

Structural and functional characterisation of a class I endochitinase of the carnivorous sundew (*Drosera rotundifolia* L.)

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Abstract

Main conclusion Chitinase gene from the carnivorous plant, *Drosera rotundifolia*, was cloned and functionally characterised.

Plant chitinases are believed to play an important role in the developmental and physiological processes and in responses to biotic and abiotic stress. In addition, there is growing evidence that carnivorous plants can use them to digest insect prey. In this study, a full-length genomic clone consisting of the 1665-bp chitinase gene (*gDrChit*) and adjacent promoter region of the 698 bp in length were isolated from *Drosera rotundifolia* L. using degenerate PCR and a genome-walking approach. The corresponding coding sequence of chitinase gene (*DrChit*) was obtained following RNA isolation from the leaves of aseptically grown in vitro plants, cDNA synthesis with a gene-specific primer and PCR amplification. The open reading frame of cDNA clone consisted of 978 nucleotides and encoded 325 amino acid residues. Sequence analysis indicated that DrChit belongs to the class I group of plant chitinases.

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Phylogenetic analysis within the Caryophyllales class I chitinases demonstrated a significant evolutionary relatedness of *DrChit* with clade Ib, which contains the extra-cellular orthologues that play a role in carnivory. Comparative expression analysis revealed that the *DrChit* is expressed predominantly in tentacles and is up-regulated by treatment with inducers that mimic insect prey. Enzymatic activity of rDrChit protein expressed in *Escherichia coli* was confirmed and purified protein exhibited a long oligomer-specific endochitinase activity on glycol-chitin and FITC-chitin. The isolation and expression profile of a chitinase gene from *D. rotundifolia* has not been reported so far. The obtained results support the role of specific chitinases in digestive processes in carnivorous plant species.

Keywords Caryophyllales · Digestive process · Chitinase · Expression profile · Pathogenesis-related proteins · Recombinant protein

Abbreviations

DrChit *Drosera rotundifolia* chitinase
FITC-chitin *N*-Fluorescein-labeled chitin
PR Proline-rich hinge
rDrChit Recombinant *Drosera rotundifolia* chitinase

Introduction

Chitinases (EC 3.2.1.14) hydrolyse chitin, a polymer of *N*-acetyl-D-glucosamine that is one of the most abundant biopolymers. It occurs in the cuticles of insects, the shells of crustaceans and the cell walls of many fungi (Kwon et al. 2007). Although plants do not contain chitin, they express various types of chitinases, which are classified

into seven classes based on the presence of specific domains: chitin-binding domain, a hinge domain and C-terminal extension, and the flanking catalytic glyco-hydro domain 18 or 19 (Meins et al. 1994; Islam et al. 2010). Class I, II, IV, VI, and VII chitinases belong to family 19, whereas classes III and V form family 18 (Neuhaus 1999; Veluthakkal and Dasgupta 2012).

The catalytic domain of chitinases consists of 220–230 amino acid residues (Liu et al. 2010) and can be followed by a C-terminal extension that is responsible for the vacuolar localisation of an expressed chitinase. Class I, IV and VI chitinases contain a chitin-binding domain (CBD) followed by a short hinge region, which both precede the catalytic domain (Collinge et al. 1993). Class II and VII chitinases lack the CBD domain that is responsible for the majority of chitinolytic activity within plant tissues (Legrand et al. 1987). Classes III and V chitinases that are members of the glycoside hydrolase family 18 also lack a CBD and share sequence similarity to fungal and/or bacterial chitinases. The processing of most plant chitinase pre-proteins occurs at the endoplasmic reticulum, where the N-terminal signal peptide is removed from the mature form.

Analyses of genome-wide sequences and microarray expression profiling show that the chitinase genes are represented by large gene families in plant genomes and the individual member genes are expressed under diverse conditions (Grover 2012). Chitinases, especially those with strong antifungal activity, respond to pathogen attack (Graham and Sticklen 1994; Kasprzewska 2003; Zhao et al. 2007). However, various types of abiotic (osmotic, salt, cold, wounding and heavy metal) stress also regulate chitinase gene expression (Takenaka et al. 2009; Wu et al. 2009; Meszaros et al. 2013). Chitinases that are capable of hydrolysing lipochitooligosaccharides are involved in symbiosis and nodule development (Salzer et al. 1997; Bonanomi et al. 2001) and those with specific hydrolytic activities that result in the production of signal molecules or morphogenic factors play a role in plant development (Domon et al. 2000; Dyachok et al. 2002; Gomez et al. 2002). Finally, a specific role of chitinases in plant carnivory has recently been demonstrated within the order Caryophyllales (Matusikova et al. 2005; Eilenberg et al. 2006).

The carnivorous genus *Drosera* contains approximately 194 species throughout the world. The leaves of this group of plants fulfil two functions: in addition to photosynthesizing, they lure, capture and digest insects using mucilaginous glands called tentacles (Juniper et al. 1989). The genomes of individual representatives of this genus, including sundew (*Drosera rotundifolia* L.), and the genes involved in digestive processes have been only poorly explored so far. In vitro-grown sundew represents a

suitable model for study of digestive processes as this species is of a small size and has accessible adhesive traps. In addition, cultivation under sterile conditions excludes microbial contamination.

In *D. rotundifolia*, the up-regulation of chitinases was detected in the tentacles upon secretion-inducing stimuli (Matusikova et al. 2005). Similarly, the presence of chitinase(s) was reported in the secreted pitcher fluid of another carnivorous genus, *Nepenthes* (Eilenberg et al. 2006; Hatano and Hamada 2008). Most studied carnivorous plants contain class I chitinase(s) in the digestive fluid (Eilenberg et al. 2006; Renner and Specht 2012; Paszota et al. 2014); however, examples exist where other chitinase classes (III, IV and V) are present in the secreted digestive enzymes (Rottloff et al. 2011; Ishisaki et al. 2012a, b; Hatano and Hamada 2012).

