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ORIGINAL ARTICLE

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Aspartic proteinases are expressed in pitchers of the carnivorous plant *Nepenthes alata* Blanco

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Abstract Carnivorous plants acquire significant amounts of nitrogen from insects. The tropical carnivorous plant *Nepenthes* accumulates acidic fluid containing aspartic proteinase (AP) in its trapping organs (pitchers), suggesting that the plant utilizes insect protein as a nitrogen source. Aspartic proteinases have been purified and characterized from sterile pitcher fluid of several species of *Nepenthes*; however, there is, as of yet, no information about sequence and expression of *Nepenthes* AP genes. To identify the pitcher AP, we cloned plant AP homologs from *N. alata* and examined their expressions. Five AP homologs (*NaAP1–NaAP5*) were obtained by reverse transcription-polymerase chain reaction with degenerate primers designed for the conserved sequences of plant APs. Alignment of deduced amino acid sequences with other plant APs demonstrated that NaAP1–NaAP4 contained a plant-specific insert (PSI), a unique sequence of plant AP. However, NaAP5 did not possess the insert, and had a shorter sequence (by >100 amino acids) than the other APs. Northern analysis using a part of the coding region of *NaAP1* as a probe showed that bands of approx. 1.8 kb corresponding to the sizes of *NaAP1–NaAP4* mRNA were present in roots, stems, leaves, tendrils, and lower part of the pitchers, but a band of approx. 1.3 kb corresponding to the size of *NaAP5* mRNA was not observed in any organs. In pitchers, highest expressions of *NaAP1–NaAP4* were seen in the lower part of open pitchers containing natural prey, suggesting that the expressions of *NaAP1–NaAP4* are coupled with prey capture. Transcripts of *NaAP2* and *NaAP4* were detected in the digestive glands, where AP secretion may occur. This result suggests that NaAP2 and NaAP4 are the possible APs secreted into the pitcher of *N. alata*.

Keywords Aspartic proteinase · Carnivorous plant · *Nepenthes* (aspartic proteinase) · Nitrogen acquisition

Abbreviations AP: aspartic proteinase · BSA: bovine serum albumin · PCMB: *p*-chloromercuribenzoic acid · PMSF: phenylmethylsulfonyl fluoride · PSI: plant-specific insert · RACE: rapid amplification of cDNA ends · RT-PCR: reverse transcription-polymerase chain reaction · TCA: trichloroacetic acid

Introduction

Nitrogen is the mineral nutrient that plants require in greatest amounts. However, nitrogen is limited in most terrestrial ecosystems (Vitousek and Howarth 1991). For instance, in some ecosystems such as tundra, boreal forests and heath lands, the mineralization rate of organic nitrogenous compounds is insufficient to meet the known rate of nitrogen uptake by vegetation (Hodge et al. 2000). To adapt to such unfavorable environments, plants have evolved various mechanisms for nitrogen acquisition. In the cases of tundra and boreal forests, plants directly take up amino acids as nitrogen sources (Chapin et al. 1993; Näsholm et al. 1998). In other cases, some plants form mycorrhizas, symbiotic associations between mycorrhizal fungi and the roots of host plants, through which plants utilize organic nitrogenous compounds (e.g. proteins, peptides, and amino acids) as nitrogen sources (Read 1991; Chalot and Brun 1998).

Carnivory is thought to be a special case of plant adaptation to soils of low nutrient availability. Carnivorous plants have evolved active and complex mechanisms by which the plants obtain nutrients from insects. More than 600 species of carnivorous plants occur throughout the world (Adamec 1997). The tropical

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carnivorous plant *Nepenthes* commonly grows in heath forests where soils are siliceous and acidic (Clarke 1997). *Nepenthes* produces trapping organs called pitchers at the tips of tendrils elongated from leaf ends (Owen and Lennon 1999). Digestive fluid is secreted in the lower part of the pitchers. The fluid is acidic and includes several hydrolytic enzymes such as protease and phosphatase (Heslop-Harrison 1975). Histochemical study suggests that the hydrolytic enzymes are secreted from the digestive glands located on the inner surface of the lower part of the pitcher (Heslop-Harrison 1975). The digestive glands are multicellular tissues of approx. 0.1 mm in diameter, and are densely localized on the inner surface of the lower part of the pitcher (Owen and Lennon 1999). Recently, the plasma membrane H^+-ATPase (An et al. 2001) and ammonium transporter (Schulze et al. 1999) were found to be expressed in the glands, further suggesting that the glands are indeed important for acid secretion and nutrient absorption.

