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Proteome analysis of digestive fluids in *Nepenthes* pitchers

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● **Background and Aims** Carnivorous plants have developed strategies to enable growth in nutrient-poor soils. For the genus *Nepenthes*, this strategy represents producing pitcher-modified leaves that can trap and digest various prey. These pitchers produce a digestive fluid composed of proteins, including hydrolytic enzymes. The focus of this study was on the identification of these proteins.
● **Methods** In order to better characterize and have an overview of these proteins, digestive fluid was sampled from pitchers at different stages of maturity from five species of *Nepenthes* (*N. mirabilis*, *N. alata*, *N. sanguinea*, *N. bicalcarata* and *N. albomarginata*) that vary in their ecological niches and grew under different conditions. Three complementary approaches based on transcriptomic resources, mass spectrometry and *in silico* analysis were used.
● **Key Results** This study permitted the identification of 29 proteins excreted in the pitchers. Twenty of these proteins were never reported in *Nepenthes* previously and included serine carboxypeptidases, α- and β-galactosidases, lipid transfer proteins and esterases/lipases. These 20 proteins display sequence signals allowing their secretion into the pitcher fluid.
● **Conclusions** *Nepenthes* pitcher plants have evolved an arsenal of enzymes to digest prey caught in their traps. The panel of new proteins identified in this study provides new insights into the digestive process of these carnivorous plants.

Key words: Carnivorous plants, digestive fluid, enzymes, mass spectrometry, *Nepenthes mirabilis*, *Nepenthes alata*, *Nepenthes sanguinea*, *Nepenthes bicalcarata*, *Nepenthes albomarginata*, pitcher plants, proteome, transcriptomic analysis.

INTRODUCTION

In nutrient-poor habitats, carnivorous plants have developed original feeding strategies based on the capture and digestion of arthropods and the assimilation of prey-derived nutrients by leaves highly specialized for trapping. Among the dozen genera of carnivorous plants recorded to date, *Nepenthes* is one of the richest, comprising nearly 160 species that are widespread in South-western Asia. The greatest diversity occurs in Borneo (Clarke, 1997), Sumatra (Clarke, 2001) and the Philippine islands (McPherson, 2009). From lowlands to mountain forests, these plants have colonized a large number of habitats comprising different substrates (e.g. sandy, peat, swampy, cliff or epiphytic substrates) (Clarke, 1997; McPherson, 2009). On these plants, leaves are extended by a tendril that bears a pitfall trap, also called a pitcher. Glandular structures have been described in the inner and basal part of the pitchers. These glands produce secretions that contain a cocktail of hydrolytic enzymes for the degradation of insects (An *et al.*, 2002).

Proteins present in the secretions of different *Nepenthes* species have been identified. Most of the proteins are enzymes related to the digestion of various prey items. In addition to the aspartic acid proteases Nepenthesin 1 and 2 isolated from *N. alata*, *N. distillatoria* and *N. gracilis* (Athauda *et al.*, 2004; Takahashi *et al.*, 2005; Hatano and Hamada, 2008; Kadek *et al.*, 2014), various hydrolytic enzymes have been identified, including S-like RNases (*N. ventricosa*) (Matthews, 1960; Stephenson and Hogan, 2006; Nishimura *et al.*, 2014), esterases (*N. hybrida*) (Higashi *et al.*, 1993), lipases (*N. macfarlanei*) (Tökés *et al.*, 1974), acid or alkaline phosphatases (*N. hybrida* and *N. macfarlanei*) (Higashi *et al.*, 1993; Płachno *et al.*, 2006), phosphoaminases (*N. hybrida*) (Higashi *et al.*, 1993), peroxidases (*N. alata*) (Hatano and Hamada, 2012) and chitinases (*N. khasiana*, *N. alata*, *N. singalana*, *N. ventricosa*, *N. gracilis*, *N. thorelii*, *N. mirabilis*, *N. ampullaria*, *N. rafflesiana* and the hybrid 'Mizuho') (Eilenberg, 2006; Hatano and Hamada, 2008; Rottloff *et al.*, 2009, 2011; Ishisaki *et al.*, 2012*a*, *b*).

Proteins that are not directly involved in prey degradation have also been reported. For example, a β-D-xylosidase in *N. alata* secretions could be involved in trap maturation according to Hatano and Hamada (2008). Other identified proteins are dedicated to plant defence against pathogens, and include a thaumatin-like protein (*N. alata*, *N. thorelii*, *N. superba*, *N. gracilis*, *N. fusca*, *N. ampullaria*, *N. mirabilis*, *N. singalana* and *N. ventricosa*) (Hatano and Hamada, 2008; Rottloff *et al.*, 2009), a PR-1 protein (*N. mirabilis*) (Buch *et al.*, 2014) and β-1,3-glucanases (*N. alata*) (Hatano and Hamada, 2008, 2012). These proteins may contribute to the inhibition of microbial growth in the pitcher fluid to prevent competition between the plant and microorganisms for the nutrients derived from the digested prey (Eilenberg, 2006; Hatano and Hamada, 2008, 2012; Mithofer; 2010; Buch *et al.*, 2013).

Given their adaptation to different habitats and spectra of prey (Kato *et al.*, 1993), the *Nepenthes* species have evolved different trapping strategies (Bonhomme *et al.*, 2011b; Bazile *et al.*, 2015). It is thus likely that each species has developed the ability to synthesize an arsenal of different and specific enzymes. Using SDS–PAGE approaches to analyse the pitcher fluids of 17 *Nepenthes* species, we previously suggested that the protein composition of the secretion might be specific to each species (Biteau *et al.*, 2013). In order to complete the study carried out by Biteau and colleagues, we further investigated the protein compositions of the digestive fluids from five *Nepenthes* species, *N. mirabilis*, *N. alata*, *N. sanguinea*, *N. bicalcarata* and *N. albomarginata*, which were grown in different conditions and collected from greenhouse cultures (*N. mirabilis, N. sanguinea* and *N. alata*), *in vitro* cultures (*N. alata* and *N mirabilis*) and from their natural habitats (*N. bicalcarata* and *N. albomaginata*). Also *N. bicalcarata* and *N. albomaginata* differ in their diet from the other species. The first one lives with *C. schmitzi* and the second one uses food hairs on the peristome to capture hundreds to thousands of termites (literature: *N. bicalcarata* versus *C. schmitzi*: Clarke and Kitching, 1995; *N. albomarginata*: Merbach *et al.*, 2002). We used a complementary three-step approach involving (1) deep sequencing of the transcriptome of tissues located in the digestive zone of an *N. mirabilis* pitcher; (2) mass spectrometry (MS)-based analyses of proteins present in the digestive fluid; and (3) an *in silico* analysis of the putative proteins.

MATERIALS AND METHODS

Plants

To construct the cDNA library, total RNA was extracted from *Nepenthes mirabilis* pitchers (1988.3.265) provided by the Montet Botanical Garden (Villers-Lès-Nancy, France).

For proteomic analyses, secretions from different *Nepenthes* species, states and cultivations were used. *Ex vitro N. alata* and *N. sanguinea* were purchased from Araflora (http://www.araflora.com) and cultivated in heated greenhouses with natural light at a temperature of 23 °C and a relative humidity of 75–85 %. *Nepenthes bicalcarata* and *N. albomarginata* were directly sampled in their natural sites in Brunei (Borneo). *Nepenthes bicalcarata* was sampled in lowlands in the peat swamp forest (4 °59'N, 114 °49'E), and *N. albomarginata* was sampled in heath forest (4 °56'N, 114 °49'E).

In vitro N. alata plants were supplied by Deroose Plants (www.derooseplants.com) and micropropagated in half-strength MS medium (Murashige and Skoog, 1962) containing 2-fold MS vitamin mixture, 2 % (w/v) sucrose, 0.05 % (w/v) casein hydrolysate, 0.07 % (w/v) MES, 0.2 % (w/v) active charcoal and 0.7 % (w/v) HP696 agar (Kalys, Bernin, France). The pH of the medium was adjusted to 5.8 before autoclaving.

In vitro stocks of *N. mirabilis* plants were established from fresh seeds supplied by Districarnivores (www.districarnivores.com). Seeds were sterilized by total immersion in a dilute commercial bleach solution of 0.25 % sodium hypochlorite for 5 min and washed three times in sterile water. After a drying step, they were sown on quarter-strength MS medium (Murashige and Skoog, 1962) containing 2-fold MS vitamin mixture, 2 % (w/v) sucrose, 0.05 % (w/v) casein hydrolysate, 0.07 % (w/v) MES, 6 mg L^{-1} ProClin® 200 (Sigma-Aldrich, St Louis, MO, USA) and 0.7 % (w/v) HP696 agar (Kalys). All cultures were cultivated under a 16 h/8 h day/night photoperiod provided by natural white fluorescent lamps at a temperature of 23 °C. Germinated plantlets were then transplanted in the same medium as *N. alata* described above, without active charcoal.

Construction of a Nepenthes RNA-seq library

An RNA sequencing (RNA-seq) library was constructed based on an RNA extraction performed on secreting tissues (from the bottom of pitchers) from an *N. mirabilis* specimen (*N. mirabilis* 1988.3.265) provided by the Montet Botanical Garden.

RNA preparation. Total RNA (40 µg) was extracted from the glandular part of pitchers 8 d after opening (wrapped in perforated bags to prevent insect contamination) using the Spectrum™ Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's instructions. To remove genomic DNA efficiently, RNA was treated with the Turbo™ DNA Free Kit (AM1907M, Ambion, Thermo Fisher Scientific, Waltham, MA, USA).

Construction of a random primed cDNA library and high-throughput sequencing. Construction of a *de novo* normalized 454-based RNA-seq library and deep sequencing were performed by Eurofins MWG Operon (Ebersberg, Germany) using GS FLX+ chemistry. Clustering and assembly of the reads were performed by the supplier.

Protein identification

Sample preparation. The digestive fluid was collected from closed pitchers using a sterile syringe. When this was not possible, pitchers were wrapped in bags before they opened in order to avoid any insect contamination. For secretions from *Nepenthes* growing in their natural habitats (*N. albomarginata* and *N. bicalcarata*), samples were collected from closed and recently opened pitchers for which we verified visually the absence of prey. Proteins present in the digestive fluid were precipitated overnight at –20 °C with 4 vols of acetone. The proteins were collected by centrifugation at 12 000 *g* for 30 min at 4 °C and used directly for MS analyses or separated by 1-D or 2-D PAGE.

