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REGULAR PAPER

Protein replenishment in pitcher fluids of *Nepenthes* × *ventrata* revealed by quantitative proteomics (SWATH-MS) informed by transcriptomics

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Abstract

Carnivorous plants capture and digest insects for nutrients, allowing them to survive in soil deprived of nitrogenous nutrients. Plants from the genus *Nepenthes* produce unique pitchers containing secretory glands, which secrete enzymes into the digestive fluid. We performed RNA-seq analysis on the pitcher tissues and liquid chromatography-tandem mass spectrometry (LC–MS/MS) analysis on the pitcher fluids of *Nepenthes* × *ventrata* to study protein expression in this carnivory organ during early days of pitcher opening. This transcriptome provides a sequence database for pitcher fluid protein identification. A total of 32 proteins of diverse functions were successfully identified in which 19 proteins can be quantified based on label-free quantitative proteomics (SWATH-MS) analysis while 16 proteins were not reported previously. Our findings show that certain proteins in the pitcher fluid were continuously secreted or replenished after pitcher opening, even without any prey or chitin induction. We also discovered a new aspartic proteinase, Nep6, secreted into pitcher fluid. This is the first SWATH-MS analysis of protein expression in *Nepenthes* pitcher fluid using a species-specific reference transcriptome. Taken together, our study using a gel-free shotgun proteomics informed by transcriptomics (PIT) approach showed the dynamics of endogenous protein secretion in the digestive organ of *N.* × *ventrata* and provides insights on protein regulation during early pitcher opening prior to prey capture.

Keywords Aspartic proteinase · Carnivorous plant · Nepenthesin · Proteomics informed by transcriptomics · RNA-seq · Shotgun proteomics · SWATH

Introduction

Plant carnivory allows trapping and digestion of insects, followed by nutrient absorption (Ellison and Gotelli 2003). This special trait enables carnivorous plants to grow in nutrient-deprived soil. *Nepenthes* L. is the only genus of carnivorous plants from the *Nepenthaceae* family which consists of more than 100 species (Mithöfer 2011). *Nepenthes*, also known as tropical pitcher plants are widely found in the Southeast Asia region, Sri Lanka, Madagascar and even Queensland in Australia (Adam and Hamid 2006). In Malaysia, pitcher plant is known as monkey cup or 'periuk kera' due to its pitcher-shaped organ.

A pitcher arises from the tip of an elongated leaf, beginning as a small, flattened structure, which later develops into a pitcher. *Nepenthes* pitchers are an enticing structure with bright and rich colors to attract insects (Givnish 2015). Hollow entrance into the pitcher (peristome) is carpeted with a waxy, slippery wall, to prevent trapped insect from escape. Pitchers undergo morphological changes after opening, especially in the pitcher shapes and curling of peristome (Owen et al. 1999). The time taken for the full lid opening or peristome curling varies between different *Nepenthes* species, though it is usually around one or 2 days after the first sign of lid opening (Bauer et al. 2009). Functionally, these changes represent pitcher maturation for insect trapping with the readiness of digestion and nutrient absorption as supported by increasing trapping efficiency from day 3 to day 6

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after opening (Bauer et al. 2009). The hydrolytic activities in the pitcher fluids also increase during pitcher maturation, which can be due to the induced secretion of endogenous enzymes by prey (Buch et al. 2015) or contribution of microbial enzymes in the pitcher fluids (Chan et al. 2016).

Digestive enzymes are secreted by the glands at the bottom of the pitcher. Acidic proteases, namely Nepenthesins are the major hydrolytic enzymes in the fluid (Ravee et al. 2018). Five genes of this protease family were found in the pitcher of *Nepenthes alata* (An et al. 2002), but only two were characterized. The characterization of Nepenthesin-1 (Nep1) showed its exceptional temperature and pH stability with a wide range of substrates (Athauda et al. 2004), which explained their activity in the extracellular environment. Hatano and Hamada (2008) successfully identified other proteins beside Nepenthesin with functions in pathogenesis-related (PR) and antibacterial activities, namely β-D-xylosidase, β-1,3-glucanase, and thaumatin-like protein from the fluid of newly opened pitchers. Therefore, the digestive system in *Nepenthes* could be derived from a plant defense mechanism via the jasmonate (JA) signaling (Yilamujiang et al. 2016).

Various other hydrolytic enzymes were found in different *Nepenthes* species, including chitinases (Eilenberg et al. 2006; Lee et al. 2016; Rottloff et al. 2016), acid/alkaline phosphatases (Lee et al. 2016; Płachno et al. 2006), esterases (Rottloff et al. 2016), lipases (Morohoshi et al. 2011; Tökés et al. 1974), and peroxidases (Hatano and Hamada 2008). A recent gel-based proteomic study based on *N. mirabilis* transcriptome reported the identification of twenty new pitcher fluid proteins, including serine carboxypeptidases, galactosidases, and transferases in *N. mirabilis*, *N. alata*, *N. sanguinea*, *N. albomarginata*, and *N. bicalcarata* (Rottloff et al. 2016). Moreover, three new Nepenthesins, two novel prolyl endoproteases, Neprosins, and a few other acid-stable enzymes were reported from the pooling of 1,000 fluid samples from pitchers of *N.* × *ventrata* Hort. ex Fleming (Gorb et al. 2004) fed with fruit fly based on a *N. rafflesiana* transcriptome (Lee et al. 2016). The newly discovered endogenous enzymes upon induction supports that *Nepenthes* species have an independent digestion mechanism for nutrient scavenging, instead of dependence on symbiotic microbes.

To date, the abovementioned studies and ongoing profiling experiments (Wan Zakaria et al. 2016a; Zulkapli et al. 2017) helped identified key proteins involved in the hydrolysis of various nutrient substrates as well as in the defense mechanism. These studies mostly focused on pitcher fluid protein profiling to elucidate the enzyme composition and digestive mechanism in *Nepenthes* upon prey capture. In control experiments, this is often simulated by insect prey feeding or treatment with a component of insects' exoskeleton, chitin. Chitin treatment in *N. alata* resulted in a gradual JA accumulation, increased expression of Nep1 and chitinase, and induced protease activity within 48 h (Yilamujiang et al. 2016).

Apart from well-known hydrolytic enzymes, information on the full protein content of pitcher fluid is still limited. A comprehensive study on pitchers of different developmental stages or the regulation of endogenous proteins in pitcher fluids is still lacking. This is due to challenges in sample acquisition confounded by low protein concentrations of the pitcher fluids, hence requires a large sample collection or an analytical instrument with high sensitivity. Protein supply in the pitcher fluid is biologically important to sustain an optimal cocktail of enzymes for readiness in prey digestion, to prevent bacterial or fungal competition for nutrients, and possibly for other roles such as signal transduction. Therefore, it is crucial to study all secreted endogenous protein components beyond hydrolytic enzymes in the pitcher fluid, which is a phenomenon unique to carnivorous plants.

To understand the dynamics of endogenous protein regulation in the pitcher fluid of *N.* × *ventrata*, a natural hybrid between *N. alata* and *N. ventricosa* (Gorb et al. 2004), we adopted a proteomics informed by transcriptomics (PIT) approach. This approach combines RNA-sequencing (RNA-seq) analysis of pitcher tissue with mass spectrometry (MS) identification of proteins from pitcher fluid based on a predicted peptide database generated from de novo transcriptome assembly. Combined with Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) analysis, which is a label-free technique in shotgun proteomics for protein quantification (Aizat et al. 2018), we provide the evidence of protein replenishment in the pitcher fluid. Furthermore, we discovered a new aspartic proteinase, namely Nepenthesin-6 (Nep6), and other proteins which may be responsible for the regulation of various metabolisms in the pitcher fluid.

