

RESEARCH PAPER

Functional characterization of a class III acid endochitinase from the traps of the carnivorous pitcher plant genus, *Nepenthes*

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Abstract

Carnivory in plants is an adaptation strategy to nutrient-poor environments and soils. Carnivorous plants obtain some additional mineral nutrients by trapping and digesting prey; the genus *Nepenthes* is helped by its specialized pitcher traps. To make the nutrients available, the caught prey needs to be digested, a process that requires the concerted activity of several hydrolytic enzymes. To identify and investigate the various enzymes involved in this process, fluid from *Nepenthes* traps has been analysed in detail. In this study, a novel type of *Nepenthes* endochitinase was identified in the digestion fluid of closed pitchers. The encoding endochitinase genes have been cloned from eight different *Nepenthes* species. Among these, the deduced amino acid sequence similarity was at least 94.9%. The corresponding cDNA from *N. rafflesiana* was heterologously expressed, and the purified protein, NrChit1, was biochemically characterized. The enzyme, classified as a class III acid endochitinase belonging to family 18 of the glycoside hydrolases, is secreted into the pitcher fluid very probably due to the presence of an N-terminal signal peptide. Transcriptome analyses using real-time PCR indicated that the presence of prey in the pitcher up-regulates the endochitinase gene not only in the glands, which are responsible for enzyme secretion, but at an even higher level, in the glands' surrounding tissue. These results suggest that in the pitchers' tissues, the endochitinase as well as other proteins from the pitcher fluid might fulfil a different, primary function as pathogenesis-related proteins.

Key words: Carnivorous plants, digestive fluid, endochitinase, *Nepenthes*, PR proteins, real-time PCR.

Introduction

Nepenthaceae are a monotypic family of carnivorous plants scattered throughout the Old World tropics. Around 130 species of *Nepenthes* have been described. This number is rapidly increasing, with several new species each year (McPherson, 2010). Their leaf morphology is very similar and consists of a photosynthetic part of the leaf (enlarged leaf base) and a tendril that carries a pitfall trap. These so-called pitchers are divided into zones which include a lid and a peristome involved in attracting and trapping prey; a waxy zone for trapping and preventing prey from escaping (Gaume *et al.*, 2002; Riedel *et al.*, 2003; Scholz *et al.*, 2010); and, at the bottom, a digestive zone. The inside of this part is covered with multicellular glands and often filled with a viscoelastic

fluid to retain and digest caught prey, mainly insects (Gorb *et al.*, 2004; Gaume and Forterre, 2007). The glands of the digestive zone fulfil various functions: the perception of chemical stimuli; the secretion of digestive enzymes; and nutrient absorption (Owen *et al.*, 1999; Schulze *et al.*, 1999). When visiting the pitchers, insects fall into the traps (Gaume *et al.*, 2002; Bohn and Federle, 2004), drown, and can be digested by the enzyme cocktail of the pitcher fluid (Mithöfer, 2011). As *Nepenthes* plants grow in nutrient-poor environments, this is an effective way to obtain additional nutrients, mainly nitrogen, that are otherwise difficult to come by.

The presence of proteolytic activities in carnivorous plants has been known since the time of Charles Darwin

(Darwin, 1875). In addition to proteases, other hydrolytic activities have been described in *Nepenthes* as well, such as RNase, esterase, phosphatase, and chitinase (Heslop-Harrison, 1975; Juniper *et al.*, 1989; Eilenberg *et al.*, 2006). The presence of additional enzymes has been suggested, such as a β -1,3-glucanase and a β -D-xylosidase (for an overview, see Hatano and Hamada, 2008; Mithöfer, 2011). All these hydrolytic enzymes are very probably employed in the digestion of the prey and help make nutrients available to the plant. Additionally, the acidification of the pitcher fluid by plasma membrane H⁺-ATPase is important for the digestion of prey and the absorption of nutrients (An *et al.*, 2001; Moran *et al.*, 2010).

Chitinases (EC 3.2.1.14) are particularly interesting plant enzymes because their substrate is not present in plant tissues *per se*. The induction of chitinases in plants upon fungal infection as well as the inhibition of fungal growth has been shown for several plant–pathogen interactions (Schlumbaum *et al.*, 1986; Theis and Stahl, 2004; Van Loon *et al.*, 2006). Thus, chitinases are thought to be necessary and involved as typical pathogenesis-related (PR) proteins in plant defence against pathogenic fungi, due to their ability to hydrolyse and degrade chitin (Theis and Stahl, 2004; Van Loon *et al.*, 2006). Most often, chitinases act as endochitinases, producing chito-oligosaccharides of 2–6 *N*-acetylglucosamine units, but they also may act as exochitinases. This large group of antifungal proteins belongs to the group of *O*-glycoside hydrolases, which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety. Based on amino acid sequence similarities, such enzymes are classified in 85 families; chitinases belong to families 18 and 19 (Henrissat, 1991).

