K^+ deficiency remains unclear. Although quantitative changes in the expression pattern have been described in sorghum roots (Alvarez-Pizarro *et al*. 2011), no evidence has been found to indicate a corresponding increase in the PM H^+-ATPase in barley roots using immunoblot assays (Samuels *et al*. 1992).

Biochemical studies have revealed changes in the kinetic parameters of ATP hydrolysis and H^+-transport by PM H^+-ATPases suggesting that specific isoforms can be induced during the adaptation to nutrient deficiency. For example, under phosphate deficiency, active proteoid roots of white lupin plants grown under both phosphorus-deficient and sufficient conditions display a higher initial rate and a steeper pH gradient for the H^+-pumping activity of PM H^+-ATPase than lateral roots (Yan *et al*. 2002). Previous results show that isoforms that more efficiently transport H^+ may be particularly important in sorghum adaptation to K^+ deficiency and can be differentially regulated depending on the predominant source of inorganic N in the root medium (Alvarez-Pizarro *et al*. 2011). In K^+-deprived sorghum, NH_4^+ stimulates significantly net proton transport across the PM and ATP hydrolysis compared with NO_3^-, but these effects were not observed under K^+-sufficient conditions. The physiological role of PM H^+-ATPase isoforms in K^+ stress in the presence of different N sources has not been elucidated yet.

In this study, we investigated the role of sorghum H^+-ATPase in the root PM during the adaptation to K^+ deficiency under the influence of two sources of inorganic N (NO_3^- and NH_4^+). To gain more information regarding the involvement of specific PM H^+-ATPase isoforms, the kinetic properties (V_{max}, K_m, K_i, and optimum pH) of the ATP hydrolytic activity were analyzed. Based on the recently published genomic sequences of sorghum (Paterson *et al*. 2009), the root expression of two genes encoding PM H^+-ATPases, *SBA1* and *SBA2*, were also investigated. This work reveals important aspects of H^+ pump regulation *via* the interaction between K^+ deficiency and N sources.

Materials and methods

Plant growth and treatments: Seeds of the sorghum [*Sorghum bicolor* (L.) Moench] genotype CSF 20, supplied by the Instituto Agronômico de Pernambuco (IPA, Brazil), were surface sterilized in a 1 % (m/v) solution of commercial bleach for 5 min and subsequently washed several times in distilled water. The seeds were germinated in plastic cups containing *Vermiculite* moistened with distilled water. After 4 d, 15 seedlings were transferred to plastic containers with 10 dm^3 of a modified 1/4 Hoagland solution containing 0.2 mM K^+ and two inorganic N sources (NO_3^- and NH_4^+) at a final concentration of 4.0 mM. K^+ was supplied as KCl, and N was supplied as either $Ca(NO_3)_2$ or NH_4Cl + $(NH_4)_2HPO_4$. In the nutrient solution containing NO_3^-, the other macronutrients were supplied as 0.25 mM $MgSO_4$ and 0.2 mM NaH_2PO_4, whereas in that containing NH_4^+, 0.25 mM $MgSO_4$, 0.2 mM $(NH_4)_2HPO_4$ and 2.0 mM $CaCl_2$ were added. To both solutions, the micronutrients were added as 50 μM $CaCl_2$, 12.5 μM H_3BO_3, 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM H_2MoO_4, and 10 μM Fe-EDTA. The pH of the growth solutions was maintained at 5.5 - 6.0 and adjusted with 1 M NaOH or HCl as needed. The K^+ concentration of the nutrient solutions was monitored daily and maintained at the originally established value. Constant aeration was also supplied. The solutions were exchanged for fresh on days 9 and 12. On day 15, the plants were transferred to identical solutions lacking K^+ and subjected to K^+ deprivation for 48 h. The plants were grown in a naturally lit greenhouse with day/night air temperatures of 32.0/22.0 ºC and relative humidities of 67.5/90.0 %.