1-D SDS–PAGE: samples were resuspended in 40 µL of electrophoresis buffer [25 mM Tris–HCl, 192 mM glycine, 0.1 % (w/v) SDS, pH 8.3 at 25 °C] and supplemented with 10 µL of

denaturation buffer [0.313 M Tris–HCl pH 6.8 at 25 °C, 10 % (w/v) SDS, 0.5 % (w/v) bromophenol blue, 50 % (v/v) glycerol, 2 M DTT (dithiothreitol)]. Proteins were heat denatured at 95 °C for 10 min and separated by 1-D SDS–PAGE on Mini-PROTEAN® TGX pre-cast gels, AnykD™ (10-well, 50 µL) (Bio-Rad, Hercules, CA, USA) for 1 h at 100 V. Protein bands were visualized by silver staining (Blum *et al.*, 1987).

2-D SDS–PAGE: to perform 2-D separation, we used the ReadyPrep™ 2D Starter Kit (Bio-Rad). To prepare samples, protein pellets were dissolved in 125 µL of Rehydration/Sample Buffer and loaded on 7 cm pH 3–6 IPG strips (ReadyStrip™ IPG Strips; Bio-Rad). The PROTEAN® i12™ IEF System (Bio-Rad) was used for isoelectric focusing with the following program: step 1 (250 V, 20 min, linear), step 2 (4000 V, 2 h, linear) and step 3 (4000 V, 5 h, rapid). After the IPG equilibration for SDS–PAGE described in the ReadyPrep™ 2D Starter Kit protocol, the strips were placed on Mini-PROTEAN® TGX™ pre-cast gels for 2-D electrophoresis (Bio-Rad). Separation was then performed for 45 min at 200 V. Spots were visualized by silver staining (Blum *et al.*, 1987).

Spot sampling: proteins were excised from 1-D or 2-D gels with a scalpel and chopped into 1 mm³ pieces. Silver-stained spots were destained with a solution containing a 1:1 mixture of 30 mM potassium ferricyanide/100 mM sodium thiosulphate until the brown colour vanished. The samples were then washed three times with distilled water (Gharahdaghi *et al.*, 1999). Next, the samples were incubated by shaking for 15 min in 200 µL of distilled water/acetonitrile (ACN) (1:1, v/v), 5 min in 200 µL of ACN, 5 min in 100 µL of 100 mM ammonium bicarbonate (BiCa) and 15 min in 100 µL of ACN. After removing the supernatant, the gel pieces were dried with a Speed Vac Concentrator Plus (Eppendorf, Hamburg, Germany). Peptides were reduced by adding 10 mM DTT in 100 mM BiCa to the gels for 45 min at 56 °C and then cooling on ice for 5 min. Peptides were alkylated by incubation with 55 mM iodoacetamide in 100 mM BiCa in the dark for 30 min. Iodoacetamide was replaced with 1 vol. of 100 mM BiCa and incubated for 15 min with shaking prior to the addition of 1 vol. of ACN. The samples were then incubated for an additional 15 min with shaking. The supernatant was removed, and the gel pieces were dried using an Eppendorf Speed Vac Concentrator Plus.

In-gel digestion with trypsin was performed as described previously (Jensen *et al.*, 1999). Proteins were treated with 10 µL of trypsin (10 ng µL⁻¹ in 50 mM BiCa) and incubated for 10 min on ice. This step was repeated until the gel pieces did not expand further. The trypsin solution was removed, 25 mM BiCa was added until the gel pieces were completely covered and the mixtures were incubated overnight at 37 °C. The samples were subjected to an Elmasonic S70 ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) for 2 min, and the supernatant was transferred to a new tube. The remaining acrylamide was incubated in 1 vol. of 25 mM BiCa for 15 min with shaking. After the addition of 1 vol. of ACN, the sample was incubated for 15 min with shaking and then transferred to an ultrasonic bath for 2 min. The supernatant was removed and added to the previously collected supernatant. Finally, the sample was incubated for 15 min with shaking in 1 vol. of 5 % (v/v) formic acid/ACN (1:1, v/v), and the supernatant was removed and added to the previously pooled supernatant. The sample was concentrated using an Eppendorf Concentrator Plus at 60 °C, and the peptides were stored at –20 °C.

NanoLC-MS/MS analyses. The peptide mixtures obtained from SDS–PAGE spots or crude protein extracts were resuspended in 15 µL of water containing 0.1 % formic acid (solvent A) and further analysed using a NanoLC-2DPlus system (with the nanoFlex ChiP module; Eksigent, ABSciex, Concord, Ontario, Canada) coupled to a TripleTOF 5600 mass spectrometer (ABSciex) operating in positive mode. A total of 5 µL of each sample (up to 1 µg for the crude extracts) was loaded on a ChIP C-18 pre-column (300 µm ID × 5 mm ChromXP; Eksigent) at 2 µL min⁻¹ in solvent A. After 10 min of desalting and concentration in the trap, the pre-column was switched online with the analytical ChIP C-18 analytical column (75 µm ID × 15 cm ChromXP; Eksigent) equilibrated in 95 % solvent A and 5 % solvent B (0.1 % formic acid in ACN). Peptides were eluted using a 5–40 % gradient of solvent B at a flow rate of 300 nL min⁻¹. The length of the LC gradient was adjusted depending on the sample complexity, from 45 min for the SDS–PAGE bands to 90 min for the crude protein extracts. The TripleTOF 5600 was operated in data-dependent acquisition mode (DDA) with Analyst software (v1.6, ABSciex). Survey MS scans were acquired during 250 ms in the 350–1250 m/z range. Up to 20 of the most intense multiply charged ions (2+ to 4+) with a threshold intensity exceeding 150 counts per second were selected for collision-induced dissociation (CID) fragmentation. Ions were fragmented using a rolling collision energy script within a 60 ms accumulation time and an exclusion time of 15 s. This so-called 'Top20' method, which had a constant cycle time of 1.5 s, was set in high-sensitivity mode.

Bioinformatics analyses

Protein data The raw data files obtained for each nanoLC-MS/MS injection were first converted to .mgf files using the MS-Data converter script (version 1.3, AB Sciex) and subsequently used by the Mascot algorithm (version 2.2, Matrix Science, London, UK) in the ProteinScape package (version 3.1, Bruker). The *Nepenthes* sub-taxonomy from the UniProt database (release from 2012-07-12 containing 235 entries) was first searched using the following parameters: (1) allowed variable peptide modifications of *N*-acetyl (protein), carbamidomethylation (C) and oxidation (M); (2) mass tolerances in MS and MS/MS of 20 ppm and 0.5 Da, respectively; and (3) trypsin as enzyme specificity with a maximum of two missed cleavages sites. Peptides were validated using the Mascot individual ions score threshold to give a P-value <0.05. The protein identifications obtained from Mascot were validated using a false discovery rate (FDR) of 1 % by employing a decoy database strategy and the Protein Assessment Configuration from Proteinscape 3.1 package (Thiele *et al.*, 2010). To create the decoy database, the makeDecoyDB.pl Pearl script from Bruker was used: a .fasta database was generated with the forward and the reversed sequences, with 'rnd' as the accession prefix associated with each decoy entry. Moreover, protein inference is taken into account by the Proteinscape package during the Protein Assessment and Protein compiling steps: for each accession number, a set of so-called Alternative Proteins is given, with the list of their shared peptides.

PEAKS Studio 5.3 (Bioinformatics Solutions Inc., Waterloo, Canada) was used in a second analysis to examine in greater depth the assignment of a peptide sequence to the maximum number of experimental MS/MS fragmentation spectra. A PEAKS identification workflow was established that consisted of an initial auto *de novo* step with the generation of ten tags per spectrum, parent and fragment mass tolerances set at 20 ppm and 0.5 Da, trypsin as the enzyme, and carbamidomethylation (C) and oxidation (M) as variable modifications. A PEAKS search was then launched with the same mass tolerances and variable modifications against three different databases: a whole SwissProt database (release from 2012-01-11 containing 533 657 entries) and the two sub-databases described above (*Viridiplantae* and *Nepenthes* taxonomic subdivisions). Spectra that remained unassigned after the PEAKS DB searches were further submitted to PTM Finder and SPIDER searches. PTM Finder was configured to search 18 additional variable modifications (including acetylation, methylation, deamidation and phosphorylation), whereas SPIDER search allowed homology matches with $L = I$ and $Q = K$. In these steps, the *de novo* score (ALC%) threshold was set at 90, and the peptide hit threshold ($-10logP$) was set at 30. Finally, peptides belonging to proteins validated at 1 % FDR were manually inspected to validate only high-quality MS/MS fragmentation spectra with a minimum of five consecutive amino acids, major peaks assigned to fragments and specific proline fragmentation patterns.

cDNA library analysis. The FASTA file provided by MWG Eurofins Operon (Ebersberg, Germany) was imported as a library into the software BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The peptide sequences obtained by MS analysis were compared with the RNA data available in the *Nepenthes* cDNA library using the Basic Local Alignment Search Tool to identify full-length or partial coding sequences. The presence of an N-terminal signal peptide (SP), which is necessary for the secretion of proteins out of the cell in eukaryotic organisms and described before for other pitcher fluid proteins (Rottloff *et al.*, 2011; Buch *et al.*, 2014), was confirmed for all identified proteins. For these analyses, we used the SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/) and the Signal-3 L (http://www.csbio.sjtu.edu.cn/bioinf/Signal-3 L/) servers. The latter contains the option to adjust the origin of the protein, e.g. plants. For proteins with a predicted SP, the molecular weight and isoelectric point (pI) of the mature protein (without SP) and the number of amino acids were calculated using the ExPASy compute pI/MW tool (http://web.expasy.org/compute_pi/). To predict potential amino acid sites for glycosylation, we used the NetNGlyc 1.0 and the NetOGlyc 4.0 servers (http://www.cbs. dtu.dk/services/NetNGlyc/ and http://www.cbs.dtu.dk/services/ NetOGlyc/) at the Center for Biological Sequence Analysis website (http://www.cbs.dtu.dk/services/).

RESULTS

cDNA library description

To characterize proteins present in the digestive fluid of *Nepenthes* pitchers, we first identified peptides corresponding to trypsin-digested proteins using MS and then fished the

complete corresponding coding sequence using a transcriptomic cDNA library. Because only a small number of coding sequences from *Nepenthes* species are available in public genomic libraries, we generated a 454-based *de novo* RNA-seq library of *N. mirabilis* pitchers cultivated in a greenhouse. This library was normalized to ensure representation of most of the genes expressed in these tissues.

After trap opening, the hydrolytic activity of *N. mirabilis* pitcher fluid requires time to become fully active. This delay is in contrast to other *Nepenthes* species and is related to the concentration of proteins detected in the pitcher fluid (Biteau *et al.*, 2013). SDS–PAGE analysis of the secreted proteins revealed a strong increase in protein amounts 5 d after trap opening compared with the first day, with a peak 8 d after opening (Biteau *et al.*, 2013). Based on these results, we used pitchers opened for 8 d as our experimental plant material because these pitchers should exhibit the greatest transcriptional activity. Total RNA was extracted from the lower part of pitchers (from a unique *N. mirabilis* specimen) covered by multicellular secretory glands that reportedly secrete the digestive enzymes (Rottloff *et al.*, 2011). Nearly 248 $\times 10^6$ bp were sequenced from the RNA sample distributed across 631 074 reads with an average length of 432 bp. Clustering and assembly of these singletons led to the identification of 43 026 contigs or 10 218 large contigs (>1000 bp). These contigs were annotated and functionally assigned using Blast2GO (https://www.blast2go.com/).