Since insects represent the major fraction of animals trapped by carnivorous plants, there may be a special role for chitinases in the process of prey digestion. However, only two reports identify chitinases from *Nepenthes* species. Four genes representing two subgroups of basic chitinases from class I, *Nkchit1b* and *Nkchit2b*, have been isolated from *N. khasiana*; these were further characterized in a heterologous system (Eilenberg *et al.*, 2006). Recently, Hatano and Hamada (2008) were able to clone another chitinase from class IV from *N. alata* but did not show any enzymatic activities. Thus, the aim of this study was to investigate the digestive fluid of *Nepenthes* plants for the presence of additional hydrolytic enzymes; the study focused on chitinases. Using a proteomic approach in combination with molecular techniques, a novel chitinase was identified and heterologously expressed in *Escherichia coli* to enable its biochemical characterization. In order to analyse whether or not this enzyme is widespread in the genus *Nepenthes*, chitinase activity in the pitchers of various species was determined and a DNA-based phylogeny was calculated. Furthermore, the regulation of the enzyme was addressed using a real-time PCR approach. The effect of the presence of the chitinase on the transcript level in the tissues of different pitchers was studied and an attempt was made to unravel possible prey-mediated regulations.

Materials and methods

Chemicals and organisms

Nepenthes species (*N. singalana*, *N. ventricosa*, *N. gracilis*, *N. thorelii*, *N. mirabilis*, *N. ampullaria*, *N. alata*, *N. rafflesiana*, and the hybrid ‘Mizuho’) were grown in the greenhouses of the Botanical Gardens in Jena and Munich. All chemicals used, unless specified, were of analytical grade and purchased from Roth (Karlsruhe, Germany).

Protein analysis

A 15 ml aliquot of fluid was collected from 18 closed pitchers of various *Nepenthes* species (*N. singalana*, *N. gracilis*, *N. mirabilis*, *N. alata*, and *N. rafflesiana*) using a sterile syringe. A 50 μ g aliquot of protein was precipitated using trichloroacetic acid (TCA) from the homogenized fluid and subjected to SDS–PAGE (10% separation gel). The limited amount of pitcher fluid proteins meant that one-dimensional SDS–PAGE with a higher protein loading capacity had to be used instead of the more frequently used two-dimensional isoelectric focusing/SDS–PAGE system, which has a higher resolution. For microsequencing, peptides obtained after in-gel digestion with trypsin (Shevchenko *et al.*, 1996) were desalted and concentrated by ZipTip columns (C18-RP, Millipore, Schwalbach, Germany). Subsequently, electrospray ionization–tandem mass spectrometry (ESI-MS/MS) was performed on a Q-TOF 1.5 hybrid mass spectrometer (Micromass, Bremen, Germany) using ‘medium’ nano ESI capillaries according to Mithöfer *et al.* (2002). Data obtained were processed using MassLynx 3.5 (Micromass) and peptide sequences were calculated manually. For the identification of the proteins, databases were used by similarity or blast searches such as Swiss-Prot (<http://www.expasy.ch/sprot/>) and EMBL (<http://www.ebi.ac.uk/Tools/fasta33/index.html>). Search parameters were set as recommended by the database programs. Alignments and homology searches were carried out with Clustal X.

The putative signal peptide was predicted using the SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>; Bendtsen *et al.*, 2004). For homology modelling, a three-dimensional model of NrChit1 was built with the automated comparative modelling program SWISS-MODEL (<http://swissmodel.expasy.org/workspace/>; Guex and Peitsch, 1997; Schwede *et al.*, 2003; Arnold *et al.*, 2006). As homologous protein template, the crystal structure of hevamine, an endochitinase from family 18 class III (Protein Data Bank entry, 1hvqA; Swiss Prot, P23472; sequence similarity with NrChit1, 65.4%, X-ray resolution, 2.2 Å; Terwisscha van Scheltinga *et al.*, 1994) from *Hevea brasiliensis*, was used. Because of the high level of sequence similarities between the templates and NrChit1, the model was evaluated to be of a high quality.

Cloning, expression, and purification of NrChit1 endochitinase cDNA

Total RNA from *N. rafflesiana* pitchers was isolated using the Concert™ Plant RNA Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. RNA was purified to eliminate genomic DNA using the Qiagen RNeasy Plant RNA kit (Qiagen, Hilden, Germany) and DNA was digested by TURBO™ DNase (Applied Biosystems/Ambion, Darmstadt, Germany). First-strand cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen, Darmstadt, Germany), oligo(dT)₂₀ primer, and 1 μ g of total RNA at 50 °C for 55 min.

Degenerate primers, designed according to conserved protein sequences of known plant endochitinases (NCBI GenBank), were used to amplify a fragmental cDNA sequence. Cloning the 5’ and 3’ end of *NrChit1* cDNA was accomplished by rapid amplification of cDNA ends (RACE) PCR using total RNA and the First-Choice® RLM-RACE Kit (Applied Biosystems/Ambion) following the manufacturer’s protocol. Primers were designed by using DNASTAR Lasergene® Software (GATC BIOTECH, Konstanz, Germany). The resulting amplified products were cloned into

a pCR[®]-TOPO[®]-vector, and the resulting plasmid was subjected to nucleotide sequencing (Eurofins MWG Operon, Ebersberg, Germany). The complete *NrChit1* cDNA sequence was amplified by PCR using *Pfu* DNA polymerase (Fermentas, St. Leon-Rot, Germany), and the primers: forward 5'-ATG AAG ACC CAT TAT TCA TCA GCA ATT C-3' and reverse 5'-TTA AAC ACT ATC CTT GAT AGC TGA G-3' (PCR: 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C; and 10 min at 72 °C).