Here, we focused on the isolation, in silico characterisation of a full-length genomic clone of chitinase gene (*gDrChit*) and its corresponding coding sequence (*DrChit*), as well as on the expression of recombinant chitinase (*rDrChit*) from *D. rotundifolia* in *Escherichia coli*. In addition, we present an expression profile of this gene in individual plant organs of non-stressed plants and demonstrate the up-regulation of this gene mainly in tentacles following chitin application to the leaves.

Materials and methods

Plant material

Plants of *D. rotundifolia* L. (Finnish cultivar “Vikilan Järvi”) were collected from open *Sphagnum fuscum* bog between *Pinus* bog and oligotrophic lake in Northern Finland (65°35'N, 28°28'E). They were provided as a gift from Terttu Kämäräinen (Department of Biology/Botany, University of Oulu, Finland) and cultivated aseptically on agar media as described previously (Bobak et al. 1995). The *DrChit* expression profiles in the leaves, leaves without tentacles, tentacles (gently abraded using a razor blade), stems and roots from non-treated plants or those treated with individual inducers were analysed using two-month-old in vitro plants. For treatment, chitin from crustacean shells (Serva), gelatine (Serva) and sand (Sigma-Aldrich), respectively, was applied to leaves for 24 h. The samples were then frozen in liquid nitrogen and stored at –80 °C.

Isolation of a genomic clone of *DrChit*

Genomic DNA of *D. rotundifolia* L. was extracted from the leaves (1 g) according to Bekesiova et al. (1999). The chitinase gene was amplified in 50 µL solution containing

100 ng DNA template, 15 pmol P1–P2 degenerate primers (Suppl. Table S1), 200 μ M dNTP, 1 \times PCR buffer and 1 U Taq DNA polymerase (Finnzymes). The first step at 94 °C for 2 min was followed by 35 cycles of denaturation at 94 °C for 30 s; annealing at 53 °C for 40 s; extension at 72 °C for 90 s and a final step was performed at 72 °C for 10 min. The PCR product was cloned into pGEM-T Easy (Promega) and commercially sequenced.

To determine the full-length continuous sequence, we used the Genome Walker kit (Clontech). To obtain the 5' upstream sequence, genomic DNA was digested by *PvuII*. Adaptor DNA provided in the kit was ligated to *PvuII*-digested genomic DNA fragments. The first PCR was conducted using an adaptor primer AP1FOR provided with the kit and a gene-specific primer GS1REV (Suppl. Table S1). The second-round PCR was performed using the nested adaptor primer AP2FOR and the GS2REV primer (Suppl. Table S1). The isolated PCR fragment with an unknown sequence at the 5' end was sequenced and used to design the gene-specific primers GS3REV and GS4REV (Suppl. Table S1). To extend the 5' upstream sequence containing the 5' regulatory sequence, Genome Walking-PCR was performed on the same library using the same forward adaptor primers and GS3REV and GS4REV primers (Suppl. Table S1) in the first- and second-round PCR, respectively. To obtain the 3' downstream DNA sequence, the genomic DNA was digested with *StuII*. The PCR and nested PCR were conducted using the GS5FOR-AP1REV and GS6FOR-AP2REV primers (Suppl. Table S1), respectively.

The PCR conditions were one cycle at 94 °C for 2 min; followed by seven cycles at 94 °C for 25 s; 72 °C for 3 min; 32 cycles at 94 °C for 25 s, 67 °C for 3 min and a final elongation step of 7 min at 67 °C. The DNA amplified by PCR was cloned into pGEM-T Easy (Promega) and sequenced. The complete chitinase sequence was amplified from genomic DNA by PCR using the P3–P4 primers (Suppl. Table S1) and the following conditions: 94 °C for 3 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 40 s and extension at 72 °C for 3 min. The final step was performed at 72 °C for 10 min.

Bioinformatic analysis of sequencing data

Sequence alignments were carried out using CLUSTALW (Thompson et al. 1994). Similarity searches for nucleotide and amino acid sequences of *DrChit* were conducted using BLASTn and BLASTp (<http://www.ncbi.nlm.nih.gov/BLAST/>) programs, respectively (Altschul et al. 1990). The structure of the *gDrChit* gene was analysed using the NetGene2 program (<http://www.cbs.dtu.dk/services/NetGene2/>) (Hebsgaard et al. 1996). The nucleotide sequence of *DrChit* was translated into the amino acid sequence

using the ExPASy Translate Tool of the proteomics server of Bioinformatics (<http://web.expasy.org/translate/>) (Gasteiger et al. 2003). Motif analysis was conducted using InterProScan software (<http://www.ebi.ac.uk/InterProScan>) (Quevillon et al. 2005) and Superfamily protein database (<http://supfam.org/SUPERFAMILY/hmm.html>) (Wilson et al. 2009). The prediction of post-translational modifications, such as the presence of signal peptides and their cleavage sites, was performed with SIGNALP 4.1 (<http://www.cbs.dtu.dk/services/SignalP/>) (Petersen et al. 2011). The deduced amino acid sequence was assessed for potential glycosylation and phosphorylation sites using GlycoEP (<http://www.imtech.res.in/raghava/glycoep/>) (Chauhan et al. 2013) and NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>) (Blom et al. 1999) softwares, respectively. The sub-cellular localisation of the protein was predicted using the TARGETP 1.1 program (<http://www.cbs.dtu.dk/services/TargetP/>) (Emanuelsson et al. 2007) and Psort program (<http://psort.hgc.jp/form.html>) (Horton et al. 2007). Predictions of eukaryotic promoter and transcription initiation sites were performed using Neural Network Promoter Prediction software (<http://www.fruitfly.org/seqtools/promoter>) (Reese 2001). In silico analysis of the 5' and 3' untranslated regions were conducted using PLACE software (<http://www.dna.affrc.go.jp/PLACE/signalscan.html/>) (Higo et al. 1999). For phylogenetic analysis, the sequences were aligned using ClustalW with default parameters and the neighbour-joining tree was constructed with MEGA6 software (<http://www.megasoftware.net>) (Tamura et al. 2013). Evolutionary distances were computed using the Poisson correction method (Zuckerkanndl and Pauling 1965). Bootstrap analysis (Felsenstein 1985) with 100 replicates was also conducted to achieve the reliability of the tree and nodes with <50 % bootstrap confidence were collapsed.