Protein analyses of in vitro N. mirabilis secretions

To establish an experimental procedure for identifying proteins present in pitcher fluid, we began our investigations with fluid sampled from 3-year-old *in vitro* cultivated *N. mirabilis* plants. Working with *in vitro* plants presents two main advantages. First, the precise culture conditions are known. Secondly, the sterile growth conditions ensured that no insect or pathogen was responsible for inducing proteins or enzymes involved in a defence reaction and that no foreign protein was present in the pitcher fluid. Whole proteins (600 µg of total proteins) were precipitated in the presence of acetone and separated by 2-D gel electrophoresis. Twenty-five different silver-stained protein spots were excised from the gel and analysed by nanoLC-MS/ MS (Supplementary Data Table S1; Fig. S1). Unfortunately, this technique only allowed the identification of two different proteins, Nepenthesin 1 and a glucanase (NgNep1 and NkGluc1) detected in different spots (Table 1; Table S2). Conclusive results could not be obtained for other spots, possibly due to (1) a loss of material during the precipitation and purification steps prior to or after migration on the acrylamide gel; and/or (2) the low concentration of most proteins present in the pitcher fluid of *in vitro* cultivated *N. mirabilis*.

We therefore modified our experimental procedure. Instead of analysing a single spot extracted from a polyacrylamide gel, we analysed a crude protein mixture followed by precipitation of all the proteins present in the pitcher fluid. Ten different proteins in *N. mirabilis* fluid were identified using this technique (Supplementary Data Table S2). The amino acid sequences of the peptides obtained were used to perform a tblastn search in public databases (GenBank) and/or the *N. mirabilis* RNA-seq database, which led to the identification of their corresponding

Table 1. *Summary of the proteins identified from digestive fluids of five* Nepenthes *species and their putative activities*

Nomenclature	Accession number	Protein description	No. of peptides
Nepenthesins			
NgNep1*	BAD07474 (*N. gracilis*)	Nepenthesin 1 (Athauda *et al.*, 2004)	3
NgNep2*	Q766C (*N. gracilis*)	Nepenthesin 2 (Athauda *et al.*, 2004)	3
NmNep2LP	Q766C2 (*N. gracilis*)	Nepenthesin 2-like protein	3
Carboxypeptidases			
NmSCP3	NP_197689 (*A. thaliana*)	Serine carboxypeptidase III	8
NmSCP20	XP_004290128 (*F. vesca*)	Serine carboxypeptidase-like 20	3
NmSCP47	NP_197689 (*A. thaliana*)	Serine carboxypeptidase-like 47	3
NmSCP51	XP_002273519 (*V. vinifera*)	Serine carboxypeptidase-like 51	2
Phosphatases			
NmNPP1	XP_003605731 (*M. truncatula*)	Nucleotide pyrophosphatase/phosphodiesterase	1
NmPP1	XP_003603267 (*M. truncatula*)	Protein phosphatase 2C	1
Peroxidases			
NmPRX27	XP_002280216 (*V. vinifera*)	Peroxidase 27	3
NmPRX-N	XP_002285642 (*V. vinifera*)	Peroxidase N	3
Lipid transfer proteins (LTPs)			
NmLTP1	XP_003531043 (*G. max*)	Lipid transfer protein 1	2
NmLTP2	ABK9681 (*O. sativa*)	Lipid transfer protein 2	2
Galactosidases			
NmAG1	XP_004297698 (*F. vesca*)	α-Galactosidase 1	4
NmAG2	XP_002279730 (*V. vinifera*)	α-Galactosidase 2	2
NmBG1	ACC7825 (*C. papaya*)	β-Galactosidase 1	2
NmBG2	CAC44500 (*F. ananassa*)	β-Galactosidase 2	1
NmBG3	EOY3046 (*T. cacao*)	β-Galactosidase 3	2
Other functions			
NaChit1*	BAF98919 (*N. alata*)	Chitinase (class IV) (Hatano and Hamada, 2008; Ishisaki *et al.*, 2012*b*)	5
NrChit1*	ABF7462 (*N. rafflesiana*)	Acidic chitinase (class III) (Rottloff *et al.*, 2011; Hatano and Hamada, 2012; Ishisaki *et al.*, 2012*a*)	3
NaGluc2*	BAM28606 (*N. alata*)	β-1,3-Glucanase (Hatano and Hamada, 2008)	2
NkGluc1*	ABB8952 (*N. khasinana*)	Glucanase (Eilenberg and Zilberstein, 2008)	12
NmAGluc1	AGA82514 (*C. sinensis*)	α-Glucosidase	1
NmBGluc7	NP_195174 (*A. thaliana*)	β-D-1,3-Glucosidase 7-like protein	3
NaPRX1*	BAM2860 (*N. alata*)	Cationic peroxidase 1 (Hatano and Hamada, 2012)	3
NgTLP*	ABC73397 (*N. gracilis*)	Thaumatin-like protein (Hatano and Hamada, 2008; Rottloff *et al.*, 2009)	13
NmPR-1*	ACT9972 (*N. mirabilis*)	PR-1 protein (Buch *et al.*, 2014)	7
NmXyl1*	AAX9296 (*O. sativa*)	β-Xylosidase (Hatano and Hamada, 2008)	1
NmLip1	XP_004232991 (*S. lycopersicum*)	Esterase/lipase	1

The protein names that are notated in bold are newly identified proteins.

Sequences identified in the NCBI database and used for protein identification were obtained from different species summarized in the 'accession number' column. The identity of peptides detected is given in Supplementary Data Table S2.

*Proteins, which were previously described in the pitcher fluid of *Nepenthes*.

full-length coding sequences. When a hit identified in a public library corresponded to a protein identified in other plants, we performed a second tblastn search on the *N. mirabilis* RNA-seq database. Four proteins had already been described in the literature: Nepenthesin 1 (Athauda *et al.*, 2004), a thaumatin-like protein (Hatano and Hamada, 2008; Rottloff *et al.*, 2009), a glucanase (Eilenberg and Zilberstein, 2008) and a class IV chitinase (Hatano and Hamada, 2008; Ishisaki *et al.*, 2012*a*). The six remaining proteins were newly identified and were putatively related to a serine carboxypeptidase III (NmSCP3), a serine carboxypeptidase-like 47 (NmSCP47), α-galactosidases 1 and 2 (NmAG1 and NmAG2), a protein phosphatase 2 C (NmPP1) and a peroxidase N (NmPRX-N) (Table 1; Supplementary Data Table S2). This first set of experiments demonstrated that a global analysis of the proteins present in the pitcher is sufficient to obtain information on several new proteins. This approach allowed us to detect proteins present in small amounts in the pitcher fluid that might otherwise be below the detection threshold of an acrylamide gel. In addition, the direct digestion leads to the identification of a more

exhaustive protein list since (1) it is not biased by the cutting of visible bands and (2) because proteins are directly exposed to the desired proteolytic digestion without the need to extract the peptides further from a gel.

In subsequent experiments, we performed both types of analyses: identification of proteins present in single protein bands sampled from polyacrylamide gels and global analyses of precipitated proteins (Supplementary Data Table S2). However, we restricted our analyses to 1-D separation, a technique that has already been efficiently used to identify new proteins from *Nepenthes* pitcher fluids (Hatano and Hamada, 2008, 2012). Although this method is not the best-adapted method for separating single proteins, it reduces the loss of protein during purification steps, a significant advantage over 2-D separation strategies.

Protein analyses of in vitro *and* ex vitro N. alata *digestive fluids*

According to Biteau *et al.* (2013), the digestive fluid protein content in pitchers varies among *Nepenthes* species. After

studying the proteins produced by *N. mirabilis*, we investigated *N. alata*. We separated the proteins present in four opened pitchers and one closed pitcher from *N. alata* grown on soil in a greenhouse (Supplementary Data Table S1). Because the electrophoresis patterns were rather similar from one gel to another, we performed the analyses on 26 bands collected from two independent gels (Fig. 1, panel 1). This investigation was completed by an analysis of a crude protein preparation collected from 4-year-old plants grown *in vitro* (one repetition) and from one opened and one closed pitcher of *N. alata* grown on soil (Table S1).

A total of 21 putative digestive enzymes were identified in *N. alata* secretions. If seven of these proteins have already been reported, others were never described in this botanical family. These proteins, identified as a lipid transfer protein 1 (NmLTP1), a serine carboxypeptidase-like 20 (NmSCP20), a serine carboxypeptidase-like 51 (NmSCP51), a PR-1 protein (NmPR-1) (Buch *et al*., 2014), a β-1,3-glucanase (NaGluc2) (Hatano and Hamada, 2008), a β-xylosidase (NmXyl1), α-galactosidases 1 and 2, β-galactosidases 1, 2 and 3 (NmBG1, 2 and 3), a β-D-1,3-glucosidase 7-like protein (NmBGluc7), a peroxidase 27 (NmPRX27) and a lipid transfer protein 2 (NmLTP2), were identified in *ex vitro* secretions (Table 1; Supplementary Data Table S2). NmXyl1 was previously described in *N. alata* by Hatano and Hamada (2008) using a proteomic approach, but complete coding cDNA sequence information was not available at the time of our study. The results of three replicates of digestive fluid collected from closed and opened pitchers highlighted a large number of different MS/MS spectra corresponding to peptides derived from Nepenthesin 1 and the thaumatin-like protein. This abundance might reflect a very high concentration of these proteins compared with others in the pitcher fluid.

Protein analyses of N. sanguinea *digestive fluids*

The whole protein mixes collected from five different pitchers were precipitated individually and separated by SDS–PAGE (Fig. 1, panel 2; Supplementary Data Table S1). The electrophoresis pattern was similar for each analysis, and six major bands were observed. Each band was excised from the gel, and the spots corresponding to proteins with the same apparent molecular weight were pooled. As observed previously, a single 1-D SDS–PAGE separation did not sufficiently resolve the proteins, and a mix of different proteins was detected in each spot. As described above, we also performed a *de novo* sequencing analysis of the fluid from opened and closed pitchers (Table S1). Using these two approaches, we detected 19 putative digestive enzymes (seven from SDS–PAGE spots and 12 from the crude extract), including a never previously described Nepenthesin 2-like protein (NmNep2LP), an α-glucosidase (NmAGluc1) and a nucleotide pyrophosphatase (NmNPP1). In addition to NgNep1, NkGluc1 and NrChit1 which were already available in databases, we could relate nine other *N. sanguinea* proteins to the *N. mirabilis* cDNA library proteins (NmSCP3, NmSCP47, NmPR-1, NgTLP, NmAG1, NmAG2, NmPRX27, NmPRX-N and NmLTP1) (Table 1; Supplementary Data Table S2).