For functional identification, cDNA was amplified with primers for an open reading frame (ORF) lacking the signal peptide. The cDNA was subcloned into the pHis8-3 expression vector (Jez *et al.*, 2000). The recombinant vector was transformed into *E. coli* BL21(DE3) already transformed with the chaperone-coding plasmid pG-Tf2 (Takara Bio Europe S.A.S., Saint-Germain-en-Laye, France). The bacterial strain was grown to $A_{600}=0.6$ at 37 °C in LB medium with kanamycin at 50 µg ml⁻¹ and chloramphenicol at 20 µg ml⁻¹. Cultures were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for *NrChit1* and with 10 ng ml⁻¹ tetracycline for chaperone co-expression. Cultures were kept overnight at 16 °C while being shaken at 200 rpm. After expression, the protein was purified following the instructions of QIAexpressionist[™] with a modification of the elution buffer (10 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8).

The respective endochitinase sequences from genomic DNA of seven additional *Nepenthes* species were also cloned. Therefore, genomic DNA was isolated from pitchers using the CTAB (cetyltrimethylammonium bromide) method (Doyle and Doyle, 1990). Amplification and cloning were done as described above.

Chitinase activity assays

The chitinolytic activity was determined by using the Chitinase Assay Kit, Fluorometric assay from Sigma-Aldrich (Taufkirchen, Germany). Substrates tested were the dimer 4-methylumbelliferyl (4MU) β-D-N-acetylglucosaminide [(GlcNAc)₂] for exochitinase, the trimer 4-methylumbelliferyl β-D-N,N'-diacetylchitobioside hydrate [4MU-(GlcNAc)₃] for chitobiosidase, or the tetramer 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotriose [4MU-(GlcNAc)₄] for endochitinase activity. Chitinase specificity was estimated by the cleavage of the β-1,4 bond that releases 4MU from the different oligomers. Chitinase reactions were done following the manufacturer's protocol. Fluorescence was measured by a fluorescence spectrophotometer (excitation at 360 nm, emission at 450 nm).

As a second substrate for endochitinase activity, the soluble polymeric substrate carboxymethyl-chitin-Remazol Brilliant Violet (CM-Chitin-RBV; LOEWE Biochemica, Sauerlach, Germany) was used. The reaction contained 50 µl of 50 mM sodium acetate buffer, pH 5, standard (chitinase from *Streptomyces griseus*, 10 µg ml⁻¹, Sigma-Aldrich) or sample (expressed endochitinase or pitcher fluid), and 150 µl of CM-Chitin-RBV (2 mg ml⁻¹). The reaction was stopped by adding 20 µl of 0.25 M HCl, and non-digested substrate was precipitated by incubation at -20 °C for 5 min and centrifugation at 4 °C for 5 min. Absorbance at 560 nm was measured by a Spectramax 250 microplate reader.

Real-time PCR

For real-time PCR analyses, total RNA was isolated from either whole pitchers, glands, or the epidermal tissue of the digestive zone of closed and open pitchers from *N. mirabilis* or *N. alata*. When pitchers were fed with *Drosophila melanogaster* (30 individuals each; three replicates), they were packed in a full-fashioned stocking to avoid contamination and harvested after 7 d. Fifty glands or surrounding epidermal tissue were isolated using the aureka platform (aura optik, Jena, Germany; Rottloff *et al.*, 2009). *NrChit1* expression levels were analysed and compared as follows: for total RNA isolation, the RNeasy[®]-Micro kit (Applied Biosystems/Ambion) was used following the manufacturer's protocol. DNA digestion and the reverse transcription reaction were done as described above. Actin was chosen as the internal control gene; the

sequence was isolated using the primers published by Van den Berg *et al.* (2004), actinF 5'-ACC GAA GCC CCT CTT AAC CC-3' and actinR 5'-GTA TGG CTG ACA CCA TCA CC-3', coding for a 180 bp fragment with 93% identity to an actin gene from *Quercus robur* (GQ339769) (PCR: 3 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 57 °C, 60 s at 72 °C; and 10 min at 72 °C).

Primers for real-time PCR were designed in order to obtain resulting PCR products of ~100 bp using the Primer3Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3-plus.cgi>): for actin, 5'-CTC TTA ACC CCA AAG CAA ACA GG-3' and 5'-GTG AGA GAA CAG CCT GGA TG-3'; and for endochitinase, 5'-AAG GGA TCA AGG TCC TCC TAT C-3' and 5'-GAG GTA GTT ATT CCA AAG GTA AGC-3'.

Real-time PCR was done on a Mx3000P Real-Time PCR System (Stratagene, La Jolla, CA, USA). The process was performed with 25 µl of reaction mixture containing 12.5 µl of 2× Brilliant II SYBR[®] Green QPCR Master Mix (Stratagene), cDNA (20–75 ng), 400 nM of each primer, and 30 nM ROX as a passive reference dye. The following protocol was used: initial polymerase activation for 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 60 s at 61 °C, and 60 s at 72 °C. Actin levels were equal in every reaction independently of tissue type. PCR conditions were determined by a non-reverse transcriptase template control and a non-template control for each primer pair. Relative RNA levels were calibrated and normalized with the level of actin mRNA by determining the efficiency of every single reaction using the method of Liu and Saint (2002). Calculation of expression ratios and statistical analyses were performed with the Relative Expression Software Tool (REST[®] 2009, <http://www.gene-quantification.de/rest.html>; Pfaffl *et al.*, 2002). Data are from triplicates.

