Planta (2001) 212: 547–555

Planta
© Springer-Verlag 2001

Plasma-membrane H^+-ATPases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco

Chung-Il An, Ei-Ichiro Fukusaki, Akio Kobayashi

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Received: 8 June 2000 / Accepted: 8 August 2000

Abstract. *Nepenthes* is a unique genus of carnivorous plants that can capture insects in trapping organs called pitchers and digest them in pitcher fluid. The pitcher fluid includes digestive enzymes and is strongly acidic. We found that the fluid pH decreased when prey accumulates in the pitcher fluid of *Nepenthes alata*. The pH decrease may be important for prey digestion and the absorption of prey-derived nutrients. To identify the proton pump involved in the acidification of pitcher fluid, plant proton-pump homologs were cloned and their expressions were examined. In the lower part of pitchers with natural prey, expression of one putative plasma-membrane (PM) H^+-ATPase gene, *NaPHA3,* was considerably higher than that of the putative vacuolar H^+-ATPase (subunit A) gene, *NaVHA1,* or the putative vacuolar H^+-pyrophosphatase gene, *NaVHP1*. Expression of one PM H^+-ATPase gene, *NaPHA1*, was detected in the head cells of digestive glands in the lower part of pitchers, where proton extrusion may occur. Involvement of the PM H^+-ATPase in the acidification of pitcher fluid was also supported by experiments with proton-pump modulators; vanadate inhibited proton extrusion from the inner surface of pitchers, whereas bafilomycin A_1 did not, and fusicoccin induced proton extrusion. These results strongly suggest that the PM H^+-ATPase is responsible for acidification of the pitcher fluid of *Nepenthes*.

Key words: *Nepenthes* (PM H^+-ATPase) – Pitcher fluid – Acidification (*Nepenthes* pitchers) – Proton pump – Plasma-membrane H^+-ATPase

This study represents a portion of the dissertation submitted by C.-I. An to Osaka University in partial fulfillment of the requirement for a Ph.D. degree

Abbreviations: FC = fusicoccin; H^+-PPase = vacuolar H^+-pyrophosphatase; MS = Murashige and Skoog; PM H^+-ATPase = plasma-membrane H^+-ATPase; RACE = rapid amplification of cDNA ends; RT-PCR = reverse transcription-polymerase chain reaction; V-ATPase = vacuolar H^+-ATPase

Correspondence to: A. Kobayashi;
E-mail: kobayashi@bio.eng.osaka-u.ac.jp; Fax: +81-6-6879-7424

Introduction

Carnivorous plants inhabit nutrient-poor environments, where they capture small animals, mainly insects, to supplement their nutrient demand. They produce special trapping organs for prey capture. The tropical carnivorous plant *Nepenthes* develops trapping organs called pitchers at the tips of tendrils (Owen and Lennon 1999). The inner surface of each pitcher is divided into two distinct parts: the upper part is coated with wax, and the slippery inside prevents prey from escaping, while the lower part is covered with multicellular glands that are thought to secrete acid and digestive enzymes (Clarke 1997), and also to be involved in the absorption of insect-derived nutrients (Lüttge 1971). The pitchers have a reservoir of acidic fluid at the bottom, and the fluid includes several digestive enzymes. In early studies, no significant amounts of the commonly occurring organic acids were detected in the pitcher fluid (Morrissey 1955); however, chloride ions were present at a concentration of ca. 20 mM (Nemček et al. 1966). These findings suggest that the acidity of pitcher fluid is due to HCl. The acidification of pitcher fluid is thought to play an important role in digestion and absorption of prey based on the following facts: (i) the pH of pitcher fluid is reduced when prey accumulates in the fluid (Lüttge 1964), (ii) the optimum pH of proteolytic enzyme in pitcher fluid is around pH 2–3 (Steckelberg et al. 1967; Nakayama and Amagase 1968; Athauda et al. 1998), and (iii) proton gradients across the plasma membrane are generally used to drive uptake of many solutes (ions, metabolites, etc.) (Michelet and Boutry 1995). Recently, the expression of transporter genes for nitrogenous compounds (ammonium, amino acids and peptides) has been reported in the pitchers of *Nepenthes alata* (Schulze et al. 1999). The mRNA of the ammonium transporter has been shown to be localized in the head cells of the digestive glands. It was reported that the transport of ammonium depends on the proton motive force (Ninnemann et al. 1994). Thus, these findings also suggest that the acidification of pitcher fluid is important for nutrient uptake in the pitchers. Since pitcher fluid

corresponds to apoplastic fluid, a plasma membrane proton pump is thought to be involved in the acidification of pitcher fluid. It has been reported that plant cells have three types of proton pump, namely, the plasma membrane H^+-ATPase (PM H^+-ATPase), the vacuolar H^+-ATPase (V-ATPase), and the vacuolar H^+-pyrophosphatase (H^+-PPase) (reviewed by Sze et al. 1999). To identify the proton pump involved in the acidification of pitcher fluid, we cloned homologous genes of these proton pumps and examined their expressions using *Nepenthes alata* as a model carnivorous plant. To collect information about the proton pump, we also examined the effects of two proton-pump inhibitors and one activator on the proton release from the pitcher's inner surface.

Materials and methods

Plant growth conditions

Nepenthes alata plants were obtained from a commercial plant distributor (Nishijima Engei Danchi, Japan). Plants were grown in a greenhouse at 25 °C, 60% humidity, and illuminated for 14 h daily at 40 μmol photons m^{-2} s^{-1}. Plants were watered daily with tap water. Outdoor plants used for experiments were grown in a garden on the campus under natural daylight.

Measurement of pitcher fluid pH of outdoor plants

Pitcher fluid samples were collected from new pitchers (7–10 d after lid opening) and old pitchers (5 weeks after lid opening). pH was measured with a Compact pH meter (B-212; Horiba, Kyoto, Japan).

