ORIGINAL RESEARCH ARTICLE

Title: **Involvement of aquaporins on nitrogen-acquisition strategies of juvenile and adult plants of an epiphytic tank-forming bromeliad**

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Author contribution statement

AM, MG and HM designed the study. MG, FC and HM supervised the project. AM, CAC, NR, FG, PTM and FCP carried out the experiments and data acquisition. All authors analyzed the data. AM wrote the paper with input from all authors.

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Main conclusion

Depending on the N source and plant ontogenetic state, the epiphytic tank-forming bromeliad *Vriesea gigantea* can modulate aquaporin expression to maximize the absorption of the most available nitrogen source.

Abstract

Epiphytic bromeliads frequently present a structure formed by the overlapping of leaf bases where water and nutrients can be accumulated and absorbed, called tank. However, this structure is not present during the juvenile ontogenetic phase, leading to differences in nutrient acquisition strategies. Recent studies have shown a high capacity of the bromeliad *Vriesea gigantea*, an epiphytic tank-forming bromeliad, to absorb urea by their leaves. Since plant aquaporins can facilitate the diffusion of urea through the membranes, we cloned three foliar aquaporin genes, *VgPIP1;1, VgPIP1;2* and *VgTIP2;1* from *V. gigantea* plants. Through functional studies, we observed that besides water, VgTIP2;1 was capable of transporting urea while VgPIP1;2 may facilitate ammonium/ammonia diffusion. Moreover, aiming at identifying urea and ammonium-induced changes in aquaporin expression in leaves of juvenile and adult-tank plants, we showed that *VgPIP1;1* and *VgPIP1;2* transcripts were up-regulated in response to either urea or ammonium only in juvenile plants, while *VgTIP2;1* was up-regulated in response to urea only in adult-tank plants. Thereby, an ontogenetic shift from juvenile to adult-tank-forming-plant appears to occur with metabolic changes regarding nitrogen metabolism regulation. Investigating urea metabolism in wild species that naturally cope with organic N sources, such as *V. gigantea*, may provide the knowledge to modify nitrogen use efficiency of crop plants.

Key-words: Ammonium/ammonia diffusion, Epiphytic bromeliads, MIPs, Urea transport, *Vriesea gigantea.*

Abbreviations

MIPs	Major intrinsic proteins
PIPs	Plasma membrane intrinsic proteins
TIPs	Tonoplast intrinsic proteins
NPA	asparagine-proline-alanine motif
FPs	Froger's positions
P_f	Osmotic water permeability coefficient

Introduction

The canopy of rainforests is an oligotrophic habitat, in which epiphytic bromeliads are highly abundant (Benzing 2000). Since epiphytic bromeliads have no access to soil resources, morphological and physiological adaptations are necessary to cope with irregular nutrient and water supply (Laube and Zotz 2003; Leroy et al. 2016). The roots of epiphytic bromeliads are poorly developed and function mainly for fixation to the host plant (Benzing and Renfrow 1974), leaving the leaves as the major part of the vegetative body and responsible for an important part of both water and nutrient absorption as well as assimilation functions. In the so-called tank bromeliads, the overlapping of the leaf bases forms a cistern that accumulates rainwater and organic compounds, which are absorbed by specialized trichomes in the leaves (Benzing 2000). Thus, the presence of the tank influences leaf metabolism, leading to functional differentiation along the length of the leaf (Takahashi and Mercier 2011; Matiz et al. 2013). Accordingly, in adult-tank bromeliads, the basal part of the leaf exhibits characteristics related to absorption functions, while the apical part of the leaf displays features related to photosynthetic functions (Takahashi and Mercier 2011; Mioto and Mercier 2013; Rodrigues et al. 2016; Kleingesinds et al. 2018). However, in order to have a functional tank, bromeliads must first reach an appropriate size, meaning that this structure is not present in small juvenile plants. As a result, some tank-bromeliad species start their life cycle as atmospheric epiphytes (i.e. obtaining water and nutrients mostly from rainfall and/or fog) and only later develop a tank (Zotz et al. 2011; Rodrigues et al. 2016; Kleingesinds et al. 2018), which may result in significant changes in the physiology of the leaves.

Epiphytic tank bromeliads obtain nitrogen from precipitation, decomposing debris and excreta of associated organisms (Stewart et al. 1995; Leroy et al. 2016). Several studies have shown the importance of amphibian, spider, and ant excreta in bromeliad nutrition (Romero et al. 2008, 2010; Leroy et al. 2013; Gonçalves et al. 2016a). Thus, organic nitrogen sources provided by animals (i.e., urea from anurans) seem to be one of the most important nitrogen sources for tank bromeliads (Benzing 2000; Romero et al. 2010; Gonçalves et al. 2016b). Furthermore, it has been described a high capacity of epiphytic bromeliads to effectively use urea as an organic nitrogen source (Endres and Mercier 2001; Inselsbacher et al. 2007; Takahashi and Mercier 2011; Matiz et al. 2017). The epiphytic bromeliad *Vriesea gigantea* exhibited a better growth when it was supplied with urea (Endres and Mercier 2001) and was able to absorb urea at high concentrations (10 mM) with no saturation in its uptake (Inselsbacher et al. 2007).

The identification of passive urea transporters in plants, such as aquaporins brought new knowledge about the molecular bases of nitrogen absorption (Liu et al. 2003; Gaspar et al. 2003; Bienert et al. 2011). Aquaporins are channels belonging to the

membrane intrinsic proteins (MIPs) superfamily. In vascular plants, MIP family is divided into 5 subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), small basic intrinsic proteins (SIPs), nodulin26-like intrinsic proteins (NIPs) and X intrinsic proteins (XIPs) (Chaumont et al. 2001; Bienert et al. 2011). Although aquaporins have initially been characterized exclusively as water channels, several functional studies performed in *Xenopus laevis* oocytes or *Saccharomyces cerevisiae* have demonstrated the capacity of some aquaporin isoforms to transport small solutes, such as urea (Liu et al. 2003; Gaspar et al. 2003; Bienert et al. 2011; Gu et al. 2012), and ammonia/ammonium (Jahn et al. 2004; Bertl and Kaldenhof 2007; Dynowski et al. 2008). Thus, urea and ammonia transport trough aquaporins highlights the importance of these channels for nitrogen metabolism.

A previous characterization of urea uptake in *V. gigantea* suggested the involvement of aquaporins since urea absorption was reduced by 78% in plants treated with $HgCl_2$, a known aquaporin inhibitor (Inselsbacher et al. 2007). Given that, *V. gigantea* represents an excellent model to study urea metabolism due to its high capacity to absorb this organic nitrogen source through their leaves and since aquaporins appear to participate in urea absorption, we wonder whether these transporters participate in nitrogen acquisition in *V. gigantea* plants. Here, we have cloned three aquaporin isoforms, *VgPIP1;1, VgPIP1;2* and *VgTIP2;1* of *V. gigantea* plants and characterized their functional capacity to facilitate urea, ammonia or water diffusion. Moreover, due to the morphological and physiological changes observed within juvenile-atmospheric and adult-tank plants and the functional specialization along its leaves, we examined whether the aquaporin expression patterns of the cloned genes change according to the ontogenetic stage in a nitrogen-dependent way. Differences in the expression patterns between atmospheric (lacking tanks) and adult-tank plants may be the reflection of a physiological strategy developed to efficiently absorb a host of nitrogen compounds that become more abundant once the tank is formed, such as urea, and also to compete with mineralizing micro-organisms that are present in the tank.

Material and methods

Plant material, growth conditions and treatments

For the experiments, *Vriesea gigantea* Gaudichaud plants were obtained from *in vitro* seed germination. The seeds were taken from stocks maintain in the Department of Botany at the University of Sao Paulo (São Paulo, Brazil). The plantlets were kept in solid culture medium with macronutrients according to Knudson (1946), micronutrients according to Murashige and Skoog (1962) and $CaCO_3$ (1 g L^{-1}) up to an average age of

1.5 years. Afterwards, the plants were transferred to and maintained in a mix of commercial organic substrate composed of pine bark and *Tropstrato*® until reaching an average age of 2.5 years (atmospheric stage) or 7 years (adult-tank stage).

One month before exposing the plants to the different treatments, atmospheric and adult-tank plants were transferred to growth chambers and kept at 25 ± 2°C with a 12 h photoperiod (200 µmoles photons m^{-2} s^{-1}) and 60±10% relative humidity. After that period, the plants were transferred to an N-free nutrient hydroponic solution containing (µM): 918 KH_2PO_4; 1038 $MgSO_4$; 50 NaFe-EDTA; 50 H_3BO_3; 50 $MnSO_4$; 18.5 $ZnSO_4$; 0.078 $CuSO_4$; 0.517 Na_2MoO_4; 2.50 KI; 0.052 $CoCl_2$ and supplemented with 1 µM $NiCl_2$ and 1.25 mM $CaSO_4$. Afterwards, N was added to N-free nutrient solution in the form of $CO(NH_2)_2$ (urea treatment) or $(NH_4)_2SO_4$ (ammonium treatment) to a final concentration of 5 mM. As a control, a group of plants was kept in the N-free solution. The pH of the solutions was adjusted to 5.8 and both roots and the basal part of the leaves were in contact with the hydroponic solution.

Analyses were performed in the apical and basal part of the leaf from four independent biological replicates. Atmospheric and adult-tank plants were exposed for up to 14 h to the different N treatments.