K^+ depletion experiments: K^+ uptake from the solutions with low-K^+ concentrations was estimated according to Claassen and Barber (1974) under a temperature of 24.8 ± 0.7 ºC, a relative humidity of 69.8 ± 5.5 %, and an irradiance provided by four fluorescent lamps (25 Watt) placed at a distance of 50 cm over the plants. After a 3-h acclimation, the roots were rinsed in deionized water for 10 min, and the plants were individually placed in plastic pots containing 130 cm^3 of a depletion solution consisting of 2.0 mM $Ca(NO_3)_2$, 0.25 mM $MgSO_4$, 0.2 mM NaH_2PO_4, and 0.1 mM KCl. The micronutrient composition was similar to that of the growth solutions. The pH of the uptake solution was adjusted to 5.8. Vanadate at 0.5 mM was added to analyze the role of the root PM H^+-ATPase in K^+ uptake. Constant aeration was supplied during the depletion assays. Samples (1 cm^3) were collected every 20 min during the first two hours of the assay and then once per hour up to five hours. The K^+ concentration of the samples was determined by a flame photometer (*B462*, *MicroNal*, SP, Brazil). After each sampling, deionized water was added to the pot to maintain a constant solution volume. For each depletion

curve, the K^+ concentration data in the first hour was fitted by simple linear regression, and the slope obtained was used to estimate the initial rate of K^+ uptake [μmol (K^+) g^{-1}(root f.m.) min^{-1}].

Plasma membrane isolation: The microsomal fraction from K^+-starved roots (15.0 g) growing in the presence of NO_3^- or NH_4^+ was isolated as described by Alvarez-Pizarro *et al*. (2011). The PM vesicles were purified by partitioning using the aqueous polymer two-phase system composed of 6.2 % (m/v) dextran *T500* (*Sigma Aldrich*, St. Louis, MO, USA) and 6.2 % (m/v) polyethylene glycol (PEG 3350, *Sigma Aldrich*) according to Widell *et al*. (1982). The protein content was determined according to Bradford (1976) using BSA as standard. The average protein yields for NO_3^- and NH_4^+-grown roots were 0.80 ± 0.06 and 0.74 ± 0.05 mg g^{-1}(f.m.), respectively.

Kinetic characteristics of plasma membrane H^+-ATPase activity: PM proteins (2 - 3 μg) were incubated in 0.5 cm^3 of a reaction medium containing a 30 mM Tris-MES (pH 6.5) buffer, 5 mM $MgSO_4$, 50 mM KCl, 1 mM $(NH_4)_6Mo_7O_{24}$, 1 mM NaN_3, and 0.05 % (m/v) *Brij 58* (*Across Organics*, Fair Lawn, NJ, USA) in the presence or absence of 0.5 mM Na_3VO_4. The ATP hydrolysis reaction was initiated by adding ATP at final concentrations ranging from 0.05 to 5 mM and proceeded at 30 °C for 30 min. The amount of inorganic phosphate liberated was determined according to Yan *et al*. (1998). The specific activity of PM H^+-ATPase was determined by the difference in activities between the assays with and without 0.5 mM vanadate. After the Lineweaver and Burk data transformation, the maximum enzymatic activity (V_{max}) and Michaelis-Menten constant (K_m) were calculated. The effect of pH on the enzyme activity was assayed in a reaction medium buffered with 30 mM MES-Tris (pH 4.5 - 6.0) and 30 mM Tris-MES (pH 6.5 - 8.0). The PM H^+-ATPase activity was examined in the presence of increasing concentrations of the inhibitor (0.005 to 1.0 mM Na_3VO_4). The data were adjusted using a decreasing hyperbolic curve, and the vanadate concentration required for the 50 % inhibition of the ATPase activity (K_i) was calculated using the equation $\nu = V_0K_i/(K_i + [I])$ described by De Souza *et al*. (2007), where ν, V_0, and [I] represent the reaction velocity in the presence of inhibitor, maximum velocity, and the concentration of Na_3VO_4, respectively.