Measurement of pH and ammonium concentration of pitcher fluid after fruit-fly feeding

Fruit-fly (*Drosophila melanogaster* Meigen) stocks were maintained at room temperature using standard honey-yeast-agar medium. Active flies were captured with a net and immediately stored at −80 °C until use. About 20 flies were fed to each pitcher within 3 d after lid opening. A cotton ball was placed in the opening of the pitcher to prevent other insects from slipping in. pH was measured with the Compact pH meter. Ammonium concentration was monitored by an enzymatic method (Ishihara et al. 1972).

cDNA cloning of plant proton-pump homologs

Amino acid sequences of known proton pumps from other plants, fungi and animals were used to design degenerate primers for reverse transcription-polymerase chain reaction (RT-PCR) cloning of homologous *Nepenthes* genes. The following degenerate primers were used (Y = C or T, R = A or G, M = A or C, N = A or C or G or T, I = inosine). Primers for the PM H^+-ATPase were: 5′ GGI ATG GAY GTI YTI TGY TCN (forward primer), 5′ T YTG YTC IGG IGC NCC YTT (reverse primer). Primers for the V-ATPase (subunit A) were: 5′ GTI GCI GCN MGN GAR GC (forward primer), 5′ C IGG RTC IGA RAA RTC NCC (reverse primer). Primers for the H^+-PPase were: 5′ GCI GAY AAY GTI GGN GAY AAY G (forward primer), 5′ C IGC IGC RTC IAR NGC RTC (reverse primer). The following sequences were used to design degenerate primers (accession numbers of Swiss-Prot and TrEMBL are shown). The PM H^+-ATPase sequences were: P05030, P19657, P24545, P49380, P28877, Q07421, P07038, P09627, P28876, P23980, P22180, Q08436, Q08435, P20649, P19456, P20431, Q03194, P54210, P54211, P54679; the V-ATPase (subunit A) sequences were: O16109, P31400, P48602, P31404, Q29048, P50516, P38606, Q40002, P49087, O23654, Q39291, P09469, P31405, P13548, Q39442, P48414, Q03498, Q26975, P38078, P11592, P17255, P31406; and the H^+-PPase sequences were: Q06572, P93410, P93409, P21616, O22124, Q43797, Q42651, Q43798, Q43801, Q42650, P31414, O68460. Total RNA was isolated from the lower part of pitchers by the hot-borate method (Wan and Wilkins 1994). The total RNA was incubated with DNase and then reverse-transcribed by AMV Reverse Transcriptase (Toyobo, Osaka, Japan) with oligo $(dT)_{12-18}$ primer (Gibco BRL, Rockville, USA). All PCR reactions were cycled 50 times for 30 s at 94 °C, 1 min at 50 °C and 2 min at 72 °C. Amplified cDNA was subcloned into the plasmid pCR 2.1 (Invitrogen, Groningen, Netherlands) and sequenced according to the instruction of the sequencing kit (ABI PRISM BigDye Terminator Cycle Sequencing Kits; PE Biosystems, Foster City, Calif., USA) using an ABI PRISM 310 Genetic Analyzer (PE Biosystems).

Northern hybridization analysis

Total RNA was isolated from roots, stems, leaves, tendrils and pitchers by the hot-borate method. Equal amounts of RNA (15 μg total RNA) were separated on 1% agarose gels containing 2.2 M formaldehyde and blotted onto nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Bucks., UK). The cloned cDNAs of the plant proton-pump homologs (*NaPHA3*, *NaVHA1* and *NaVHP1*) were used as probes (the probe regions are indicated by gray lines in Fig. 2). Hybridization and washing were carried out with AlkPhos Direct (Amersham Pharmacia Biotech) following the manufacturer's instructions. CDP-Star (Amersham Pharmacia Biotech) was used for signal detection. Results were reproduced in an independent experiment.

Isolation of 3′-untranslated regions of PM H^+-ATPase genes

Gene-specific probes for PM H^+-ATPase genes were obtained by PCR amplification of the 3′-untranslated regions. The 3′-untranslated regions were obtained by 3′-Full RACE Core Set (TaKaRa, Kyoto, Japan). The following primers were used for PCR amplification. Primers for *NaPHA1* were: 5′ GGAAACTTTCATTCCCGCTACA (forward primer) and 5′ TGCAGCATAGTTTGAAAGAACGC (reverse primer). Primers for *NaPHA3* were: 5′ TTCTTTTTCCTCTTTGTTTGGGG (forward primer) and 5′ ACCAGTAAACGCAGAGTGACAGG (reverse primer). Primers for *NaPHA4* were: 5′ GAGTCTGAAGGTTCTTCAGCATG (forward primer) and 5′ TTTACTCTCACCACATTCCATTC (reverse primer). All PCR reactions were cycled 35 times for 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C. Subcloning and sequencing were carried out as described above.

Expression analysis by in situ hybridization

In situ hybridization to mRNA was performed as in Lincoln et al. (1994). Newly opened *Nepenthes* pitchers were harvested, rinsed quickly in distilled water and then fixed immediately in phosphate-buffered 4% paraformaldehyde under vacuum. After fixation, the tissue was dehydrated in serial dilutions of ethanol and embedded in Paraplast Plus (Sigma, St. Louis, Mo., USA). Sections (4 μm) were mounted on pre-coated slides (Micro Slide Glass; Matsunami, Osaka, Japan). Slides were de-waxed, re-hydrated in increasing dilutions of ethanol, and protein was digested in proteinase K (1 μg/ml) at 37 °C for 30 min. After washing the slides in 0.2%

phosphate-buffered glycine solution and phosphate-buffered saline (PBS: 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 130 mM NaCl, pH 7.0), the tissue was re-fixed in 4% paraformaldehyde. Positive charges were masked by treatment with 16 mM acetic anhydrate in 0.1 M triethanolamine buffer. Digoxigenin-labeled sense and antisense transcripts of 3′ gene-specific regions were used for hybridization at a concentration of 2.5 ng μl^{-1} kb^{-1} in a volume of 200 µl per slide. The probe was detected by an alkaline-phosphatase-coupled anti-digoxigenin-antibody and visualized by the fluorescent dye 2-hydroxy-3-naphthoic acid-2′-phenylanilide phosphate (HNPP) and FastRed TR (Roche Molecular Biochemicals, Mannheim, Germany). Fluorescence was viewed under blue excitation (excitation filter: BP470–490, absorption filter: BA510) with a fluorescence microscope (BX-FLA; Olympus, Tokyo, Japan).

Induction of proton release from the inner surface of the lower part of pitchers by administration of ammonium ions

Pitcher sections (approx. 1 cm^2) of the lower part of new pitchers (within 3 d after lid opening) were used for experiments. The sections, which were placed inner surface down, were floated on the acclimation fluid [20 mM KCl, pH 4.4, supplemented with inorganic salts (1/1000 MS medium; Murashige and Skoog 1962)] and then incubated for 24 h in a chamber at 25 °C, 60% humidity under 40 µmol m^{-2} s^{-1} illumination for 14 h. We employed the cap of a 1.5-ml microcentrifuge tube (QSP, Fairburn, USA) as a cup to contain the acclimation fluid. The acclimation fluid (200 µl) supplemented with 10 mM NH_4Cl was loaded into the cavity at the back of the cap and the acclimated pitcher sections were mounted in the fluid. Acclimation fluid without NH_4Cl was used as a control. Fluid pH was measured at 6 h using a Φ320 pH Meter (Beckman Coulter, Fullerton, Calif., USA) with calomel electrodes (5 mm × 178 mm). Proton concentration was calculated from the pH value of the fluid.