Cloning of *VgPIP1;1*, *VgPIP1;2* and *VgTIP2;1* cDNA

In order to isolate aquaporin genes, which may be related to urea transport, two-year old *in vitro* plants of *V. gigantea* were cultivated during 10 days in N-free conditions and then transferred to a new culture medium containing 5 mM of urea. After 16 hours of urea treatment, the leaves were collected and immediately frozen in liquid nitrogen. Total RNA was isolated using TRIzol Reagent (Invitrogen) according to manufacturer's instructions. cDNA was synthesized with the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen). Degenerate primers designed on two conserved regions within *PIP* and *TIP* genes from Poaceae were used for the isolation of partial cDNAs (Electronic Suppl. Table S1). PCRs were carried out with Platinum® Pfx DNA Polymerase (Invitrogen) using the following amplification conditions: 94°C for 4 min, 35 cycles of 94°C for 45 s, 58.1°C (*PIP*) or 61.9°C (*TIP*) for 1 min, 68°C for 1 min; and a final extension at 68°C for 10 min. Amplification products were separated by electrophoresis in 1.0% (w/v) agarose gels. The partial cDNA fragments were purified by QIAquick® Gel Extraction Kit (Qiagen). Addition of 3′-A overhangs post-amplification was carried out by using a thermostable Taq DNA polymerase (Invitrogen). The fragments were cloned into pGEM® -T Easy vector system (Promega) and used to transform JM109 Competent Cells (Promega). Recombinant clones were selected and sequenced using Big Dye Terminator v.3.1 (Applied Biosystems) and the M13 Forward and Reverse Sequencing

Primers. After sequencing randomly selected clones, two different *PIP1* genes and one *TIP2* gene were identified. The missing 5´ and 3´ ends of cDNA fragments were obtained by inverse PCR as described by Sambrook and Russell (2001), modified for circular cDNA template with specific primers for *PIP1;1*, *PIP1;2, and TIP2;1* (Electronic Suppl. Table S1). The full-length sequences were then amplified by primer pairs annealing at the start and stop codon of the genes (Electronic Suppl. Table S1). Full-length cDNAs were cloned as previously described.

Phylogenetic and *in silico* analysis

A phylogenetic tree was constructed using the sequences of *Ananas comosus*, *Arabidopsis thaliana*, *Physcomitrella patens*, *Solanum lycopersicum* and *Zea mays* obtained from the family 92441764 from Phytozome v12.1 (Electronic Suppl. Table S2). The retrieved protein sequences were aligned by Clustal W in MEGA 6.0 software (Tamura et al. 2013) using default parameters. The tree reconstruction from the obtained alignment was performed using the PHYML 3.0 algorithm (Guindon et al. 2010) hosted at *http://phylogeny.lirmm.fr* (Dereeper et al. 2008) with LG substitution model. The numbers in the nodes are bootstrap support in percentage. Nodes with less than 50% support were collapsed.

Subcellular localization of *Vriesea* aquaporins was carried out at LocTree3 server *https://rostlab.org/services/loctree3* (Goldberg et al. 2012, 2014). Several studies indicated that the two conserved asparagine-proline-alanine motifs (NPA), the ar/R (aromatic/arginine) filter and the five residues (P1-P5) at Froger's positions (FPs) affect the substrate specificity of the aquaporins (Froger et al. 1998; Hove and Bhave 2011). In order to identify the residues of the primary selective filters related to non-aqua substrate transport, VgPIP1;1, VgPIP1;2 and VgTIP2;1 protein sequences were align to AQP1 and GlpF as proposed by Hove and Bhave (2011) together with SoPIP2;1 and AtTIP2;1 (Electronic Suppl. Fig.S1). Our prediction of non-aqua substrates transport was based on the consensus sequences described by these authors who, in turn, based their predictions on relating these motifs to those of aquaporins that were functionally proven to transport non-aqua substrates.

cDNA cloning into Xenopus or yeast vectors

For heterologous expression in *Xenopus* oocytes, *VgPIP1;1*, *VgPIP1;2* and *VgTIP2;1* cDNAs were amplified using specific primers (Electronic Suppl. Table S1). PCR products were directionally sub-cloned through the uracil-specific excision reagent (USER) technique, into the USER-compatible *Xenopus* expression vectors pNB1u and pNB1YFPu (Nour-Eldin et al. 2006). Capped complementary RNAs were synthesized *in*

vitro as described by Preston et al. (1992), in order to be injected into *Xenopus* oocytes. The cRNAs were quantified with a Nanodrop 1000 spectrophotometer (NanoDrop Technologies) and its integrity checked on an agarose gel. For yeast complementation assay, cDNAs were cloned into the yeast expression vector pYeDP60u (Hamann and Moller 2007).

Oocyte transport assays and confocal imaging

Xenopus laevis frogs were obtained from Xenopus 1 research frog company (Dexter, MI, USA). *Xenopus* oocytes (Dumont stages V or VI) were isolated and defolliculated by using 4mg mL^{-1} collagenase A (0.271 U/mg lyophilized) kept in Barth´s solution (88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 10 mM Hepes, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$; pH 7.4 and 200 mOsmol L^{-1}) for 1.5 h at room temperature. After this period, 50 nL of *in vitro* transcripts were injected into *Xenopus* oocytes. Injected oocytes were kept in Barth´s solution supplemented with 50 ug/mL gentamycin sulfate at 18°C. After 3 days of injection, oocytes were used to test water and urea transport.

To measure the osmotic water permeability coefficient (Pf), oocytes were transferred from Barth's solution (200 mOsmol L^{-1}) to the same solution diluted two times (100 mOsmol L^{-1}) with distilled water. Changes in oocyte volume were followed with a video microscope system. Images were captured at 5 s intervals until complete 15 frames. P_f was calculated according to Fetter et al. (2004). As a positive control was used PIP2;5 from maize, an aquaporin exhibiting a significant water transport activity.

Urea permeability was measured according to Bienert et al. (2011) and Gaspar et al. (2003). Urea uptake was performed 3 days after injection. Oocytes were incubated at room temperature in 1 mL of modified iso-osmotic Barth´s solution containing 1 mM unlabeled urea supplemented with [14C] urea (2.11x 106 Bq mol-1; Amersham Pharmacia Biotech). After 0, 5 and 10 min of incubation, oocytes were washed rapidly four times in ice-cold iso-osmotic Barth´s solution containing 100 mM unlabeled urea. In order to prepare an iso-osmotic Barth's solution containing 100 mM urea, it was used a 1x Barth's solution without NaCl and complemented with urea (to a final concentration of 100 mM). Then, the osmolarity of the solution (with urea) was measured and adjusted with NaCl 5M to the osmolarity of the complete Barth's solution in which the injected oocytes were kept. Individual oocytes were lysed for 2 to 3 h in 1 mL of 5% (w/v) SDS for scintillation counting. As a positive control was used AQP9 from rabbit, a well-known aquaporin transporting urea. Solute permeability (P_{sol}) was calculated by means of the equation: P_{sol}(pmol/oocyte/10 min)= (CPM_{sample} - CPM_{blank}) x [solute]/STD, where CPM_{sample} and CPM_{blank} is the CPM per oocyte incubated with or without the labeled solute during 10 min, respectively. [solute] is the urea concentration (pmol/L) in which

the oocytes were incubated and STD is the difference between the CPM of the solute and the CPM of the blank.

Fixation of oocytes for confocal imaging was performed as described by Sayers et al. (1997). Oocytes injected with *YFP-VgPIP1;1, YFP-VgPIP1;2 and YFP-VgTIP2;1* cRNAs were kept during 3 days in Barth's solution (200 mOsmol L^{-1}) supplemented with gentamycin sulfate. After this period, the oocytes were fixed with 4% (v/v) paraformaldehyde prepared in PBS buffer (pH 7.4) for 2 h at room temperature, and then they were washed five times with cold PBS buffer. The oocytes were split in two halves and analyzed with a confocal microscope (Zeiss LSM710). YFP was excited at 514 nm and the emitted fluorescence recorded from 520 to 620 nm. The images were analyzed using the ImageJ software (*http://imagej.nih.gov/ij*).

Yeast strains and growth assays

In addition to urea transport assays in oocytes, urea permeability was also tested through a second heterologous expression assay using *Saccharomyces cerevisiae* strain YNVW1 (Liu et al. 2003), while the strain 31019b was used for ammonia permeability assays (Marini et al. 1997; Jahn et al. 2004). Transformants were grown on synthetic medium with 2% galactose, 0.17% yeast nitrogen base without amino acids and NH_4^+ (Difco) supplemented with either different concentrations of urea (urea permeability assay) or $(NH_4)_2SO_4$ (ammonia permeability assay) as the sole nitrogen sources at pH 5.5.