Southern hybridisation

For Southern-blot analysis, 10 μ g total DNA was digested with the restriction enzymes *KpnI/BamHI* and *KpnI/EcoRI*, separated on a 1 % (w/v) agarose gel and blotted by capillary transfer with 20 \times saline sodium citrate (SSC) on a positively charged nylon membrane (Roche Applied Science). The 1.2 kb-long chitinase-specific probe was amplified by PCR using the P5–P6 primers (Suppl. Table S1), isolated from an agarose gel and non-radioactively labelled using the digoxigenin (DIG) Probe Synthesis kit (Roche Applied Science). The hybridisation was performed in a 100-mL DIG Easy Hyb solution containing 2 μ g DIG-labelled probe at 42 °C. Hybridisation signals were visualised after the binding of anti-DIG-AP to hybridised DIG-labelled nucleic acids and a colour reaction with BCIP (Roche Applied Science).

Isolation of *DrChit* coding sequence and sequencing

Total RNA was isolated from the leaves of in vitro cultivated sundew plants using the protocol described previously (Bekesiova et al. 1999). Genomic DNA was eliminated from RNA with RNase-free DNaseI (Thermo Fischer Scientific). The quality of RNA was checked by resolving the RNA on a 1 % agarose gel stained with ethidium bromide and the quantification was performed using a BioSpec-nano spectrophotometer (Shimadzu). First-strand cDNA was synthesised using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fischer Scientific), according to the manufacturer's instructions. Two micrograms of total RNA were converted to cDNA in a 20- μ L reaction mixture containing 5 μ M P8 (REV) primer and 1 μ L Maxima H Minus Enzyme Mix. The chitinase coding sequence (*DrChit*) was amplified using P7–P8 primers (Suppl. Table S1), cloned into pGEM-T Easy vector (Promega) and commercially sequenced.

RT-qPCR

Total RNA was isolated from different organs (stem, root, leaves, leaves without tentacles and tentacles) of treated and non-treated in vitro cultivated sundew plants as described previously (Bekesiova et al. 1999) and genomic DNA was removed by RNase-free DNaseI treatment (Thermo Fischer Scientific). The integrity of the RNA was checked on a 1 % agarose gel and RNA quantification was performed using a BioSpec-nano spectrophotometer (Shimadzu). First-strand cDNA was synthesised using the Maxima H Minus First Strand cDNA Synthesis Kit (Thermo Fischer Scientific), according to the manufacturer's instructions. Total RNA (1–2 μ g) was converted to cDNA in a 20- μ L reaction mixture containing 5 μ M oligo (dT)₁₈ primer and 1 μ L Maxima H Minus Enzyme Mix. The mixture was incubated at 50 °C for 30 min and the reaction was terminated by incubation at 85 °C for 5 min. Real-time PCR was performed using a Light Cycler nano (Roche Applied Science) and the Luminaris Color HiGreen qPCR Master Mix (Thermo Fischer Scientific) on 1 μ L cDNA (diluted 1:4) per 10 μ L reaction volume containing 0.3 μ M of P9–P10 (actin, GQ339775.1) and P11–P12 (chitinase) primers, respectively (Suppl. Table S1). The reaction was initiated by a uracil-DNA glycosylase step at 50 °C for 2 min, followed by one cycle at 95 °C for 10 min and 40 cycles at 95 °C for 15 s, 60 °C for 60 s, followed by a melting curve analysis step to confirm the specificity of the amplified products. For each treated and non-treated sample, three independent biological and technical repetitions were performed. The obtained data were evaluated using the XLSTAT version 2014.5.3. All sample groups exhibited a normal distribution according to the

Kolmogorov–Smirnov test. Differences between individual sample groups were analysed by a two-tail *t* test at a statistical significance level of $P < 0.05$ (*) and $P < 0.005$ (**).

Expression of fusion rDrChit in *E. coli* and its purification

Open reading frame of *DrChit* gene lacking the sequence of signal peptide was amplified by PCR using the primers P13–P14 with *Nco*I and *Eco*RI restriction sites, respectively (Suppl. Table S1). The *DrChit* coding sequence cloned into pGEM-T Easy vector was used as a template. The PCR program ran at 95 °C for 3 min, followed by 32 cycles of denaturation at 95 °C for 30 s; annealing at 54 °C for 30 s; extension at 72 °C for 90 s; the cycle ended with 10-min extension at 72 °C. The PCR product was digested with *Nco*I and *Eco*RI, ligated to pET32a(+) expression vector (Millipore) digested with the same restriction enzymes and used for transformation of *E. coli* DH5 α . After sequencing of the *T7/DrChit* expression region of pET32aDrChit, this plasmid was introduced into *E. coli* BL21-CodonPlus (DE3) RIL expression strain (Agilent). The expression of fusion rDrChit protein (TrxA-rDrChit) was induced by adding 1 mM IPTG to the bacterial culture at OD₆₀₀ of 0.6 and followed by incubation for 3 h at 37 °C. The cells were collected by centrifugation at 4 °C and frozen at –80 °C.

The expression of fusion rDrChit protein was checked on 12 % SDS–polyacrylamide gel using total cell protein extracts from bacterial pellet (1 mL of bacterial culture) of induced and non-induced *E. coli* BL21-CodonPlus (DE3) RIL/pET32aDrChit resuspended directly in 1 \times SDS-PAGE sample buffer [45 mM Tris–HCl (pH 6.8), 10 % glycerol, 1 % SDS, 0.01 % bromophenol blue, 50 mM DTT]. The cleared *E. coli* lysate was prepared by two freezing–thawing cycles of cells and resuspending in 4 mL of lysis buffer [50 mM Tris–HCl (pH 7.8), 5 mM MgCl₂, 2.5 mM β -mercaptoethanol, 0.1 % (w/v) Tween 20, 8 M urea] as described previously (Kirubakaran and Sakthivel 2007). Following centrifugation at 10,000g for 20 min at 4 °C, 1 mL of Ni-NTA agarose (Qiagen) was added to the supernatant and gently mixed for 30 min at 4 °C. The adsorbed fusion rDrChit protein was washed with the same buffer and released from Ni-NTA agarose with elution buffer [100 mM Na₂HPO₄, 10 mM Tris–HCl, 8 M urea (pH 5.9)]. The elution fractions (6 \times 0.5 mL) were pooled, desalted using the Econo-Pac 10DG Columns (Bio-Rad) and concentrated to 0.5 mL by centrifugation in the Amicon[®] Ultra-4 10K centrifugal filter devices (Millipore).