Effects of proton pump modulators on proton release

Two proton pump inhibitors (vanadate and bafilomycin A_1) and one activator (fusicoccin) were employed as proton-pump modulators. Each of the modulators was tested under the same conditions as mentioned above.

Results

Determining the pH of the pitcher fluid of outdoor Nepenthes

Fluid samples were collected from pitchers where prey is naturally captured and immediately used for pH measurement. The fluid of new pitchers (7–10 d after lid opening) was around pH 3 (Table 1). However, the fluid of the closed pitchers had a higher pH (ca. pH 4.9), and

Table 1. Pitcher fluid pH of outdoor *Nepenthes* plants. Three new pitchers (7–10 d after lid opening) and two old pitchers (5 weeks after lid opening) were used for pH measurement. The numbers in the second row indicate individual pitchers. All pitchers had captured a large quantity of insects (mainly ants)

	New pitchers			Old pitchers	
	1	2	3	1	2
pH	2.9	3.3	2.8	7.0	7.8

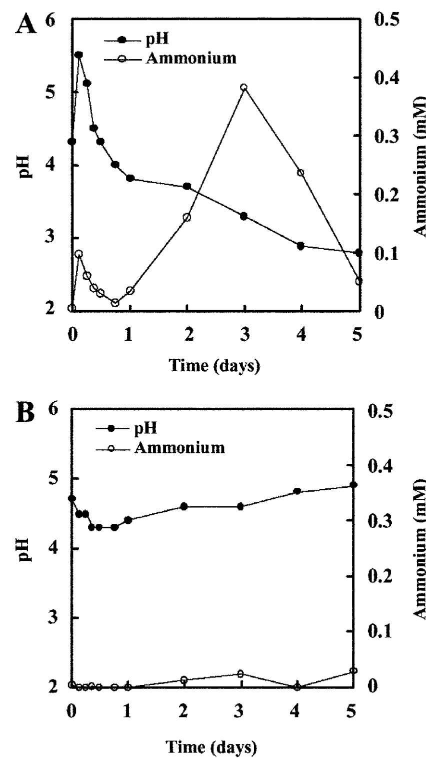


Fig. 1. Time courses of pH and ammonium concentrations of pitcher fluid after feeding on fruit flies **A** or not feeding **B**. The results are representative of three independent experiments

the pH of fluid obtained from young pitchers immediately after lid opening was ca. 4.4. These levels suggest that the fluid pH of new pitchers is lowered in response to prey capture. In contrast, old pitchers (5 weeks after lid opening) had higher pH values (> pH 7) and clearly could not sustain the low pH (Table 1).

Time course of pitcher-fluid pH after feeding on fruit flies

As shown in Fig. 1A, the pH of the pitcher fluid became rapidly elevated after flies were fed to the pitcher, and then declined to the original level within 12 h. The pH continued to decline during the period of the experiment and finally reached pH 2.8 at 5 d after feeding.

Sequence analysis of Nepenthes proton-pump homologs

Among the cloned products, six PM H^+-ATPases (*NaPHA1-6*), one V-ATPase subunit A (*NaVHA1*), and one H^+-PPase (*NaVHP1*) were identified. Six homologs of the PM H^+-ATPase shared 83–96% identity at the amino acid level (Fig. 2A). NaPHA1 (DDBJ accession number: AB033371) and NaPHA2 (DDBJ accession number: AB033372) were 91% identical at the amino acid level to LHA2, the PM H^+-ATPase from tomatoes (Ewing et al. 1990). NaPHA3 (DDBJ accession number: AB033373) showed 88% identity to AHA2, the PM H^+-ATPase from *Arabidopsis* (Harper et al. 1990). NaPHA4 (DDBJ accession