Protein analyses of N. albomarginata *digestive fluids*

Pitcher fluid from 1–4 plants was collected from closed (three pitchers) and just-opened pitchers (two pitchers) (Supplementary Data Table S1). These samples were pooled and protein precipitated with acetone. The resulting mixes were separated using an SDS–PAGE approach. Because large smears encompassing more precise bands could be observed on the gel, we divided the gel into 22 parts that were analysed by nanoLC-MS/MS approaches. Nine gel pieces were collected from SDS–PAGE profiles generated with closed pitchers, and 13 spots were collected from two SDS–PAGE patterns corresponding to opened pitchers (Fig. 1, pnel 3). These experiments allowed us to identify four different proteins including three corresponding to proteins whose orthologues have already been reported in libraries (NgTLP and NkGluc1). The two remaining proteins could be identified as NmAG1 and NmAG2 in the *N. mirabilis* cDNA library. A complementary analysis was carried out on crude extracts of a single just-opened pitcher which allowed NmAG2, NkGluc1, NgTLP and an additional chitinase named NrChit1 to be discriminated (Table 1; Table S2).

Protein analyses of N. bicalcarata *digestive fluids*

We collected and pooled the contents of four pitchers. NanoLC-MS/MS analyses were performed on 22 bands collected from *N. bicalcarata* SDS–PAGE patterns obtained for relatively mature closed pitchers and one pitcher that had been open for an extended period and inhabited by *C. schmitzi* (Fig. 1, panel 4). In addition to these analyses, we performed an investigation of crude digestive fluid from just-opened pitchers (Supplementary Data Table S1). We identified peptides belonging to four different proteins in the analyses of the bands, and five additional proteins were identified using the global approach. Among those nine putative enzymes, seven had already been described for the other *Nepenthes* species (NgNep1, NkGkuc1, NmAG1, NMAG2, NmBGluc7, NmPRX-N and NmLTP2). The two remaining proteins were identified as an esterase/lipase (NmLip1) and a cationic peroxidase 1 (NaPRX1), and were observed only in this species so far (Table 1; Table S2). Interestingly, the secretions collected from opened pitchers inhabited by *C. schmitzi* exhibit a low amount of protein in comparison with closed pitchers without these ants.

Detailed in silico *analysis of the protein sequences deduced from the* N. mirabilis *RNA-seq library*

For detailed information on the identified proteins, we used various bioinformatical tools. Starting with identifying the complete coding sequence in the RNA-seq library, we were able to determine the predicted SP, the theoretical molecular weight ranging from 9.6 to 66.6 kDa, the pI, as well as putative post-translational modifications such as *N*- or *O*-glycosylation.

Nepenthesin. We could identify three aspartic proteinases. NmNep1 and NmNep2 are similar to the two Nepenthesins that were already described in *N. alata* (97 % identity with NmNep1) and *N. gracilis* (97 % identity with NmNep2). They both harbour 12 cysteine residues, which could lead to the formation of six disulphide bonds contributing to the stability of
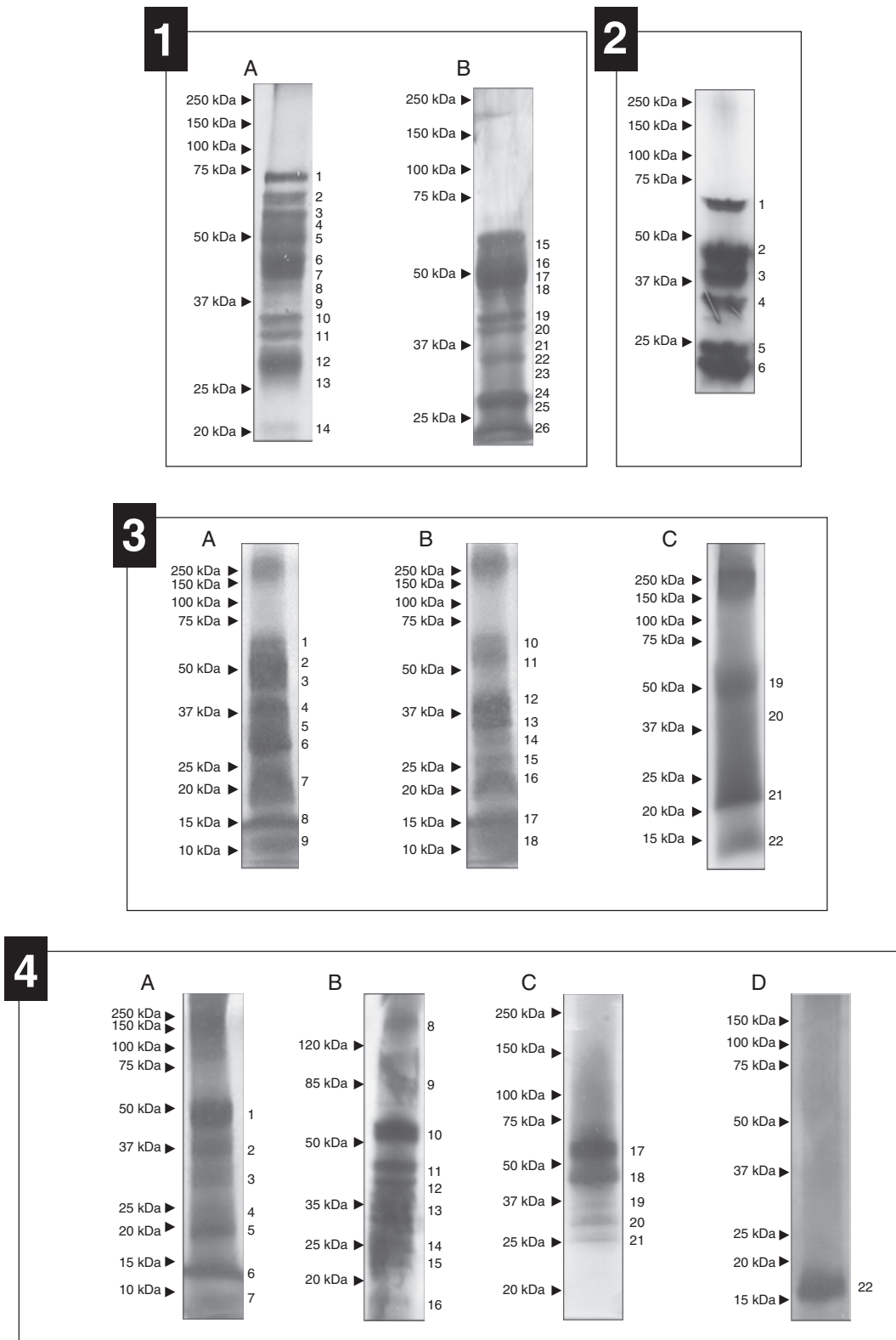
FIG. 1. SDS–PAGE analyses of *N. alata*, *N. sanguinea*, *N. albomarginata* and *N. bicalcarata* secretions. (1) *Nepenthes alata* secretions from closed (A) and opened pitchers (B). 1, 3–8, 10, 12, 15–25: NgNep1; 2: NgNep1, NmSCP3 and NmXyl; 11: not detectable (ND); 13: NrChit1 and NgTLP; 14: NgTLP; 26: NgNep1 and NgTLP. (2) *Nepenthes sanguinea* secretions from closed pitchers. 1: NgNep1 and NaGluc2; 2: NgNep1, NgNep2, NmAG1 and/or NmAG2, and NkGluc1; 3: NgNep1, NgNep2 and NkGluc1; 4: NgNep2 and NkGluc1; 5: NgNep2, NgTLP and NkGluc1; 6: NgTLP, NkGluc1 and NaChit1. (3) *Nepenthes albomarginata* secretions from closed pitchers (A), just-opened pitchers (B) and pitchers open for an extended period (C). 1, 2, 4, 6, 8, 12, 14, 15, 18–22: ND; 3: NmAG2; 5, 13: NkGluc1; 7, 16: NgTLP; 9, 11, 17: no putative enzyme detected; 10: NmAG1 and/or NmAG2. (4) *Nepenthes bicalcarata* secretions from closed pitchers (A–C) and pitchers opened for an extended period and inhabited by *C. schmitzi* (D). 1–8, 11, 13–17, 20–22: ND; 9, 10, 12: NmPRX-N; 18: NgNep1; 19: NgNep1, NkGluc1 and NmLip1.

A NmN2LP

```
MASSLYSFIIPLAIAFIFVTPPTSTSKISLNHEREETEPSFRIMLKRVDAGKNLTTSERI    60
RRALQRDRKRLQMFKTMKLSSSGIEAPVFPGPGEFLMQLAIGTPPQYYPAIMDTGSDLI   120
                     *    * *
WTQCSPCVQCFSQPTPVFDPSKSDSFESLSCSSKFCQDLSSACIGNCYYTYAYGDSSFTI   180
                *
GYMATETFAFNGSGSPVSVQNIAFGCGQFDQGTFTNCSGLVGMGRGPLSLPSQLPVSTFS   240
  *
YCLTSTDSSNDSTLYLGPSPYELPQGTPVTSTLVKNTYQPSFYYFTLTGISVGATRLDIP   300
                              *
SDGFAIGKNGTNGIIIDSGTTLTYIPSNYYDMAKDEFVRQTKLSPFPNSTSLIGLDCYEL   360
                               *
PQSPSTVQFPILVFHFDGGDLMVKDYLITGSYSNYELVCLAMGSSDTLGISIFGNIMQVN   420
                  *
KYIVYDLEREVLTFIPTNCNGK*  442
```

B NmSCP3

```
MEKLVFLPTLFFLLQSPLSTARLLSSGVHPVPWGPKRPSSQAEALIRALNLFPREPVNIV   60
DGPDSSWADGPRLVEKRFKFPSLDDDNGTASVEELGHHAGYYKIQDSYDARMFYFFFESR   120
                           *
GSSNDPVVLWLTGGPGCSSELAVFYENGPFKITDNLSLVWNEYGWDQASNIIYVDQPTGT   180
GFSYSNDSRDIRDTEVGVSNDLYDFIQAFFAAHPDYQNNDFYITGESYAGHYIPAFAARV   240
                                              *
YQGNQANEGIQINLKGFAIGNGLTEPGIQYQAYPDFALMMGLIDQQTYDSINQTVPECEN   300
    *         *            *               *      *
EANQCGTNGTETCLNALSTCSTIFNSILQVVGNINVYDIRKQCEGSLCYNFSNAQYFLNE   360
                   *
QSVRNALGVGDIDWVPCSSTVYQAMEVDWMRDLEVGIPALLDDGIRLLVYAGEYDLICNW   420
LGNSRWVAAMEWSGQQNFTAAPEVPFLVDGNEAGVLTSYGPLSFLKVYNAGHMVPMDQPK   480
AALAMLKGWIQGTLG*  495
```