Immunoblotting: For SDS-PAGE, PM proteins were transferred on a nitrocellulose membrane (*Hybond-C Extra*, *GE Healthcare*, Little Chalfont, UK) as described previously (Alvarez-Pizarro *et al*. 2011). The blot was probed with a polyclonal antibody raised against *Arabidopsis thaliana* PM H^+-ATPase (AHA3), and immunodetection was performed with a secondary antibody (anti-rabbit IgG, *Sigma Aldrich*) conjugated to alkaline phosphatase. A standard 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT) protocol (*Bio-Rad*, Hercules, CA, USA) was used for the development of the alkaline phosphatase reaction.

Design of specific primers: A non-redundant genome database search using the *TBLASTN* was performed to identify PM H^+-ATPase nucleotide sequences from sorghum. The query sequences used were *MHA2* from *Zea mays* (GenBank accession NM_001112000) and *AHA4* from *Arabidopsis thaliana* (GenBank accession NM_114664). The scores of the hits were used as selection criteria, and two full-length cDNA sequences from sorghum were selected. The sequences were named *SBA1* and *SBA2* (*Sorghum bicolor* H^+-ATPase 1 and 2), and their GenBank accession numbers are XM_002447204 and XM_002442573, respectively. Both sequences were aligned using the *ClustalW* program from the National Center for Biotechnology Information (*NCBI*) to design specific primers for each H^+-ATPase gene. The primers used for a gene amplification were as follows: *SBA1* forward, 5' GTCCTTACTAGCAGA-TGC 3'; *SBA1* reverse, 5' CCTGATTGACCTC ACACCG 3'; *SBA2* forward, 5' GTGCTTACTAGT CGCGCT 3'; and *SBA2* reverse, 5' TCAAGGCTTTCG ACATGA 3'. The specificity of the primer sequences was analyzed using the *Primer-BLAST* at NCBI, and the products of the PCR amplification (expected fragments of 300 bp) were analyzed by agarose gel electrophoresis.

Total RNA extraction, cDNA synthesis, and real-time PCR: Total RNA was isolated from whole roots of two sorghum plants using an *RNeasy* plant mini kit (*Qiagen*, Hilden, Germany). The quality of RNA was evaluated on an agarose gel (1 %, m/v) with ethidium bromide staining, and quantified spectrophotometrically (*Nanodrop ND-1000*, *Thermo Scientific*, Wilmington, DE, USA). Approximately 2 μg of RNA from each sample was treated with RNAase-free *DNase I* (*Invitrogen*, Carlsbad, USA) and used as template for first-strand cDNA synthesis which was performed with a *Superscript*^{III} kit (*Invitrogen*) following the manufacturer's instructions. The cDNA synthesis reaction mixture was diluted 50 times with *Milli-Q* (*Merck*, Billerica, MA, USA) water. For the real-time PCR, each reaction contained 3.75 mm^3 of *SYBR Green Rox Plus* (*LGC Biotechnology*, Cotia, SP, Brazil), 0.5 mm^3 of sense and antisense primers (10 μM), 2 mm^3 of diluted cDNA, and 8.25 mm^3 of *Milli-Q* water. Each sample was analyzed in triplicate. The cDNA was amplified using a *Mastercycler realplex ep* gradient (*Eppendorf*, Hamburg, Germany) under the following conditions: 95 °C for 30 min followed by 40 amplification cycles at 95 °C for 15 s, 53 °C for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 5 min. After each PCR, the dissociation curve of the PCR products was analyzed. Two housekeeping genes, *actin* (Shih *et al*. 2006) and *18S* (Monreal *et al*. 2007), were used as internal standards to normalize the *SBA* expression data assuming 100 % reaction efficiency (E = 2). The transcript accumulation was estimated using the $2^{-\Delta\Delta Ct}$ method.

Phylogenetic tree construction: The complete cDNA sequences of PM H^+-ATPases from different plant species that were available in the *NCBI* database were aligned using the *ClustalW* program. An unrooted phylogenetic tree was constructed using *ClustalW*, bootstrapped with 1 000 replications, and viewed with the *MEGA* program.