Phylogenetic analyses

To infer phylogenetic relationships, a maximum parsimony analysis of the endochitinase sequence data set that had been obtained from genomic DNA as described above was carried out in PAUP* 4.0b10 (Swofford, 2003) using the default exhaustive search settings. The 50% bootstrap majority rule consensus trees (Felsenstein, 1981) were calculated from 10 000 replicates under the default heuristic search settings with no maxtrees limit, and character states specified as unordered and equally weighted.

Additionally, a Bayesian analysis was conducted using the Markov-Chain-Monte-Carlo algorithm of MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001) under the assumption of the HKY model (Hasegawa *et al.*, 1985) and invariable sites (HKY+I), which was determined to be the best-fit model of sequence evolution by the likelihood ratio tests implemented in MrModeltest 2.2 (Nylander, 2004). Four Markov chains were calculated simultaneously, according to MrBayes' default setting. Analysis was terminated after 1 000 000 generations; trees were summarized in a 50% majority rule consensus tree after discarding burn-in trees yielded before reaching likelihood stationarity.

Results and Discussion

Protein identification

Based on the peptide sequence YYDNGYSSA(I/L)K that was determined from a 30 kDa protein band, high similarities of this protein with acid endochitinases from other plant species, *Nicotiana tabacum* (NCBI accession no. CAA77656), *Vitis vinifera* (BAC65326), *Hevea brasiliensis* (CAA09110), *Malus domestica* (AAG25709), and *Beta vulgaris* (AAB28479), were detected by blast searches. This was possible because this N-terminal-localized 12 amino acid long peptide was specific enough to suggest that the

protein was very likely to be an acid endochitinase. In particular, the GYS tripeptide is a highly conserved motif which can be found all over these proteins (Supplementary Fig. S1 available at *JXB* online).

Cloning of *NrChit1* from *Nepenthes rafflesiana* and its heterologous expression

In order to isolate the corresponding cDNA, degenerated primers based on the peptide sequence and on a conserved peptide stretch close to the N-terminus of the consensus sequence of the various endochitinases were synthesized and applied in a reverse transcription-PCR (RT-PCR) using RNA isolated from *N. rafflesiana* pitchers. However, no PCR fragment was amplified. Only the usage of oligonucleotides, which have been deduced from the consensus sequence of the cDNAs of the endochitinases mentioned above, resulted in the amplification of a 474 bp fragment. RACE PCR (Frohman *et al.*, 1988) was further used to isolate the missing 5' and 3' regions of the cDNA, again by using RNA isolated from *N. rafflesiana*. The isolated full-length cDNA (GenBank accession no. GQ338257) encoding the putative endochitinase (*NrChit1*) showed an ORF of 879 bp corresponding to 292 amino acid residues (Fig. 1A). The deduced amino acid sequence comprised the sequenced peptide from position ²⁷⁹Y to ²⁸⁹K, indicating an isoleucine at position 288. Moreover, *NrChit1* also contained the conserved sequence of the catalytic domain of family 18 glycoside hydrolases (Karlsson and Stenlid, 2009), LDGIDFDIE, from position 145 to 154 (Fig. 1A). Its primary structure suggested a class III chitinase (Collinge *et al.*, 1993), and it shares significant homology with plant class III chitinases (Supplementary Fig. S1 at *JXB* online), including the presence of six cysteine residues at conserved positions in all class III chitinases (Kim *et al.*, 1999)

(Fig. 1A). This classification is strongly supported by further sequence analyses that predicted the presence of an N-terminal region representing a signal peptide; this peptide in turn directs the protein to the apoplasm (Bendtsen *et al.*, 2004). These signal peptides are cleaved off the mature protein. Most probably, the cleavage site is between amino acid positions 26 and 27 (Fig. 1A) (Bendtsen *et al.*, 2004).

In addition, the endochitinase sequences from genomic DNA of *N. ventricosa* (GenBank accession no. GQ338254), *N. mirabilis* (GQ338258), *N. thorelii* (GQ338255), *N. ampullaria* (GQ338261), *Nepenthes* hybrid 'Mizuho' (GQ338259), *N. gracilis* (GQ338260), and *N. singalana* (GQ338256) were also cloned. Interestingly, no introns could be found for any of them. The significance of single exon genes in plants is still unknown. Genes without introns are often retrogenes and formed by retroposition, a cellular process in which spliced mRNAs are reverse transcribed and inserted into new genomic positions. Typically, they become non-expressed pseudogenes, because of the lack of regulatory elements (Wang *et al.*, 2006). However, many retrogenes were identified in *Populus* species and *Arabidopsis thaliana*, and most were functional (Wang *et al.*, 2006; Zuh *et al.*, 2009). Although some genes encoding PR proteins do not have introns, such as the *PR-1* gene from *Oryza sativa* (Liu and Xue, 2006) and *TLP* from *N. singalana* (Rottloff *et al.*, 2009), this is not a general phenomenon; such a general phenomenon would include, for example, four genes encoding basic chitinases class I (*Nkchit1b-1*, -2 and *Nkchit2b-1*, -2) of pitchers from *N. khasiana* which possess two introns (Eilenberg *et al.*, 2006). However, it is conceivable that the single exon structure is typical for class III acidic chitinases, as genomic sequence analysis with other plant species coding for this enzyme [*M. domestica* (AF309514), *Medicago truncatula* (AY238969), and *V. vinifera* (AB105374)] also showed no introns in the coding region.