Nepenthes is thought to take up substantial amounts of nitrogen from insects. In *N. mirabilis*, the proportion of insect-derived nitrogen reaches approx. 60% of total nitrogen intake (Schulze et al. 1997). Considering the presence of protease in the pitcher fluid, insect proteins should be an important nitrogen source for *Nepenthes*. To date, proteases have been purified and characterized from sterile pitcher fluids of several species of *Nepenthes* (Steckelberg et al. 1967; Jentsch 1972; Tökés et al. 1974). These studies provide evidence that aspartic proteinases (APs) are secreted into the pitcher. However, no information about the sequence and expression of *Nepenthes* AP genes has been presented. For an understanding of the nitrogen-acquisition mechanism of *Nepenthes*, it is essential to collect information about the AP genes. To identify the genes encoding APs secreted into the pitcher, we cloned homologous genes of plant APs and examined their expressions using *Nepenthes alata* as a model carnivorous plant.

Materials and methods

Plant material

Nepenthes alata Blanco plants were obtained from a commercial plant distributor (Nishijima Engei Danchi, Kochi, Japan). Plants were grown in a greenhouse at Osaka University without fertilizer. Plants were watered daily with tap water.

Protease assay

The pitcher fluid samples used were collected from newly opened pitchers so that the insides of the pitchers were nearly sterile. Proteolytic activity was determined by the method of Bohak (1970). A 50-µl aliquot of pitcher fluid was mixed with 50 µl of 2% (w/v) bovine serum albumin (BSA) in 200 mM glycine-HCl (pH 3.0), and incubated at 37 °C for 1 h. The reaction was stopped by the addition of 150 µl of 5% (w/v) trichloroacetic acid (TCA). The mixture was incubated on ice for 10 min, and centrifuged at 20,000 *g* for 10 min. Absorbance of the supernatant at 280 nm was measured. One unit of activity is defined as the amount of enzyme that causes an increase of 0.001 per min in the absorbance at 280 nm. To determine the optimum pH for proteolytic activity, the following buffers were used; pH 1.5, 2.0, 2.5, 3.0: glycine-HCl; pH 3.0, 3.5, 4.0: formate-NaOH; pH 4.0, 4.5, 5.0, 5.5: acetate-NaOH; pH 5.5, 6.0, 6.5: MES-NaOH; pH 6.5, 7.0: PIPES-NaOH. To examine the effect of protease inhibitors, the following inhibitors were added to the above assay system: 1 mM phenylmethylsulfonyl fluoride (PMSF; serine protease inhibitor), 1 mM *p*-chloromercuribenzoic acid (PCMB; cysteine proteinase inhibitor), 1 mM ethylenediaminetetraacetic acid (EDTA; metalloproteinase inhibitor), 10 µg/ml Pepstatin A (aspartic proteinase inhibitor).

cDNA cloning

Amino acid sequences of APs from plants 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):

- 5′ TAY ATG AAY GCI CAR TAY TWY GG (forward primer 1),
- 5′ CAR AAR TTY ACI GTI RTI TTY GAY AC (forward primer 2),
- 5′ CC RAA IAC IAI YTC ICC NCC YTC (reverse primer 1),
- 5′ CC CAT ITY RAA YTG CCA RTA NCC (reverse primer 2).

The following sequences were used to design degenerate primers. Q38934, Q39311, O04057, P40782, Q40140, P42210, Q42456 (SWISS-PROT and TrEMBL accession numbers), T11686 (PIR accession number), CAA70340 (EMBL accession number), BAA76870 (DDBJ accession number), AAD29758, AAC34854 (GenBank accession numbers). Total RNA was isolated from the lower part of closed 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 (Life Technologies, Rockville, Md., 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 sequencing-kit instructions (ABI PRISM BigDye Terminator Cycle Sequencing Kits; Applied Biosystems, Foster City, Calif., USA) using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Based on the sequencing results, the full-length cDNAs of *Nepenthes* AP homologs were cloned by 3′-Full Race Core Set and 5′-Full RACE Core Set (Takara Shuzo, Kyoto, Japan).