RNA extraction and quantitative PCR analysis

Total RNA was extracted from 200 mg of either frozen apical or basal part of the leaves with ReliaPrep™ RNA Tissue Miniprep System kit (Promega) according to the manufacturer's instructions. After quantification by Nanodrop spectrophotometer (Thermo Fisher Scientific) and integrity check by agarose-gel electrophoresis, RNA samples were treated with DNAse I (Invitrogen). cDNA was synthesized from 1 µg of total RNA using random primers and the SuperScript IV enzyme (Thermo Fisher Scientific) together with RNAse OUT™ ribonuclease inhibitor (Invitrogen) in a final volume of 20 µL. Each cDNA synthetized was checked for gDNA contamination using an intron-flanking pair of primers for the *actin2* gene (forward: 5´-CCTCTCCCTCTATGCCAGTG-3´, reverse: 5´-CTCCTTAATGTCACGCACGA-3´). cDNA samples were 1/10 diluted to a final concentration of 5 ng reverse-transcribed RNA/µL and subsequently used for qPCR reactions. Primer sequences used are detailed in Supplementary Table S1. The optimal concentration of qRT-PCR primer pairs was determined in a 100–600 nM range based on the lowest Ct values and absence of primer

dimerization. qRT-PCR reactions were performed in a StepOnePlus PCR Real-Time thermocycler (Applied Biosystems) in a final volume of 10 µl using 2X SYBR Green Master Mix reagent (Thermo Fisher Scientific). The qRT-PCR conditions were 10 min at 95 C, followed by 40 cycles of 15 s at 95° C, 30 s at 60°/65° C (Electronic Suppl. Table S1), and 30 s at 72° C. The amplification of single products was confirmed by melting curve analysis and by agarose-gels electrophoresis. Fluorescence data were analyzed using the LinRegPCR software package (Ruijter et al. 2009) to calculate primer efficiency. Transcript abundances were normalized against the geometric mean of two reference genes, *ACTIN2* and *GPDH*, previously tested for gene stability with BestKeeper software (Pfaffl et al. 2004). Relative gene expression was calculated by the $^{\Delta\Delta}$Ct method proposed by Hellemans et al. (2007). The mean relative expression was calculated from means of two technical replicates of at least three biological replicates and normalized against the control.

Statistical analyses

The statistical analyses of the data were performed using the software JMP 10.0.0 (*http://www.jmp.com*). The results of water and urea transport in *X. laevis* oocytes were compared using Student's *t*-test, while analysis of variance followed by Tukey's test was used for comparisons of aquaporin expression levels. Differences were considered significant at $P<0.05$. In order to statistically test synergistic effect between PIP1s and PIP2s on Pf, we performed a two-way ANOVA, considering each of our VgMIPs as one factor (each separately) and ZmPIP2 as another, as described by Slinker (1998). Significant positive interaction supporting the hypothesis of synergism was considered at $P<0.0001$.

Results

Three *MIP*s were cloned from urea-treated *V. gigantea* plants

Sequence analysis confirmed that one *TIP2* and two different *PIP1* genes were isolated from plants of *V. gigantea*. A phylogenetic tree of the protein deduced sequences including the MIPs of *V. gigantea* and representative members of other known dicot and monocot MIP subfamilies (PIPs and TIPs) from *A. thaliana*, *S. licopersicum*, *A. comosus*, *Z. mays* and the monilophyte *P. patens* was generated (Fig. 1). All cloned *Vriesea* PIPs gathered together with PIP1s and grouped separately from the PIP2s and TIPs subfamilies. VgPIP1;2 showed 94.5 % identity with AcoPIP1;2 while VgTIP2;1 clustered with TIP2s subfamily, particularly, with dicot TIP2s subfamily (SlTIP2s).

VgPIP1;1, VgPIP1;2 and VgTIP2;1 channel water

In order to determine whether *V. gigantea* aquaporins were permeable to water, the P_f was measured in oocytes injected with *VgPIP1;1, VgPIP1;2* and *VgTIP2;1* cRNAs. Injection of both PIP1s alone showed no effect on the P_f when compared to oocytes injected with water (control). However, VgTIP2;1 showed a significant capacity to facilitate water diffusion, with the P_f of VgTIP2;1-expressing oocytes being 5-fold higher than the P_f of control oocytes (Fig. 2). Previous experiments have shown that co-expression with PIP2 isoforms is required in order to bring PIP1 isoforms to the oocyte plasma membrane and test its water channel activity (Fetter et al. 2004). Thus, we tested whether it was the case for VgPIP1;1 and VgPIP1;2. Since *PIP2*-type aquaporins have not been cloned from *V. gigantea*, we co-expressed VgPIP1s with ZmPIP2;5. In addition, the subcellular localization of VgPIP1;1 and VgPIP1;2 expressed alone or co-expressed with ZmPIP2;5 was checked by fusing them with the YFP (Fig. 3).

As shown in Fig. 3, YFP-VgPIP1;1 and YFP-VgPIP1;2 expressed alone did not label the plasma membrane (Fig. 3b-c). Nevertheless, when these aquaporins were co-expressed with ZmPIP2;5, a synergistic effect on the P_f (Fig. 2) was observed and the YFP-PIP1s proteins were detected in the plasma membrane (Fig. 3e-f), indicating that PIP2s are required for the targeting of PIP1s to the plasma membrane.

VgTIP2;1 facilitates urea transport while VgPIP1;2 seems to facilitate NH_4^+/NH_3 transport

Besides water transport, aquaporins are able of facilitating the diffusion of small solutes. Based on *in silico* structural analysis of the primary selectivity-related motifs, VgPIP1s have highly conserved NPA regions, ar/R filter with F-H-T-R residues, typical for the PIP1 subfamily, and the P2 to P5 Froger's positions (S-A-F-W) (Table 1). In contrast, the P1 position is variable, containing an E or Q residue, which confers a different transport profile of non-aqua substrates (Table 1). In addition, VgTIP2;1 has NPA motifs different from VgPIP1s as well as ar/R filter (H-I-G-R) and FPs (T-S-A-Y-W) (Table 1). VgPIP1;1 and VgTIP2;1 were predicted as capable of transporting urea.

In order to investigate urea transport activity of *V. gigantea* aquaporins we used expression in *Xenopus* oocytes and yeast complementation approaches. Urea uptake was approximately 3-fold higher in oocytes expressing VgTIP2;1 than in control oocytes. On the other hand, neither VgPIP1;1 nor VgPIP1;2 facilitated urea diffusion when expressed alone or co-expressed with ZmPIP2;5 (Fig. 4). Therefore, VgTIP2;1 was the only tested aquaporin able to facilitate the diffusion of urea through the oocyte membrane.

These results were confirmed by a functional complementation assay in the urea uptake-defective yeast *S. cerevisiae* YNVW1 (*Δdur3 and ura3*) strain. This yeast mutant carries a deletion in the DUR3 urea transporter gene, making it incapable of growing on medium containing less than 5 mM of urea as the sole nitrogen source (Liu et al. 2003). All yeast transformants were able to grow on 20 mM of urea. Both rAQP9 (positive control) and VgTIP2;1 were able to complement the growth of the yeast strain YNVW1 on 2, 4 and 5 mM of urea as a sole N source (Fig. 5), confirming the ability of VgTIP2;1 to facilitate urea diffusion. WT strain was also transformed with the empty vector or the vector containing *VgPIP1;1, VgPIP1;2* or *VgTIP2;1* in order to show that the transformation itself did not affect yeast viability (Fig. 5).

To determine whether *V. gigantea* aquaporins facilitate the diffusion of NH_4^+/NH_3 across membranes, yeast complementation assays were performed. The yeast mutant 31019b strain (Δ*mep 1-3, ura3*) carries a deletion in the *Mep1*, *Mep2* and *Mep3* ammonium transporter genes from *S. cerevisiae*, restricting growth on media containing less than 5 mM NH_4^+ but allowing normal growth at high ammonium concentrations (Marini et al. 1997). 31019b strain was transformed with *VgPIP1;1, VgPIP1;2, VgTIP2;1* cDNAs or with the empty vector.

The transformants expressing *VgPIP1;2* grew better than those with the empty vector (control) at concentrations from 10 to 1 mM of ammonium at pH 5.5 (Fig. 6). At acid pH, ammonium in the medium is favored over ammonia, suggesting that VgPIP1;2 may facilitate N transport from ammonium. WT strain was also transformed with different *AQP* cDNAs, with the purpose of showing that the transformation itself did not affect the viability of the yeast (Fig. 6).

V. gigantea aquaporin expression in response to urea or ammonium availability depends highly on the developmental stage of the plant

qPCR analyses were performed to investigate how urea or ammonium exposition after a N-starving period affect aquaporin expression in two different ontogenetic stages (atmospheric and adult plants) of *V. gigantea*. In atmospheric plants, *VgPIP1;1* and *VgPIP1;2* gene expressions were up-regulated in response to both urea and ammonium treatments. However, *VgPIP1;1* was more highly expressed in urea than in ammonium in both apical and basal part of the leaves (Fig. 7). In contrast, *VgPIP1;2* was highly up-regulated in response to ammonium. *VgTIP2;1* showed no responses to either urea or ammonium in atmospheric plants.

On the other hand, in adult-tank plants, *VgPIP1;1* and *VgPIP1;2* had low expression levels after a mid-term exposure of either urea or ammonium. In these plants,

VgTIP2;1 was the only aquaporin gene up-regulated in both portions of the leaves, exclusively under urea exposition.

Discussion

In this study, we isolated and functionally characterized aquaporins of *V. gigantea* belonging to the PIP and TIP subfamilies. Through phylogenetic analysis it was possible to determine that VgPIP1;1 and VgPIP1;2 grouped preferentially with members of the PIP1 subclass, such as PIP1;2 from *A. comosus*, a terrestrial bromeliad (Fig. 1). Although AcoPIP1;2 has not been functionally characterized, other PIP1 members have been functionally determined as facilitators of water, glycerol, urea and CO_2 (Biela et al. 1999; Gaspar et al. 2003; Heckwolf et al. 2011). On the other hand, VgTIP2;1 grouped with TIP2 from dicots, such as TIP2;1 from tomato and Arabidopsis (Fig. 1). Other TIP2s including AtTIP2;1 have shown to be able to transport water, ammonia/ammonium and urea (Liu et al. 2003; Holm et al. 2005; Kirscht et al. 2016). Ohrui et al. (2007) cloned four PIPs from *Tillandsia ionantha*, an epiphytic bromeliad, and showed that they were important in facilitating water transport into the plant but to date, we have not found specific data related to the physiological role in nutrient transport of aquaporins of epiphytic bromeliads.