Statistical analysis: The experimental design was completely randomized. Two independent experiments with five plants each were set up for the analysis of K^+ depletion. Three independent experiments were set up for the kinetic characterization of PM H^+-ATPase and the immunoblot assays. Transcription data were obtained from one single experiment. Data regarding the initial rate of K^+ depletion, root growth, and kinetic parameters (V_{max}, K_m, and K_i) of PM H^+-ATPase were analyzed using the one-way analysis of variance (*ANOVA*). The values are expressed as means ± SE and compared using the Tukey's test ($P = 0.05$).

Results

After the K^+ starvation for 48 h, the sorghum plants grown in the presence of NO_3^- or NH_4^+ exhibited similar abilities to uptake K^+ when measured in depletion solutions containing NO_3^- (Fig. 1). There was no significant difference in the initial rate of K^+ uptake of plants grown with both N sources (Table 1). When 0.5 mM Na_3VO_4, an inhibitor of PM H^+-ATPase, was added, K^+ uptake was significantly reduced (Fig. 1). After 4 h, when 90.0 % of the external K^+ had been depleted by the roots in the vanadate-free solutions, the inclusion of vanadate inhibited the depletion to 65.0 and 52.5 % by plants grown at NO_3^- and NH_4^+, respectively. The initial rate of K^+ uptake in the presence of vanadate was significantly reduced only in the plants grown with NH_4^+ (Table 1). Significant differences in root growth were not induced either by the N source or the vanadate (Table 1).

It was verified that the membrane preparations were enriched (approximately 90.0 %, data not shown) with plasma membrane. The effect of an increasing ATP concentration on the ATPase activity of PM H^+-ATPase from sorghum roots revealed typical Michaelis-Menten kinetics (Fig. 2*A*). A higher PM H^+-ATPase V_{max} (2.8-fold) and a lower affinity for ATP (2.6-fold) were achieved in samples from the NH_4^+-grown roots compared with the NO_3^--grown roots (Fig. 2, Table 2). The optimum pH for the PM H^+-ATPase isolated from the NO_3^--grown roots was slightly more acidic (6.0) than that of the PM H^+-ATPase from the NH_4^+-grown roots (6.0 - 6.5) (Fig. 3*A*, Table 2). The vanadate sensitivity of the sorghum root PM H^+-ATPase was similar regardless of the N source (Fig. 3*B*, Table 2). The concentration required for a half-maximum inhibition (K_i) estimated at Na_3VO_4 concentrations between 5 and 50 μM (Fig. 3*B*) was approximately 15.0 μM (Table 2).

Protein blots with membrane preparations show that the PM H^+-ATPase protein content was slightly increased in sorghum roots grown with NH_4^+ *versus* those grown with NO_3^- (data not shown).

The phylogenetic analysis with the full-length cDNA sequences of PM H^+-ATPases from monocots and dicots

Table 1. Initial rate of K^+ uptake [μmol(K^+) g^{-1}(root f.m.) min^{-1}] and root growth [g $plant^{-1}$] of sorghum plants grown in nutrient solutions containing NO_3^- or NH_4^+ and subjected to K^+ deficiency for two days. Means ± SE were calculated from 10 plants collected in two independent experiments. The significant differences ($P \leq 0.05$) due to the nitrogen source are indicated with different uppercase letters; the significant differences due to the inclusion of vanadate are indicated with lowercase letters.

N source	Na_3VO_4	K^+ uptake	Root growth
NO_3^-	-	0.63 ± 0.08Aa	0.68 ± 0.06Aa
NO_3^-	+	0.49 ± 0.06Aa	0.69 ± 0.11Aa
NH_4^+	-	0.89 ± 0.06Aa	0.52 ± 0.04Aa
NH_4^+	+	0.57 ± 0.07Ab	0.54 ± 0.09Aa