Materials and methods

Plant materials and sample preparation

N. × *ventrata* Hort. ex Fleming [=(*N. ventricosa* Blanco × *N. alata* Blanco) Phil.] plants were grown under shade in a nursery (2°55′09.0″N, 101°47′04.8″E) at Universiti Kebangsaan Malaysia, Bangi. Pitcher tissue and fluid samples were collected from newly opened pitchers (within 24 h of lid opening) as day 0 control. For RNA-seq analysis, whole pitcher samples were emptied, and flash-frozen with liquid nitrogen before storing at − 80 °C for further use. For protein depletion experiment through the removal of endogenous proteins, emptied pitchers from day 0 control were replenished with the filtrate depleted of proteins greater than 10 kDa and sealed with parafilm for 3 days to avoid insect contamination. For the chitin treatment experiment,

30 mM of chitin (Sigma-Aldrich) were added into newly opened pitchers (within 24 h of pitcher opening) and sealed for 3 days using parafilm. After 3 days, pitchers were rinsed with distilled water to remove partially digested chitin sample, before flash-frozen in liquid nitrogen.

For pitcher fluid study, collected pitcher fluid samples were processed according to Hatano and Hamada (2008). Briefly, fluids were syringe filtered through 25 mm Acrodisc syringe filter with 0.2 µm PVDF membrane (PALL, USA) and ultraconcentrated at 10 kDa molecular weight cut off (MWCO) using Microsep Advance Centrifugal Devices with Omega membrane (PALL, USA).

RNA-sequencing analysis

RNA extraction using modified CTAB method (Abdul-Rahman et al. 2017), cDNA library construction, sequencing, and read processing were performed as described in a previous report (Wan Zakaria et al. 2016b). Briefly, the assembled and annotated transcriptome reference was analyzed using Trinity (v2.0.6) (Haas et al. 2013) and annotated with standard Trinotate (v2.0.0) annotation pipeline (Bryant et al. 2017). Gene ontology (GO) analysis was conducted using WEGO database (http://wego.genomics.org.cn/) (Ye et al. 2006). Predicted peptide sequences (Supplementary Data 1) were generated by Transdecoder version v2.0.1 as a part of Trinity analysis pipeline. These predicted peptide sequences were used as a reference for protein identification in this study. Transcript abundance was estimated by RSEM with the mapping of reads against reference transcriptome sequences as part of the Trinity analysis pipeline. Transcript abundance in normalized Transcripts Per Million (TPM) provides an estimation of transcript expression level (Supplementary Data 2). This Transcriptome Shotgun Assembly (TSA) project has been deposited at DDBJ/EMBL/GenBank under the accession GFAD00000000. The version described in this paper is the first version, GFAD01000000.

Pitcher fluid study

For protein identification, pitcher fluids from the control and endogenous protein depletion experiment were used. Sample preparations were described above with at least three biological replicates. As protein preparation only consists of filtration step, other impurities that remained in the sample may hinder the correct A_{280} absorbance reading. Hence, an equal volume of concentrated samples was used for SDS-PAGE analysis due to low protein concentration and a limited amount of pitcher fluid samples. A 1-D SDS-PAGE was performed using 20 µL of standardized fluid samples in Laemmli buffer (Laemmli 1970) with heat denaturation at 95 °C for 10 min and separated by 12.5% SDS-PAGE for 25 min at 75 V followed by 125 V for 90 min. Protein bands were then visualized by silver staining (Chevallet et al. 2006) with 11 mM silver nitrate and 0.15% (v/v) formaldehyde. The protein band sizes were estimated using the PageRuler prestained protein ladder (10–170 kDa) (Thermo Scientific).

Solid-phase extraction

Proteins from pitcher fluid were separated from other impurities using commercially available 1 cc solid-phase extraction (SPE) cartridges with Waters Oasis HLB sorbent (Waters, USA) prior to LC–MS. Protein samples were diluted with 1 mL formic acid (FA) before running through the column. The SPE cartridges were conditioned subsequently with 1 mL methanol. Then, the column was equilibrated with 1 mL of 0.1% FA. After sample loading, the cartridge was washed with 1 mL of 1% methanol in 0.1% FA. The analytes were eluted with 200 µL of 80% methanol. Samples were then lyophilized.

Trypsin digestion

Lyophilized samples were reconstituted in 150 µL of 50 mM NH_4HCO_3. Samples were reduced by incubation in dithiothreitol (DTT) solution for 20 min in 60 °C. Then, iodoacetamide was added to alkylate free cysteine residue and incubated in dark at room temperature for 45 min, before trypsin was added for overnight digestion at 37 °C. Three biological replicates were pooled and dried using vacuum concentrator (RVC 2-18 MZ2C, Christ, Germany).

LC–MS/MS analysis

Protein digests were analyzed using the TripleTOF 5600 system (AB SCIEX, Framingham, MA, USA) coupled with an Eksigent NanoLC-Ultra 2D + with Nanoflex cHiPLC system (AB SCIEX). Dried samples were reconstituted in 20 µL of 1% FA and 2 µL of samples were injected into an Eksigent nano cHiPLC Trap column (200 µm × 0.5 mm ChromXP C18-CL 3 µm 120 Å) with 95% solvent A (2% acetonitrile (ACN) containing 0.1% FA) and 5% solvent B (98% ACN containing 0.1% FA) at a flow rate of 300 nL min^{-1} for 10 min before resolved on a cHiPLC analytical column (75 µm × 15 cm ChromXP C18-CL 3 µm 120 Å) with a linear gradients of solvent B from 5 to 40% in 90 min; 40–90% in 5 min, followed by a gradient of solvent A from 60 to 10% in 10 min. The nanoLC column was rinsed with 90% solvent B for 5 min and equilibrated with 95% solvent A for 15 min.

For information-dependent acquisition (IDA), 250 ms survey scan (TOF–MS) of *m/z*: 350–1250 and forty 50 ms automated MS/MS product ion scan of *m/z*: 100–1800 for the top 20 ions with the highest intensity was performed with a cycle time of 2.3 s. The MS/MS triggering criteria for parent ions were as follows: precursor intensity (> 125

counts per second), charge state (2–5) with dynamic exclusion time of 12 s and collision energy (CE) set as rolling CE script based on *m/z* and charged state of the precursors. Triplicate LC–MS/MS runs per sample were performed.

For Sequential Window Acquisition of All Theoretical Mass Spectra (SWATH-MS) analysis (Aizat et al. 2018), the same LC condition was applied as IDA experiment described above and SWATH analysis was performed with the following settings: TOF–MS *m/z*: 350–1250, SWATH-MS *m/z*: 100–1800, SWATH window 36 (25 Da), CE 35 + 15 CES with 75 ms accumulation time and 2.8 s cycle time.