C NmSCP20

```
MSRIYSFIQTASIFVSLSLVFSQSAPQTALVTQVPGFNGTLPSKQYAGYVTIDESHGKEL   60
                          *
YYYFVESERNSSEDPVVLWLNGGPGCSSFDGFVYEHGPFNFEAPKKQGDLPTLHPNPYSW   120
SKVSNIIYLDSPAGVGFSYSRNTSDYITGDLKTASDTHTFLLKWFELYQEFLANPFFVAG   180
ESYAGVYVPTLASNIVKGLDSGITPAINFKGYMVGNGVTDEVFDGNALVPFTHGMGLISN   240
                 *            *                     *
DLYEEVVNKCQGNYYAPTTDNCQKLLSEVDNEVDGLNIYDILEPCYHGSGSRSTSTDSLR   300
LPSSFKKLGGTERPLPVRKRMFGRAWPLRAPVRAGIIPTWPQLSKNNNVPCMSDEVATLW   360
*                      *
CNNAAVRKAIHAAEESVAGPWNLCTDRISYTHDAGSMIPYHKNLTSQGFRALIYSGDHDM   420
*
CVPFTGTEAWTRSIGYKIISLWRPWISQDQVAGYTQEYDKNLTFLTVKGAGHTVPEYKPQ   480
EALAFFSRWLAGKSI*  495
```

FIG. 2. Amino acid sequences of new putative digestive enzymes of *N. mirabilis*. Nepenthesin 2-like protein (NmN2LP) (A), serine carboxypeptidase III (NmSCP3) (B), serine carboxypeptidase-like 20 (NmSCP20) (C), serine carboxypeptidase-like 47 (NmSCP47) (D), protein phosphatase 2C (NmPP1) I, nucleotide pyrophosphatase (NmNPP1) (F), peroxidase 27 (NmPRX-27) (G), peroxidase N (NmPRX-N) (H), lipid transfer protein 2 (NmLTP2) (I), α-galactosidase 1 (NmAG1) (J) and α-galactosidase 2 (NmAG2) (K), β-galactosidase 1 (NmBG1) (L) and β-galactosidase 13 (NmBG3) (M). Matched peptides are shown in bold. Putative transit peptides are highlighted in black. Putative *N*-glycosylation sites are underlined, and putative *O*-glycosylation sites are double underlined. Cysteine residues are indicated by an asterisk. (A) The active site sequence motifs and flap tyrosine residue are boxed. (B) Residues indicated in italics correspond to putative propeptides, and conserved residues involved in the catalytic activity are boxed. (C and D) Conserved residues involved in the activity are boxed. (E) The Mn^{2+}/Mg^{2+} aspartate-binding site is highlighted in grey, and conserved residues of the catalytic core related to metal co-ordination and phosphate binding are boxed. (F) Blocks of conserved amino acid residues required for metal co-ordination are boxed. (G and H) The peroxidase active site and peroxidase haem ligand site are highlighted in grey, and residues forming calcium-binding sites are boxed. (I) Conserved residues are boxed, and two consensus pentapeptides are highlighted in grey. (J and K) α-Galactosidase patterns are highlighted in grey, and catalytic aspartate residues specific to glycosyl hydrolase family 27 are boxed. (L and M) The putative active site-containing consensus sequences of the glycosyl hydrolase family 35 are highlighted in grey, with glutamate as a proton donor residue identified by a box.

D **NmSCP47**

```
MIFTAMPPHSNNNYYNYISLRSLCLLVLAAVPLVSPSSSSSSELLGQNYGDLRLSSTANF      60
PKLEAERLIRQLNLFPKHPFNRAVAVEESSAAGRLVERRFKISQLVAGGGDSDGTSLEDF     120
GHHAGYYRLPRSKDARMFYFFFESRNKKEEDPVVIWLTGGPGCSSELALLYENGPFHISD     180
NLSLVWNDFGWDKASNLIYVDQPIGTGFSYTSDSSDIRHDEDGVSNDLYDFLQAFFKEHP     240
QYVKNDFFITGESYAGHYIPAFAARVHRGNKANEGIHINLKGFAMGNGLTNPWIQYAAYA     300
DYALDNGLISASDYKSINQMVPPCQEAIKLCGTDGDQACLSAYIVCTNIFNKIMDIAGQT     360
NYYDIRKKCETDLCYDFSKMEKLLNEKSVRQALGVGNIDFVSCSSTVYEAMQSDWMKNLE     420
TGIPTLLEDGINLLVYAGEYDLICNWLGNSRWVHAMEWSGQKQFSASPTVSFVVDGTEAG     480
LLKGHGPLSFLKVHDAGHMVPMDQPKASLEMLRRWMHGTLAAAETKDEVVPQ*  532
```

E **NmPP1**

```
MGYLDSVISSSKQVHAEELPVSGGGLSQNGKFSYGYASFPGKRSSMEDFYETRIDGVDGE      60
IVGLFGVFDGHGGARAAEYVKQHLFSNLIEHPKFISDTKSAIAEAYNRTDTEFLKSDNSQ     120
NRDAGSTASTAILVGDRLLVANVGDSRAVICRRGEAIAVSRDHKPDQSDERQRIEDAGGF     180
VMWAGTWRVGGVLAVSRAFGDKLLKQYVVADPEIQEEKVDGSLEFLILASDGLWDAVSNE     240
DAVAMIKSEQDPEAAAKKLMQEAYQRGSADNITCVVVRFLANGGDSVRVTERLYLRRDXL     300
XLASSYLGR*  309
```

F **NmNPP1**

```
MIQSTIKYSMMDRRNPSLQVLIITILVLSLTSSSYSHRLYPHGHEYKIEDYDHTMISDFR      60
LINRMSLIKCPIESPYIQINTSATSALPNEGNITVTVSGVLHPATGDWVAMVSPSTADIG     120
SCPLDAVMYMQTGDLASLPLLCHYPVKAQYVSNDPGYLSCQNSACQDSSCLIKTCSGSIT     180
FHYTNIRTDLIFVFFAGGVKTPCILATTQPIQFANPASPLYGHLSSIDSTATSMTLTWVS     240
GDQVPQQVLYGNNQSQTSVVTTFTQNDMCTTTLLPSPAKDFGWHDPGYVHSAVMTGLLPS     300
TTYSYQYGSNSVGWSNQIQFRTPPAGGSNQLTFLAFGDMGKTPLDPSIEHYIQPGAESVI     360
QAMASEVAAGNIDAIYHIGDISYATGFLVEWDFFLNLIEYIASSVSYMTSIGNHERDYIN     420
SGSVYITPDSGGECGVPYETYFPMPTPAKDKPWYSIEQGPVHFTVISTEHPWSVNSEQYN     480
WMQSDMASVNRSRTPWLIFAGHRPMYSSIKGLEILNVDPRFVSAVEPLLLQYQVDLALFG     540
HVHNYERLCAVYQKQCLGMPIKNANGIDTYDNGNYTAPVHVIIGMAGFTLDSFTSNPSSW     600
SLVRISEFGYSKVQATPNSLLFQFVNGHTRQIDDSFYITK*  640
```

Fɪɢ. 2. Continued

this enzyme family. Their putative active sites are typical for aspartic proteases and contain Asp–Thr–Gly (at position 113–115 for NmNep1 and position 114–116 for NmNep1), Asp–Ser–Gly (at position 315–317) and the flap tyrosine (Tyr174 for NmNep1 and Tyr175 for NmNep2) (Table 2) (Athauda *et al.*, 2004). NmNep2 contains three predicted sites for *N*-glycosylation and five for *O*-glycosylation.

The third *N. mirabilis* Nepenthesin identified was named Nepenthesin 2-like protein (NmNep2LP). Interestingly, this protein shares only 52 % identity with NmNep1 and 51 % with NmNep2, as detected in our cDNA library. NmNep2LP is composed of 442 amino acids, including a 417 amino acid mature enzyme and a 25 residue putative signal sequence (Table 2; Fig. 2A). The predicted cleavage site for the SP is located between Thr25 and Ser26. In contrast to NmNep1 and NmNep2, no putative propeptide was predicted for NmNep2LP. The calculated molecular weight was 45.7 kDa and the pI value 4.9. Like typical nepenthesin-aspartic proteases, NmNep2LP does

G   **NmPRX27**

```
MSALKISVLFFLELVIQLAIVANSQELVLGFYNDTCPEAENIVNQVTALYIYNDSTLAPA      60
LLRLTFHDCFVRGCDASILLEPTNANNATEITAPPNLTLRGFEVIDAIKTKLEEYCPGVV     120
SCADIIQLAGRDAIQTAYGASWVVLTGRRDGTVSLASEALEDLPSPYSNFDALKQKFEAV     180
GLSVKDLVVLSAAHTVGVAHCPSFSGRLYNFSGEGGTDPSLDPNYAKFLKQKCPPNDQLT     240
TVYQDPITPNKFDIGYYVAVDQHEGLFESDAALLDDNETRGYLESQLWTMGWTWLEDFGV     300
SMMKMYKFKVLTGTEGEIRKKCGYVN*  326
```

H   **NmPRX-N**

```
MHAVAFIMKKSGSNACLLLVGFFVFAAGVRSQLTTDYYKTTCPNLPKIVRKVVQSAIMNE      60
TRMAASLLRLHFHDCFVNGCDASILLDGSDGEKYAFPNVNSVRGFEVVDAIKSAVESACN     120
ETVSCADILAIAARDSVVLSGGPSWRVSLGRRDGLVANQTGANNNLPSPTDALDTIISKF     180
AAVGLNVTDVVTLSGGHTIGQARCTTFSTRLYNFSGTDAADTTLNSNLASQLEALCPENG     240
DGNVTTSLDQNSTDLFDNHYFKNLLSNKGILFSDQELYSSDAAASTTMSLVESYSNSSYL     300
FFQNFVNSMIKMGNISPLTGSNGEIRTNCRVINS*  334
```

I   **NmLTP2**

```
MASSVLFKVACLVLICMLVAAPQAAATITCGQVVQYLLPCLSYLRNGGRIPPRCCPGVRG      60
LNSAARTTPDRQAACNCLKTAARSYRINYGNGAALPGKCGVSIGFKIALNTDCSKVH*  117
```

J   **NmAG1**

```
MEKRTEVPMLFVCLSIFIVIMFSDDASASRHHHDTFRRYLLSNGLGLTPPMGWNSWNHFA      60
CHISENMIRETADALVSTGLSKLGYKYVNIDDCWAELTRDGKGNLVPKNSTFPSGIKALA     120
DYVHSKGLKLGIYSDAGYFTCSKTMPGSLGHEEQDAQTFASWGIDYLKYDNCYNDGSKPT     180
VRYPVMTRAIMKTGRPIFFSLCEWGDLHPALWGYAVGNSWRTTNDISDNWASMVSRADMN     240
EYYAELARPGGWNDPDMLEIGNGGMTTDEYIVHFSIWAISKAPLLIGCDVRNLTKETIEI     300
IGNSEVIAVNQDPLGVQAKKVRMEGDREVWAGPLSRYRVALLLINRAALRTSITANWDDI     360
GIPPXSVVXARDLWKHETLRQKFVGNLTATVDSRASKMYILTPIS*  405
```

Fɪɢ. 2. Continued

not present a plant-specific insertion properly to plant vacuolar aspartic proteases, but contains 'the nepenthesine-type AP (NAP)-specific insertion' (Athauda *et al*., 2004) upstream of the flap tyrosine residue (Tyr173). It is made up by 20 residues (at position 149–168) and contains four cysteine residues (Cys151, Cys156, Cys163 and Cys167). Asp–Thr–Gly and Asp–Ser–Gly corresponding to putative active sites are located at positions 114–116 and 317–319 respectively.