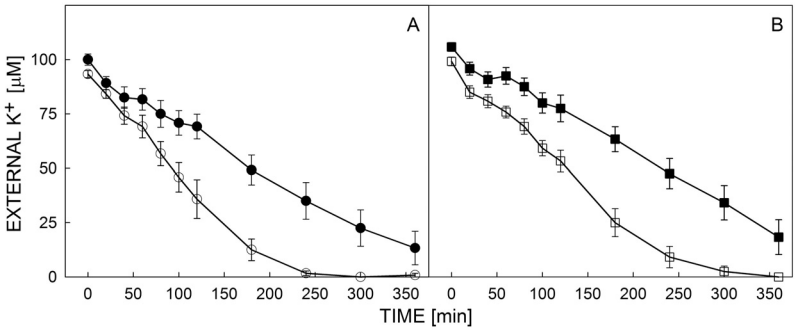

Fig. 1. The depletion of external K^+ by K^+-starved roots of sorghum grown in nutrient solutions containing NO_3^- (*A*) or NH_4^+ (*B*) as the N source. The depletion assays were performed in the absence (○ and □) and presence (● and ■) of vanadate. The K^+ concentration at each sampling time represents a mean ± SE calculated from 10 plants collected in two independent experiments.

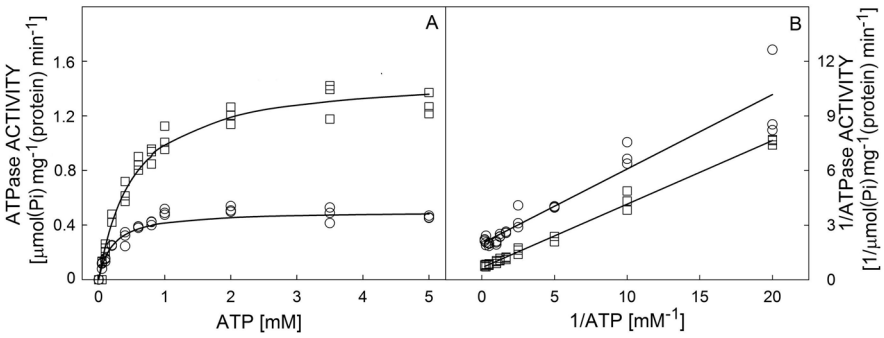

Fig. 2. *A* - The kinetic analysis of the ATPase activity of PM H^+-ATPase from K^+-starved roots from sorghum grown in nutrient solutions containing NO_3^- (○) or NH_4^+ (□). The data were obtained from three representative experiments, and both lines were fitted to the Michaelis-Menten equation. *B* - The Lineweaver-Burk plot of the data presented in part *A*. The data were fitted by the linear regression analysis. The linear equations obtained were $Y_{○} = 0.4091x+1.9931$ and $Y_{□} = 0.3492x+0.6613$ ($r > 0.98$).

Table 2. The kinetic parameters of PM H^+-ATPase and the relative expression of transcripts *SBA1* and *SBA2* from sorghum roots grown in nutrient solutions with NO_3^- or NH_4^+ and submitted to K^+ deficiency. For kinetic parameters, means ± SE of three independent experiments. Significant differences among the treatments are indicated with different letters ($P \leq 0.05$). Transcription was measured only in one experiment.

N source	V_{max} [µmol(Pi) mg^{-1}(prot.) min^{-1}]	K_m [mM]	K_i [µM]	Optimum pH	*SBA1*	*SBA2*
NO_3^-	0.50 ± 0.09b	0.17 ± 0.02b	16.67 ± 2.69a	6.0	1.00 ± 0.15	1.00 ± 0.13
NH_4^+	1.38 ± 0.06a	0.45 ± 0.09a	14.57 ± 2.35a	6.0 - 6.5	1.51 ± 0.28	3.28 ± 0.48

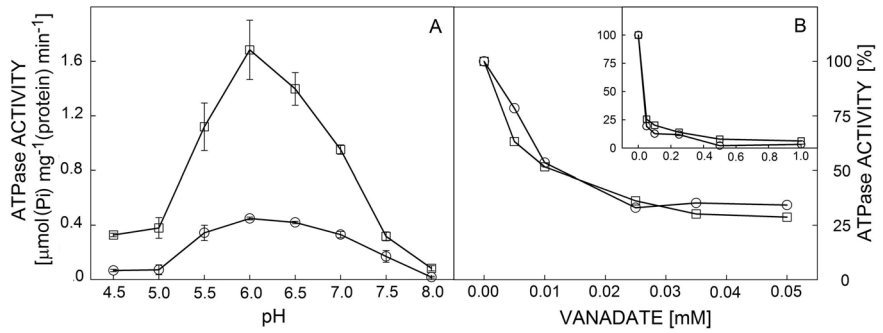

Fig. 3. The effect of pH (*A*) and vanadate (*B*) on the ATPase activity of PM H^+-ATPase from K^+-starved roots from sorghum grown in nutrient solutions containing NO_3^- (○) or NH_4^+ (□). The assays with vanadate were performed with concentrations ranging from 0.005 - 0.05 mM and 0.05 - 1.0 mM. The data shown were obtained from a representative experiment and represent means ± SE.