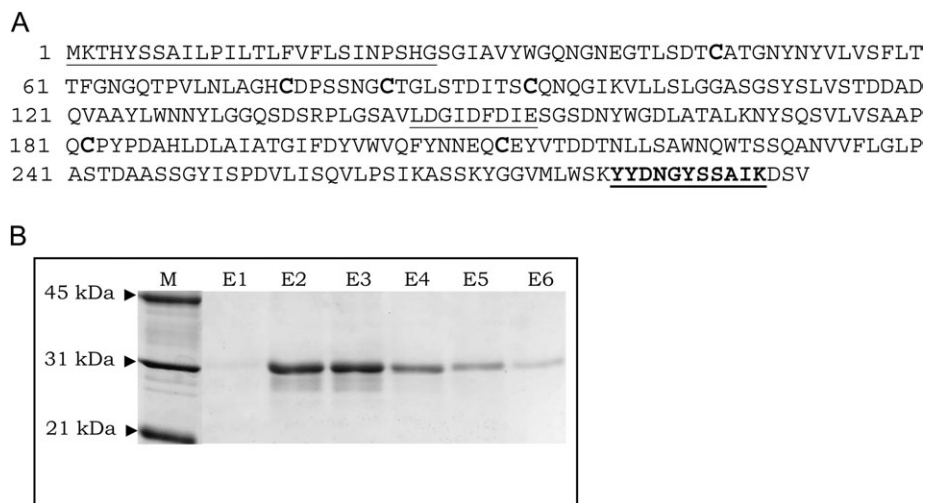


Fig. 1. (A) Deduced amino acid sequence of the endochitinase gene from *Nepenthes rafflesiana*. The predicted signal peptide (amino acids 1–26) is indicated in italics and underlined, the conserved sequence of the catalytic domain of family 18 glycoside hydrolases is underlined (amino acids 145–153), the sequenced peptide (amino acids 279–189) is indicated in bold and underlined, and conserved cysteines are in bold. (B) Coomassie-stained SDS-PAGE of recombinant endochitinase *NrChit1* heterologously expressed in *E. coli* and isolated using His-Tag-based purification. M, broad range protein marker (Biorad); E1–6, different fractions eluted with imidazole.

Biochemical characterization

The recombinant NrChit1 protein, which was heterologously expressed in *E. coli* under chaperone co-expression and purified using a His-tag of eight histidines (Fig. 1B), was further used to characterize catalytic activities (Table 1). Among the various compounds that have been tested to elucidate the substrate specificity—4MU-(GlcNAc)₁ for exochitinase activity, 4MU-(GlcNAc)₂ for chitobiosidase activity, and 4MU-(GlcNAc)₃ and CM-chitin-RBV for endochitinase activity—only CM-chitin-RBV could be hydrolysed. Using this substrate, basic enzymatic properties have been determined (Table 1). However, the specific activity of NrChit1 was quite low, ΔE_{550} : 0.08 AU (h μ g protein)⁻¹. Thus, a detailed kinetic analysis of the enzymatic reaction was not feasible because CM-chitin-RBV concentrations >1.5 μ g ml⁻¹ were not applicable and, as a consequence, substrate saturation was impossible to reach. In spite of this handicap, the endochitinase activity of NrChit1 could be demonstrated with the recombinant enzyme. Thus, clearly NrChit1 is able to hydrolyse longer chitin polymers occurring in the exoskeleton of arthropods or fungal cell walls. This might happen together with other endochitinases in the digestion fluid, described by Eilenberg *et al.* (2006). However, the question remains: why have no chitobiosidases or exochitinases been identified up to now? Such enzymes release and provide dimeric or even monomeric products which easily can be further metabolized by the plant.

As several crystal structures were determined for chitinases from family 18 class III, the spatial structure of the NrChit1 was modelled by comparative modelling. The modelled residue range was from amino acids 1 to 266 (i.e. without a signal peptide). The sequence of NrChit1 was then structurally compatible with the fold of chitinases from class III. Similar to other chitinases from this class, the predicted NrChit1 structure was composed of a ($\beta\alpha$)₈-barrel folding motif, which is the typical architecture of chitinases from family 18 (Terwisscha van Scheltinga *et al.*, 1994) and consists of an eight-stranded parallel β -barrel surrounded by eight α -helices. Two additional β -strands can be found outside the ($\beta\alpha$)₈-barrel (Fig. 2).

The widespread occurrence of this particular type of chitinase in the genus *Nepenthes* was demonstrated by the identification of its enzymatic activities in several species (Fig. 3). This demonstrates that in all tested *Nepenthes*

species the encoding gene is indeed not a pseudogene but expresses an active enzyme. The endochitinase is detectable in closed pitchers, suggesting it is constitutively expressed. In *N. rafflesiana* and *N. mirabilis*, the activities in closed pitchers are even higher compared with the open ones. On the other hand, at least for *N. thorelii*, it may be that some proteins were induced. In any case, the enzymatic activities were calculated on the basis of protein concentration; thus dilution effects due to different volumes of fluid in the pitchers can be ruled out. The differences in the activities can be explained by a lack of information about the relative participation of the endochitinase with respect to the total amount of protein in the fluid of the particular species. Thus, the ratio of endochitinase to total protein might vary in different species.