Carboxypeptidase. Four putative serine carboxypeptidases (NmSCP3, NmSCP20, NmSCP47 and NmSCP51) were identified in our study. The last, NmSCP51, is incomplete in our RNA-seq library. The molecular masses of NmSCP3, NmSCP20 and NmSCP47 range from 52.4 to 55.3 kDa, with a

pI between 4.3 and 5.6 (Table 2). Each protein contains a 22–36 residue SP at the N-terminus (Table 2). Of the NmSCPs, only NmSCP3 has a 171 amino acid long sequence, which corresponds to a potential propeptide (Fig. 2.B). Each identified putative serine carboxypeptidase belongs to the S10 family of serine proteases, which are only active at an acidic pH. The catalytic activity of SCPs is induced through a charge relay system involving a catalytic triad composed of an aspartic acid residue linked to a histidine (hydrogen bond) and a serine (Liao and Remington, 1990). We identified these residues in each of the NmSCPs, with the exception of the incomplete Nm SCP51, using the peptidase database MEROPS, (http://merops.sanger. ac.uk): Ser227, Asp441 and His498 for NmSCP3 (Fig. 2B), Ser182, Asp419 and His472 for NmSCP20 (Fig.2C) and

```
K      NmAG2
MANTDRAEAWACVLLLSLSAISLAIAAGRGVPLLDKQRIFSSSFHRIYDNSKYGKLQLHN        60
                                          *
GLAQTPQMGWNSWNFFACNINETVIKETADAIVSTGLADLGYVHVNIDDCWSNASRNSKG       120
                            *
ELVPDPKTFPSGIKALADYVHDKGLKLGIYSDAGVFTCEVRPGSLFYENIDAELFASWGV      180
            *                        *
WIIKYDNCYNLGIKPEERYPPMRDALNATKRRIFYSICEWGVDDPALWAGQVGNSWRTTD      240
DINDSWVSMTAIADINDKWAAYAGPGGWNDPDMLEVGNGGMTYEEYRAHFSIWALMKAPL      300
   *                                       *
LIGCDVRNISSETMEIVSNKEIIAVNQDPLGVQGRKVYVSGPDGCQQVWAGPLSGNRIAV      360
      *                                             *
ALWNRCSTATIITASWEVLGLQSSIGVSIRDLWQGKEITRDAVASFGVRVDSHDCAMYIF      420
TPDPGALSNM*    430

L      NmBG1
MGSIWKMLRANILTLLFLCSLVSSVTASVSYDRKAIIINGQRRILLSGSIHYPRSTPEMW      60
PDLIQKAKNGGLDVIQTYVFWNGHEPSPGQYYFEGRYDLVKFIKLVQQAGLLVHLRIGPY     120
   *
VCAEWNFGGFPVWLKYIPEIEFRTDNGPFKEKMQGFTGKIVNMMKSERLFQSQGGPIIMS     180
QIENEFGPVEWEIGAPGKVYTEMGSADGRWIEYWSSMGYVQTG*    223

M      NmBG3
LIQISEASYSISFLLYICLTNAGENARYFTGKIVNMMKSERLFQSQGGPIIMSQIENEFG      60
                                    *         *    *
PVEWEIGAPGKVYTKWAAQMAVGLNTGVPWVMCKQDDAPDPVINTCNGFYCEDFTPNKNY     120
KPKMWTENWTGWYTKFGGAVPYRPAEDVAFSVLRFIQNGGSFVNYYMYHGGTNFGTTSGR     180
                                            *
FVATSYDYDAPIDEYGLLREPKYTHLKYLHNAIRMCEPALVSADPTVTKLGSNQEAHVFS     240
         *                               *
SSSGACAAFLANYDTKWSVKVAFGGMQYDLPRWSISVLADCKKEVFNTARVISQSFHAKM     300
TPVSSFSWQSYVDETPSAEYDTSFSATGLYEQLNVTRDTSDYLWYMTDVTIGANEGFLKN     360
GEYPVLTVMSAGHALHVFINGQQAGTVYGSLANPKLTFSENVRLGPGINKISLLSVAVGL     420
ANVGTHFETWNQGVLGPVTLKGLNEGTRDLTWQKWSYKVGMKGENLGLHTVSGSSSVEWM     480
EGSLTVQRQPLTWYKTSFNSPAGNDPLALDMNSMGKGQVWVNGQSIGRHWPAYKASGSCG     540
                                                         *
QCDYAGFFNENKCLSNCGQASQRWYHVPRSWLQPSGNLLVIFEEWGGDPSEISLVKRTV*   599
```

FIG. 2. Continued

Ser253, Asp441 and His498 for NmSCP47 (Fig/ 2D). Various putative glycosylation sites could be predicted: nine for NmSCP47, 11 for NmSCP3 and 13 for NmSCP20 (Table 2).

Phosphatases. NmPP1, which has a molecular mass of 32 kDa and a pI of 5 (Table 2), belongs to the 2C family of serine/threonine phosphatases and may be an Mn^{2+}- or Mg^{2+}-dependent phosphatase. This protein has a 16 residue SP in the N-terminal position and three putative glycosylation sites (Table 2). An Mn^{2+}/Mg^{2+} aspartate-binding site was detected at amino acids 64–72 (Fig. 2E). The predicted catalytic core harbours several amino acids that contribute to metal coordination (Asp48, Asp69–Gly70, Asp231 and Asp270) and phosphate binding (Arg43) (Table 2 Supplementary Data Fig. S1) (Shi, 2009) (Fig. 2E).

NmNPP1 is a putative digestive enzyme of 66.5 kDa with a pI of 5.1, a 36 residue SP and seven putative glycosylation sites (Table 2). This protein is an apparent acidic phosphatase of the metallophosphatase superfamily, which is known to hydrolyse phosphate esters and anhydrides under acidic conditions.

NmNPP1 contains a putative active site consisting of five blocks of conserved residues required for metal coordination: Gly337–**Asp**–Met–Gly340, Gly379–**Asp**–Ile–Ser–**Tyr**383, Gly412–**Asn**–His–Glu415, **His**502 and Gly540–**His**–Val–**His**543 (bold residues are conserved metal-binding sites) (Fig. 2F) (Schenk *et al.*, 2000).

Peroxidases. Peroxidase 27 (33.4 kDa and pI 4.6) and peroxidase N-like protein (32.5 kDa and pI 4.9) belong to class III of plant secretory peroxidases and have an SP of 24 and 31 amino acids, respectively (Table 2). Class III plant peroxidases are glycoproteins. NmPrx-27 and NmPRX-N harbour 13 and 10 putative glycosylation sites, respectively (Table 2). NmPRX-27 contains a putative peroxidase active site between position 58 and 69 and a peroxidase haem ligand site between position 186 and 196 (Fig. 2G). NmPRX-N contains a putative peroxidase active site between position 64 and 75 and a peroxidase haem ligand site between position 189 and 199 (Fig. 2H). The general structure of class III peroxidases contains four conserved disulphide bonds (Hiraga *et al.*, 2001), which are present in the

Table 2. *Bioinformatic analyses of 20 putative new pitcher fluid proteins*

Nomenclature	Full-length protein	Signal peptide length (no. of amino acids)	No. of N-glycosylation sites	No. of O-glycosylation sites	No. of amino acids*	Mol. wt (kDa)	pI	Accession number
NmNep2LP	x	25	3	5	417	45.7	4.9	KT209998
NmSCP3	x	21	7	4	474	52.5	4.3	KT209999
NmSCP20	x	22	3	10	473	52.4	5.6	KT210000
NmSCP47	x	36	1	8	496	55.3	5.2	KT210001
NmSCP51		26						KT210002
NmXyl1		26						KT210017
NmAGluc1								KT210003
NmAG1	x	28	2	1	377	42.2	6.6	KT210004
NmAG2	x	26	2	0	405	44.7	5.0	KT210005
NmBG1	x	27	0	0	196	22.5	6.8	KT210006
NmBG2		24						KT210007
NmBG3			2	6	577	64	6.2	KT210008
NmBGluc7		25						KT210009
NmPP1	x	16	2	1	293	32.0	5.0	KT210010
NmNPP1	x	36	5	3	604	66.6	5.1	KT210011
NmPRX27	x	24	5	8	302	33.3	4.6	KT210012
NmPRX-N	x	31	5	5	303	32.5	4.9	KT210013
NmLTP1	x	20	1	20	165	16.4	6.1	KT210014
NmLTP2	x	26	0	3	91	9.6	9.6	KT210015
NmLip1								KT210016

Sequence information was obtained from the cDNA library of *N. mirabilis*, and the corresponding peptides were identified in various *Nepenthes* species.
We determined the molecular weight and isoelectric point (pI) of the mature form of each protein, and predicted the size of the signal peptide and number of *N*- and *O*-glycosylation sites

peroxidases isolated from *N. mirabilis*: Cys36–Cys116, Cys69–Cys74, Cys122–Cys322, Cys201–Cys233 for NmPRX-27 (Fig. 2G); and Cys42–Cys119, Cys75–Cys80, Cys125–Cys329 and Cys125–Cys204 for NmPRX-N (Fig. 2H). The structure also includes two conserved calcium-binding sites in distal and proximal positions: the calcium-binding sites of NmPRX27 are formed by the distal cluster Asp68–Val71–Gly73–Asp75–Ser77 and the proximal cluster Thr195–Asp245–Thr248–Lys251–Asp253 (Fig. 2G). The binding sites of NmPRX-N are composed of the groups Asp74–Val77–Gly79–Asp81–Ser83 and Thr198–Asp249–Ser252–Leu255–Asp257 (Fig. 2H).