Data processing and analysis

All spectra generated from IDA acquisitions were first searched against known *Nepenthes* sequences (retrieved from NCBI nr protein database on Sept 2016) using ProteinPilot™ v4.5 (AB SCIEX) for protein and peptide identification. User-defined parameters were configured as follows: (i) Sample Type: identification; (ii) Modifications: methionine oxidation (variable) and cysteine alkylation (fixed); (iii) Digestion: none/trypsin; (iv) Instrument: TripleTOF 5600; (v) Species: none; (vi) ID Focus: biological modifications and amino acid substitutions; (vii) Search effort: thorough. List of proteins and peptides identified were generated for further analysis. All spectra were also searched against predicted peptide database from *N.* × *ventrata* transcriptome reference using the same parameters as stated above. Peptide detected was filtered by the false discovery rate, FDR < 0.01.

SWATH-MS analysis for protein expression comparison was performed on ProteinPilot™ v4.5 (AB SCIEX) using default parameters. Up to four peptides with three transitions were selected for XIC peak area generation using PeakView software (AB SCIEX) with ≥ 95% confidence phospho-/acetylated peptides, excluding shared peptides. Statistical analysis (*T* test) of normalized MS data was performed using MarkerView software (AB SCIEX). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Vizcaíno et al. 2016) partner repository with the dataset identifier PXD007251 (Wan Zakaria et al. 2018).

Reverse transcription-PCR sequence validation of Nep6

cDNA was prepared from isolated total RNA from control pitcher sample using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). Primers used for amplification of Nep6 were: forward primer 5′-CAGCATCTTTCT CTGTCTTCA-3′ and reverse primer 5′-CTATGTGTGTGT GTCTGTGGTA-3′ in 25 µL, reaction volume with 0.5 unit of KAPA HiFi DNA Polymerase (Kapa Biosystems). PCR conditions for Nep6 comprised of initial denaturation at 95 °C for 3 min, 35 cycles of 98 °C for 20 s, 64 °C for 15 s and 72 °C for 1 min, and a final extension step of 72 °C for 5 min. PCR products were sent for sequence validation with Sanger sequencing and deposited into GenBank with accession number MG700593.

Results

Early pitcher maturation

Upon opening, the shape of *N.* × *ventrata* pitcher became curvier and peristome curled inwards (Fig. 1). These changes are believed to help increase trapping efficiency, as shown by the increasing number of trapped insects from day 3 onwards (Bauer et al. 2009). Based on these observations, pitchers from day 3 after opening were chosen to study the changes in the pitcher fluid protein composition through proteomics informed by transcriptomics. A schematic diagram of the workflow is shown in Fig. 2.

RNA-sequencing analysis of *N.* × *ventrata*

To investigate global gene expression changes in *N.* × *ventrata* pitchers after 3 days of opening, we performed endogenous protein depletion and chitin treatment experiments (Wan Zakaria et al. 2016b) to maximize transcript discovery. Samples acquired from each experiment were sequenced using the Illumina HiSeq 2500 sequencing platform, generating three transcriptome libraries which were merged to form a reference transcriptome. In total, circa 125 million of 125 bp paired-end clean reads after read processing were analyzed using Trinity analysis pipeline. All of the RNA-seq reads were de novo assembled into a total of 137,138 unigenes and 181,810 transcripts with the length between 224 and 13,720 bp, and N50 of 1,207 bp (Wan Zakaria et al. 2016b). A total of 57,833 predicted peptide sequences were generated using Transdecoder based on predicted open reading frame from the transcripts (Supplementary Data 1). This serves as a database for spectra analysis in peptide identification.

To functionally annotate assembled transcript sequences, similarity search was performed against NCBI non-redundant database, Swiss-Prot, Pfam, eggNOG, GO, and SignalP using BLASTx and BLASTp from Trinotate software with E-value cutoff of 1e-5 (Supplementary Data 2). From a total of 59,335 (32.6%) annotated transcripts, 52,722 transcripts were classified according to three GO categories: molecular function, biological process, and cellular component (Fig. 3). For biological process, transcripts involved in cellular and metabolic processes were highly represented, apart from biological regulation, response to stimuli, and pigmentation. For molecular function, transcripts were mostly with

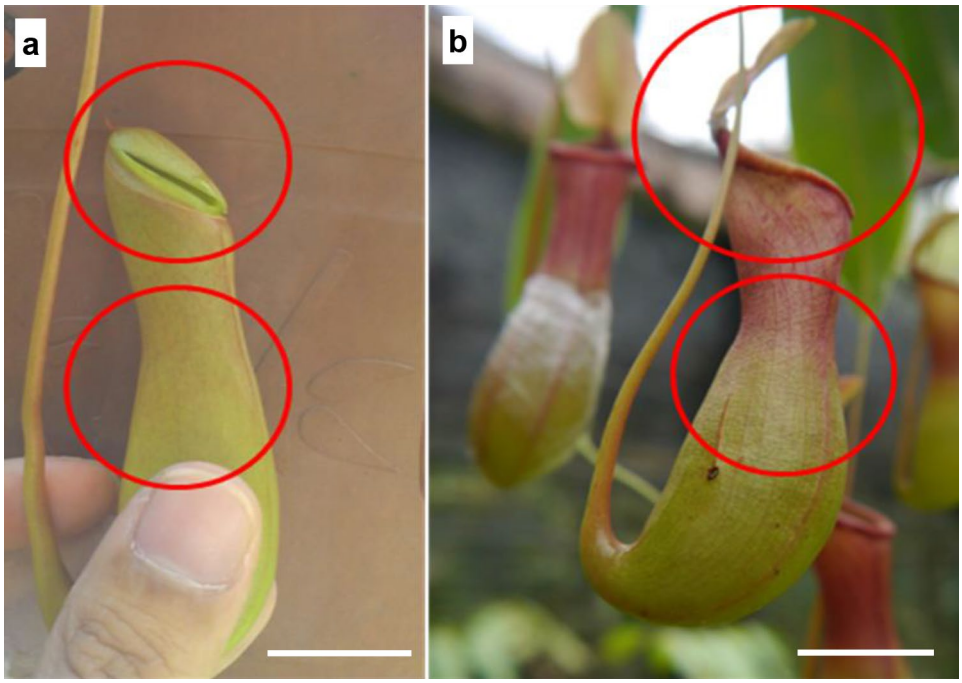

Fig. 1 Pitchers of *N.* × *ventrata*. **a** Day 0 opened pitcher of *N.* × *ventrata.* **b** Mature pitcher of *N.* × *ventrata.* Differences in pitcher morphology and coloration due to maturing process can be seen in lid opening, peristome curling, and pitcher shape (circles). Bars = 2 cm

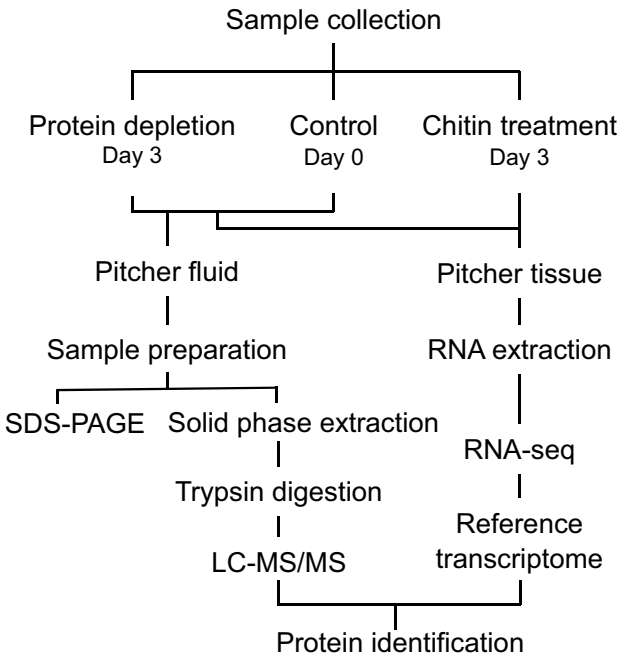

Fig. 2 Proteomics informed by transcriptomics approach in the identification of *N.* × *ventrata* pitcher fluid proteins

binding and catalytic functions, apart from being transporter and transcription regulator.