As shown by functional analysis in *Xenopus* oocytes expressing *V. gigantea* aquaporins, VgPIP1;1 and VgPIP1;2 channel water when associated to PIP2 subclass (Fig. 2). Several studies have shown that when expressed alone, some proteins belonging to PIP1 subclass do not increase the water permeability of oocyte membrane (Chaumont et al. 2000; Bots et al. 2005) since they are not able to reach the plasma membrane; unless they are co-expressed with members of PIP2 subclass (Fetter et al. 2004; Jozefkowicz et al. 2016). This was also observed when VgPIP1;1 and VgPIP1;2 were co-expressed with ZmPIP2;5 in oocytes. A synergistic increase in P_f was observed together with a relocalization of VgPIP1s from internal membranes to the plasma membrane (Fig. 3).

Despite the fact that PIP2s have not been isolated from *V. gigantea*, the results obtained with ZmPIP2;5 suggest that *V. gigantea* PIP2s may interact with VgPIP1;1 and VgPIP1;2 in order to allow its trafficking to the plasma membrane and consequently, increase cell membrane permeability. In fact, VgPIP1;1 and VgPIP1;2 show 92% and 89% sequence identity with ZmPIP1;1 and ZmPIP1;2, respectively, and this high similarity could account for a successful interaction between orthologous PIP1s and PIP2s, suggesting that changes on few residues in the protein sequence does not prevent the heteromerization of orthologous proteins. Thus, VgPIP1;1 and VgPIP1;2, besides having the conserved residues F-H-T-R at ar/R filter (Table 1, Fig S1) to

transport water, also need a posttranslational regulation through the association with PIP2 subclass to act as water channels. On the other hand, VgTIP2;1 showed a great water permeability (Fig. 2) and was able to reach the plasma membrane when expressed alone in oocytes (Fig. 3).

In order to verify whether *V. gigantea* aquaporins also facilitate the diffusion of non-aqua substrates, such as urea and NH_3/NH_4^+, we performed an *in silico*-analysis and functionally characterized them in heterologous expression systems. VgPIP1;1 and VgPIP1;2 exhibited highly conserved NPA motifs and ar/R filters, therefore their differentiate selectivity profiles to non-aqua substrates might be in part due to the residues at FPs. VgPIP1;1 and VgPIP1;2 showed conserved P2-P5 residues but at P1 position they displayed Q or E residues, respectively (Table 1). Interestingly, while in *in silico* analyses of VgPIP1;2 did not render any probable non-aqua substrate transport due to the residues present at P2 to P5 positions, its heterologous expression was able to restore growth of the ammonium uptake-defective yeast strain (31019b), indicating that VgPIP1;2 may be a NH_3/NH_4^+ facilitator (Fig. 6). Thus, our results suggest that substrate transport selectivity of VgPIP1s might be more related to the variability at P1 than at P2-P5. Even if NH_4^+ transport by aquaporins such as VgPIP1;2 may seem unusual because its charged nature, it was recently suggested that plant aquaporins might participate in the deprotonation of NH_4^+ and allow the NH_3 generated to permeate (Kirscht et al. 2016). Thus, N derived from ammonium may be transported through aquaporins with deprotonation capacity.

On the other hand, VgTIP2;1 has residues at ar/R and P2-P4 FPs that may be related to ammonia/ammonium transport (Jahn et al. 2004; Bertl and Kaldenhoff 2007). Nevertheless, VgTIP2;1 was unable to facilitate NH_3/NH_4^+ diffusion (Fig. 6) but was highly permeable to urea (Fig. 4 and 5). Several studies have shown that members from TIP subfamilies are urea transporters (Liu et al. 2003; Gu et al. 2012), suggesting a role in urea uptake, relocation, and vacuolar storage. Despite TIPs being mainly localized in the tonoplast, we wondered whether VgTIP2 could be also localized in the plasma membrane, as reported for other TIP isoforms (Gattolin et al. 2011), and in this way, not only mediate urea vacuolar storage but also its entry into the cell. However, VgTIP2;1 was partially located at the plasma membrane when YFP was fused to the N-terminal or C-terminal end of VgTIP2 and transiently expressed in tobacco cells (Electronic Suppl. Fig. S2).

In addition, urea can also be hydrolyzed by urease, generating ammonium and CO_2, which can be readily assimilated (Wang et al. 2008). Previous studies in *V. gigantea* showed a high proportion of urease in cell walls and membranes (Cambui et al. 2009), suggesting that aquaporins also might assist with NH_3/NH_4^+ and CO_2 diffusion into the

cells. The ammonium produced by urea hydrolysis can be assimilated into amino acids while CO_2 may be transported into the chloroplast to form organic molecules (Matiz et al. 2017) (Fig. 8). Despite not having been functionally tested, *in silico* analysis predicted that VgPIP1;1 transports CO_2 (Table 1). Several PIPs have been demonstrated to mediate CO_2 conductance in leaves (Uehlein et al. 2012; Heinen et al. 2014) and the signatures of CO_2 transporter appears to be all present in VgPIP1;1 protein sequence (Table 1). These results suggest that *V. gigantea* may have a very efficient system not only for transporting urea but also for its hydrolysis products, NH_3/NH_4^+ and CO_2 (through VgPIP1;1 and VgPIP1;2, respectively – see Fig. 8).

In order to gain further information on nitrogen metabolism and its relationship to aquaporins, gene expression analyses of *VgPIP1;1, VgPIP1;2 and VgTIP2;1* were conducted in juvenile (atmospheric) and adult plants of *V. gigantea* (Fig. 7). By comparing the transcript abundance of VgMIPs in urea- and ammonium-treated plants, we found that *VgPIP1;1* and *VgPIP1;2* (which were shown to be water and maybe ammonium/ammonia transporters) were predominantly expressed in juvenile plants in both treatments, whereas *VgTIP2;1* (which is a urea transporter) was highly expressed in adult-tank plants treated with urea. Therefore, the differential expression of *VgPIP1s* and *VgTIP2;1* genes in the atmospheric and adult-tank plants suggest that their encoding proteins may be directly associated with the form of N (ammonium or urea) that different ontogenetical stages of *V. gigantea* are exposed to. Accordingly, inorganic nitrogen sources, such as ammonium are obtained by epiphytic bromeliads mainly through the rainfall and mist, whilst organic nitrogen, such as urea, is mostly derived from inputs by animals (e.g. excreta of frogs) (Benzing 2000; Stewart et al. 1995; Hietz et al. 1999).

Thus, adult-tank plants of *V. gigantea* may be more likely to receive urea at high concentrations once its tank allows sustaining biological association with animals that use these plants as a shelter. Moreover, nutrient absorption and storage above immediate requirements is a behavior known as “luxury consumption” that is observed in epiphytic bromeliads, such as *V. gigantea* (Gonçalves et al. 2016b). Thus, the excess of nutrients can be used to support growth when the availability of nutrients is scarce (Chapin 1980). As a result, *VgTIP2* up-regulation in adult plants treated with urea may indicate that these plants are effectively absorbing and storing urea into the vacuoles for future usage (Fig. 7 and 8) as well as efficiently compete with mineralizing micro-organisms present in the tank for urea uptake since the highest expression of VgTIP2;1 is observed in the basal part of the leaves in relation to the apex (Electronic Suppl. Fig. S3).

On the other hand, juvenile stages that lack a tank are unable to hold large quantities of nutrients coming from animals, being more dependent on atmospheric

deposition and rainfall inputs, which seemingly consist of predominantly inorganic nitrogen sources (Stewart et al. 1995). Inselsbacher et al. (2007) and Cambui et al. (2009) suggested that at low concentrations of urea, *V. gigantea* plants hydrolyze it outside the leaves (either by releasing plant ureases or through microorganism action) and, consequently, absorb the ammonia/ammonium produced rather than taking up urea directly. Alongside with these pieces of evidence, we observed an up-regulation of *VgPIP1;1* and *VgPIP1;2* (in both urea and ammonium-treated plants) which may indicate that atmospheric plants absorb the products of urea hydrolysis, NH_3/NH_4^+ and CO_2, instead of the intact molecule, while adult plants may directly uptake urea when present at high concentrations (Fig. 7). Based on our results, we propose an initial model (Fig. 8). Although informative, this model needs additional investigation to clarify whether VgPIP1;1 can facilitate CO_2 transport, whether channel activities of VgMIPs are in accordance with dual localizations of VgTIP2;1 (plasma membrane and tonoplast) and VgPIP1;1 (plasma membrane and chloroplast), and whether other potential MIPs and/or permeases play an additional role in nitrogen transport processes in *Vriesea*.

Finally, it is important to highlight that urea has been increasingly characterized as a very important source of organic nitrogen and carbon for plants. Moreover, it is frequently employed as a low-cost fertilizer for several crops. However, more than half of the nitrogen applied in agriculture is not absorbed by plants resulting in pollution of waterbodies and atmosphere. Therefore, in a scenario in which plants were more efficiently to absorb and assimilate this organic source of nitrogen, urea application could be substantially reduced. A way to achieve such scenario could be through the inclusion of MIPs with high rates of N transport, such as VgTIP2;1 into crops. Consistent with this, it is of great importance to know the elements involved in these processes in plants that naturally receive urea, such as *V. gigantea*. Our results revealed that VgPIP1;1, VgPIP1;2 and VgTIP2;1 may play a vital role on nitrogen metabolism.