Lipid transfer protein (LTP) According to Carvalho and Gomes (2007), the LTPs belonging to family 1 have molecular masses close to 10 kDa and are basic with pIs of 9–10. These 90–95 amino acid long LTPs feature cysteines conserved in similar positions along the primary structure. NmLTP2 seems to belong to this family of LTPs. Its molecular mass is 9.6 kDa, its pI is 9.6 (Table 2), and the amino acid sequence contains eight cysteines, which could form four disulphide bonds (Cys30–Cys77, Cys40–Cys54, Cys55–Cys99 and Cys75–Cys113) (Fig. 2I). Other residues are highly conserved, including Val33, Tyr43, Gly57, Leu78, Lys79, Ala93, Val101 and Ile103, as well as the two consensus pentapeptides Thr67–Thr68–Pro69–Asp70–Arg71 and Gly104–Phe105–Lys106–Ile107–Ala108 (Fig. 2I) (Carvalho and Gomes, 2007). As a member of the LTP family 1, NmLTP2 contains an internal hydrophobic cavity that may be the binding site for lipid-like molecules. This cavity is composed of 18 residues, including Leu37, Leu41, Leu44, Val58 or Ala61. Finally, this protein harbours a 26 residue SP.

Galactosidases. NmAG1 and NmAG2 are putative α-galactosidases of 42.2 and 44.7 kDa with pI values of 6.6 and 5.0, respectively. They contain 28 or 26 amino acid long SPs, as well as three and two putative glycosylation sites, respectively

(Table 2). NmAG1 features the α-galactosidase pattern from position 84 to 100 (α-galactosidase signature: G-[LIVMFY]-x-x-[LIVMFY]-x-[LIVM]-D-[DF]-x(1,2)-W-x(3,7)-[RV]-[DNSF]; x = any amino acid). NmAG1 belongs to the glycosyl hydrolase family 27 and contains two catalytic aspartate residues, which function as a nucleophile (Asp170) and an acid/base catalyst (Asp226) (Fig. 2J). The α-galactosidase consensus pattern of NmAG2 is located between positions 101 and 117, and two aspartate residues acting as a nucleophile and acid/base catalyst are located at positions 186 and 241, respectively (Fig. 2K) (http://prosite.expasy.org/PDOC00443).

β-Galactosidases, a widespread family of glycosyl hydrolases, can hydrolyse terminal, non-reducing β-D-galactosyl residues from β-D-galactosides. NmBG1 is a putative β-galactosidase of 22.5 kDa with a pI of 6.8 and a 22 residue SP (Table 2). It contains a putative active site containing the consensus sequence pattern G-G-P-[LIVM]-x-Q-x-E-N-E-[FY], which is found in glycosyl hydrolases. The active site is localized between positions 174 and 186, and Glu185 serves as a proton donor residue (Fig. 2L) (Smith and Gross, 2000). NmBG3 is also a putative β-galactosidase with a mol. wt of 64 kDa and a pI of 6.2 (Table 2). The sequence of its SP is incomplete in our RNA-seq database. However, it has the putative active site-containing consensus sequence pattern of the glycosyl hydrolase family 35 between positions 47 and 59, with Glu58 serving as the proton donor residue (Fig. 2M) (Smith and Gross, 2000).

DISCUSSION

To digest prey, *Nepenthes* species secrete an arsenal of enzymes which are mostly proteolytic enzymes. Some of these proteins have already been identified through many specific studies and their sequences are available in public libraries. In

this report we extended the list of proteins through a study performed on five different *Nepenthes* species displaying different ecological behaviour. Since only a few accession are available in public databases (GenBank), we compared the peptide sequences obtained by a nano-LC/MS approach with a cDNA library based on RNA extracted from *N. mirabilis*. This study, however, is not exhaustive. It is likely that some proteins have not been identified by classical proteomics approaches because they are secreted in too low concentrations. The SDS–PAGE profile differences might also be linked to post-translational modifications. For example, a proteolytic digestion or maturation of proteins excreted in the pitcher fluid can lead to the finding of peptides of one protein in smaller bands than its actual size. This has been observed for Nepenthesin by Buch *et al.* (2015). Other post-translational modifications such as glycosylation also impact the SDS–PAGE pattern. Buch showed in 2014 that the highly glycosylated PR-1 protein migrates at double the size of its original molecular weight.

A total of 29 proteins were identified in the secretome of closed and opened pitchers harvested from five different *Nepenthes* species (*N. mirabilis*, *N. alata*, *N. sanguinea*, *N. bicalcarata* and *N. albomarginata*). Of these, nine hydrolytic enzymes had been previously reported: the aspartic proteases Nepenthesin 1 and 2 from *N. gracilis* and *N. distillatoria* (NgNep1 and NgNep2; Athauda *et al.*, 2004), two glucanases isolated from *N. khasiana* (NkGluc1; Eilenberg and Zilberstein, 2008) and *N. alata* (NaGluc2; Hatano and Hamada, 2012), a cationic peroxidase (NaPRX1; Hatano and Hamada, 2012), and class I (NkChit1; Eilenberg, 2006), class III (NrChit1 or NaChit3; Rottloff *et al.*, 2011) and class IV chitinases (NaChit1; Hatano and Hamada, 2008; Ishisaki *et al.*, 2012*b*). Two additional previously identified putative antimicrobial proteins were also detected in our study: a PR-1 protein (NmPR-1; Buch *et al.*, 2014) and a thaumatin-like protein (NgTLP; Hatano and Hamada, 2008; Rottloff *et al.*, 2009). Beside these proteins, we could identify peptides which, when compared with the *Nepenthes* nucleotide database, correspond to 13 new putative digestive enzymes: a third aspartic protease (NmNep2LP), three serine carboxypeptidases of types 3, 20 and 47 (NmSCP3, NmSCP20 and NmSCP47), two α- (NmAG1 and NmAG2) and one β-galactosidase (NmBG1), a protein phosphatase (NmPP1), a nucleotide pyrophosphatase/phosphodiesterase (NmNPP1), two new peroxidases of type 27 (NmPRX27) and type N (NmPRX-N) and two lipid transfer proteins (NmLTP1 and NmLTP2). This work also led to the identification of partial sequences of seven additional putative enzymes: a serine carboxypeptidase (NmSCP51), a β-xylosidase (NmXyl1), an α-glucosidase (NmAGluc1), two β-galactosidases (NmBG2 and NmBG3), a β-D-1,3-glucosidase 7-like protein (NmBGluc7) and a lipase (NmLip1).

The carnivorous system, including trap formation, prey digestion and nutrient assimilation, is an important energetic investment for plants (Givnish *et al.*, 1984; Ellison, 2006; Pavlovič and Saganová, 2015). The pool of digestive enzymes has to be efficient so that the benefits obtained from digested prey outweigh the cost of carnivory. A complex association of enzymes acting at different levels and favouring the gain of the greatest amount of nutrients from prey degradation is therefore expected. We demonstrated that the protease content of several of the studied species is composed of two classes of protein-degrading enzymes: aspartic proteases [Nepenthesin 1 and 2 and Nepenthesin 2-like protein (NmN2LP)] and serine carboxypeptidases (NmSCP3, NmSCP20, NmSCP47 and NmSCP51). The serine carboxypeptidases identified in this work putatively belong to the S10 family of serine proteases. These enzymes are only active at an acidic pH (Schulze *et al.*, 2012), as is the case for the two Nepenthesin enzymes (with optimal activity at pH 2.6) (Athauda *et al.*, 2004; Takahashi *et al.*, 2005). A similar diversity of protease activities was found in the digestive fluid of the Venus flytrap, with cysteine proteases (Dionain), aspartic proteases and serine carboxypeptidases (Schulze *et al.*, 2012; Libiakova *et al.*, 2014). The frequency of proteases in carnivorous plants, such as *Nepenthes* and *Dionaea*, suggests a strong dependence on nutrients gained from digested prey.

The assimilation of products derived from protein degradation is an important step in the carnivory process. Our results support that *N. mirabilis*, *N. alata* and *N. sanguinea* absorb protein-derived nutrients by two different strategies. First, Nepenthesin 1 (in cooperation with Nepenthesin 2) degrades prey-derived proteins into peptides, which can then be directly absorbed by the digestive glands (Schulze *et al.*, 1999). Alternatively, proteins may be degraded by serine carboxypeptidases starting at the C-terminus, and the resulting amino acids could be delivered into plant tissues by transporters expressed in the digestive glands (Schulze *et al.*, 1999). Free radicals released during the generation of reactive oxygen species (ROS) by peroxidases increase the degradation rate of proteases (Chia *et al.*, 2004; Hatano and Hamada, 2012). In addition to the cationic peroxidase 1 described by Hatano and Hamada, we identified two new sub-classes of putative peroxidases: peroxidase 27-like protein (NmPRX27) in *N. alata* and *N. sanguinea* fluids, and peroxidase N-like protein (NmPRX-N) in *N. mirabilis*, *N. alata*, *N. sanguinea* and *N. bicalcarata* fluids. These enzymes belong to the class III peroxidases.

Chitinases found in the fluids of *Drosera* sp., *Dionaea muscipula* and *Triphyophyllum peltatum* contribute to the degradation of prey exoskeletons (Matušíková *et al.*, 2005; Schulze *et al.*, 2012; Paszota *et al.*, 2014). A two-step mechanism involving different types of chitinases may be implemented to degrade chitin present in the insect cuticle. First, chitin polymers derived from prey might be fragmented into shorter polymers by a class III endochitinase (NrChit1 or NaChit3). Secondly, the resulting shorter chitin chains might be hydrolysed to *N*-acetylglucosamine residues by the class I endochitinases (NkChit1; Eilenberg, 2006) and the class IV endochitinases (NaChit1; Hatano and Hamada, 2008). Chitinase activity has been detected in *Nepenthes* digestive fluid. Eilenberg (2006) reported chitinase activity in the digestive fluid of *N. khasiana* on both a long chitin polymer and a tetrameric substrate, 4MU-(GlcNAc)$_3$. The latter was described to be hydrolysed by the chitinases isolated from *N. khasiana*. Another class III endochitinase (NrChit1) hydrolyses a long-chain chitin polymer (colloidal CM-chitin-RBV) (Rottloff *et al.*, 2011), and ethylene glycol chitin or shorter chains of *N*-acetylglucosamine residues [(GlcNAc)$_n$; $n = 5$, 6] (Ishisaki *et al.*, 2012*a*)]. Hatano and Hamada (2012) identified a class IV chitinase (NaChit1) in *N. alata* secretions that also hydrolyses (GlcNAc)$_n$ and generates a chain length of 3–6 units. In this work, we reported the presence of class III and IV chitinases in the digestive fluid of

N. sanguinea collected from closed and opened pitchers similar to those identified by Hatano and Hamada in *N. alata* secretions after chitin addition.