We searched the transcript annotation for a list of previously reported proteins in pitcher fluid. Glucosidase showed the highest number of unigenes, followed by peroxidase, esterase/lipase, serine carboxypeptidase, galactosidase, and glucanase (Table 1, Supplementary Data 2). These unigenes with signal peptide sequences were the potential candidates coding for secreted proteins in the pitcher fluid.

Analysis of *N.* × *ventrata* pitcher fluid

Pitcher fluids were collected within 24 h after pitcher opening as a day 0 control. All fluid samples collected were in acidic condition (pH 3–4) with an average fluid volume of 2.13 ± 0.85 mL (mean ± SEM, $N = 4$). The experiment was performed to investigate the effect of endogenous protein depletion on protein secretion during early pitcher maturation. Day 0 emptied pitchers were sealed after replenished with respective filtered pitcher fluids deprived of proteins (> 10 kDa) and left for 3 days before day-3 sample collection. This helped to minimize the confounding effect from refilling with distilled water or buffers (Eilenberg et al. 2006; Hatano and Hamada 2012) in the study of pitcher fluid protein replenishment. From our observations, the emptied pitcher will senesce if not replenished with fluid. The average volume of fluids collected on day 3 (2.75 ± 1.19 mL, mean ± SEM, $N = 4$) increased significantly (paired t test, $P < 0.05$) with similar acidic pH 3–4. The fluid samples from chitin treatment experiment were contaminated with unknown polymers in addition to low protein concentrations which hindered further LC–MS/MS analysis. Therefore, fluid protein analysis was focused on control (day-0) and protein depleted (day-3) samples.

Equal volumes of concentrated pitcher fluid from individual samples were analyzed by SDS-PAGE with silver staining to detect the proteins in the fluid. Protein bands

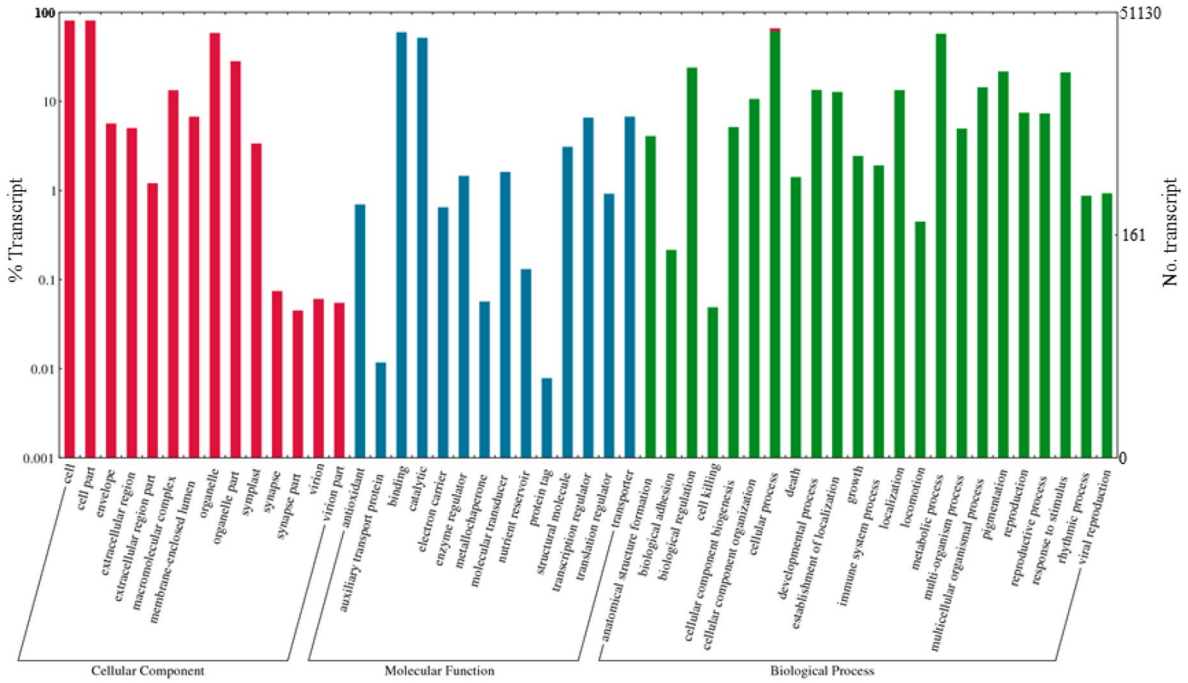

Fig. 3 Functional annotation of transcriptome sequences according to gene ontology (GO) classification. GO analysis was performed using WEGO database at level three for cellular component, molecular function, and biological process

Table 1 Number of unigene identified from *N.* × *ventrata* reference transcriptome of proteins previously reported in *Nepenthes* pitcher fluid

Protein	No. unigene
Glucosidase	174 (39)
Peroxidase	138 (21)
Esterase/lipase	99 (30)
Serine carboxypeptidase	72 (18)
Galactosidase	64 (14)
Glucanase	42 (9)
Purple acid phosphatase	39 (7)
Thaumatin-like protein	28 (6)
Chitinase	20 (7)
Pathogenesis-related protein	20 (4)
Xylosidase	21 (4)
Nepenthesin	12 (7)
Lipid transfer protein	12 (0)

Number in brackets indicates the number of unigene with signal peptide

were detected with size between 15 and 100 kDa (Fig. 4). Samples from day 3, albeit most proteins (> 10 kDa) were filtered on day 0, showed similar protein bands with different intensities compared to control. Despite higher sensitivity of staining method with an increased detection range, the presence of oxidative aldehyde in high concentration modified covalent bond in proteins (Chevallet et al. 2006), which rendered protein bands detected from SDS-PAGE unsuitable for gel-based MS analysis (Shevchenko et al. 2006). Therefore, further protein analysis of *N.* × *ventrata* pitcher fluid was performed by gel-free approach.

Protein identification in *N.* × *ventrata* pitcher fluid

A gel-free approach of protein identification was taken by pooling 3 biological replicates as one sample and analyzed using nanoLC–MS/MS. We first searched the MS spectra against all *Nepenthes* protein sequences from Swiss-Prot and identified 12 proteins (Table 2). These include two aspartic proteinases, one β-1,3-glucanases, one H^+-ATPase, one ATPase with unknown function, one NADH-plastoquinone oxidoreductase, one ATP synthase, two defense-related proteins namely pathogenesis-related and thaumatin-like proteins, two transporter proteins, and one maturase K.