Northern analysis

Total RNA was isolated from roots, stems, leaves, tendrils, closed pitchers, and opened pitchers (0 and 7 days after lid opening) with RNAqueous-Midi Kit (Ambion, Austin, Tex., USA) supplemented with Plant RNA Isolation Aid (Ambion). Equal amounts of RNA (5 µg total RNA) were separated on 1% agarose gels containing 2.2 M formaldehyde and blotted onto nylon membranes (Hybond-N+; Amersham Pharmacia Biotech, Uppsala, Sweden). A 440-bp fragment corresponding to position 423–862 of the *NaAP1* cDNA (DDBJ accession number BAB20969) was used as a probe. Hybridization, washing and signal detection were carried out with ECL Direct (Amersham Pharmacia Biotech) following the manufacturer's instructions.

In situ hybridization

The 3′-untranslated regions (3′-UTRs) of *NaAP1–NaAP5* were used as gene-specific probes for in situ hybridization. As shown in Fig. 1, the 3′-UTRs showed low (less than 37%) identity between each homolog, indicating that the 3′-UTRs can be used as gene-specific probes. The lower parts of the pitchers were excised from newly opened pitchers, and immediately fixed in phosphate-buffered 4% paraformaldehyde. Digoxigenin-labeled sense and

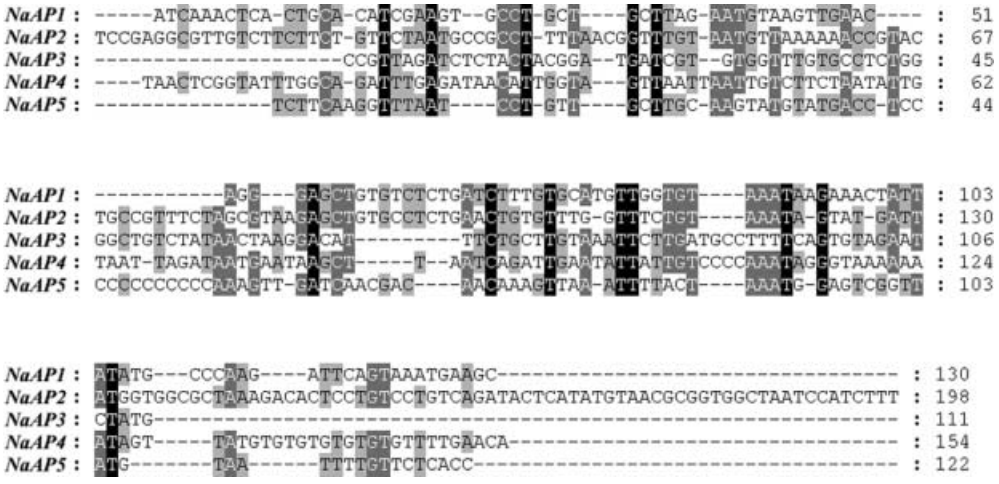

Fig. 1 Alignment of the 3′-untranslated regions (3′-UTRs) of *Nepenthes alata NaAP1–NaAP5* used as gene-specific probes for in situ hybridization. Only probe regions in the whole 3′-UTR are shown. The four levels of shading used are: *black* 100% sequence identity, *mid-gray* 80–90% identity, *light gray* 60–79% identity, *no shading* < 60% identity

antisense RNA probes were prepared by using the DIG RNA labeling kit (SP6/T7) (Roche Diagnostics, Mannheim, Germany), and used for hybridization at a concentration of 5 ng μl^{-1} kb^{-1} in a volume of 200 µl per slide. In situ hybridization to 4-µm-thick Paraplast (Sigma, St. Louis, Mo., USA) sections of paraformaldehyde-fixed tissues was performed as described previously (An et al. 2001).