One hundred micrograms of fusion rDrChit protein were cleaved with 2 units of enterokinase (New England Biolabs) in 30 μ L of 20 mM Tris–HCl, 50 mM NaCl, 2 mM

CaCl₂ (pH 8.0) at 23 °C for 16 h. The reaction mixture was added to 100 µL of Ni-NTA agarose and incubated at 4 °C for 30 min. The mixture was centrifuged at 4 °C for 10 s at 15,000g and the supernatant collected. The protein fractions taken from individual steps covering the process of expression, isolation and purification of recombinant chitinase were analysed on 12 % (w/v) SDS-PAGE.

Chitinase activity assay

The same protein samples as analysed above, but without the heat treatment were separated on 12 % (w/v) SDS-PAGE containing 0.01 % glycol chitin at 8 °C and constant voltage of 120 V. The glycol chitin was prepared by acetylation of glycol chitosan (Sigma-Aldrich) as described previously (Trudel and Asselin 1989) After electrophoresis and re-naturation of separated proteins in the solution containing 50 mM sodium acetate (pH 5.2), and 1 % Triton, the bands with chitinase activity were detected as dark zones (Fig. 6b) after staining the gel with 0.01 % (w/v) Fluorescent Brightener 28 for 15 min and UV illumination (Pan et al. 1991; Matusikova et al. 2005).

The substrate specificity of sundew chitinase was tested by fluorometric assay with the *N*-fluorescein-labeled chitin (FITC-chitin) (Tikhonov et al. 2004), 4-methylumbelliferyl β-D-*N,N',N''*-triacylchitotrioside [4MU-(GlcNAc)₃] (Sigma-Aldrich) and 4-methylumbelliferyl β-D-*N,N'*-diacylchitobioside [4MU-(GlcNAc)₂] (Sigma-Aldrich). In case of FITC-chitin, each incubation mixture consisted of 100 mg FITC-chitin dissolved in 500 µL 0.1 M sodium acetate buffer (pH 5.2) and 1 µg of the protein sample. After incubation at 37 °C for 120 min, the samples were centrifuged for 1 min, 10 µL of supernatant was transferred into 90 µL 0.5 M Tris-HCl (pH 8.9), mixed and centrifuged for 15 min. Next, 5 µL of supernatant was added to 100 µL of 0.5 M Tris-HCl (pH 8.9) and used for fluorimetry. In case of 4MU-(GlcNAc)₂ and 4MU-(GlcNAc)₃, each reaction mixture (50 µL) contained 300 µM substrate in 0.1 M sodium acetate buffer (pH 5.2) and 0.1 µg of protein sample. Incubation was performed in the thermocycler at 37 °C for 1 h. Next, 5 µL of each analysed sample was diluted 76 times in 0.5 M Tris-HCl (pH 8.9), and 100 µL of diluted sample was used for fluorimetry.

Finally the samples were transferred in 96-well black-sides assay plates and the measurement of released MU or FITC was performed in SynergyTM H1 microplate reader (BioTek, Winooski, VT, USA) using 360/470 nm and 490/520 nm excitation and emission filters for 4MU-(GlcNAc)₂, 4MU-(GlcNAc)₃ and FITC-chitin, respectively. Each sample was analysed in three technical and biological replicates. As a positive control, the chitinase of *Streptomyces griseus* (Sigma-Aldrich) was used.

Results

Cloning and sequence analysis of *gDrChit*

Here, we focused on the isolation of the chitinase gene from the carnivorous plant *D. rotundifolia* and the characterisation of its expression profile in different tissues. As the genome of this plant species is poorly characterised, a homology-based strategy was deployed to identify the chitinase gene, taking advantage of highly conserved amino acid motifs present in plant chitinases. Degenerate primers were designed according to the conserved amino acid sequences of known plant class I chitinases (NCBI GeneBank) and were used for the amplification of the 994-bp long conserved genomic region. Subsequently, a genome-walking approach was used to amplify the 5' and 3' overlapping gene fragments of 1,480 bp and 746 bp, respectively. From the overlapping sequences of PCR fragments, a 2,674-bp contig of DNA sequence was obtained. In silico analysis using NetGene2 software revealed the presence of complete chitinase gene of 1665 bp in length (designated as a *gDrChit*), including the translation ATG start and TAG stop codons. The 108-bp long, 3' untranslated region, was terminated by the putative polyadenylation signal (AATTAA) which differed from the plant consensus sequence AATAAA by a single base (Joshi 1987; Hunt 1994). Program prediction identified the presence of two introns of 115 and 572 bp in length, with TG/GA and TA/CA splice junction sites, respectively (Fig. 1). Intron splicing was confirmed by the alignment of the gDNA sequence and its corresponding coding sequence (*DrChit*) that was obtained following RNA isolation from leaves of aseptically grown in vitro plants, cDNA synthesis and PCR with P7–P8 primers (Suppl. Table S1). Each exon of *gDrChit* encodes at least one amino acid residue necessary for catalysis. Exon 1 starts with ATG and includes regions that encode the entire signal peptide, chitin-binding domain, proline-rich (PR) hinge and ends with the sequence encoding the SHET motif, typical for class I chitinases. The glutamic acid (E) residue in this motif is crucial for enzymatic activity. Exon 2 encodes the second part of the catalytic domain, with a second glutamic acid (E) residue required for enzymatic activity. Exon 3 encodes the remaining part of the chitinase catalytic domain with the tyrosine (Y) amino acid residue that is important for enzyme substrate binding. The full-length *gDrChit* sequence was cloned, sequenced and deposited in GeneBank with the accession number KU516826.