Phylogenetic analyses

To investigate whether the encoding genes and proteins of the endochitinases from various *Nepenthes* species are homologues within this genus as well as compared with other species, a phylogenetic analysis was performed. A comparison of the whole amino acid sequence of NrChit1 with protein sequences of endochitinases from other plants (Supplementary Fig. S1 at *JXB* online) revealed a similarity, always in the range from ~61.6% with signal peptide and 65.2% without signal peptide (*V. vinifera*) to 66.0% and 69.5% (*B. vulgaris*), respectively. Within the additional endochitinases of seven other *Nepenthes* species that have been cloned as well, the similarity on the amino acid level was never lower than 94.9%.

A maximum parsimony analysis of the corresponding DNA sequence data set resulted in one tree with a length of 78 steps (consistency index=0.95, retention index=0.95). The topology of the bootstrap 50% majority rule consensus tree (identical values) corresponded to the topology of the Bayesian consensus tree which was computed from 1 000 000 generations (burn-in: 10 000). This is shown in Fig. 4, including Bayesian posterior probabilities (PPs) and bootstrap support (BS) values from the parsimony analysis. The topology indicates three major lineages, each with high statistical support. One lineage consists of *N. ventricosa* and *N. mirabilis* (PP=1.00, BS=100) and the second (PP=1.00, BS=100) consists of *N. thorelii* and—as a sister group—a monophyly comprising *N. rafflesiana* and *N. ampullaria* (PP=1.00, BS=100). The third lineage (PP=0.99, BS=95) contains the hybrid 'Mizuho', which is a sister taxon of a monophyly that comprises *N. gracilis* and *N. singalana* (PP=1.00, BS=99).

A previous molecular study divided the genus *Nepenthes* into three major evolutionary lineages based on a phylogenetic reconstruction from chloroplast *trnK* intron sequence data (Meimberg *et al.*, 2001). Six of the eight taxa investigated in the present study were present in the sampling of this chloroplast DNA (cpDNA) analysis; five were found to be members of the same lineage (*N. ampullaria*, *N. gracilis*, *N. mirabilis*, *N. rafflesiana*, and *N. thorelii*). Considering the moderate statistical support within the clades inferred by Meimberg *et al.* (2001), it cannot be said that there is a major conflict with the present

Table 1. Properties of the heterologously expressed endochitinase from *Nepenthes rafflesiana*

Protein characteristics	Specificities
Enzyme activity	Endochitinase
Glycosyl hydrolase family	18
Classification	Class III
Substrate specificity	CM-chitin-RBV
Temperature optimum	41 °C
pH optimum	3–4
Molecular mass	31.1 kDa
Isoelectric point	3.86

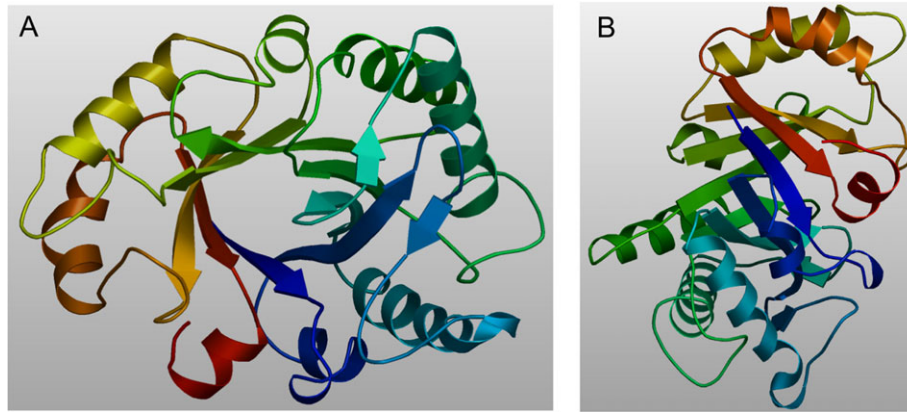


Fig. 2. Calculated 3D structure of the endochitinase NrChit1 from *Nepenthes rafflesiana*. The structure was suggested by the SWISS-MODEL web server upon comparative modelling using an endochitinase sequence from *Hevea brasiliensis* (NCPI, CAA09110; Swiss Prot, P23472) as the homologous template structure. The modelled residue range is from amino acids 1 to 266. A and B represent different views of the modelled structure.