Results

Proteolytic activity of the pitcher fluid

The proteolytic activity of the pitcher fluid from newly opened pitchers was highest at pH 3.0 (Fig. 2). Based on this result, the effects of protease inhibitors were examined at pH 3.0. Among the four types of inhibitor, only pepstatin A strongly (approx. 80%) inhibited the proteolytic activity (Fig. 3). This suggests that aspartic proteinase (AP) is included in the pitcher fluid of newly opened pitchers of *N. alata*.

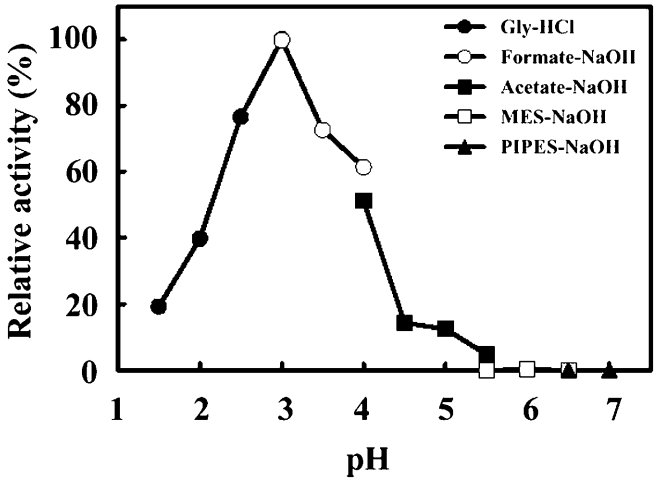

Fig. 2 pH dependency of proteolytic activity of *N. alata* pitcher fluid. Pitcher fluid collected from newly opened pitchers was incubated with BSA (substrate) in an appropriate buffer at 37 °C for 1 h. After addition of 5% TCA, cooling, and centrifugation, the absorbance of the supernatant was measured at 280 nm (for details, see *Materials and methods*). Data are expressed as a percentage of the highest activity over the pH range examined, and are representative of three independent experiments

Sequences of the *Nepenthes* AP homologs

Five plant AP homologs (named *NaAP1–NaAP5*: DDBJ accession numbers are BAB20969, BAB20970, BAB20971, BAB20972 and BAB20973, respectively) were obtained by RT-PCR using degenerate primers. Alignments of deduced amino acid sequences of *Nepenthes* AP homologs with other plant and animal (human) APs are shown in Fig. 4. The conserved sequences, including two aspartic acid residues (asterisks in Fig. 4) in the active center, are recognized in the five homologs. SignalP (http://www.cbs.dtu.dk/services/SignalP-2.0/; Nielsen et al. 1997) predicted that the five homologs have signal peptides at the N-terminus (boxed

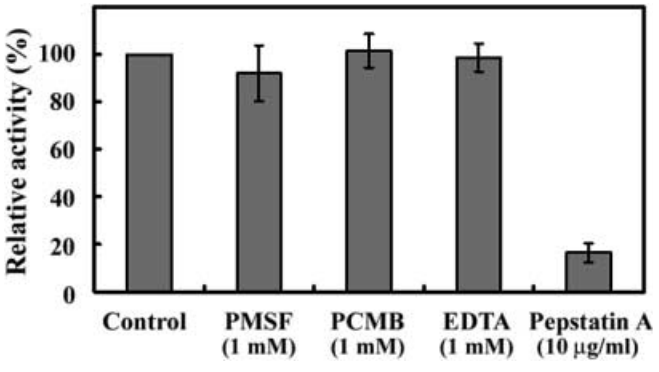

Fig. 3 Effect of inhibitors on proteolytic activity of *N. alata* pitcher fluid. Pitcher fluid collected from newly opened pitchers was incubated with BSA (substrate) and an inhibitor of indicated concentration in glycine-HCl buffer at 37 °C for 1 h. After addition of 5% TCA, cooling, and centrifugation, the absorbance of the supernatant was measured at 280 nm (for details, see *Materials and methods*). Data obtained from three independent samples are expressed as a percentage of control value (without inhibitors). The means ± SD are shown as bar graphs and error bars, respectively ($n = 3$)