Next, predicted *N.* × *ventrata* protein sequences from reference transcriptome (Supplementary Data 1) was used for protein identification in which 32 proteins were identified from the fluid, including all the proteins identified based on Swiss-Prot database. Sixteen of the identified proteins were not reported previously, including proteins involved in lipoate biosynthesis, DNA replication, gene and protein regulations, and a fasciclin-like arabinogalactan protein (Table 2). We cannot exclude the possibility that certain identified protein, such as DNA gyrase, might be of

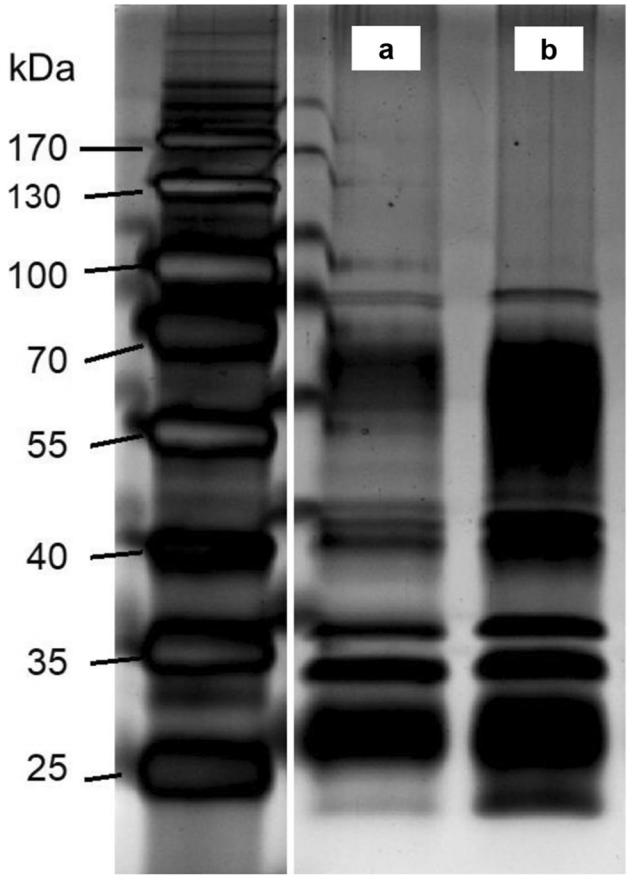

Fig. 4 Non-MS-compatible silver stained SDS-PAGE of *N.* × *ventrata* pitcher fluid. **a** Pitcher fluid sample collected from day-0 opened pitcher (control), **b** Pitcher fluid sample from day 3 (endogenous protein depletion). The SDS-PAGE has been cropped for clarity. For full SDS-PAGE gel, can refer to Fig. S2

microbial origin, which might also be captured by the transcriptome reference due to contamination.

Of the 32 identified proteins in pitcher fluid, the expression of 19 proteins can be quantified based on SWATH analysis (Table 2), in which 11 proteins showed significant ($P < 0.05$) differences between day-0 and day-3 samples with eight upregulated and three downregulated proteins.

A new secreted aspartic protease in the pitcher fluid

Our results were consistent with Lee et al. (2016), whereby only serine carboxypeptidases and aspartic proteases were found in the fluid of *N.* × *ventrata*. A prolyl endopeptidase, Neprosin-1 (Npr1) which was first reported by Lee et al. (2016), was also found in our samples. No cysteine protease was found in the pitcher fluid despite the presence of cysteine protease family in *N.* × *ventrata* transcriptome (Supplementary Data 2).

Two out of three aspartic proteinases which are > 90% identical to deposited sequences of nepenthesins were identified as Nepenthesin-1 (TR40217_c0_g1_i1) and Nepenthesin-2 (TR19440_c0_g1_i1) in *N.* × *ventrata*. Another protein (TR22561_c0_g2_i4) related to Nep1 and Nep2 was discovered in the pitcher fluid with specific peptide sequence from LC–MS/MS analysis (Fig. 5). To investigate further, the sequence validated by Sanger sequencing was aligned with other known nepenthesins to identify conserved domains and sequence similarity (Fig. S1). All sequences were aligned at active-site sequence motifs, Asp-Thr-Gly and Asp-Ser-Gly. These motifs are marked as flap tyrosine residues, which are common to ordinary aspartic proteinase (Simões and Faro 2004). Other criteria of nepenthesin-type aspartic protease (NAP) are the lack of a plant-specific insertion and the occurrence of a NAP insertion site as described by Athauda et al. (2004). There are also four cysteine residues responsible for the increased stability of this enzyme at the insertion site. However, there is a misalignment of the third cysteine residues among the nepenthesins within NAP insertion site (Fig. 5).

The existence of another nepenthesin in the fluid was already hinted by Hatano and Hamada (2008)when three out of seven bands from pitcher fluid matched to nepenthesin. Rottloff et al. (2016) also reported a Nep2-like protein found from *N. mirabilis* whereas Lee et al. (2016) suggested the existence of three other nepenthesins (Nep3-5), which can also be found in our transcriptome reference (Fig. 5). However, alignment between transcript TR22561_c0_g2_i4 and Nep 3-5 from Lee et al. (2016) showed only 20.9–25.1% similarity (Fig. S1). The alignment between all these sequences strongly suggests TR22561_c0_g2_i4 as a new member of nepenthesin in *N.* × *ventrata*, which is proposed here as Nepenthesin-6 (Nep6).

Discussion

Differential expression between day 0 and day 3 protein profiles

For SDS-PAGE analysis, a greater range of protein bands can be detected due to higher sensitivity with aldehyde compared to the previous study which reported only protein bands between 25 and 75 kDa based on 1,000 pooled *N.* × *ventrata* fluid samples (Lee et al. 2016). Interestingly, most protein bands can be found in newly opened pitchers compared to those fed with Drosophila fruit flies (Lee et al. 2016). Similar protein bands between day-0 and day-3 samples (Fig. 4) showed that most but not all proteins in the opened pitcher were continuously produced and secreted into the pitcher fluid even without prey after its opening. Our finding also corroborates with the previous report that protein content in the fluid increased in newly opened pitchers (Hatano and Hamada 2008). There were more protein

Table 2 List of proteins identified from pitcher fluid of day-0 and day-3 samples through search against Swiss-Prot and *N.* × *ventrata* transcriptome