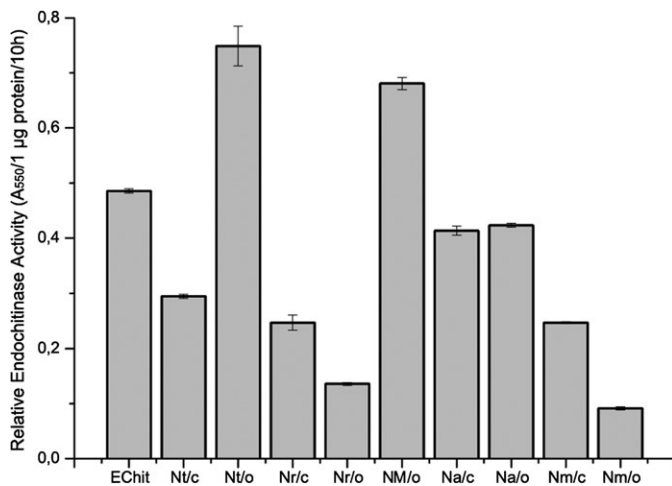


Fig. 3. Endochitinase activities in the pitcher fluid of various *Nepenthes* species and the heterologously expressed NrChit1 using CM-chitin-RBV as substrate. Echit, *E. coli*-expressed NrChit1 from *N. rafflesiana*; Nt, *N. thorelli*; Nr, *N. rafflesiana*; NM, *N. hybrid* 'Mizuho'; Na: *N. ampullaria*; Nm, *N. mirabilis*; o, open pitchers; c, closed pitchers. Bars represent means calculated from $n=3$ pitchers \pm SD.

phylogenetic reconstruction, but it is evident that the present data provide a significantly higher resolution among these five taxa. The sixth species, *N. ventricosa*, is nested within a different lineage in the cpDNA phylogeny; the results, however, indicate a close relationship to *N. mirabilis* with high statistical support (Fig. 4). This grouping receives support from phylogeographic considerations (McPherson, 2010), as both species occur in the Philippines. Biogeographic congruence is also true for two other indicated subgroups, *N. gracilis* and *N. singalana* (both are found in Sumatra), as well as *N. ampullaria* and *N. rafflesiana* (found in Borneo, Peninsular Malaysia, Singapore, Sulawesi, Sumatra, and Thailand).

The difference in resolution in comparison with cpDNA data and the inconsistency described above strongly

indicate the crucial effect of the marker employed for phylogenetic analyses on the results. This suggests that a thorough re-examination of the phylogenetic relationships within *Nepenthes*, based on a multiple marker analysis or on fingerprinting methodology, might be rewarding.

Endochitinase transcript abundance

To gain insight into its molecular regulation, endochitinase gene expression was measured in pitchers of *N. mirabilis*. In order to investigate whether or not the presence of prey affects the transcript level, pitchers fed with fruit flies were analysed. As shown in Table 2, a slight but significant up-regulation was found (1.40-fold) in open pitchers due to the presence of the flies, suggesting that the endochitinase gene is regulated on the mRNA level. In addition, in the glands' surrounding tissue, a significantly higher level of endochitinase transcripts was detected in open compared with closed pitchers (7.52-fold). This was not the case in the glands themselves, although a trend (not significant) towards this condition was observed [1.76-fold; $P(HI)$: 0.16]. These results can be interpreted as an induction of the respective gene in the presence of prey. Recently, the injection of colloidal chitin into the trap of *N. khasiana* was shown to induce a type I basic chitinase, NkChit1b, which is very probably secreted into the pitcher fluid (Eilenberg et al., 2006). Analysing glands and their surrounding tissue in open pitchers fed with flies demonstrated that in the tissue, a striking higher level of endochitinase transcripts (10.04-fold) occurred compared with in the glands (Table 2). This result was somewhat surprising if it is assumed that the endochitinase functions in the digestion of prey and is therefore synthesized and secreted mainly by the glands. However, the results of Owen et al. (1999) showed that at least in *N. alata*, the glands of mature, open pitchers are less able to secrete a fluorescent dye, 6(5)-carboxyfluorescein, than glands of younger pitchers. Consequently, it might be that the reduced secretion ability diminishes transcript accumulation in glands but not in the surrounding tissues.