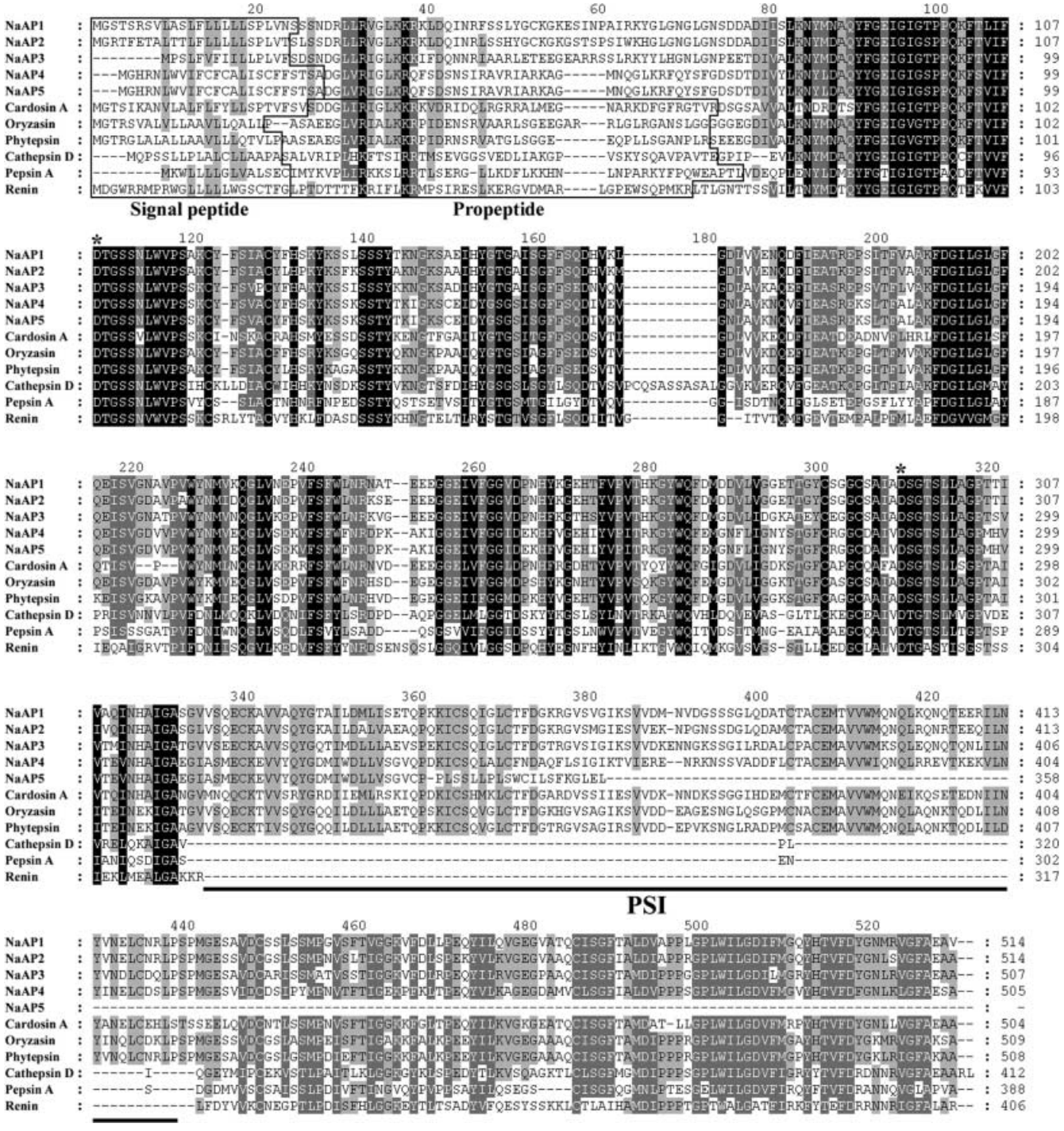

Fig. 4 Alignment of deduced amino acid sequences of putative *Nepenthes* APs (NaAP1–NaAP5) with other plant APs (cardosin A from cardoon, oryzasin from rice, and phytepsin from barley) and animal (human) APs (cathepsin D, pepsin A, and renin). Amino acids are shown in one-letter code. The four levels of shading used are: *black* 100% sequence identity, *mid-gray* 80–90% identity, *light gray* 60–79% identity, *no shading* <60% identity. Position of plant-specific insert (PSI) is indicated by a *black underline*. Aspartic acid residues of the active center are indicated by *asterisks*. Regions of signal peptides and propeptides are *boxed* (for NaAP1–NaAP5, putative regions are indicated)