To verify the presence and organisation of the *gDrChit* gene in the sundew genome, a Southern-blot was performed using the 1247 bp fragment as a probe, containing the intron–exon structure of the *DrChit* gene and genomic

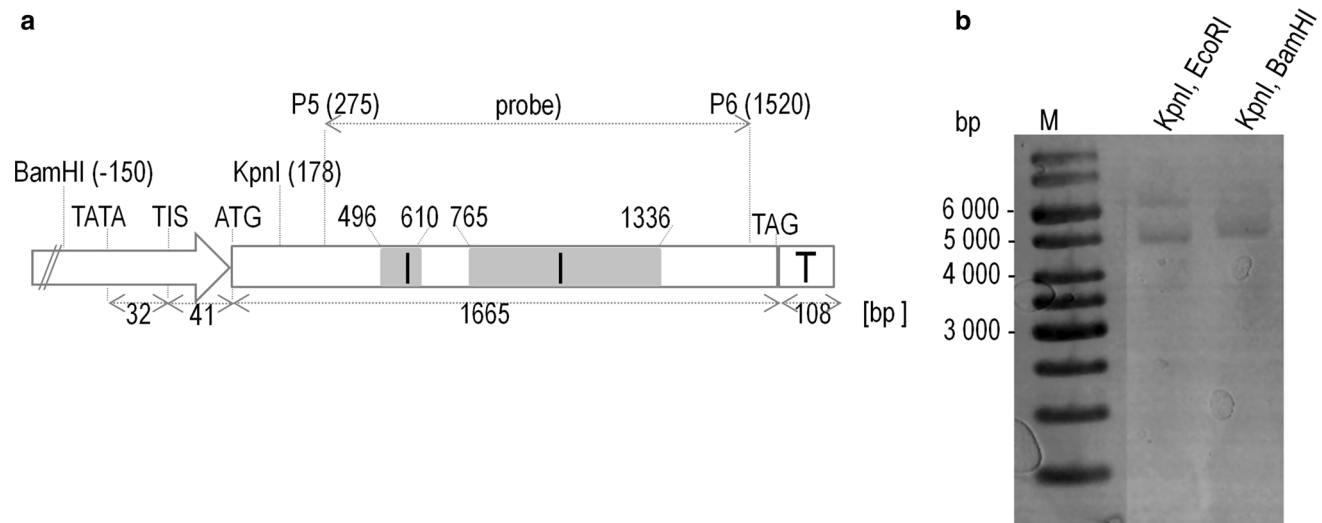


Fig. 1 **a** Scheme of the *DrChit* gene structure. The predicted TATA box was identified 32 nucleotides upstream of the transcription start site (TIS) followed by 41 bp of the 5' untranslated region. Numbering of the introns (I), P5–P6 primers and *Bam*HI and *Kpn*I enzymes is related to the transcription initiation site (TIS). Restriction endonuclease *Eco*RI does not occur in the *DrChit* gene. The putative polyA

signal was identified 108 nucleotides behind the TAG stop codon, within the 3' untranslated region. **b** Detection of the presence of the chitinase gene by Southern blotting in the genome of *D. rotundifolia*. The blot was hybridised with the 1.2-kb PCR DIG-labelled *DrChit* fragment. Lane M the GeneRuler 1 kb DNA Ladder (Life Technologies) was used as a marker

DNA digested with *Kpn*I/*Bam*HI and *Kpn*I/*Eco*RI restriction enzymes. As shown in Fig. 1, one and two DNA fragments hybridised to the probe in the case of DNA digestion with *Kpn*I/*Bam*HI and *Kpn*I/*Eco*RI restriction enzymes, respectively. This suggests that probably two copies of the chitinase gene occur in the genome of *D. rotundifolia*, but it is unknown whether both copies are functional.

When the 5' upstream sequence from the ATG was analysed using the Neural Network Promoter Prediction program, the presence of the promoter was determined with two possible transcription initiation sequences (TIS). One of these, shown in Fig. 1, correlated well with the 5'-RACE analysis and was located 41 bp upstream of the translation ATG start codon (Đurechová et al. 2015). The putative TATA region (CCGTATATATAG) required for precise transcription initiation was detected 32 bp upstream from the TIS. The promoter region possessed a typically high A + T content (65.6 %), which is commonly found in other plant promoters (Bhat et al. 2014). To explore the presence of potential *cis*-acting regulatory elements implicated in the expression of the isolated chitinase gene, the sequence of a putative promoter of 698 bp in length was investigated by the PLACE program (Đurechová et al. 2014). The CAAT box element identified immediately upstream of the core TATA box of the *DrChit* promoter (Fig. 2) may function as an enhancer of the gene transcription (Sawant et al. 1999). However, we have identified 12 copies of this element within both analysed strands (Suppl. Table S2). An abundant occurrence of this element

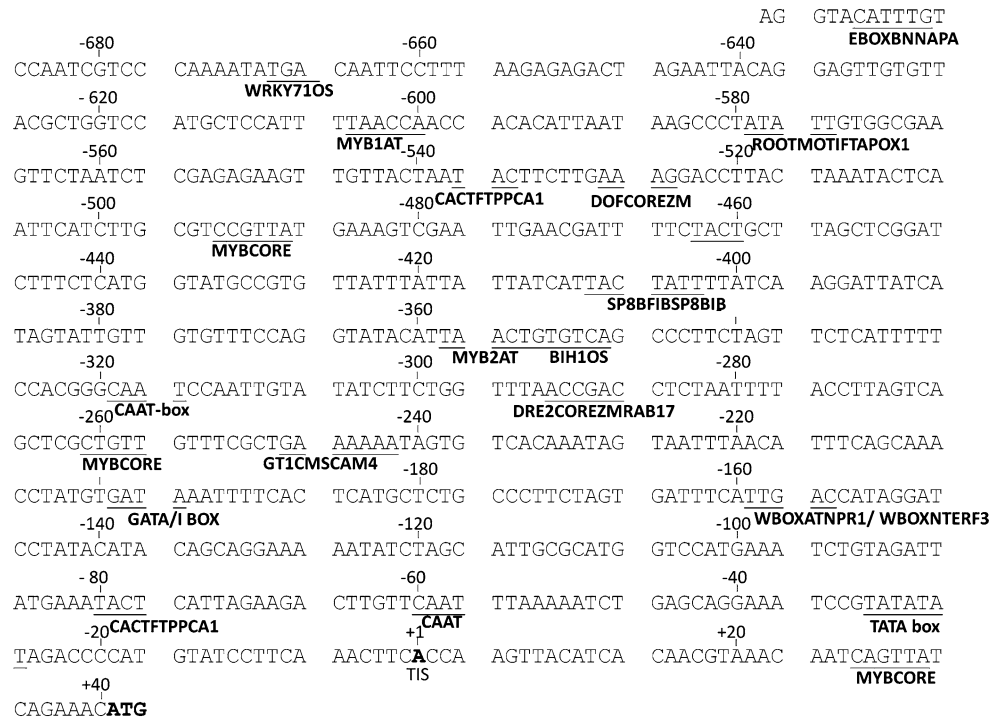
within the promoter sequences was previously referred to the tissue specificity (Razdan et al. 2013). The scanning of promoter boxes resulted in the detection of functionally significant *cis*-acting regulatory elements that can be associated with expression in leaves and roots. The second, very numerous group of *cis*-elements is associated with biotic and abiotic stress responses. In addition, *cis*-regulatory elements that play a role in abscisic acid (ABA) hormonal regulation were also detected, which is also important in the response to environmental stress and plant pathogens (Seo and Koshiba 2002). Key promoter elements and their putative function are listed in Suppl. Table S2.