allowed us to distinguish five clusters (subfamilies I, II, III, IV, and V). The *SBA1* and *SBA2* genes from sorghum clustered into groups II and I, respectively (Fig. 4). *SBA1* shared a sequence similarity of 92 % with the *MHA2* isoform from maize, whereas *SBA2* shared a sequence similarity of 90 % with the *OSA3* isoform from rice. Under K^+ deficiency, the *SBA1* and *SBA2* transcript levels were 1.5- and 3.3-fold higher, respectively, in the sorghum plants grown in the presence of NH_4^+ compared with those grown in the presence of NO_3^- (Table 2).

Discussion

In this work, we observed that long-term nutrition with different inorganic forms of N (NO_3^- and NH_4^+) may lead to the induction of specific PM H^+-ATPase isoforms in sorghum roots during K^+ starvation. After 48 h of K^+ withdrawal, the kinetic parameters (V_{max}, K_m, and optimum pH) of PM H^+-ATPase hydrolytic activity in the NH_4^+-fed sorghum roots were significantly different from those in the NO_3^--fed sorghum roots (Figs. 2 and 3*A*, Table 2). Additionally, the presence of NH_4^+ during K^+ deficiency induced the root expression of the *SBA1* and *SBA2* genes, with the latter showing a higher expression (Table 3). Together with the increased amount of H^+-ATPase protein in the PM (data not shown), the results suggest that a transcriptional regulatory

mechanism of PM H^+-ATPase might be mediated by the interaction between the K^+ stress and the source of inorganic N that predominates in a given root environment. Although the root medium pH is also considered to be a determining factor in the regulation of PM H^+-ATPase (Yan *et al*. 1998, Zhu *et al*. 2009), this variable was ruled out given the continuous adjustment of the growth solution pH which varied according to the typical patterns of acidification and alkalinization induced by NH_4^+ and NO_3^-, respectively.

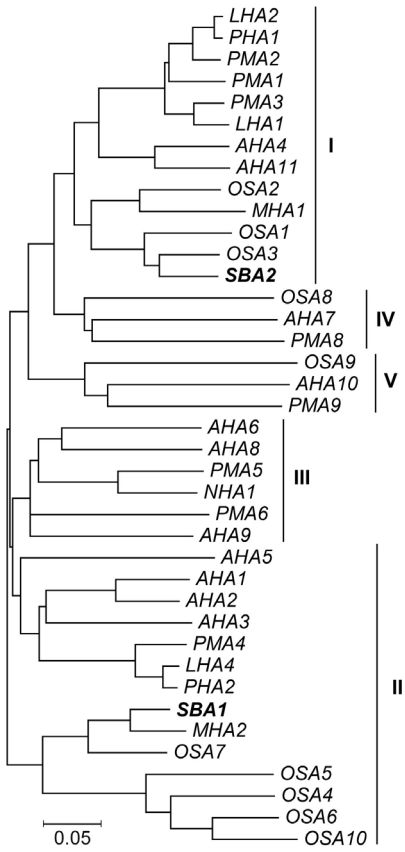

Fig. 4. Phylogenetic relationships between PM H^+-ATPases identified in various plant species. The tree was generated using the complete cDNA sequences of *Arabidopsis thaliana* (*AHA1-11*), *Oryza sativa* (*OSA1-10*), *Nicotiana plumbaginifolia* (*PMA1-9*), *Solanum tuberosum* (*PHA1-2*), *Lycopersicon esculentum* (*LHA1-2,4*), *Zea mays* (*MHA1-2*), and *Nicotiana tabacum* (*NHA1*). The PM H^+-ATPases from sorghum roots (*SBA1* and *SBA2*) are *in bold*. The different sub-families are numbered with Roman numerals in accordance with the family subdivision by Palmgren (2001) and Arango *et al*. (2003).