in Fig. 4). Among the five homologs, *NaAP1–NaAP4* encoded open reading frames (ORFs) with similar lengths (approx. 510 amino acids) to those of other plant APs, and contained the plant-specific insert (PSI), a unique sequence found only in plant APs (black underline in Fig. 4). In contrast, *NaAP5* encoded an ORF of 358 amino acids, which is >100 amino acids shorter than those of *NaAP1–NaAP4*, and contained only the N-terminal region of PSI (Fig. 4). The N-terminal 336 amino acids of NaAP4 and NaAP5 were identical (5′-1111 nucleotides of their cDNAs were

identical), suggesting that NaAP4 and NaAP5 are generated by alternative splicing.

Expression of the *Nepenthes* AP homologs

Northern hybridization was carried out to examine expression of the *NaAP* genes in roots, stems, leaves, tendrils, closed pitchers, and open pitchers. A part of the coding region of *NaAP1*, which shared 70–89% identity with *NaAP2–NaAP5*, was used as a probe (for details, see *Materials and methods* section). As shown in Fig. 5, bands of approx. 1.8 kb, corresponding to the size of *NaAP1–NaAP4* mRNAs, were detected in roots, stems, leaves, and the lower part of pitchers. In contrast, a band of approx. 1.3 kb, corresponding to the size of *NaAP5* mRNA, was not observed in any organs (Fig. 5). Among the all organs tested, expression levels of *NaAP1–NaAP4* were highest in leaves; and in pitchers, the highest expression was observed in the lower part of open pitchers containing natural prey (7 days after lid opening, Fig. 5). To investigate expression of *NaAP1–NaAP5* in the digestive glands, the putative secretory tissues of digestive enzymes, in situ hybridization was performed using digoxigenin-labeled transcripts of the 3′-UTR as gene-specific probes. Among the five homologs, *NaAP2* and *NaAP4* were expressed clearly in the digestive glands (orange fluorescent signals in Fig. 6, parts d and h, respectively; control in Fig. 6, parts e and i, respectively) as well as parenchymatic cells and sclerenchymatic bundle sheath cells surrounding the vascular tissue (data not shown). In contrast, obvious expressions of *NaAP1*, *NaAP3* and *NaAP5* were not seen in the digestive glands (Fig. 6, parts b, f and j, respectively); expressions of these genes were observed only in parenchymatic cells and sclerenchymatic bundle-sheath cells surrounding the vascular tissue (data not shown).

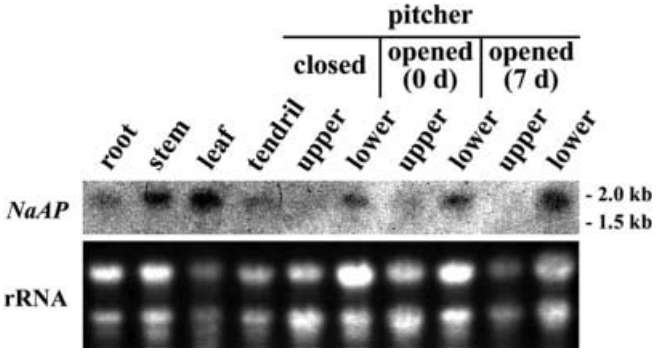

Fig. 5 Northern analysis of the expression of *NaAP* genes in various organs of *N. alata*. A part of the coding region of *NaAP1*, which shared 70–89% identities with *NaAP2–NaAP5*, was used as a probe (for details, see *Materials and methods*). Total RNA was isolated from the indicated tissues, and 5 μg of RNA was loaded into each lane. The length of RNA size markers is given on the right. The pitchers that had had their lids open for 7 days contained captured insects (mainly ants). Results were reproduced in an independent experiment