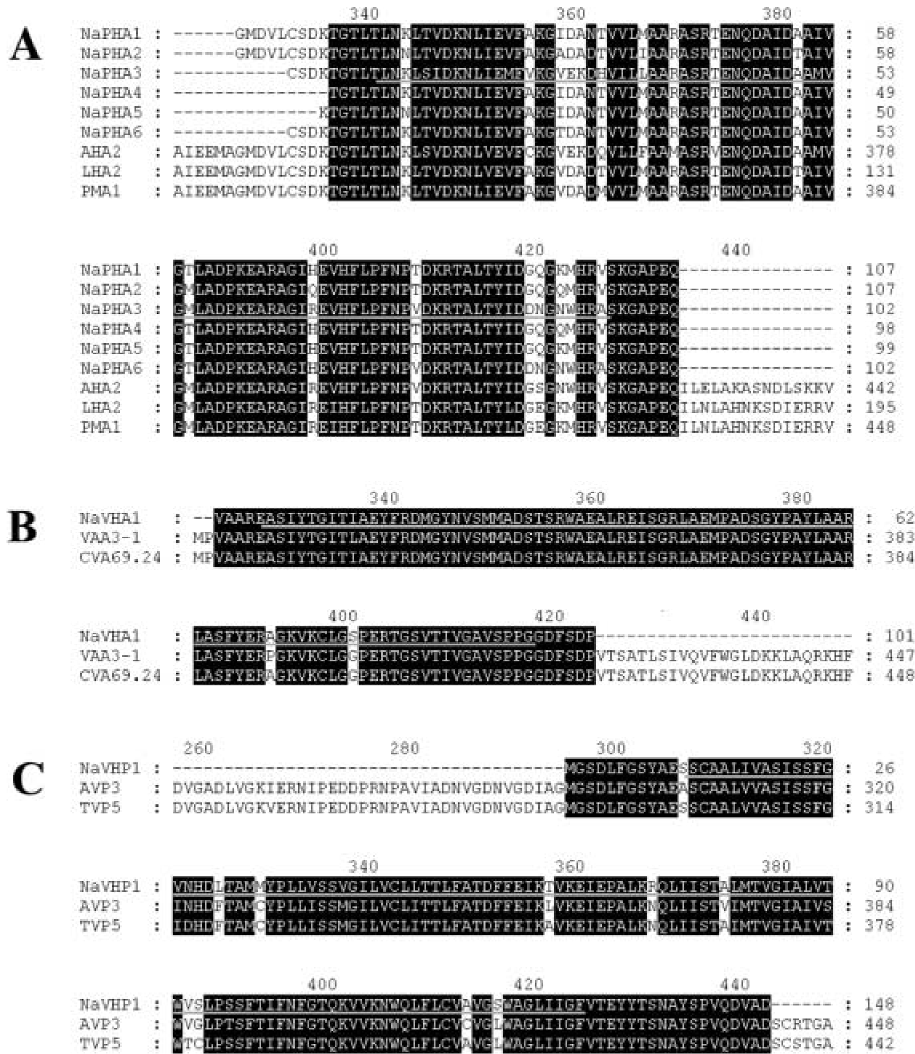


Fig. 2A–C. Alignment of amino acid sequences of the putative *Nepenthes* proton pump genes with those of their closest homologs. Identical residues are shown in *black*. The probe regions used for Northern hybridization are indicated by *gray lines*. **A** *NaPHA1* is 91% identical to LHA2, *NaPHA2* is 91% identical to LHA2, *NaPHA3* is 88% identical to AHA2, *NaPHA4* is 90% identical to PMA1, *NaPHA5* is 90% identical to PMA1, and *NaPHA6* is 87% identical to PMA1. **B** *NaVHA1* is 97% identical to VAA3-1 and 99% identical to CVA69.24. **C** *NaVHP1* is 87% identical to AVP3, and 89% identical to TVP5

number: AB033374) and NaPHA5 (DDBJ accession number: AB033375) were 90% identical to PMA1, the PM H^+-ATPase from tobacco (Perez et al. 1992). NaPHA6 (DDBJ accession number: AB033376) also showed 87% identity to PMA1. NaVHA1 (DDBJ accession number: AB033377) showed 97% identity to VAA3-1, the V-ATPase subunit A from *Phaseolus aureus* (mung beans) (Chiu et al. 1995) (Fig. 2B). NaVHP1 (DDBJ accession number: AB033378) was 87% identical to AVP3, the H^+-PPase from *Arabidopsis* (Sarafian et al. 1992) (Fig. 2C).

Northern hybridization analysis

Northern hybridization was carried out to examine expression of the *Nepenthes* putative proton-pump genes in roots, stems, leaves, tendrils, closed pitchers, and opened pitchers with natural prey. *NaPHA3*, one of the PM H^+-ATPase gene homologs, hybridized with the RNA from tendrils and pitchers (Fig. 3). In the pitchers, *NaPHA3* was expressed more in the lower part than in the upper part, and the highest expression was observed in the lower part of the opened pitcher with natural prey (Fig. 3). High *NaVHA1* expression was detected in tendrils and the upper part of closed pitchers but not in the stems (Fig. 3). *NaVHP1* was expressed in all the tissues tested, and no obvious difference in expression was observed (Fig. 3).

Sequence analysis of 3′-untranslated regions

To analyze the expression of each of six PM H^+-ATPase homologs, gene-specific probes were required. The 3′-untranslated regions of the PM H^+-ATPase have gene-specific sequences in *Arabidopsis* (Harper et al. 1990). Therefore, we isolated 3′-untranslated regions by 3′-RACE. As shown in Fig. 4, the 3′-untranslated regions of the genes *NaPHA1* and *NaPHA5*, *NaPHA2* and *NaPHA4*, and *NaPHA1* and *NaPHA5* have similar sequences. *NaPHA1* showed 92% identity to *NaPHA5*, *NaPHA2* showed 94% identity to *NaPHA4*, and *NaPHA3* showed 83% identity to *NaPHA6*. Due to the sequence similarities, we used 3′-untranslated regions of *NaPHA1*, *NaPHA3*, and *NaPHA4* as gene-specific probes for in situ hybridization.

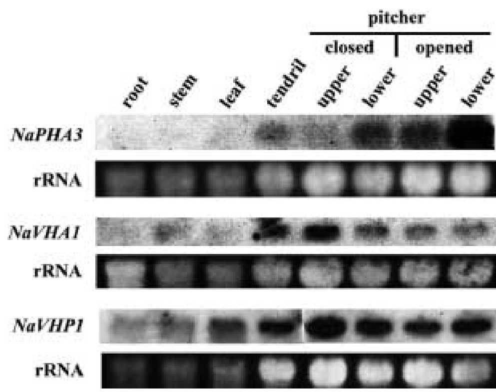


Fig. 3. Northern analysis of the expression of putative *Nepenthes* proton-pump genes *NaPHA3*, *NaVHA1* and *NaVHP1*. The cloned cDNAs of the plant proton-pump homologs (*NaPHA3*, *NaVHA1* and *NaVHP1*) were used as probes (the probe regions are indicated by gray lines in Fig. 2). Total RNA was isolated from the indicated tissues. Open pitchers had captured a large quantity of insects (mainly ants)

Expression analysis by in situ hybridization

To determine the site of expression of PM H^+-ATPase genes in the lower part of pitchers, their local expression was analyzed by in situ hybridization using the gene-specific probes. *NaPHA1* was expressed in the digestive glands (orange fluorescent signal in Fig. 5A; control in Fig. 5B), parenchyma cells and sclerenchymatic bundle sheath cells surrounding the vascular tissue (data not shown). In contrast, *NaPHA3* and *NaPHA4* expression was not detected in the digestive glands (Fig. 5C and 5E, respectively); these genes were expressed in parenchyma cells and sclerenchymatic bundle sheath cells surrounding the vascular tissue (data not shown).

The effect of proton-pump modulators on proton release from the inner surface of the lower part of pitchers

To classify the type of proton pump involved in the acidification of pitcher fluid, we referred to a previous report showing that the pH of pitcher fluid rapidly declined in response to ammonium (Higashi et al. 1993). Sections excised from the lower part of pitchers were used for the experiments. To evaluate the adequacy of this method, we first examined the response of pitcher sections to ammonium. For a pre-treatment, pitcher sections were floated for 24 h on acclimation fluid consisting of KCl and diluted MS salts. This artificial fluid was designed to correspond to the mineral composition (Nemček et al. 1966), osmolarity, and pH [osmolarity: 36.3 ± 10.7 mOsmol/kg (mean $\pm$ SD), corresponds to 19.4 mM KCl; pH: 4.4 ± 0.2, $n = 15$] of the fluid of pitchers immediately after lid opening. The pitcher sections showed a clear reaction to ammonium (Fig. 6), and this acclimation promised to offer us reproducible results. Using the artificial fluid, we tested the effect of two proton-pump inhibitors and one activator on proton release. Vanadate inhibits several phosphate-metabolizing enzymes such as all members of the P-type ATPase family and phosphatases (Macara 1980). Vanadate acts as a phosphate analogue and produces a stable intermediate state similar to that of the phosphoenzyme (Lewis and Thomas 1986). As shown in Fig. 7A, vanadate inhibited proton release in a concentration-dependent manner. When 1 mM vanadate was applied, 32% of proton release was inhibited. Bafilomycin A_1 is a specific inhibitor of the V-ATPase (Bowman et al. 1988). This inhibitor did not inhibit proton release (Fig. 7B). On the contrary, proton release was induced by this inhibitor in a concentration-dependent manner. Fusicoccin (FC), a fungal toxin, functions as an