Analysis of the deduced amino acid sequence of DrChit

The *DrChit* open reading frame consisted of 978 nucleotides that encoded 325 amino acid residues. Analysis using the computer program SignalP indicated that the sequence of the first 20 amino acids represents a putative signal peptide for transport to the endoplasmic reticulum. The remaining 305 amino acids were considered to constitute the mature protein, with an approximate molecular mass 31.9 kDa and a theoretical isoelectric point of 7.49.

InterProScan software predicted that the encoded protein is chitinase (*DrChit*) with a glyco_hydro_19 domain (IPR000726) from position 87 to 318. In addition, a chitin-binding domain (IPR001002) and a lysozyme-like superfamily domain (IPR023346) were detected, at positions 22–70 and 83–324, respectively. The presence and the

Fig. 2 Sequence of the *DrChit* promoter. The nucleotide at the position +1 corresponds to the transcription initiation site (TIS). The nucleotides upstream from the +1 position are negatively numbered. To illustrate, some *cis*-regulatory elements revealed by PLACE program as well as the core of the promoter (TATA box) located 32 bp upstream of the TIS are *underlined*



length of putative domains were confirmed by the Superfamily protein database as well. The *DrChit* amino acid sequence showed strong homology to class I chitinases of *Dionaea muscipula* (No. AHB62682.1), *Nepenthes khasiana 3* (No. AAT40732.1), *Allium sativum* (No. AAA32640.1) and *N. khasiana 2* (No. AAT40738.1), which ranged from 78 to 64 %. As shown in Fig. 3, highly conserved regions were detected in the chitin-binding- and catalytic domains, but not in the signal peptide and proline-rich (PR) hinge. The occurrence of three potential glycosylation sites for amino acid residues at positions 70, 72 and 74 was predicted within the PR hinge using the GlycoEP program with a defined threshold of 0.5. Potential phosphorylation sites using the NetPhos program were also investigated. In total, 12, 6 and 5 phosphorylation sites for serine, threonine and tyrosine, respectively, were revealed at the same threshold as in previous in silico analysis.

As shown in Fig. 3, the catalytic domain of *DrChit* starts with a string of three glycine and valine residues, which are distinctive for carnivorous *N. khasiana* extracellular chitinases as well as monocot class I chitinases and encompasses all seven active sites (Renner and Specht 2012). As expected, the role of the conserved family 19 “signature sequence” QTSHETTG in substrate binding and catalysis is also part of this domain (Garcia-Casado et al. 1998; Tiffin 2004).

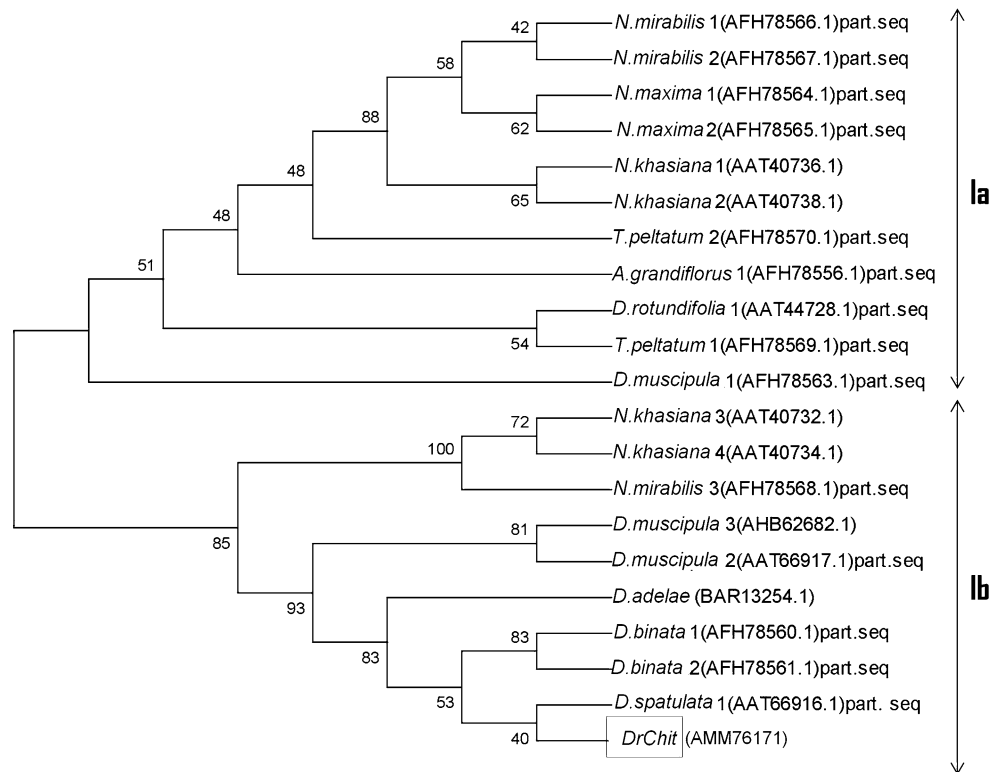
The active site IV, which contains a conserved tyrosine (Y) residue at position 205 is essential for substrate binding to the catalytic cleft (Verburg et al. 1993). The other amino acid residues identified by Passarinho and de