Discussion

The pitcher fluid of the carnivorous plant *Nepenthes* includes several hydrolases responsible for prey digestion. Among the hydrolases, protease is expected to be important for nitrogen acquisition from insect proteins. To elucidate the nitrogen-acquisition mechanism from insects, we focused on protease in the pitcher fluid. The proteolytic activity of the pitcher fluid of *N. alata* showed a pH optimum of 3.0 (Fig. 2). Our previous report demonstrated that the pH of the fluid decreased from 4.3 to 2.8 within 5 days when insects were artificially added to the pitcher fluid of newly opened pitchers of *N. alata* (An et al. 2001). This finding implies that *Nepenthes* pitchers have a mechanism by which the fluid pH decreases to the optimum pH of protease in response to prey capture. Pepstatin A strongly (approx. 80%) inhibited the proteolytic activity, suggesting that AP is included in the pitcher fluid of newly opened pitchers of *N. alata*. Strong inhibition of proteolytic activity by pepstatin A has also been reported in other species of *Nepenthes* (Tökés et al. 1974; Athauda et al. 1998). To identify the candidate genes encoding APs secreted into the pitcher, plant AP homologs were cloned and their expressions were examined.

RT-PCR with degenerate primers enabled us to clone five plant AP homologs (*NaAP1–NaAP5*). These homologs exhibited high similarity to other plant and animal APs, and signal peptides at their N-terminal were predicted in all of the homologs (Fig. 4). Four homologs, NaAP1–NaAP4, had a similar length of amino acids to other plant APs, and contained homologous sequence to the plant-specific insert (PSI). PSIs of NaAP1–NaAP4 shared 48–70% identities with that of phytepsin (cf. Fig. 4). Although PSI is a unique sequence of plant APs, its function and physiological role is poorly understood. Based on the structural similarity with saposins, lysosomal sphingolipid-activating proteins in mammalian cells (Guruprasad et al. 1994; Kervinen et al. 1999), it has been proposed that the PSI may function as a targeting signal to the vacuole. Indeed, some plant APs containing PSI localize to the vacuole (Runeberg-Roos et al. 1994; Ramalho-Santos et al. 1997). However, cardosin B, a PSI-containing AP of cardoon, has been detected in an extracellular matrix (Faro et al. 1998), indicating that the role of PSI remains unclear. One of the proposed functions of PSI is to promote association of plant AP precursors with cell membranes (Faro et al. 1999). A recent study showed that PSI of cardosin A was able to interact with phospholipid vesicles and induce leakage of their contents (Egas et al. 2000). This report therefore suggested that PSI might function as a defensive agent against pathogens (Egas et al. 2000). If NaAPs are secreted into the pitcher fluid as precursors and their PSI has a similar