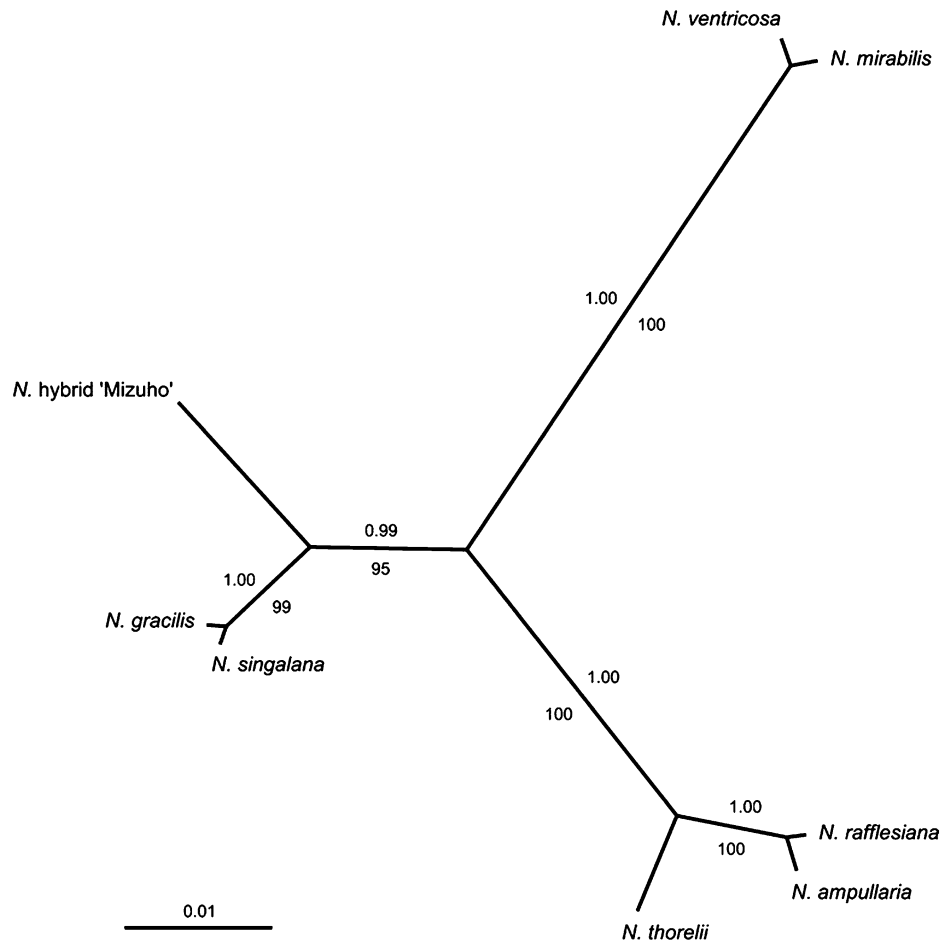


Fig. 4. The 50% majority rule consensus tree resulting from the Bayesian analysis of the DNA sequence data set, shown as a phylogram. For each node, posterior probabilities are given above the corresponding branch. Bootstrap values from 10 000 MP replicates are given below the branches.

Table 2. Real-time PCR analyses for the *NrChit1* level in *Nepenthes* pitchers under various conditions

Whole tissue of open or closed pitchers was investigated or the indicated parts: glands only (gl) and tissues surrounding the glands only (ts), respectively. Some pitchers were treated with prey (*Drosophila melanogaster*, *Dm*) for 7 d before harvesting.

Plant	Comparison	Expression ratio	$P(H1)^a$
<i>N. mirabilis</i>	Open + <i>Dm</i> versus open	1.40	<0.001
<i>N. mirabilis</i> , gl	Open + <i>Dm</i> versus closed	1.76	0.160
<i>N. mirabilis</i> , ts	Open + <i>Dm</i> versus closed	7.52	<0.001
<i>N. mirabilis</i>	Open + <i>Dm</i> , tissue versus glands	10.04	<0.001
<i>N. mirabilis</i>	Closed, tissue versus glands	2.97	<0.001
<i>N. alata</i>	Closed, tissue versus glands	2.98	<0.001

^a The hypothesis test $P(H1)$ represents the probability of the alternative hypothesis that the difference between the sample and control group is due only to chance. $P(H1) < 0.001$ indicates significant differences between the groups compared.

Thus, to investigate further whether this distribution of endochitinase transcripts in glands and surrounding tissue is typical for *Nepenthes* and present even in developing traps, the abundance of endochitinase transcript in glands and in the tissue of closed pitchers of two species, *N. mirabilis* and

N. alata, was analysed. In principle, higher levels of endochitinase transcripts have usually been found in *N. alata* compared with the respective part of *N. mirabilis* (whole pitcher, glands, and surrounding tissue). However, this difference (5.89-fold) was significant only for the tissue. However, even more interesting, the results obtained revealed significantly higher expression levels (~3-fold) in the tissue than in glands for both plants (Table 2). Thus, given that reduced secretion ability is typical for mature but not developing pitchers, it is unlikely that the main site for transcript accumulation shifts from glands to their surrounding tissue. Although a fast transcript turnover in the glands could explain the findings, it is also conceivable that the glands are indeed responsible for protein secretion but not necessarily for their biosynthesis. All pitcher tissues might be employed in the generation of enzymes for the digestion fluid, but only the glands can secrete them. In any case, more experiments addressing these questions should be performed.

Conclusions

Several lines of evidence are consistent with the hypothesis that chitinases as PR proteins are an important component

of plant defence systems against pathogenic fungi. However, the presence of such a hydrolytic activity in the digestion fluid of carnivorous plants also makes sense as it is employed to break down captured insect prey. That means the plant is taking a given enzyme and using it in a new, different context. This represents a sophisticated and highly efficient way of overcoming an environmental handicap. The fact that the endochitinase is inducible supports the hypothesis that this enzyme actually is a PR protein because inducibility is a typical property of PR proteins (Van Loon *et al.*, 2006). Moreover, the occurrence of this protein not only in the pitcher fluid or the secreting glands but also in the tissue of the pitchers, as indicated by the detection of transcripts, suggests that the endochitinase might be employed in defence. It is hoped to prove this hypothesis by further experiments with non-pitcher tissues of *Nepenthes*. If a pathogenic infection causes the induction of the endochitinase in these parts of the plant, it is very likely to be a real PR protein. The signal peptide suggests that the endochitinase is located in the apoplast. Similar findings have been reported for a thaumatin-like protein from *Nepenthes* pitchers (Rottloff *et al.*, 2009). Further experiments will study the exact localization of such enzymes in the pitchers' tissue as well as how their genes are regulated on the molecular level. Thus, although the activity of the enzyme remains the same, its function has shifted from defence to digestion as postulated recently (Mithöfer, 2011).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Amino acid sequence alignment of endochitinases from various species. The amino acid sequence alignment and the deduced consensus sequence were generated using Clustal X involving the plant species *Pyrus pyrifolia*, *Lupinus albus*, *Cucumis sativus*, *Nepenthes rafflesiana*, *Beta vulgaris*, *Vitis vinifera*, and *Oryza sativa*.

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