Process	Annotation	Class/function	MW (kDa)	SP	Reference		Quantification[b]	
					GenBank ID	Transcriptome ID	Log_2FC	*P* value
Protein metabolism	Aspartic proteinase nepenthesin-1	Aspartic proteinase	46.3	1–24	AFV26024.1	TR40217_c0_g1_i1_m.38162	1.59	0.000
	Aspartic proteinase nepenthesin-2	Aspartic proteinase	46.0	1–24	Q766C2.1	TR19440_c0_g1_i1_m.16733	0.92	0.006
	Aspartic proteinase nepenthesin-6[a]	Aspartic proteinase	56.9	1–35		TR22561_c0_g2_i4_m.18901	−0.70	0.653
	Neprosin 1	Prolyl endopeptidase	42.9	1–31		TR80436_c1_g1_i4_m.78310	0.74	0.000
	Serine carboxypeptidase-like 51	Serine carboxypeptidase	58.0	1–26		TR9354_c0_g1_i2_m.9287	−2.55	0.000
	Serine carboxypeptidase-like 49	Serine carboxypeptidase	49.3	1–21		TR12326_c0_g2_i1_m.12043	1.06	0.000
Nucleic acid metabolism	Purple acid phosphatase 27	Metallophosphatase	61.1	–		TR26566_c0_g1_i1_m.22346	−2.00	0.000
Polysaccharide metabolism	β-1,3-glucanase	Glycoside hydrolase 17/defense	37.3	1–41	BAM28606.1, BAM28611.1	TR63442_c0_g1_i1_m.59872, TR25775_c0_g1_i3_m.21177	2.79	0.000
Energy metabolism	Plasma membrane H + -ATPase	ATP synthesis/proton pump	20.9	–	BAA94374.1	TR43910_c1_g1_i2_m.42163	−0.24	0.724
	Protein Ycf2	ATPase of unknown function	267.8	–	AEK78240.1	TR44684_c1_g5_i3_m.43748	–	–
	ATP-dependent zinc metalloprotease FTSH4[a]	Oxidative phosphorylation system	72.6	–		TR43903_c0_g3_i1_m.42128	–	–
	NADH-plastoquinone oxidoreductase subunit 2	ATP synthesis coupled electron transport	56.7	–	AEK78241.1	TR29120_c1_g8_i6_m.26766	−0.47	0.001
	ATP synthase subunit beta	ATP synthesis coupled proton transport	53.0	–	AAF01642.1, CAB89942.1	TR59195_c0_g2_i1_m.55970, TR55157_c3_g1_i2_m.52927	–	–
Metabolic process	Lipoyl synthase[a]	Lipoate biosynthesis	45.5	–		TR47266_c0_g1_i1_m.45625	–	–
	Putative UDP-rhamnose:rhamnosyltransferase 1[a]	Glycosyltransferase	52.0	1–29		TR36309_c0_g1_i7_m.34063	–	–
Plant defense	Pathogenesis-related protein 1	Defense	18.3	1–30	ACT99721.1	TR65252_c0_g1_i1_m.61269	5.20	0.000

Table 2 (continued)

Process	Annotation	Class/function	MW (kDa)	SP	Reference		Quantification[b]	
					GenBank ID	Transcriptome ID	Log_2FC	*P* value
	Thaumatin-like protein	Antifungal activity/pathogen defense	23.9	1–24	BAF98917.1, ACU31843.1	TR46175_c0_g1_i1_m.44574, TR46175_c0_g1_i5_m.44578	2.16	0.003
Transport	Amino acid transporter	Amino acid transport	41.6	–	AAD16013.1	TR80104_c0_g1_i2_m.77906	–	–
	Peptide transporter	Peptide transport	33.2	–	AAD16016.1	TR14216_c0_g1_i1_m.13424	−4.50	0.098
	Syntaxin-binding protein 5[a]	Protein transport	121.7	–		TR83992_c0_g1_i2_m.81801	6.25	0.058
	Kinesin KP1[a]	Microtubule-based movement	18.3	–		TR2431_c0_g1_i1_m.2295	3.08	0.005
DNA replication	DNA replication complex GINS protein PSF3[a]	DNA replication initiation	20.9	–		TR10618_c0_g1_i2_m.10917	–	–
	DNA polymerase delta catalytic subunit[a]	DNA replication/repair	52.0	–		TR26681_c0_g1_i3_m.22428	4.33	0.145
	DNA gyrase subunit B[a]	DNA topoisomerase	81.0	–		TR9241_c1_g1_i1_m.8675	1.91	0.189
Gene regulation	Maturase K	Intron splicing	60.7		AAY58086.1	TR91110_c0_g2_i1_m.85955	−2.86	0.265
	Pentatricopeptide repeat-containing protein[a]	RNA modification	74.3	–		TR84752_c0_g1_i2_m.82798	–	–
	Cyclic Dof factor 2[a]	Transcription factor	21.5	–		TR17712_c0_g2_i3_m.15521	–	–
	Probable protein arginine *N*-methyltransferase 3[a]	Transcriptional regulation	57.3	–		TR9994_c0_g1_i2_m.10234	2.08	0.176
Protein regulation	Plant U-box protein 4[a]	Protein ubiquitination	60.4	–		TR9929_c0_g1_i1_m.9886	–	–
	ARM repeat protein interacting with ABF2[a]	Protein ubiquitination	80.1	1–23		TR32001_c0_g1_i1_m.28644	–	–
	Probable serine/threonine-protein kinase[a]	Protein phosphorylation	66.0	–		TR66134_c1_g1_i1_m.62450	–	–
Other	Fasciclin-like arabinogalactan protein 8[a]	Cell surface adhesion protein	43.9	1–20		TR59253_c0_g2_i2_m.56310	–	–

SP Signal peptide position if present as predicted by SignalP or Phobius via InterPro analysis

[a]Protein not reported previously

[b]Protein quantification analysis is based on ProteinPilot software. Log_2FC: Log_2 fold change value