Vries (2002) which putatively contribute to the correct substrate–enzyme interaction, were also defined in the catalytic domain. They involve threonine (T) 150 in the SHET sequence, glutamic acid (E) 171, serine (S) 202, asparagine (N) 206, glutamine (Q) 244, lysine (K) 247, asparagine (N) 281 and arginine (R) 297. Only tryptophan (W) 186 was substituted by tyrosine (Y). Two glutamic acid (E) residues at positions 149 and 171 probably play vital roles in the catalytic function of the *DrChit* protein. However, the glutamine (Q) 200, serine (S) 202 and asparagine (N) 281 residues were also shown to be involved in the depolymerisation of the chitin substrate (Graham and Sticklen 1994; Passarinho and de Vries 2002; Veluthakkal and Dasgupta 2012).

The *DrChit* protein has extracellular targeting, as the C-terminal extension (CTE) required for localization to the vacuole at the end of *DrChit* protein is absent (Fig. 3). The 82 % probability of extracellular localisation was also predicted in silico, using the Psort program.

Next, we attempted to cluster the *DrChit* protein together with the deduced amino acid sequences of Caryophyllales class I chitinase orthologues using the Mega 6 program, as the coding regions are considered to be relatively conserved (Rottloff et al. 2011). For this, we used available sequences of Caryophyllales class I chitinases (Suppl. Table S3) (Renner and Specht 2012). As the cladogram shows (Fig. 4), the *DrChit* protein groups together with the extracellular Ib chitinases that are presumed to be involved in carnivory (Renner and Specht 2012).

Fig. 4 Phylogenetic tree showing the relatedness of the deduced full-length amino acid sequence of the *D. rotundifolia* *DrChit* gene and 20 class I chitinase proteins of the order Caryophyllales. Phylogenetic analysis was performed using ClustalW and Mega 6 software based on the neighbour-joining method. Clusters Ia and Ib involve the chitinases with vacuolar and apoplastic localisations, respectively



Spatial expression profile of *DrChit* in sundew

A prerequisite for RT-qPCR analysis of gene expression is an efficient method for obtaining high quality total RNA with an optical density ratio of $A_{260/230}$ and $A_{260/280}$ of approximately 1.9 and 1.7, respectively, suggesting little contamination with polysaccharides and proteins. To unravel the spatial transcriptional profile of *DrChit* in sundew, total RNA was isolated from leaves, leaves without tentacles, tentacles, stems and roots, and tissue-specific cDNAs were generated. These were used as a template for qPCR analysis of *DrChit* expression, which was normalised to the gene expression level of the housekeeping gene β -ACTIN. ACTIN expression was successfully used as an internal reference in a similar study dealing with chitinase III expression in tissues and glands of carnivorous pitcher plants of the genus *Nepenthes* (Rottloff et al. 2011). In non-treated plants, *DrChit* expression was highest in leaves, whereas stem and root tissues showed extremely low levels of *DrChit* mRNA (~ 500 times lower than in leaves) (Fig. 5). A detailed analysis of leaves revealed that *DrChit* is mainly expressed in tentacles, because the expression level in leaves without tentacles was about 70 times lower.

Expression of the *DrChit* gene in response to mimicking the presence of insect prey

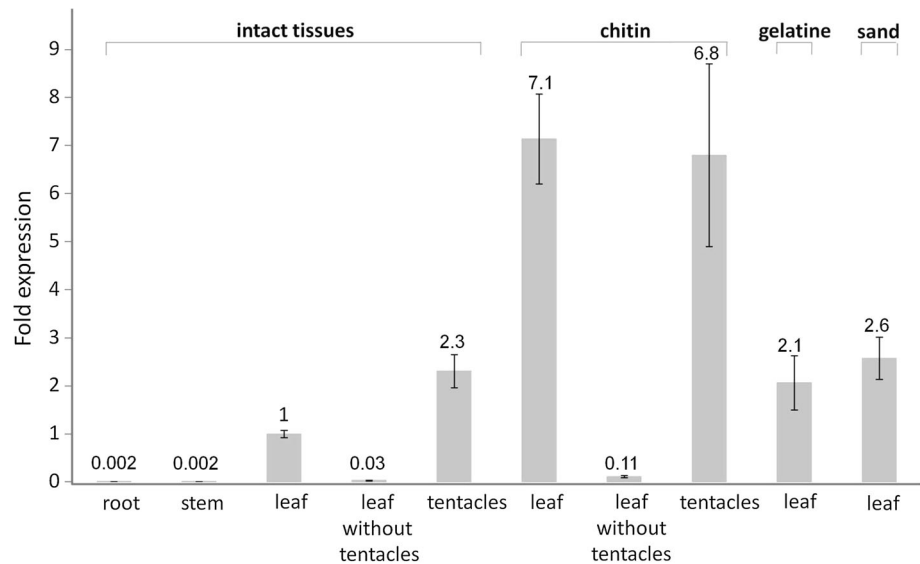
To further elaborate the function of the *DrChit* apoplastic chitinase we analysed its transcription expression patterns

in intact (control) leaves as well as in leaves exposed to sand, gelatine, and chitin for 24 h. Sand represents the mechanical stimulation of tentacles and can be considered as a signal of approaching potential prey or pathogen. The gelatine protein and chitin were used to mimic the trapped insect prey (Hatano and Hamada 2012). The results of RT-qPCR analysis on leaves showed that *DrChit* expression was up-regulated by chitin by sevenfold followed by sand (2.5-fold) and gelatine (twofold), compared with expression in the non-treated (control) leaves ($P < 0.005$ for sand and chitin, $P < 0.05$ for gelatine). To address the question whether the studied chitinase might come into direct contact with the captured insect prey, we analysed leaves with and without tentacles separately. Chitin induction resulted in a ~ 60 times higher *DrChit* transcript level in tentacles than in leaves without tentacles (Fig. 5).