Conclusions

We cloned three aquaporins, *VgPIP1;1, VgPIP1;2* and *VgTIP2;1* from *Vriesea gigantea*, a tank-forming epiphytic bromeliad with high capacity to absorb urea. All these proteins were able to transport water but PIP1s needed to be co-expressed with PIP2s in order to be targeted to the plasma membrane. Besides water, *VgTIP2;1* was capable of transporting urea, while *VgPIP1;2* appears to facilitate NH_3/NH_4^+ transport. Since the presence of the tank changes the physiological characteristics of the plant, it seems that young atmospheric plants preferably transport urea hydrolysis products, such as ammonium/ammonia, while adult-tank plants prefer taking up urea, as evidenced through differential recruitment of *VgPIP1s* and *VgTIP2;1* genes in response to

ammonium and urea treatments. Thus, VgTIP2;1 probably regulates the urea stock in the vacuole and consequently, may act on “luxury consumption” processes in adult-tank plants. Therefore, the data currently available suggest that, depending on the N source and plant ontogenetic state, *V. gigantea* can modulate aquaporin expression to maximize absorption of the most available nitrogen source.

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Table and figure captions

Table 1 Prediction of subcellular localization and non-aqua substrate transport according to specificity determining positions (SDPs) in NPA regions, ar/R selectivity filter and FP (P1-P5) of V. gigantea aquaporins. LB, Loop B; LE, Loop E; H2, Helix 2; H5, Helix 5; PM, Plasma membrane; VM, Vacuolar membrane; PSCL, prediction of subcellular localization by LocTree. Parenthesis indicates the percentage of expected accuracy

Fig. 1 Phylogenetic analysis of the deduced amino acid sequences of *V. gigantea* aquaporins with PIPs and TIPs members of other plants. Deduced amino acid sequences were aligned using Clustal W. Naming of *V. gigantea* aquaporins were done based on sequence similarities and phylogenetic relation with orthologous genes from *A. thaliana* (At), *S. licopersicum* (Sl), *Z. mays* (Zm), *A. comosus* (Aco) and the monilophyte *P. patens* (Pp). PIP1s and TIP2s subfamilies are denoted by gray and blue areas, respectively. The robustness of the nodes is denoted with bootstrap support in percentage and nodes with less than 50% support are collapsed

Fig. 2 Osmotic water permeability coefficient of *X. laevis* oocytes expressing *V. gigantea* aquaporins. The oocytes were injected with water, 12.5 ng of Vg*PIP1;1* or Vg*PIP1;2* cRNA, or 4 ng of Vg*TIP2;1* or *ZmPIP2;5* cRNA. The analyses were performed three days after the injections. The results are expressed as the mean ± SD of 10 to 15 cells from one of three independent assays. Asterisks indicate significant differences between oocytes injected with water (negative control) and oocytes injected with aquaporin cRNAs (Student's t-test; $P < 0.05$)

Fig. 3 Confocal microscopic images of oocytes showing the localization of YFP-VgPIP1;1, YFP-VgPIP1;1 or YFP-VgTIP2;1. The oocytes were injected with water (**a**), 25 ng of YFP–*VgPIP1;2* (**b**) or YFP–*VgPIP1;1* (**c**), or 8 ng of YFP–*VgTIP2* cRNA (**d**). Additionally, 4 ng of *ZmPIP2;5* was injected along with 25 ng of YFP–*VgPIP1;2* (**e**) or YFP–*VgPIP1;1* (**f**). The analyses were performed three days after the injections. Bars=100 µm

Fig. 4 Urea transport assay in *X. laevis* oocytes expressing *V. gigantea* aquaporins. The oocytes were injected with water, 12.5 ng of *rAQP9* (rabbit aquaporin, positive control), Vg*PIP1;1* or Vg*PIP1;2* cRNA, 5 ng of Vg*TIP2;1* or 6 ng of *ZmPIP2;5* cRNA. The analyses were performed three days after the injections when oocytes were incubated in an iso-osmotic solution supplemented with $37x10^3$ Bq. mL^{-1} [^{14}C]-urea. The results are expressed as the mean ± SD of 10 cells from a pool of two independent assays. Asterisks indicate significant differences between oocytes injected with water (negative control) and oocytes injected with aquaporin cRNAs (Student's t-test; $P< 0.05$)

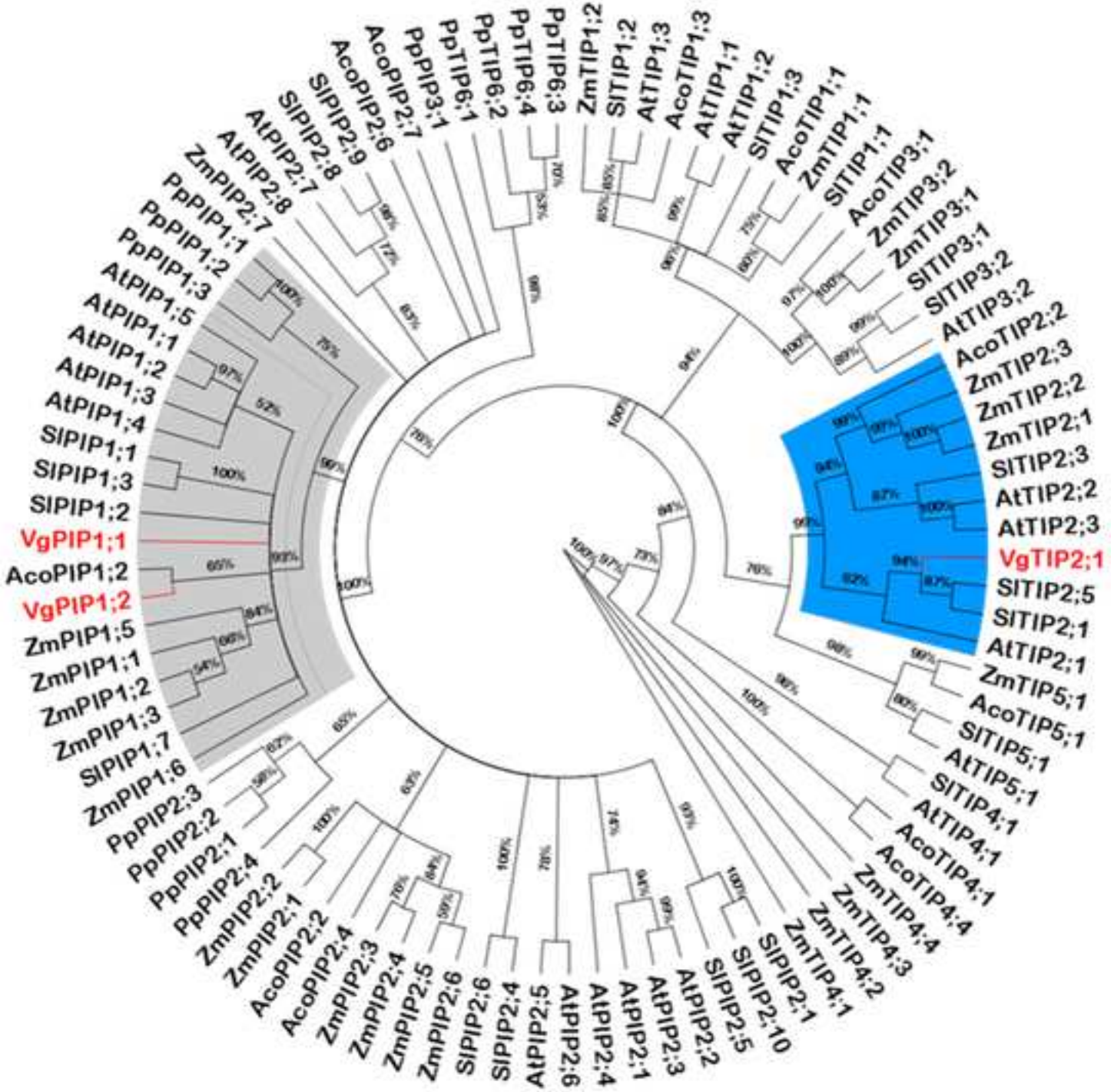
Fig. 5 Urea transport assay in *S. cerevisiae* transformed with *V. gigantea* aquaporins. The urea uptake-defective yeast YNVW1 (Δ*dur3* and *ura3*) or WT strain were transformed with the empty vector pYeDP60u or pYeDP60u containing the indicated *AQP* cDNA. Transformants were spotted at an OD_{600} of 10^{-2} or 10^{-5} on medium containing 2, 4, 5 or 20 mM of urea as the sole nitrogen source at pH 5.5. Growth was recorded after 8 days