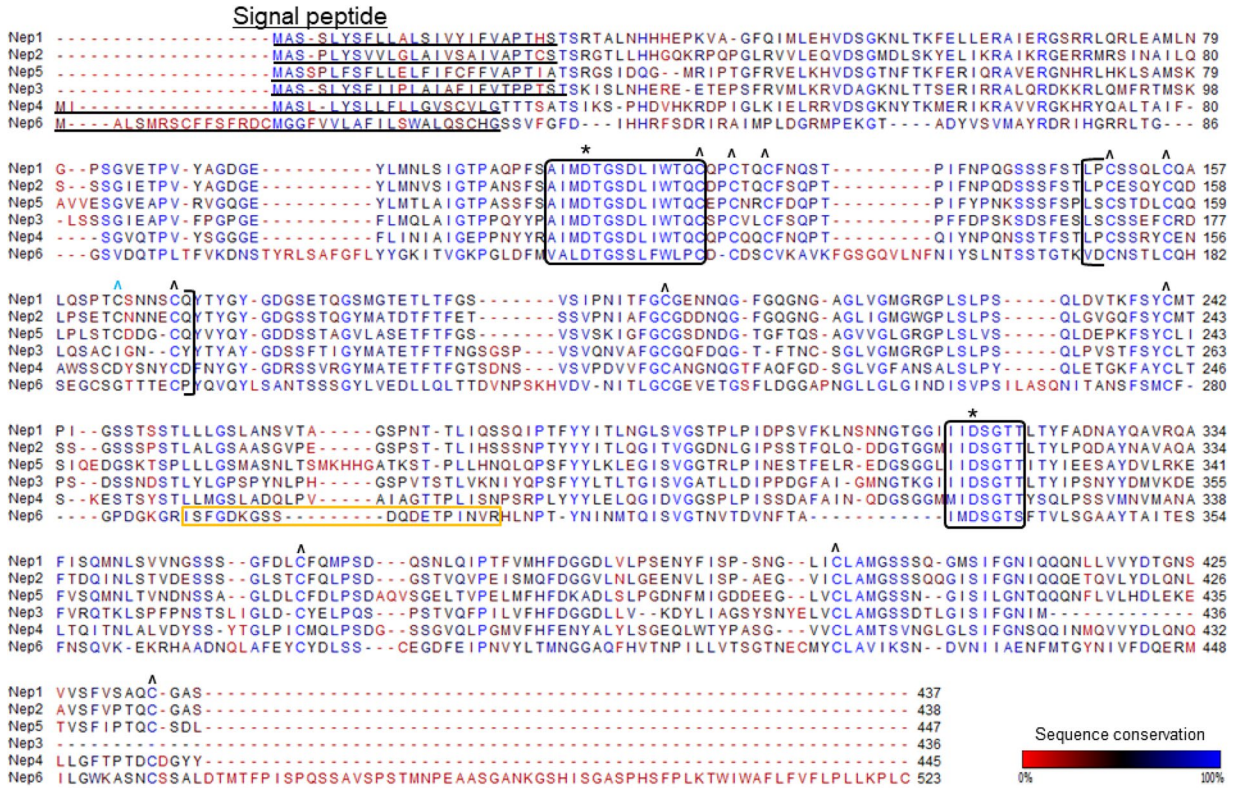

Fig. 5 The alignment of the predicted open reading frame of proposed Nep6 with five other nepenthesins from *N.* × *ventrata.* Signal peptide sequences are underlined. Two active site sequence motifs Asp-Thr-Gly (DTG) and Asp-Ser-Gly (DSG) were boxed with the conserved catalytic aspartate residues marked by asterisks. Twelve conserved cysteine (C) residues are indicated by circumflex accent, while nepenthesin-type aspartic protease (NAP)-specific insertion were marked with brackets. Peptide sequence for the identification of Nep6 in pitcher fluid is indicated by box with lighter border

bands with higher intensities in day-3 samples compared to the control, indicating increased secretion of certain proteins after pitcher opening.

Based on the functional annotation of pitcher fluid secretome in this study, hydrolase is the main group of enzymes (Fig. 6). This is consistent with the proposed digestive and defense functions of enzymes in the pitcher fluid. Although only three biological samples were pooled for LC–MS/MS analysis, the number of identified proteins (32) was comparable to two recent reports, namely Rottloff et al. (2016) with 29 proteins identified from five different *Nepenthes* species based on *N. mirabilis* transcriptome, and a total of 36 proteins from *N.* × *ventrata* pitchers based on *N. rafflesiana* transcriptome by Lee et al. (2016). This may be due to a higher sensitivity of SWATH-MS employed in this study as well as using an *N.* × *ventrata* species-specific sequence database.

Common proteins, such as aspartic proteinases, β-1,3-glucanase, serine carboxypeptidase (SCP), purple acid phosphatase (PAP), pathogenesis-related protein, and thaumatin-like protein (TLP), were consistently found in studies of *Nepenthes* pitcher fluid (Lee et al. 2016; Rottloff et al. 2016). Nonetheless, differences in protein profiles are expected in different *Nepenthes* species and different developmental stages (Biteau et al. 2013). However, no study to date has comprehensively compared the differential expression of proteins in the pitcher fluid during early pitcher opening.

Functional analysis of protein secretion in the pitcher fluid

From the LC–MS/MS analysis, all proteins identified from day-0 samples were also found in day-3 samples. This supports SDS-PAGE analysis, which showed a similar pattern of protein bands. The proteins in the pitcher fluid were quantified to investigate the relative differential expression between the samples (Table 2). Pathogenesis-related protein 1 (PR-1) showed the highest upregulation, followed by kinesin, β-1,3-glucanase, thaumatin-like protein (TLP), nepenthesin-1 (Nep1), serine carboxypeptidase (SCP)-like 49, nepenthesin-2 (Nep2), and neprosin-1 (Npr1). These eight proteins found to be significantly more abundant on day 3 are likely to be continuously secreted proteins, perhaps higher abundance due to the induced effect of protein

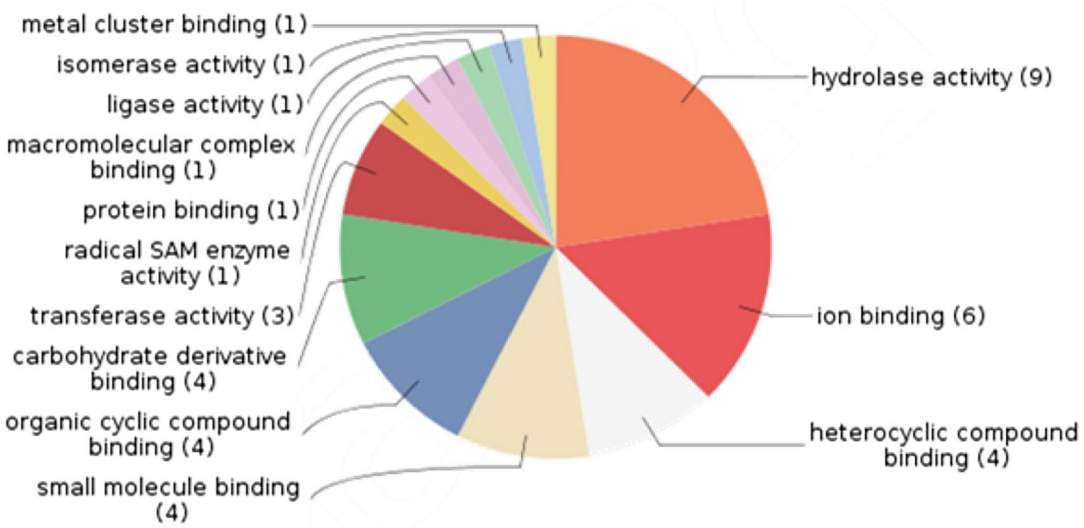

Fig. 6 Gene ontology distribution of proteins identified from pitcher fluid based on level 3 molecular function

depletion on day 0. Notably, all these proteins apart from kinesin have been reported to be abundant in other Nepenthes studies, which play key biological roles in prey digestion and defense mechanism. This demonstrates that certain enzymes essential for the functionality of pitchers are continuously secreted into the pitcher fluid, at least within the 3 days of pitcher opening. This observation further suggests a signaling or feedback mechanism exists in the pitcher that is responsible for protein replenishment. On the other hand, kinesin together with syntaxin-binding protein 5 might play a role in vesicular protein trafficking and protein secretion (Bennett et al. 1993). This might compensate for the loss of endogenous proteins during protein depletion experiment.

The proteins found to be in significantly lower abundance on day 3 include serine carboxypeptidase-like 51, purple acid phosphatase 27 (PAP), and NADH-plastoquinone oxidoreductase subunit 2. These three proteins were not replenished to the level of day 0. PAP is one of the most abundantly secreted proteins in the pitcher fluid, which is the largest group of acid phosphatase enzyme with bacteria-killing property (Schenk et al. 2008). Low phosphate level in pitcher fluid is essential to prevent bacterial growth that leads to nutrient competition (Buch et al. 2013). On the other hand, PAP plays a key role in phosphate acquisition, through the production, transportation, and recycling of phosphate (Tran et al. 2010). This extracellular enzyme hydrolyzes Pi from external organophosphates in Pi-starved plant (Olczak 2003). Carnivorous plants have been shown to absorb phosphate from prey (Buch et al. 2013; Ibarra-Laclette et al. 2011), which helps to increase leaf photosynthesis (Pavlovič et al. 2010). Therefore, PAP is related to defense against pathogen and Pi deprivation in the pitcher fluid. The lack of replenishment might indicate that PAP secretion requires prey induction. Similarly, SCP-like 51 might also require prey induction compared with SCP-like 49 which was constitutively secreted.