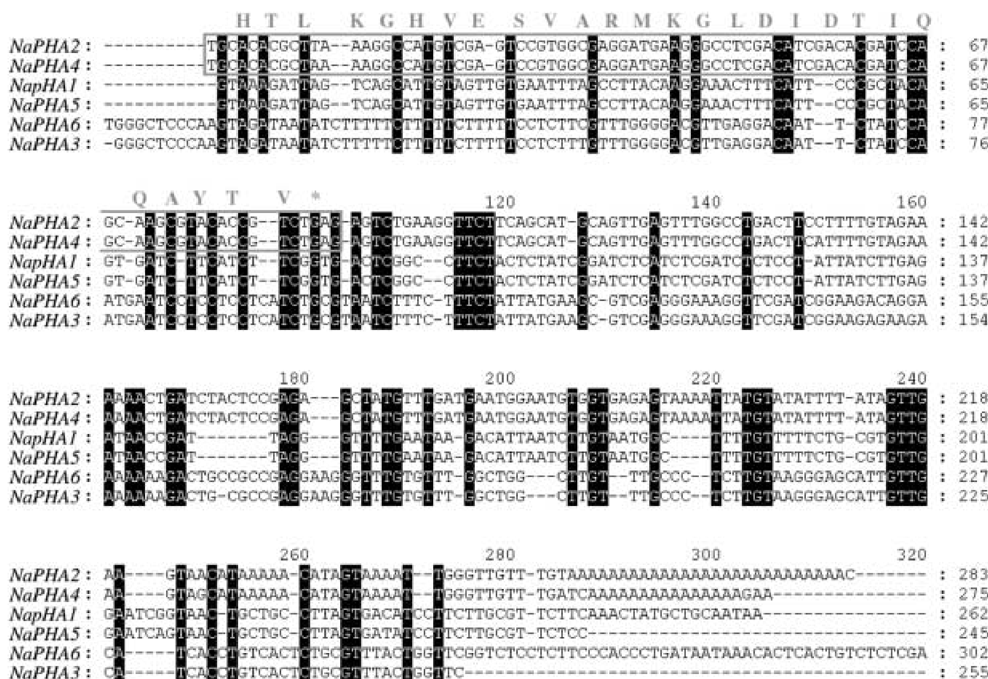


Fig. 4. 3′-Untranslated regions of the putative PM H^+-ATPase genes of *Nepenthes alata*. Identical sequences are shown in *black*. The conserved region of the plant PM H^+-ATPase is *boxed*. The deduced amino acid sequence is indicated by *single letters* and the stop codon is indicated by an *asterisk*. *NaPHA1* is 92% identical to *NaPHA5*, *NaPHA2* is 94% identical to *NaPHA4*, *NaPHA3* is 83% identical to *NaPHA6*

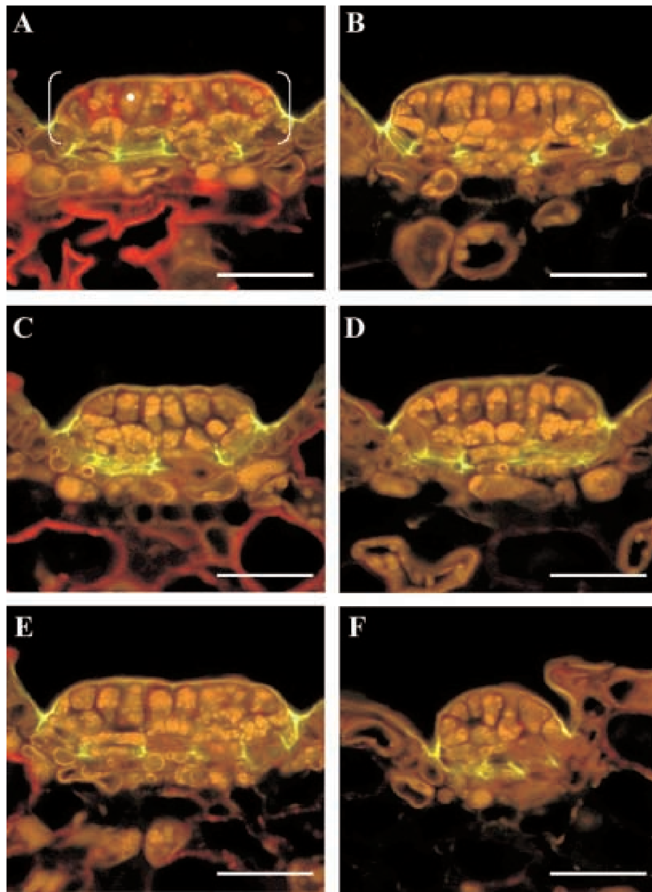


Fig. 5A–F. In situ hybridization of *NaPHA1*, *NaPHA3* and *NaPHA4* in the lower part of pitchers of *Nepenthes alata*. The 3′-untranslated region of each gene was used as a probe. **A** Longitudinal section of the lower part of a pitcher hybridized with *NaPHA1* antisense riboprobe. The digestive gland is indicated by *parentheses* and a head cell is indicated by a *white dot*. **B** Equivalent section hybridized with *NaPHA1* sense riboprobe. **C** Equivalent section hybridized with *NaPHA3* antisense riboprobe. **D** Equivalent section hybridized with *NaPHA3* sense riboprobe. **E** Equivalent section hybridized with *NaPHA4* antisense riboprobe. **F** Equivalent section hybridized with *NaPHA4* sense riboprobe. Bar = 50 μm