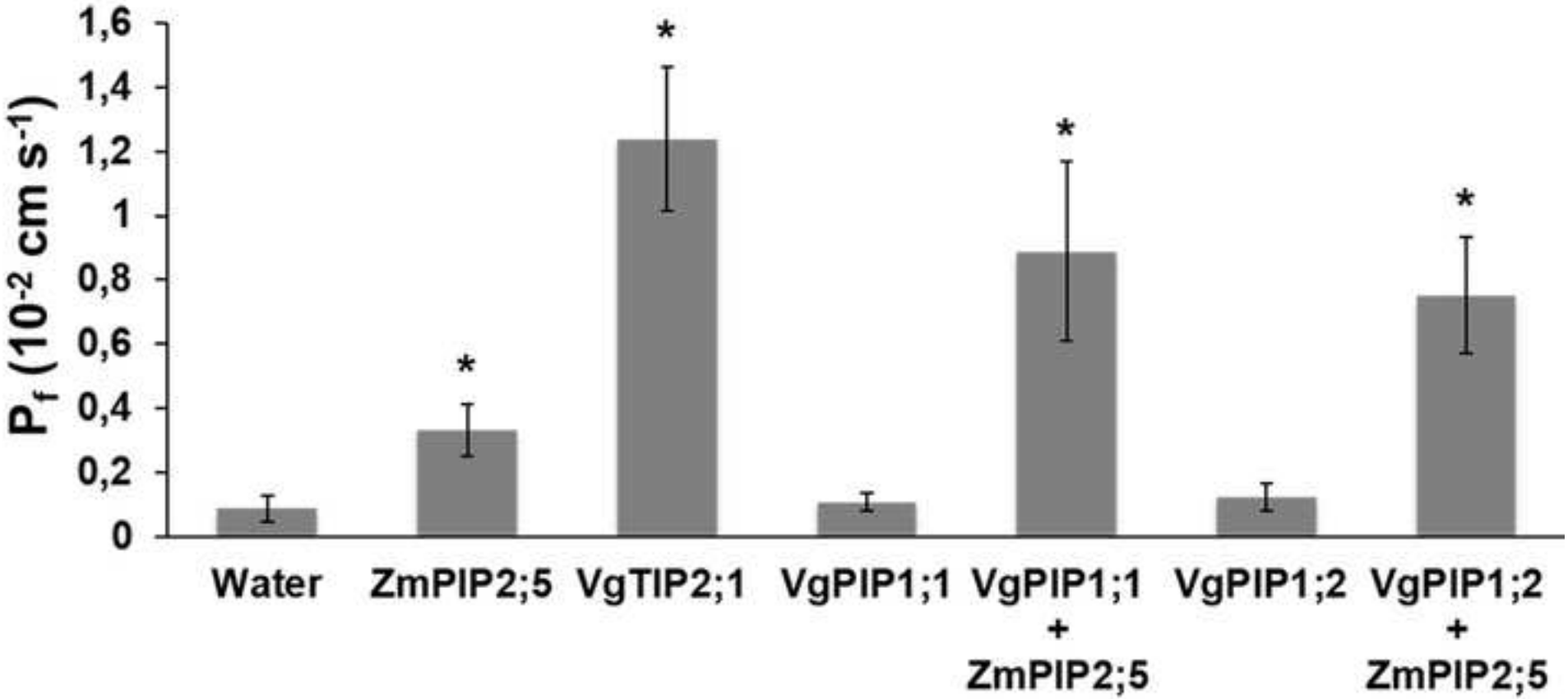
Fig. 6 NH_4^+/NH_3 transport assay in *S. cerevisiae* transformed with *V. gigantea* aquaporins. The ammonium uptake-defective yeast 31019b (Δ*mep1-3* and *ura3*) or WT strain were transformed with the empty vector pYeDP60u or pYeDP60u containing the indicated *AQP* cDNA. Transformants were spotted at anOD_{600} of 10^{-2} or 10^{-5} on medium containing 1, 2, 5 or 10 mM of $(NH_4)_2SO_4$ as the sole nitrogen source at pH 5.5. Growth was recorded after 10 days

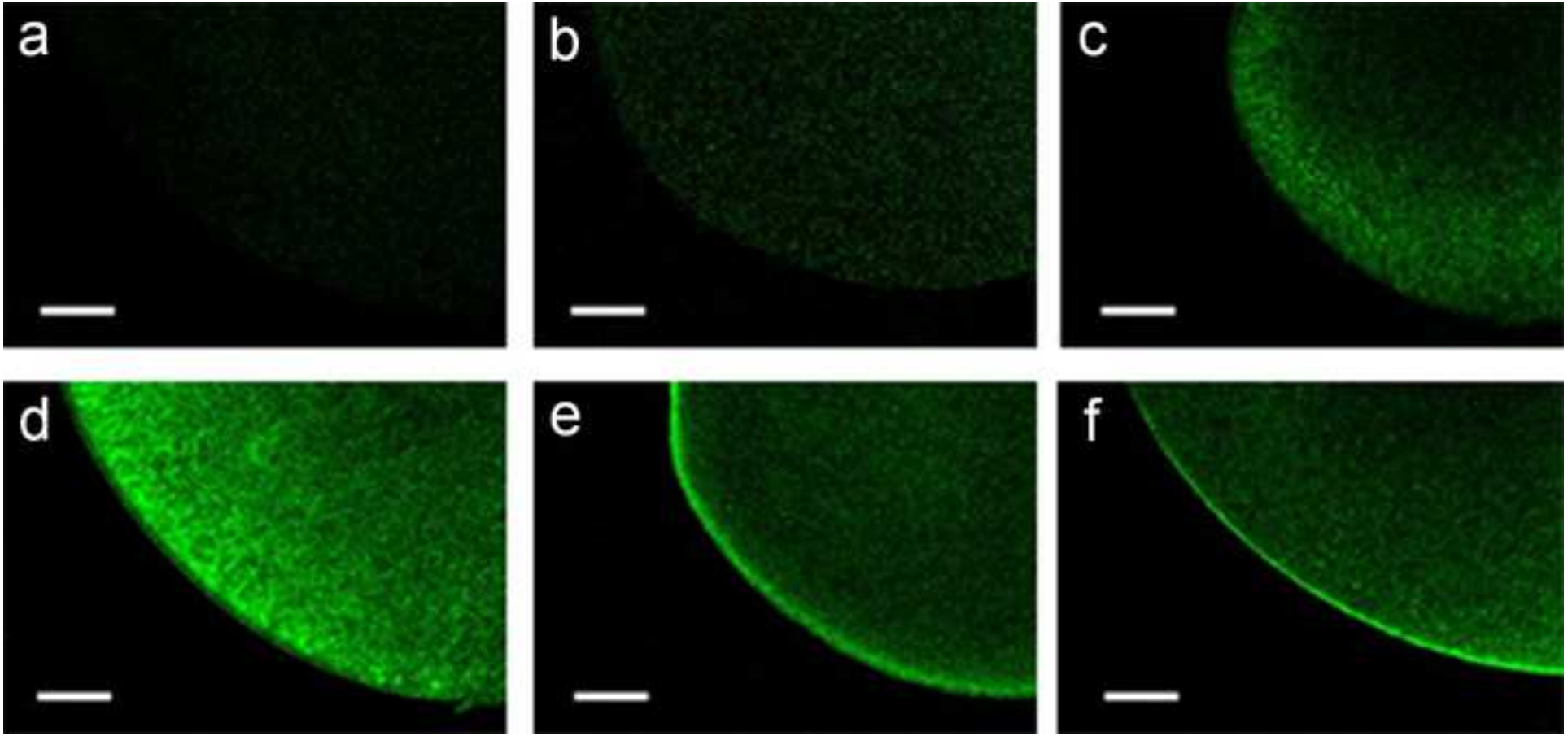
Fig. 7 Relative transcript abundance of *VgPIP1;1, VgPIP1;2* and *VgTIP2;1* genes in the apical (**a**) and basal (**b**) part of the leaves. Values shown are mean ± SE. The mean relative expression was calculated from means of two technical replicates of at least three biological replicates and normalized against N-free treatment (control) in atmospheric and adult developmental stages. Different letters indicate significant statistic differences (Tukey's test, $P< 0.05$) between the treatment and the control at each developmental stage

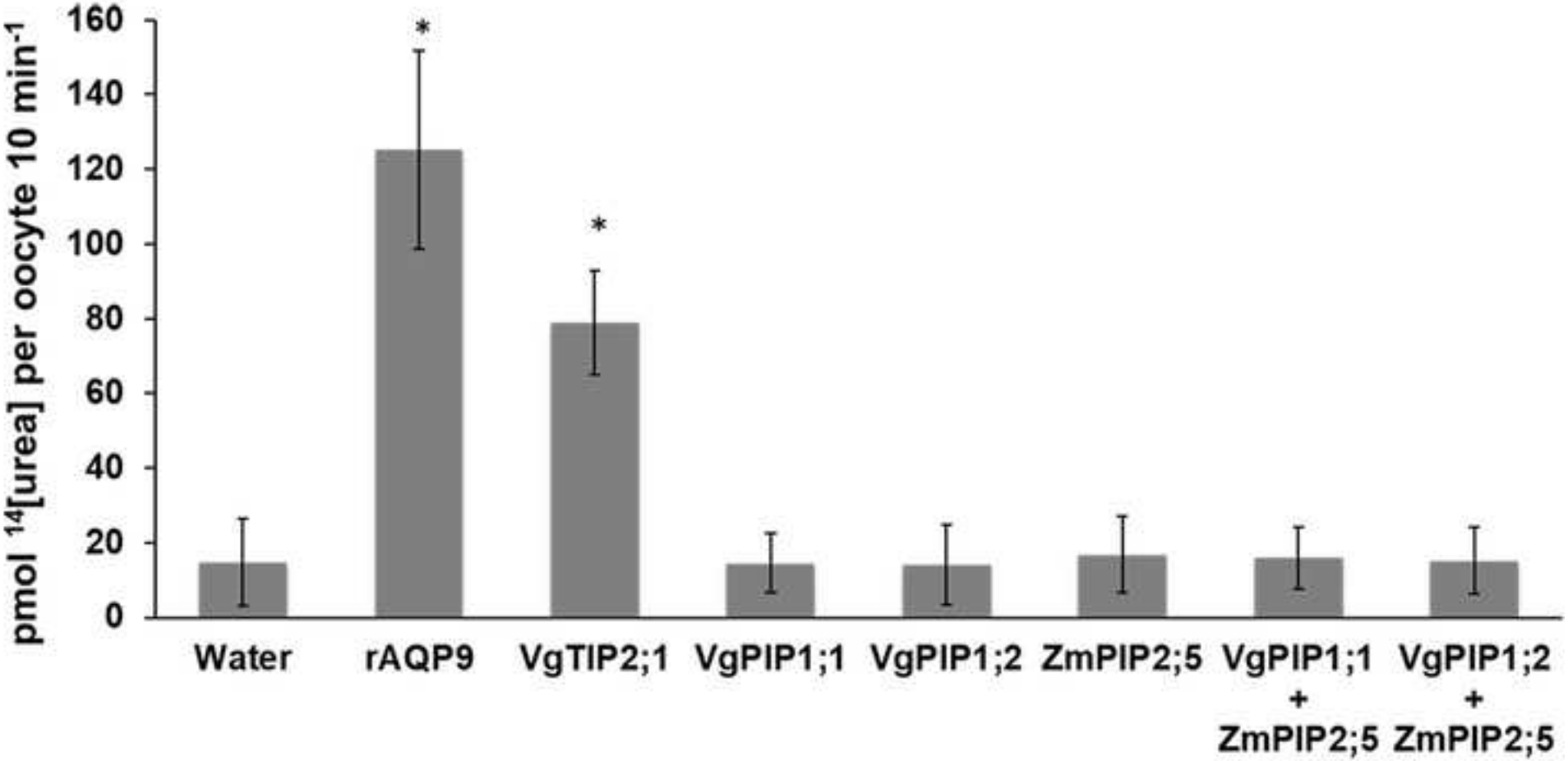
Fig. 8: Hypothetical diagram of urea transport in *V. gigantea* focused on the functional characterization of VgTIP2;1, VgPIP1;1, VgPIP1;2. The main N input in atmospheric plants seems to come from rain, while the presence of the tank in adult plants allows the association with animals that result in inputs of organic nitrogen sources, such as urea. Urea may be transported through VgTIP2;1 and accumulated in the vacuole for future