Meanwhile, the other eight quantified proteins found to be not significantly different ($P > 0.05$) were likely to be proteins which are also continuously replenished in the pitcher fluid. These include nepenthesin-6 (Nep6), plasma membrane H + -ATPase, peptide transporter, syntaxin-binding protein, DNA polymerase, DNA gyrase, maturase K, and arginine *N*-methyltransferase. Furthermore, the molecular weights of the more abundant proteins were consistent with the observed range of band sizes with increased intensities in the SDS-PAGE analysis (Fig. 4).

The presence of multiple proteins involved in energy metabolisms, such as ATPases, ATP-dependent zinc metalloprotease FTSH4, NADH-plastoquinone oxidoreductase, and ATP synthase showed that energy-intensive biological processes were occurring in the pitcher fluid. The uptakes of peptides and amino acids from protein metabolism are possible through peptide and amino acid transporters that also require metabolic energy. Furthermore, the discovery of enzymes involved in metabolic processes, such as lipoyl synthase and UDP-rhamnose:rhamnosyltransferase 1, suggests possible metabolite biosynthesis previously not reported.

Plant U-box protein 4 (PUB4) is an E3 ubiquitin ligase involved in protein ubiquitylation and degradation through proteasome pathway activity (Kinoshita et al. 2015). A PUB protein in Arabidopsis, AtPUB, mediates ubiquitin-directed protein degradation in plant response to various environmental stresses (Liu et al. 2011). The upregulation of another PUB, AtCHIP, was found to increase plant sensitivity towards temperature stress and ABA hormone (Yan et al. 2003). On the other hand, armadillo (ARM) repeat

protein interacting with ABF2 (ARIA) is a transcription factor which controls the abscisic acid (ABA)-dependent gene expression via the G-box type ABA-responsive elements (Kim et al. 2004). ARIA protein is also known as a positive regulator of ABA response as its overexpression results in ABA hypersensitivity. The discovery of PUB4 and ARIA suggests the possibility of ABA involvement in the regulation of pitcher fluid.

ABA is a phytohormone with a pivotal role in developmental processes and adaptive stress responses to environmental stimuli (Fujita et al. 2011). This hormone is also responsible for the regulation of water relation in plants in drought stress by adjusting cellular osmotic pressure and stomata closure, which is important for gland secretion (Daszkowska-Golec and Szarejko 2013). In *N.* × *hookeriana*, the presence of ABA is found to stimulate the net outflow of K^+ and Cl^- ions from pitcher gland into the pitcher (Jung and Lüttge 1980). In *Dioneae* species, ABA helps in regulating gland secretion together with 12-oxo-phytodienoic acid (OPDA) (Escalante-Pérez et al. 2011). A serine/threonine-protein kinase newly discovered in this study could facilitate ABA signal transduction by phosphorylating ABA response element-binding factors (Kobayashi et al. 2005). However, this remains highly speculative and the relationship between ABA and fluid protein regulation in *Nepenthes* species needs to be further explored.

It was also surprising to discover these regulatory factors inside the pitcher fluids. Even though the transcriptome used for protein identification was derived from the pitcher tissue, we cannot exclude the possibility that some of the transcript sequences might be of microbial or fungal origins. This might explain the presence of DNA replication complex GINS protein, which was functionally annotated to a fungal protein (Supplementary Data 2). However, other proteins were functionally annotated to plants, which include DNA polymerase delta catalytic subunit, arginine *N*-methyltransferase, and DNA gyrase, a topoisomerase commonly found in bacteria but also present in plastids. Therefore, these proteins could be legitimate in the pitcher fluid as supported by the presence of plant-specific regulatory factors, such as plastidial maturase K and pentatricopeptide repeat-containing protein. Nonetheless, it was puzzling to find cyclic Dof factor, a transcription factor of photoperiodic flowering response (Fornara et al. 2009). The roles of these novel regulatory factors and plastidial proteins in the pitcher fluids remain to be elucidated in future studies.

On the other hand, fasciclin-like arabinogalactan 8 (FLA8) protein is a subclass of arabinogalactan protein (AGP) family with two fasciclin conserved regions (Seifert and Roberts 2007). AGP is a proteoglycan with various functions in plant growth and development process. Amphiphilic molecular structure of this protein enables them to be a soluble signal and acts as modulators and co-receptors of apoplastic morphogens between the cell wall, plasma membrane, and cytoplasm (Seifert and Roberts 2007). This protein also functions in cell-to-cell interaction and communication for environmental signaling during plant development. The expression of FLA8 is also found to be downregulated in an ABA-dependent pathway, suggesting their role in abiotic stress response (Johnson et al. 2003). Furthermore, arabinogalactan protein might also contribute to the viscosity of the pitcher fluid.

Glucosidase, peroxidase, esterase/lipase, galactosidase, chitinase, xylosidase, and lipid transfer protein were not detected in the fluid samples despite their expression in the pitcher tissue (Table 1). Evidence of these enzymes present in pitcher fluids of different species of *Nepenthes* was reported previously (Ravee et al. 2018). The secretion of chitinase is induced by the presence of chitin in *N. khasiana* pitcher fluid (Eilenberg et al. 2006) and not detected in all species of *Nepenthes* (Rottloff et al. 2016), which indicates that chitinase is not always secreted into the pitcher fluid. Chitinases have also been found in symbiotic bacteria from the pitcher fluid (Chan et al. 2016). In the current experiment, pitchers were sealed by parafilm and fluid samples were not induced by prey or chitin, which might explain why chitinase or other prey-induced enzymes were found to be absent or not detected due to low abundance.

Conclusions

This study employed an integration of high throughput technologies in transcriptomic and proteomic analyses of *N.* × *ventrata* pitcher fluid proteins. The usage of predicted protein sequences from a species-specific transcriptome database successfully identified more proteins without genome information. Quantitative proteomics through SWATH analysis coupled with SDS-PAGE analysis on the differential protein expression in day-0 and day-3 samples supports that many proteins were continuously secreted into the pitcher fluid. The identification of many endogenous proteases in pitcher fluid suggests that protein metabolism occurs mainly in the fluid, with evidence of a new aspartic protease Nepenthesin-6. The discovery of 16 previously unreported proteins related to gene and protein regulations in the pitcher fluid provides unprecedented insights into the molecular regulation of proteins in *Nepenthes* pitcher fluids with possible involvement of ABA signaling. Furthermore, this study demonstrates that endogenous proteins involved in plant defense mechanism were highly replenished during early pitcher opening, which supports the origin of plant carnivory from plant defenses. *Nepenthes* pitchers could serve as a unique model system for the cost–benefit investigation of plant protein turnover and replenishment in future studies.

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Author contributions HHG and WNAWZ designed the experiments and prepared the figures/tables, WNAWZ performed the experiments, HHG, WMA and WNAWZ analyzed the data, all authors drafted the work and reviewed the manuscript.

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Data availability statement Predicted protein sequences are provided in a fasta file as Supplementary Data 1 and transcriptome annotation is provided as Supplementary Data 2 in this manuscript. The datasets generated in the current study are available in the public repositories as stated above, namely NCBI SRA and TSA, and PRIDE through the following links: www.ncbi.nlm.nih.gov/sra/SRR2854733. www.ncbi.nlm.nih.gov/sra/SRR2854734. www.ncbi.nlm.nih.gov/sra/SRR2854735. www.ncbi.nlm.nih.gov/nuccore/GFAD00000000. ftp.pride.ebi.ac.uk/pride/data/archive/2017/08/PXD007251

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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