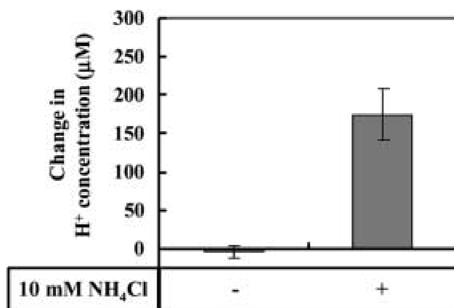
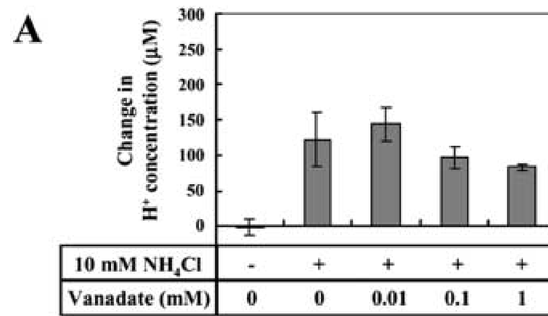
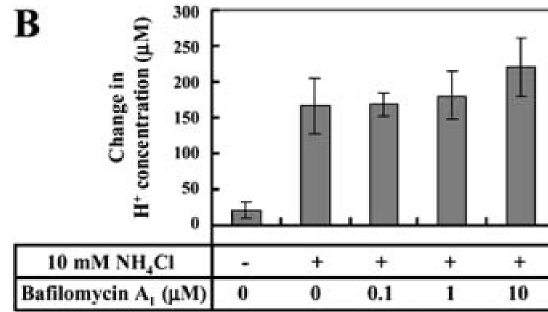


Fig. 6. Proton release from inner surfaces of pitchers induced by ammonium. Pitcher sections excised from lower parts of pitchers were mounted in the cavity at the back of the microcentrifuge tube cap which was filled with acclimation fluid only or acclimation fluid plus 10 mM NH_4Cl. The fluid pH was measured at 6 h and the H^+ concentration calculated. The changes in H^+ concentration during 6 h incubation are shown as bar graphs. Data are means ±SD ($n = 3$)





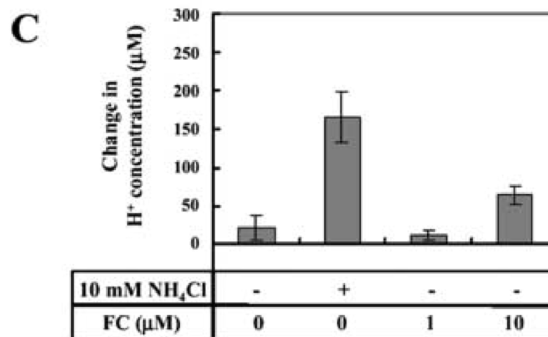


Fig. 7A–C. Effect of proton-pump modulators on H^+ release from the inner surface of a *Nepenthes* pitcher. Proton release was induced by 10 mM NH_4Cl. Sections excised from the lower part of pitchers were mounted in the cavity at the back of the microcentrifuge tube cap, which was filled with acclimation fluid containing 10 mM NH_4Cl and an inhibitor, or an activator, at the indicated concentrations. The fluid pH was measured after 6 h and the H^+ concentration calculated. The changes in H^+ concentration during 6 h incubation are shown as bar graphs. Effects of vanadate **A**, bafilomycin A_1 **B** and FC **C** are shown. Data are means ±SD ($n = 3$)

activator of the PM H^+-ATPase (Palmgren 1999). Proton release was effectively induced by 10 μM FC (Fig. 7C).

Discussion

Nepenthes plants develop vase-like trapping organs called pitchers at the tips of their tendrils. The pitchers store acidic fluid at the bottom. Since pitcher fluid corresponds to apoplastic fluid, its acidity is thought to drive the uptake of insect-derived nutrients. Generally, apoplastic space is acidified (Marschner 1995), and the acidification, i.e., the formation of proton gradients across the plasma membrane, is used to drive the uptake

of many solutes (ions, metabolites, etc.) (Michelet and Boutry 1995). However, there is no report on the mechanism or the role of acidification in the digestion and absorption of prey. In this study, we focused on identification of the proton pump involved in the acidification of pitcher fluid.

Acidification of pitcher fluid in response to prey capture

The acidity of the fluid in new pitchers was found to decrease to around pH 3 in response to the presence of natural prey (Table 1). The pH decrease was also seen in the pitchers fed artificially with prey. These findings suggest that the pH decrease occurs in response to prey capture. The acidification of pitcher fluid is also induced by ammonium (Higashi et al. 1993), and in the artificially fed pitchers, the fluid pH declined according to the increase in ammonium concentration. This result suggests that the fluid pH declined in response to ammonium derived from prey. Recently, Schulze et al. (1999) demonstrated that the ammonium transporter gene is expressed in the head cells of the glands of the lower part of pitchers. The transporter is thought to take up ammonium from the pitcher fluid into the head cells. The fluid pH rose 3 h after feeding on flies, and a concomitant increase in ammonium concentration was observed, which suggests that the pH elevation is due to alkalinization of the fluid by ammonia derived from the prey. The fluid of the old pitchers was found to be of a higher pH (>pH 7), and it did not trap new insects at all. This observation suggests that the older pitchers with high pH do not sustain their function as nutrient-uptake organs.

Sequence of putative Nepenthes proton-pump genes

Six putative PM H^+-ATPase genes (*NaPHA1-6*), one putative V-ATPase subunit A gene (*NaVHA1*), and one putative H^+-PPase gene (*NaVHP1*) were obtained by degenerate PCR (Fig. 2). *NaPHA1-6* showed high identity with *AHA2*, which is known to encode a functional PM H^+-ATPase in *Arabidopsis* (Palmgren and Christensen 1994). NaVHA1 was 97% identical to VAA3-1, a functional V-ATPase subunit A of mung beans (Matsuura-Endo et al. 1990). NaVHP1 was 87% identical to AVP3, a functional H^+-PPase of *Arabidopsis* (Kim et al. 1994). The high homologies of *Nepenthes* genes to these functional proton pumps strongly suggested that the cloned *Nepenthes* proton-pump gene homologs would encode functional proton pumps. Among the obtained six homologs of PM H^+-ATPase, 83–96% identity at the amino acid level was observed. This high identity between isoforms has been reported in several plants. In the model plant *Arabidopsis thaliana*, 11 PM H^+-ATPase genes, which shared 72–94% identity at the amino acid level, have been identified (Palmgren 1998). The 3′-untranslated regions of *NaPHA1* and *NaPHA5*, *NaPHA2* and *NaPHA4*, and *NaPHA1* and *NaPHA5* have highly similar sequences in the respective pairs (Fig. 4). These similarities might be due to the duplication of ancestral genes, but we cannot discuss the similarities further because information on ploidy in *Nepenthes alata* is not available.