In this work, the PM H^+-ATPase activity could be solely regulated by the N-induced alterations on the cytoplasmic pH. NH_4^+-fed plants often show a reduction in intracellular pH as result of H^+ production during NH_4^+ assimilation (Van Beusichem *et al*. 1988, Marschner 1995). The maintenance of the cytoplasmic pH of root cells grown with NH_4^+ would require the efflux of H^+, therefore requiring the activation of PM H^+-ATPase. A strong correlation between H^+ extrusion and the stimulation of PM H^+-ATPase was found in barley roots after exposure to NH_4^+ (but not NO_3^-) for 1 d (Yamashita *et al*. 1995). However, it has been also demonstrated that NO_3^- and NH_4^+ in nutrient solutions at pH 6.0 - 6.5 (a pH similar to that used in the present work) had little effect on the cytoplasmic pH in maize roots (Gerendás *et al*. 1993) and on the hydrolytic and H^+ pumping activities of H^+-ATPase in rice roots (Zhu *et al*. 2009). In a previous paper, we showed that the activities of PM H^+-ATPases are not significantly different when sorghum roots were grown in complete nutrient solutions at pH 6.0 containing NO_3^- or NH_4^+ as the nitrogen source (Alvarez-Pizarro *et al*. 2011). In this species, the stimulation of PM H^+-ATPase activities in root cells occurred in K^+-deficient plants grown with NH_4^+ and not in those grown with NO_3. These data and our results suggest that NH_4^+ could regulate PM H^+-ATPase during the adaptation to K^+ deficiency.

Under deficiency of different minerals, alterations in kinetic parameters of PM H^+-ATPase are interpreted as the result of the induction of specific isoforms. For example, increases in the V_{max} of ATP hydrolysis, a reduction in the affinity for ATP, and a shift in the optimum pH toward more acidic values were described in *Lupinus albus* roots under phosphorus deficiency suggesting the involvement of different PM H^+-ATPase isoforms in the adaptation to P-stress (Yan *et al*. 2002). Based on these results, the differences in the K_m (Fig. 2, Table 2) and optimum pH (Fig. 3*A*, Table 2) for the PM H^+-ATPase isolated from the K^+-starved sorghum roots grown with NH_4^+ or NO_3^- would suggest that different isoforms were induced. Although enzyme isoforms from other plant species exhibit different sensitivity to vanadate (Palmgren and Christensen 1994, Yan *et al*. 2002), the isoforms found in the sorghum roots appeared to be equally sensitive (Fig. 3*B*, Table 2). However, in our K^+-depletion assays (Fig. 1, Table 1), a significant reduction in the initial rate of K^+ uptake in the roots grown with NH_4^+ (compared with the roots grown with NO_3^-) in the presence of vanadate was observed. This discordance between the biochemical assays and depletion assays could be attributed, at least in part, to the higher capacity of the sorghum roots cultivated with NH_4^+ to deplete low K^+ concentrations as compared with those cultivated with NO_3^- (Alvarez-Pizarro *et al*. 2011).

The data of expression analysis of the two studied genes of PM H^+-ATPase in the sorghum roots, *SBA1* and *SBA2*, (Table 2) do not allow the conclusion that the differences in the kinetic properties resulted from the expression of specific enzyme isoforms. Both the genes were expressed under K^+ deficiency and in the presence of NO_3^- and NH_4^+, but a higher expression was observed in the roots grown with NH_4^+ compared with those grown in the presence of NO_3^- (Table 3). It is likely that the differences in the kinetic parameters of the PM H^+-ATPases from sorghum roots could be attributed to the expression of other isoforms, which were not studied in this work. However, based on the phylogenetic

relationships of *SBA1* and *SBA2* with the PM ATPases from other plant species, it can be deduced that *SBA1* and *SBA2* belong to sub-families II and I, respectively (Fig. 4). Similarly, the *SBA1* ortholog *MHA2* and the *SBA2* ortholog *OSA3* have been described as major isoforms of PM H^+-ATPase with a high expression in various plant tissues including roots (Frias *et al*. 1996, Zhu *et al*. 2009, Sperandio *et al*. 2011). Additionally, their involvement in mineral nutrition has been suggested. Although the amount of *SBA1* and *SBA2* transcripts were not analyzed in other sorghum tissues, the detection of their high transcription in roots suggested their roles in nutrient acquisition.