Phosphatases represent a broad group of enzymes that catalyse the hydrolysis of phosphate esters. They may play a role in phosphorus mobilization from prey and its uptake by the plant tissue (Płachno *et al.*, 2006). Together with nitrogen, phosphorus limits the growth of carnivorous plants (Ellison, 2006). Recently, Pavlovič *et al.* (2014) showed that phosphatase activity was induced 24 h after prey capture in *Drosera capensis*. Therefore, phosphatases are crucial enzymes that enable prey-derived phosphorus acquisition in carnivorous plants (Plachno *et al.*, 2009). We identified two putative phosphatases: protein phosphatase 2C (NmPP1) detected in *N. mirabilis* and a nucleotide pyrophosphatase/phosphodiesterase (NmNPP1) in *N. sanguinea*. These two enzymes represent the first putative phosphatases identified in *Nepenthes* species.

Given the high energy cost of the carnivorous strategy, it is likely that additional nutritional sources such as glucan-rich particles from pollen grains, fungal spores, seeds or plant detritus are utilized by members of the *Nepenthes* genus. This was previously observed in *N. ampullaria* (Moran *et al.*, 2003; Adlassnig *et al.*, 2011; Pavlovič *et al.*, 2011) and other carnivorous plants such as *Drosera* (Michalko *et al.*, 2013). A study of *Drosera* demonstrated that a β-1,3-glucanase is involved in the degradation of laminarin, a β-(1–3)-glucan polymer with β-(1–6)-branches, the products of which are taken up by the trap tissue. We observed putative glucanase activity in each *Nepenthes* species studied (NkGluc1 and NaGluc2). Other enzymes identified in *Nepenthes* secretions may also be involved in carbohydrate metabolism. For example, the thaumatin-like protein identified in *N. mirabilis*, *N. alata* and *N. sanguinea* (Hatano and Hamada, 2008; Rottloff *et al.*, 2009, 2011) displays glucanase activity in other plants (Liu *et al.*, 2010), although these proteins were also described to have osmotic function leading to cell wall degradation (Yun *et al.*, 1997).

In addition to their role in food acquisition through degrading macromolecules, such enzymes might also be involved in the formation of a polysaccharidic network responsible for the viscoelasticity of the secretion. Although the exact composition of the *Nepenthes* polysaccharide remains to be elucidated, Gaume and Forterre (2007) speculated that the *Nepenthes* fluid could exhibit an acidic polysaccharide mucilage similar to that of the secretions produced by sundew. The polysaccharide of *D. capensis* is composed of L-arabinose, D-xylose, D-galactose, D-mannose and D-glucuronic acid in a molar ratio of 3.6:1.0:4.9:8.4:8.2 (Gowda *et al.*, 1983) and constitutes 4 % of *D. capensis* adhesive (Rost and Schauer, 1977). *Drosera binata* polysaccharide has a different ratio, 8:1:10:18:17 (Gowda *et al.*, 1982). Recently, Bauer and Bazile demonstrated that the viscoelastic properties of *N. rafflesiana* fluid decrease significantly after pitcher opening (Bauer and Federle, 2009; Bazile *et al.*, 2015). These properties contribute significantly to the capture and retention of prey (Gaume and Forterre, 2007; Bonhomme *et al.*, 2011*b*). Enzymes that may be involved in this process were highlighted in the present analyses. Two types of glucosidases were identified: an α-glucosidase (NmAGluc1 in *N. sanguinea*) and a β-D-1,3-glucosidase 7-like protein (NmBGluc7 in *N. alata*, *N. sanguinea* and *bicalcarata*). In addition to glucosidases, we also detected galactosidases such as α-galactosidases 1 and 2 (NmAG1 and NmAG2) in *N. mirabilis*, *N. alata*, *N. sanguinea*, *N. bicalcarata* and *N. albomarginata* secretions, and β-galactosidase 1, 2 and 3 (NmBG1, NmBG2 and NmBG3) in *N. alata*. At the end of pitcher life, the polysaccharide might be removed by saccharide-cleaving enzymes to facilitate the digestion and absorption of nutrients.

To recover most nutrients coming from prey digestion it is necessary to limit the proliferation of micro-organisms that could metabolize part of them. Many studies have highlighted the antimicrobial properties of the digestive fluid of *Nepenthes* after opening of the pitcher (Adlassnig *et al.*, 2011; Chou *et al.*, 2014). The study carried out by Buch and co-workers (2013) showed that the microbial proliferation is limited because of the naturally poor mineral content of the secretions, the presence of some secondary metabolites, the acidic pH and the presence of pathogenesis-related (PR) proteins. Six different PR protein families (PR-1, 3, 5, 7, 8 and 9) were identified in *Nepenthes* fluids by Mithöfer (2010). In our study we extended this list in *N. alata*, *N. sanguinea* and *N. bicalcarata* to other PR proteins belonging to the PR-14 family: NmLTP1 and NmLTP2. Although never described in Nepenthes, these proteins related to LTPs were previously detected in the secretions of another carnivorous plant, *Dionaea muscipula* (Schulze *et al.*, 2012). Some LTPs were also described to exhibit antifungal and antibacterial activities (Van Loon *et al.*, 2006) and might therefore be part of the antimicrobial strategy of *Nepenthes*. They might also be involved in the formation of cutin barriers in the digestive zone of the pitchers to prevent the entry of pathogens into the tissue (Heredia, 2003; Carvalho and Gomes, 2007).

At the species level, there is an increasing body of evidence showing that species differ in their trapping strategies (Gaume and Di Giusto, 2009; Benz *et al.*, 2011; Bonhomme *et al.*, 2011*a*; Bauer *et al.*, 2012) as well as in their nutrient sequestration strategies, with nitrogen derived from prey in truly carnivorous species (the majority of species), but also from vegetal detritus in more detritivorous species (Moran *et al.*, 2003; Pavlovič *et al.*, 2011), from faeces of mammals (Clarke *et al.*, 2009; Chin *et al.*, 2010) or from symbiotically associated ants (Bonhomme *et al.*, 2011*a*; Bazile *et al.*, 2012) in more coprophagous species. Our work demonstrated that although several enzymes were detected in all considered species (NkGluc1, NmAG1 and NmAG2), the protein composition in digestive fluid could differ according to the considered species and development stage (Fig. 3). These differences might reflect trapping strategies established by the plant. For example, *N. bicalcarata* develops an outstanding strategy of nutrient sequestration thanks to a mutualistic relationship with the symbiotic ant species, *Camponotus schmitzi*. These ants assist the plant by the catching and the digestion of the prey (Bonhomme *et al.*, 2011*a*; Bazile *et al.*, 2012), prevent nutrient export by fluid inhabitants (Scharmann *et al.*, 2013), clean pitcher trapping surfaces (Thornham *et al.*, 2011) or protect the host plant against damage caused by *Alcidodes* sp. (Merbach *et al.*, 2007). This relationship considerably increases the foliar nitrogen in adult plants (Bazile *et al.*, 2012). Surprisingly, ants can live and swim in the digestive fluid (Clarke and Kitching, 1995; Bohn *et al.*, 2012) without being damaged by the hydrolytic enzymes. The analyses of *N. bicalcarata* pitcher fluids enabled us to visualize 22 SDS–PAGE bands which were characterized as only nine different putative proteins. Among all proteases, we

N. mirabilis

N. albomarginata

N. alata

N. bicalcarata

N. sanguinea

Protein phosphatase 2C

Chitinase

Lipid transfer protein 2

Serine carboxypeptidase III
Serine carboxypeptidase-like 47

β-1,3-Glucanase
β-Xylosidase
Serine carboxypeptidase-like 51
β-Galactosidase 1
β-Galactosidase 2
β-Galactosidase 3

Peroxidase N
Nepenthesin 1

Thaumatin-like protein

β-D-1,3-Glucosidase 7-like protein

PR1 protein
Peroxidase 27
Lipid transfer protein 1
Serine carboxypeptidase-like 20

Glucanase
α-Galactosidase 1
α-Galactosidase 2

Acidic chitinase

Nepenthesin 2
Nepenthesin 2-like protein
Nucleotide pyrophosphatase/phosphodiesterase
α-Glucosidase

Cationic peroxidase 1

FIG. 3. Summary of the proteins identified from the digestive fluid of five *Nepenthes* species. Already identified proteins are underlined. Proteins found only in closed pitchers are marked by red symbols and proteins found only in open pitchers are marked by green symbols. Proteins identified thanks to one peptide are coloured in orange, two or three peptides in blue, four or five peptides in green, 5–10 peptides in black and >10 peptides in red.

detected only the presence of Nepenthesin 1 active only at an acidic pH (Athauda *et al*., 2004) and the pH of the *N. bicalcarata* fluid has been described to be at around pH 5 (Bazile *et al*., 2012). Therefore, Nepenthesin 1 might be less active, which could protect the ants against the proteolytic activity. These observations are correlated with a low amount of protein in secretions of opened pitchers inhabited by *C. schmitzi* in comparison with opened pitchers without ants. Another example concerns *N. sanguinea*, *N. alata* and *N. mirabilis* which display a conventional nitrogen acquisition strategy of carnivorous plants by trapping and digesting arthropods. According to our results, they produce a blend of digestive enzymes which, in conjunction with acidic pH conditions, ensures essential functions of prey degradation (protein degradation through abundant aspartic proteases, carboxypeptidases and peroxidases, and exoskeleton degradation through chitinases) and potential use of sugar-rich particles as a supplementary nutritional source (through sugar-cleaving enzymes) (Fig. 3).

The present characterization of the fluid protein content of *Nepenthes* based on deep sequencing of the transcriptome in combination with proteomic analyses provides new insights into the composition of *Nepenthes* digestive fluids. The *Nepenthes* secretome reveals an important diversity of hydrolytic enzymes that differ among species, hypothetically in response to particular ecological pressures. In addition to nine digestive enzymes described previously in the literature, 20 new putative proteins were identified as hydrolytic enzymes. The plant origin of enzymes identified in secretions coming from plants cultivated in greenhouses or living in natural sites remains to be confirmed because the experimental conditions

did not exclude microbial contamination in most of the fluids. This study is, however, not exhaustive and needs to be extended to other species that vary in their nitrogen source and diet. A recent report published by Buch *et al.* (2015) describes the first evidence of direct interaction between Nepenthesin 2 and prey. Such an analysis might be extended to many other proteins and therefore provide valuable information on the evolution of digestive processes in these fascinating plant species.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Figure S1: 2-D analysis of proteins present in the pitcher fluid of two *in vitro N. mirabilis* plants. Table S1: summary of growth conditions, origin of *Nepenthes* plant, studied pitcher development stage and the proteomic approach used for each condition. Table S2: summary of the proteins identified from digestive fluids of five *Nepenthes* species.

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