Expressions of putative Nepenthes proton-pump genes

In the lower part of pitchers, *NaPHA3* showed higher expression in opened pitchers (with prey) than in closed pitchers (without prey) (Fig. 3). In the experiment, we used the coding region of *NaPHA3* (indicated by the gray line in Fig. 2A) as a probe. Since the coding regions of six PM H^+-ATPase homologs share high (87–92%) similarity at the nucleotide level (cf. Fig. 2A), the result of Northern analysis is thought to reflect a net expression profile of six PM H^+-ATPase homologs. In contrast, there were only minor differences in the expressions of *NaVHA1* and *NaVHP1* between the opened pitchers and the closed pitchers. Recently, Shulze et al. (1999) reported that the presence of prey induces the expression of the ammonium transporter gene. However, they also reported that ammonium itself did not induce the expression of the ammonium transporter gene. Their results seem to imply the presence of additional signals that allow the plant to perceive the presence of prey. However, our results suggest that ammonium derived from prey induces the expression of the PM H^+-ATPase (see Fig. 1A and Fig. 3). Previously, we found that ammonium not only induced the acidification of pitcher fluid, but also induced secretion of protease (unpublished data). Neither pH decrease nor protease secretion was effectively induced by other nitrogenous compounds (bovine serum albumin, amino acids, urea and uric acid). In the case of the carnivorous plant *Dionaea muscipula* Ellis (Venus's Flytrap), a wide range of low-molecularweight nitrogenous compounds (ammonium, urea, allantoic acid etc.) were found to induce secretion of both Cl^- and protease (Rea 1982). Furthermore, inductions of both pH decrease and protease secretion by ammonium were also reported in the pitcher plant *Sarracenia purpurea* (Gallie and Chang 1997). These findings suggest that ammonium is a factor that allows the plant to perceive the presence of prey. However, the possibility still remains that other factors are involved in prey perception. Although the mechanism of induction is still unknown, the phenomenon can be interpreted as follows. The PM H^+-ATPase generates an H^+ electrochemical gradient, which is used to drive solute uptake across the PM. If the H^+ gradient is used for the uptake of insect-derived nutrients (e.g. by H^+-coupled symporters), the cytoplasmic pH will decline and the H^+ gradient will be lost. These changes may activate the H^+-ATPase at a post-translational level (e.g. by phosphorylation of the C-terminus; Portillo 2000) and induce its expression, thus increasing the capacity of cells to extrude protons. This idea is rationalized by the general knowledge that the PM H^+-ATPase is involved in both nutrient uptake and intracellular pH homeostasis (Marschner 1995). *NaVHA1* is highly expressed in

tendrils and the upper part of closed pitchers (Fig. 3). This might be due to involvement of the V-ATPase in specific functions of the tendrils and the upper part of closed pitchers.

Glandular expression of putative Nepenthes PM H+-ATPase genes

Glands of the lower part of pitchers are thought to be responsible for the secretion of acid and digestive enzymes and the absorption of prey-derived nutrients. Expression analysis by in situ hybridization demonstrated that *NaPHA1* is expressed in the head cells of digestive glands (Fig. 5A), indicating that *NaPHA1* or its closest homolog, *NaPHA5*, is involved in the acidification of pitcher fluid. However, since expression of *NaPHA1* was observed in the entire lower part of pitchers, including the digestive glands, it is unlikely that *NaPHA1* (or *NaPHA5*) is the proton pump gene that is specifically expressed only in the head cells of digestive glands.

Effect of proton pump modulators

To classify the type of proton pump involved in the acidification of the pitcher fluid, two proton-pump inhibitors (vanadate and bafilomycin A_1) and one proton pump activator (FC) were used. Vanadate inhibits P-type proton pumps by binding to the phosphorylation site of the cytoplasmic domain. Vanadate was not an effective inhibitor of proton release; only 32% inhibition was seen at a concentration of 1 mM (Fig. 7A). Vanadate is taken up by the phosphate transport system in *Neurospora* (Bowman 1983); however, vanadate (HVO_4^{2-}) tends to aggregate into polynuclear complexes at physiological pH and orange-colored decavanadate, $V_{10}O_{28}H^{5-}$, preferentially formed at the lower pH values (Macara 1980). Its polymerization can interfere with its uptake into cells. Thus, our result may not reflect the net vanadate inhibitory action on the proton pump because a large molecule such as decavanadate is not effectively conveyed to the site of its action. Bafilomycin A_1, a specific inhibitor of the V-ATPase, facilitated proton extrusion in a concentration-dependent manner (Fig. 7B). Bafilomycin induced cytoplasmic acidification in *Arabidopsis* root columella cells (Scott and Allen 1999). The cytoplasmic acidification due to inactivation of the V-ATPase may cause the proton extrusion to the outside of cells. Fusicoccin is a toxin produced by *Fusicoccum amygdali* Del. The major effect of fusicoccin is the stimulation of electrogenic, energy-linked H^+ extrusion, associated with the uptake of K^+ (Marrè 1979). Fusicoccin is thought to stabilize the interaction between 14-3-3 protein and the PM H^+-ATPase, causing a conformational change and enzyme activation (Baunsgaard et al. 1998). Fusicoccin was able to induce proton release in the absence of ammonium (Fig. 7C). That result suggests that the PM H^+-ATPase of digestive glands is involved in the acidification of the pitcher fluid. The PM H^+-ATPase is the only proton pump so far found in the PM of plant cells. Thus, we conclude that the PM H^+-ATPase of the head cells of digestive glands is involved in the acidification of pitcher fluid in *Nepenthes*. At present, the role of acidification of pitcher fluid may be deduced as follows. The optimum pH of proteolytic enzymes is around pH 2–3 (Steckelberg et al. 1967; Nakayama and Amagase 1968; Athauda et al. 1998), and such a low pH should be favorable to the digestive enzymes in the pitcher fluid. Higashi et al. (1993) previously reported that 26 strains of bacteria were isolated from *Nepenthes* pitchers and that 10 of the 26 strains had casein hydrolase activity. We isolated only one acid-resistant strain of yeast from pitchers of *Nepenthes alata* grown in a garden and confirmed that the yeast had casein hydrolase activity (unpublished data). This finding indicates the possibility that microbes residing in pitchers may be involved in the process of digestion of the prey. We found that as long as the yeast grows predominantly in the pitcher, proliferation of microbes, for example, *Bacillus subtilis* and *Escherichia coli*, artificially added to it is strongly suppressed (unpublished data). This suggests that the acid-tolerant microbes may form a unique ecosystem that is favorable to the carnivorous plant. Further work on clarifying the role of acidification of the pitcher fluid is now in progress.