The transcriptional control of PM H^+-ATPase expression in sorghum roots during the interaction between K^+ deficiency and N source was also confirmed by immunoblotting using a polyclonal antibody against the PM H^+-ATPase isoform *AHA3* from *Arabidopsis thaliana* which revealed an increase in protein amount when plants were grown in the presence of NH_4^+ (data not shown). This antibody recognizes epitopes conserved in different isoforms of subfamily II of *Arabidopsis* (AHA1, AHA2, and AHA3), and it has been employed for the identification and localization of PM H^+-ATPase in other plant species (Palmgren *et al*. 1991, Campos *et al*. 1996). Our results showed a significant increase in the expression level of the *SBA2* isoform (Table 2), belonging to PM H^+-ATPase subfamily I which could have been recognized by the anti-*AHA3* antibody used. This hypothesis is based on the fact that a polyclonal antibody against PM H^+-ATPase might detect different isoforms of this enzyme due to the high similarity of their amino acid sequences (Roldán *et al*. 1991, Campos *et al*. 1996, Lefebvre *et al*. 2005). Interestingly, PM H^+-ATPase sequences in the sorghum genome with a high similarity to *AHA2*, *AHA*3, and *AHA5* isoforms of *Arabidopsis* have not been identified (data not shown). Therefore, it is likely that increases in both the amount of protein in the plasma membrane and the ATPase activity (Fig. 2) of sorghum root H^+-ATPase under K^+ deficiency and in the presence of NH_4^+ have resulted, at least in part, from the increased expression of the *SBA2* isoform.

The induction of PM H^+-ATPase may be essential for K^+ uptake under a low K^+ concentration. In the presence of NH_4^+, the possible induction of PM H^+-ATPase would explain similar initial rates of K^+ influx as in plants grown at NO_3^- (Fig. 1, Table 1). However, the physiological significance of this enzyme is not in agreement with its low affinity for ATP (Fig. 2, Table 2). Perhaps, such a PM H^+-ATPase isoform is more efficient when expressed in root cells with elevated concentrations of ATP. When exposed to high NH_4^+ concentrations, some plant species exhibit increased respiratory rates (Hadži-Tašković Šukalović and Vuletić 2001, Britto and Kronzucker 2002). Also, an increase in ATP content in barley roots grown under NH_4^+ nutrition has been described (Lang and Kaiser 1994). Accordingly, the maintenance of elevated ATP content in root cells of NH_4^+-fed sorghum would allow PM H^+-ATPase with a low affinity for ATP to operate at a significant rate. However, the initial rate of K^+ net uptake in the NO_3^--fed sorghum roots with the low activity of PM H^+-ATPase (Fig. 2, Table 2) was not lower than in the NH_4^+-fed sorghum roots (Fig. 1, Table 1). The K^+ uptake rate was likely promoted to counterbalance the excess negative charge from the NO_3^- taken up and accumulated in plant tissue. A large accumulation of cations (such as K^+) in plants grown with NO_3^- has been previously reported (Van Beusichem *et al*. 1988, Ruan *et al*. 2007).

In conclusion, the PM H^+-ATPase activity could be differentially modulated during the interaction between K^+ deficiency and the N source. Under such growth conditions, the gene expression and isoforms with different kinetic parameters were induced according to the energetic requirements of the K^+ transport system. Isoforms that are more efficient in ATP hydrolysis could be required to create a steeper pH gradient and drive K^+ influx through specific K^+ channels which are the predominant means of K^+ acquisition when plant growth occurs in the presence of NH_4^+.

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