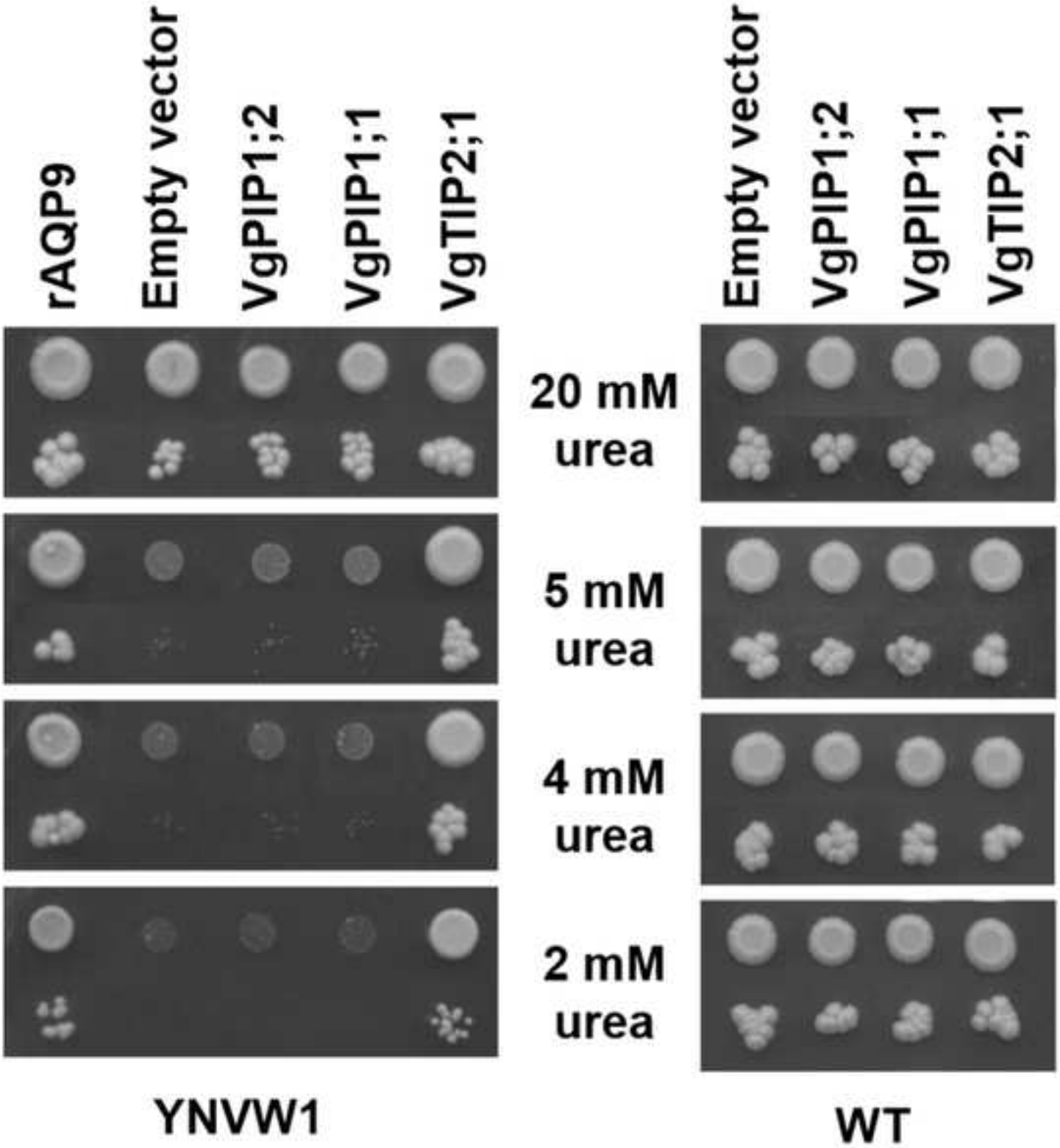
use, particularly in adult plants. Urea hydrolysis (curved arrows) generates NH_3/NH_4^+ and CO_2. In atmospheric plants, ammonia/ammonium is potentially transported by VgPIP1;2 while, based on *in silico* analysis, CO_2 might be transported by VgPIP1;1 (illustrated by the question mark). VgTIP2;1, VgPIP1;1, VgPIP1;2 are capable of transporting water (blue arrows) whenever PIP1s are associated with PIP2s. Further analyses are required to determine whether other symport-type transporters/permeases may be involved in urea and NH_3/NH_4^+ transport processes (dashed arrows and barrels) as well as the dual localization of VgPIP1;1 and VgTIP2;1. Dashed squares indicate non-canonical localization of VgPIP1;1 and VgTIP2;1. Gray square represents the plant plasma membrane. Black arrows indicate transport of non-aqua substrates. AA indicates the amino acids produced by ammonium assimilation. The scale bars in the pictures correspond to 1 and 10 cm for the atmospheric and adult-tank plants, respectively