We thank Prof. M. Maeda (Laboratory of Biochemistry and Molecular Biology, Graduate School of Pharmaceutical Sciences, Osaka University) for advice on cloning of proton pumps, and Dr. M. Hayashi from our department for advice and support in setting up in situ hybridization experiments. We also thank Dr. S. Kajiyama and Dr. A. Okazawa from our department for valuable comments and suggestions. This research was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (A.K.).

References

Athauda SB, Inoue H, Iwamatsu A, Takahashi K (1998) Acid proteinase from *Nepenthes distillatoria* (Badura). Adv Exp Med Biol 436: 453–458

Baunsgaard L, Fuglsang AT, Jahn T, Korthout HA, de Boer AH, Palmgren MG (1998) The 14-3-3 proteins associate with the plant plasma membrane H^+-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. Plant J 13: 661–671

Bowman BJ (1983) Vanadate uptake in *Neurospora crassa* occurs via phosphate transport system II. J Bacteriol 153: 286–291

Bowman EJ, Siebers A, Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc Natl Acad Sci USA 85: 7972–7976

Chiu S-J, Hung S-H, Lin L-Y, Pan RL (1995) A cDNA clone encoding the A subunit of the vacuolar H^+-ATPase (accession no. U26709) from etiolated mung bean seedlings (PGR95-083). Plant Physiol 109: 1125

Clarke C (1997) *Nepenthes* of Borneo. Natural History Publications, Kota Kinabalu

Ewing NN, Wimmers LE, Meyer DJ, Chetelat RT, Bennett AB (1990) Molecular cloning of tomato plasma membrane H^+-ATPase. Plant Physiol 94: 1874–1881

Gallie DR, Chang SC (1997) Signal transduction in the carnivorous plant *Sarracenia purpurea*. Regulation of secretory hydrolase expression during development and in response to resources. Plant Physiol 115: 1461–1471

Harper JF, Manney L, DeWitt ND, Yoo MH, Sussman MR (1990) The *Arabidopsis thaliana* plasma membrane H^+-ATPase multigene family. Genomic sequence and expression of a third isoform. [Erratum published in J Biol Chem 1990; 265: 22569] J Biol Chem 265: 13601–13608

Higashi S, Nakashima A, Ozaki H, Abe M, Uchiumi T (1993) Analysis of feeding mechanism in a pitcher of *Nepenthes hybrida*. J Plant Res 106: 47–54

Ishihara A, Kurahasi K, Uehara H (1972) Enzymatic determination of ammonia in blood plasma. Clin Chim Acta 41: 255–261

Kim EJ, Zhen RG, Rea PA (1994) Heterologous expression of plant vacuolar pyrophosphatase in yeast demonstrates sufficiency of the substrate-binding subunit for proton transport. Proc Natl Acad Sci USA 91: 6128–6132

Lewis SM, Thomas DD (1986) Effects of vanadate on the rotational dynamics of spin-labeled calcium adenosinetriphosphatase in sarcoplasmic reticulum membranes. Biochemistry 25: 4615–4621

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S (1994) A knotted-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell 6: 1859–1876

Lüttge U (1964) Untersuchungen zur Physiologie der Carnivoren-Drüsen. I. Mitteilung. Die an den Verdauungsvorgängen beteiligten Enzyme. Planta 63: 103–117

Lüttge U (1971) Structure and function of plant glands. Annu Rev Plant Physiol 22: 23–44

Macara IG (1980) Vanadium – an element in search for a role. Trends Biochem Sci 5: 92–94

Marrè E (1979) Fusicoccin: a tool in plant physiology. Annu Rev Plant Physiol 30: 273–288

Marschner H (1995) Mineral nutrition of higher plants. Academic Press, London

Matsuura-Endo C, Maeshima M, Yoshida S (1990) Subunit composition of vacuolar membrane H^+-ATPase from mung bean. Eur J Biochem 187: 745–751

Michelet B, Boutry M (1995) The plasma membrane H^+-ATPase. A highly regulated enzyme with multiple physiological functions. Plant Physiol 108: 1–6

Morrissey S (1955) Chloride ions in the secretion of the pitcher plant. Nature 176: 1220–1221

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nakayama S, Amagase S (1968) Acid protease in *Nepenthes*. Partial purification and properties of the enzyme. Proc Japan Acad 44: 358–362

Nemček O, Sigler K, Kleinzeller A (1966) Ion transport in the pitcher of *Nepenthes henryana*. Biochim Biophys Acta 126: 73–80

Ninnemann O, Jauniaux JC, Frommer WB (1994) Identification of a high affinity NH_4^+ transporter from plants. EMBO J 13: 3464–3471

Owen TP Jr., Lennon KA (1999) Structure and development of the pitchers from the carnivorous plant *Nepenthes alata* (Nepenthaceae). Am J Bot 86: 1382

Palmgren MG (1998) Proton gradients and plant growth. Adv Bot Res 28: 1–70

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

Palmgren MG, Christensen G (1994) Functional comparisons between plant plasma membrane H^+-ATPase isoforms expressed in yeast. J Biol Chem 269: 3027–3033

Perez C, Michelet B, Ferrant V, Bogaerts P, Boutry M (1992) Differential expression within a three-gene subfamily encoding a plasma membrane H^+-ATPase in *Nicotiana plumbaginifolia*. J Biol Chem 267: 1204–1211

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

Rea PA (1982) Fluid composition and factors that elicit secretion by the trap lobes of *Dionaea muscipula* Ellis. Z Pflanzenphysiol Bd 108: 255–272

Sarafian V, Kim Y, Poole RJ, Rea PA (1992) Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump of *Arabidopsis thaliana*. Proc Natl Acad Sci USA 89: 1775–1779

Schulze W, Frommer WB, Ward JM (1999) Transporters for ammonium, amino acids and peptides are expressed in pitchers of the carnivorous plant *Nepenthes*. Plant J 17: 637–646

Scott AC, Allen NS (1999) Changes in cytosolic pH within *Arabidopsis* root columella cells play a key role in the early signaling pathway for root gravitropism. Plant Physiol 121: 1291–1298

Steckelberg R, Lüttge U, Weigl J (1967) Reinigung der Proteinase aus *Nepenthes*-Kannensaft. Planta 76: 238–241

Sze H, Li X, Palmgren MG (1999) Energization of plant cell membranes by H^+-pumping ATPases. Regulation and biosynthesis. Plant Cell 11: 677–690

Wan CY, Wilkins TA (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). Anal Biochem 223: 7–12