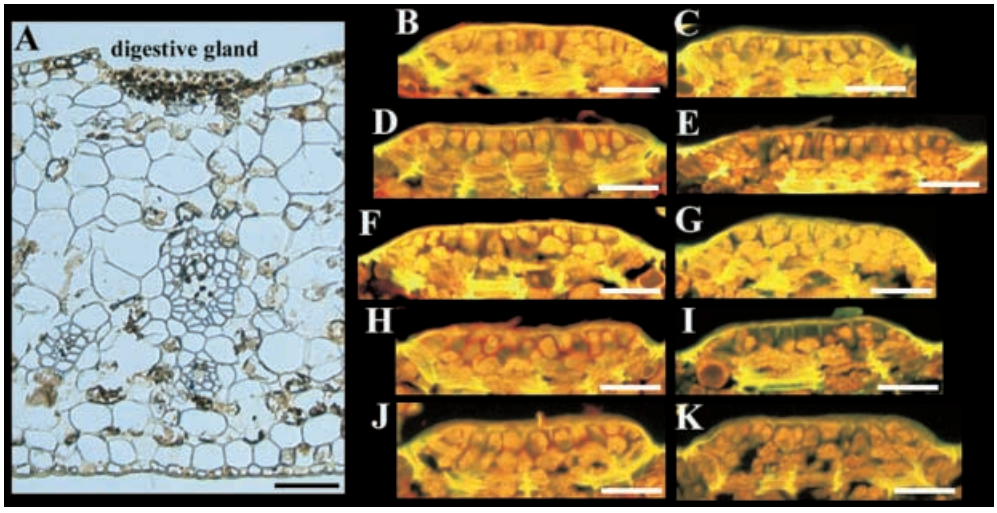

Fig. 6a–k Expression of *NaAP* genes in the digestive glands. Longitudinal sections of the lower part of newly opened *N. alata* pitchers were hybridized to digoxigenin-labeled transcripts. Expression was detected as an orange fluorescent signal. **a** Light-microscopic view of a longitudinal section of the lower part of a pitcher. **b** Longitudinal section of the lower part of a pitcher hybridized with *NaAP1* antisense riboprobe. **c** Equivalent section hybridized with *NaAP1* sense riboprobe. **d** Longitudinal section of the lower part of a pitcher hybridized with *NaAP2* antisense riboprobe. **e** Equivalent section hybridized with *NaAP2* sense riboprobe. **f** Longitudinal section of the lower part of a pitcher hybridized with *NaAP3* antisense riboprobe. **g** Equivalent section hybridized with *NaAP3* sense riboprobe. **h** Longitudinal section of the lower part of a pitcher hybridized with *NaAP4* antisense riboprobe. **i** Equivalent section hybridized with *NaAP4* sense riboprobe. **j** Longitudinal section of the lower part of a pitcher hybridized with *NaAP5* antisense riboprobe. **k** Equivalent section hybridized with *NaAP5* sense riboprobe. Bars = 50 μm (**a**), 20 μm (**b–k**)

function to that of cardosin A, it is possible that the PSI may contribute to prey digestion by destroying prey cell membranes.

Among the cloned APs, NaAP5 has the following remarkable features that are lacking in most plant APs: (i) NaAP5 lacked the majority of the PSI sequence, (ii) the amino acid sequence of NaAP5 was shorter (>100 amino acids) than those of NaAP1–NaAP4, and (iii) the N-terminal 336 amino acids of NaAP4 and NaAP5 were identical (5′-1111 nucleotides of their cDNAs were identical). Although we cannot currently rule out a possibility that *NaAP5* is a cloning artifact, point iii mentioned above suggests that *NaAP4* and *NaAP5* are generated by alternative splicing. Expression of *NaAP5* could not be detected by Northern analysis. Expression analysis by quantitative PCR with GeneAmp 5700 (Applied Biosystems) showed that the expression level of *NaAP5* in the lower part of pitchers was more than 10-times less than those of *NaAP1–NaAP4* (data not shown). This result suggests that NaAP5 may not be important for prey digestion even if it is secreted into the pitcher.

Northern analysis showed that the expression levels of *NaAP1–NaAP4* were highest in leaves (Fig. 5). In plants, long-distance transport of proteins through sieve elements can occur (Imlau et al. 1999). It is therefore possible that NaAP1–NaAP4 expressed in leaves or stems could be transported to pitchers, and then secreted into the pitcher fluid. In fact, it is suggested that the endodermal barriers beneath the digestive glands are immature during pitcher development, and molecules can freely traffic through the barriers (Owen et al. 1999). If so, protease detected in the pitcher fluid of unopened (developing) pitchers might be derived from multiple organs such as leaves and stems in addition to the pitchers.

In pitchers, expression levels of *NaAP1–NaAP4* were highest in the lower part of open pitchers containing natural prey (7 days after lid opening; Fig. 5). This result suggests that the expressions of *NaAP1–NaAP4* might be induced by prey capture. In situ hybridization with gene-specific primers demonstrated that *NaAP2* and *NaAP4* are expressed clearly in the digestive glands of the lower part of pitchers (Fig. 6). Considering that the digestive glands are putative secretory tissues, NaAP2 and NaAP4 are currently the candidate APs secreted into the pitcher of *N. alata*. Further studies on identifying the protease in the pitcher fluid are in progress.

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