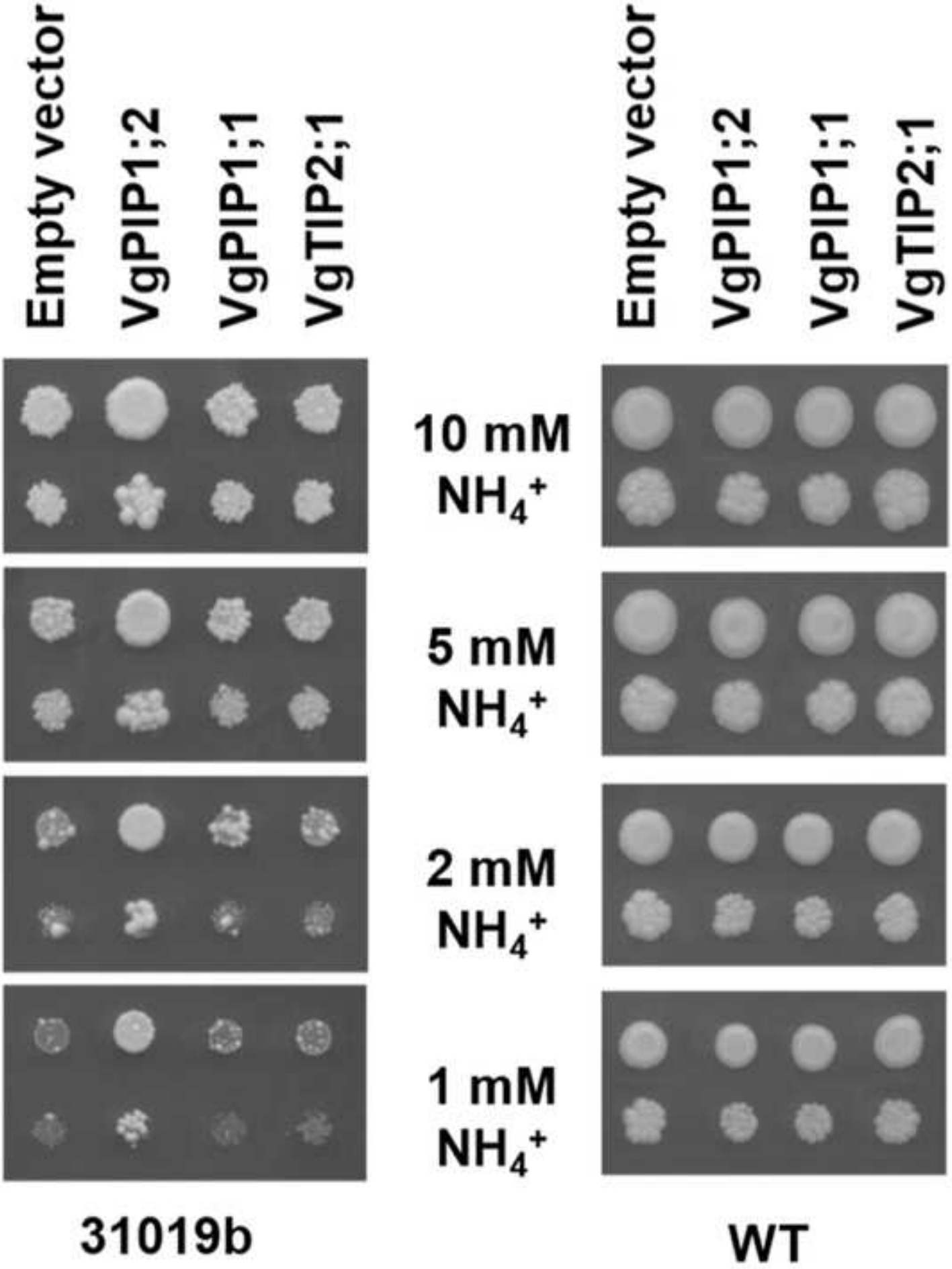


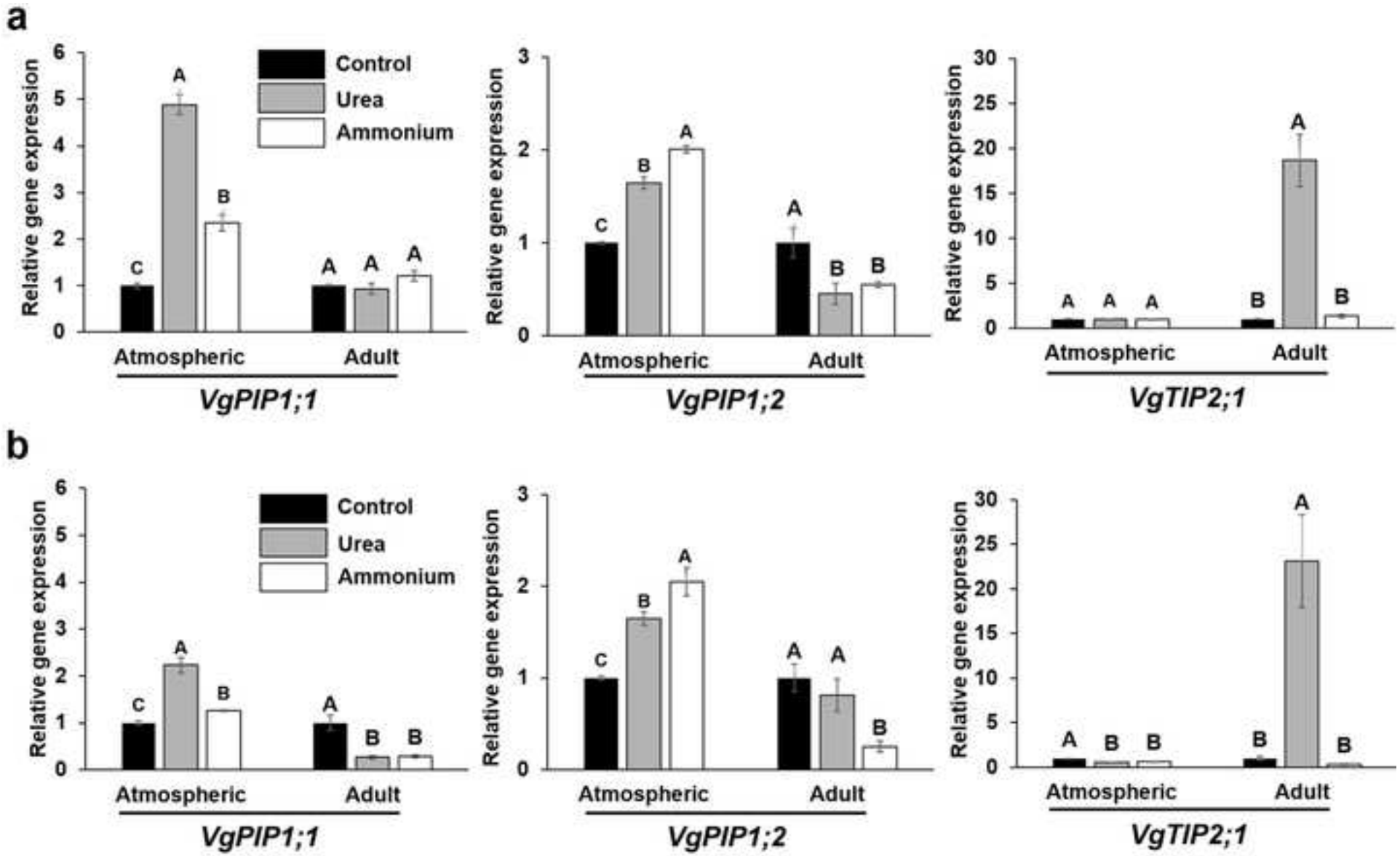












Figure

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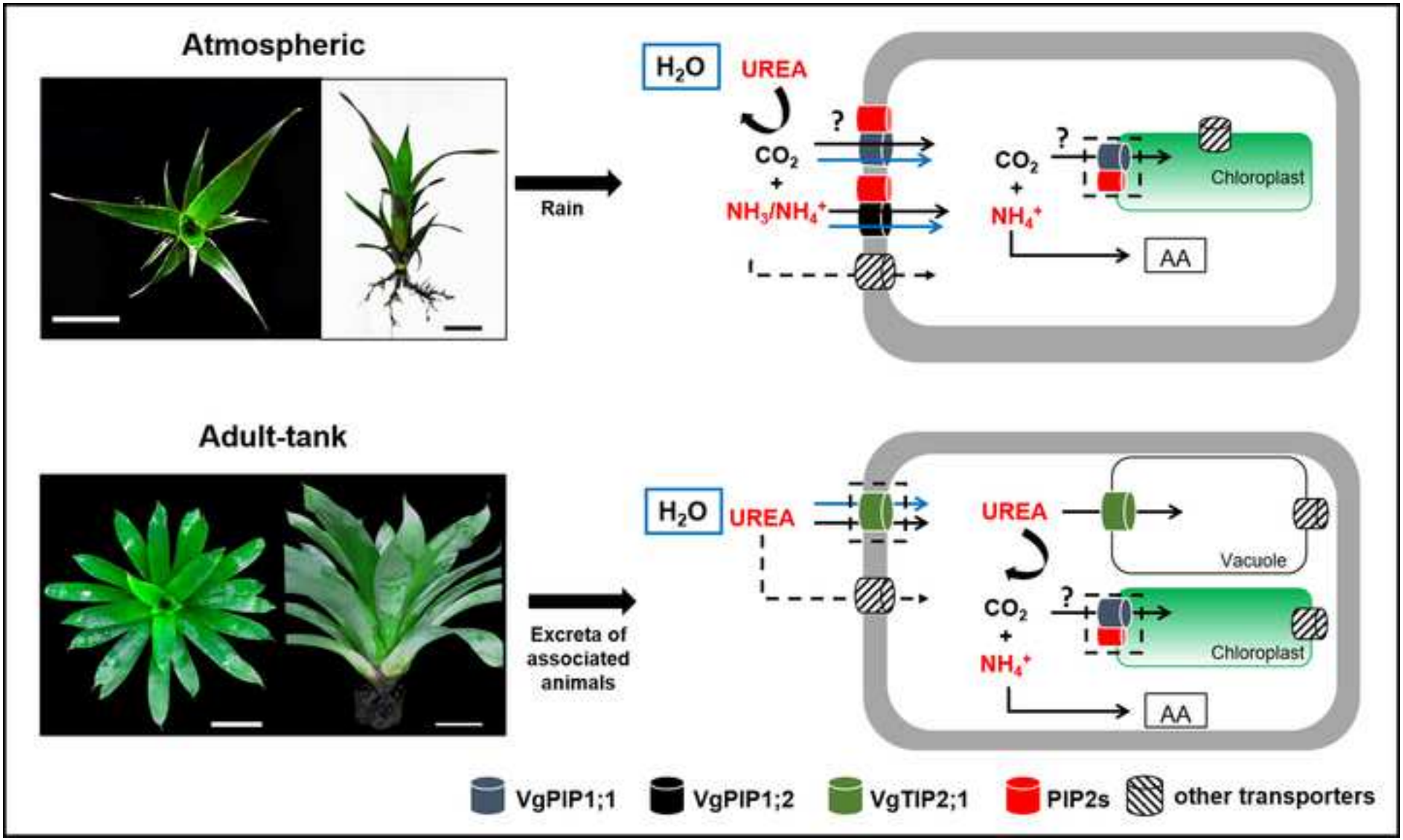

Table

MIPs	Signature sequences											Substrate	PSCL
	NPA		Ar/R				FPs						
	LB	LE	H2	H5	LE		P1	P2	P3	P4	P5		
VgPIP1;1	SGGHI (NPA) VT	GTGI (NPA) RSLG	F	H	T	R	Q	S	A	F	W	H_2O_2, CO_2, boron, urea	PM (97%)
VgPIP1;2	SGGHI (NPA) VT	GTGI (NPA) RSLG	F	H	T	R	E	S	A	F	W	None	PM (97%)
VgTIP2;1	SGGHV (NPA) VT	GGSM (NPA) RSFG	H	I	G	R	T	S	A	Y	W	H_2O_2, ammonia, urea	VM (95%)

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