Expression of r*DrChit* protein in *E. coli* and testing its chitinolytic activity

To characterise the enzyme activity of the *DrChit* protein, the open reading frame of *DrChit* gene without the putative signal peptide was PCR-amplified, cloned into pET32a(+) vector and introduced into *E. coli* BL21-CodonPlus (DE3) RIL strain. SDS-PAGE analysis of protein extract from IPTG-induced bacterial culture revealed the presence of a predominant band of approximately 50 kDa, corresponding to the fusion of r*DrChit* protein with thioredoxin (TrxA),

Fig. 5 Expression levels of *DrChit* shown as fold-expression relative to that of intact (non-treated) leaves of sundew. Data from RT-qPCR were normalised relative to the abundance of the endogenous control β -*ACTIN* gene. Standard errors indicated by bars were calculated from three independent biological samples, each with three technical replicates



6xHis-Tag and S-Tag sequences (Fig. 6a, lane 3). The fusion recombinant protein was soluble in the presence of 8 M urea in the lysis buffer (Fig. 6a, lane 4). Following the purification on Ni-NTA agarose and buffer exchange, the purified fusion protein was incubated with enterokinase to remove the fusion tags from rDrChit. As shown in Fig. 6a, lane 6, the band corresponding to the fusion rDrChit disappeared after digestion, while the rDrChit band with a predicted molecular weight of approximately 32 kDa was clearly detected. Moreover, the samples of total cell protein extracts containing fusion rDrChit protein (Fig. 6b, lane 3), purified fusion rDrChit (Fig. 6b, lane 5), and rDrChit

protein without fusion tags (Fig. 6b, lane 6) showed chitinolytic activity in polyacrylamide gels containing glycol chitin as a substrate. As expected, the chitinolytic activity was not detected in the control samples containing the separated total cell proteins from uninduced *E. coli* BL21-CodonPlus (DE3) RIL/pET32aDrChit and induced *E. coli* BL21-CodonPlus (DE3) RIL/pET32a (Fig. 6b, lanes 1 and 2). Similarly, no chitinolytic activity was detected in the sample of the cleared cell lysate before purification on Ni-NTA agarose as the cell lysis was performed in the buffer containing urea (Fig. 6b, lane 4). For further testing of sundew chitinase substrate specificity we

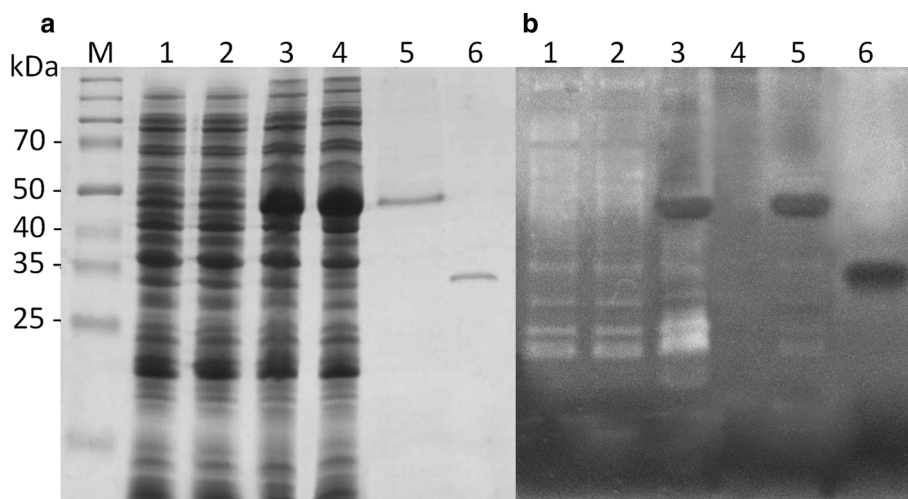


Fig. 6 a SDS-PAGE analysis of recombinant DrChit protein expressed in *E. coli* and isolated using His-Tag based purification. **b** Detection of endochitinase activity for long polymers in the gel containing glycol chitin as substrate in the gel. After re-naturation and staining the gel with Fluorescent Brightener 28, the bands with chitinase activity appeared as dark zones after UV illumination. Lanes: M, Spectra™ Multicolor Broad Range Protein Ladder

(Thermo Fischer Scientific); 1 total cell proteins from uninduced *E. coli* BL21-CodonPlus (DE3) RIL/pET32aDrChit, 2 total cell proteins from induced *E. coli* BL21-CodonPlus (DE3) RIL/pET32a, 3 total cell proteins from induced *E. coli* BL21-CodonPlus (DE3) RIL/pET32aDrChit, 4 cleared cell lysate before purification on Ni-NTA agarose, 5 Ni-NTA agarose-purified fusion rDrChit protein, 6 purified rDrChit protein after enterokinase digestion

have used FITC-chitin, 4MU-(GlcNAc)₃ and 4MU-(GlcNAc)₂ as specific substrates for long oligomer-specific endochitinases, short oligomer-specific endochitinases and chitobiosidases, respectively. Fluorometric assays showed the hydrolysis only of FITC-chitin indicating a long oligomer-specific endochitinase activity of sundew chitinase. Based on the amount of releasing fluorescein, the specific activity of the enzyme was estimated approximately three times lower (1012 ± 180 RFU) than control chitinase from *S. griseus* (3254 ± 347 RFU) (Suppl. Table S4).

Discussion

The diversity and the physiological roles of chitinolytic enzymes, which are distributed among all plant and animal kingdoms, has led to the hypothesis that chitinolytic enzymes are living examples of gene evolution (Adrangi and Faramarzi 2013). Carnivory specialisation within the Caryophyllales was recently demonstrated by the phylogenetic analysis conducted on 49 angiosperm class I chitinase homologues (Renner and Specht 2012).

Since only a limited number of complete chitinase gene sequences are available within the order Caryophyllales, we focused on the isolation and characterisation of a full-length genomic clone consisting of the 1665-bp chitinase gene (*gDrChit*) with adjacent promoter from *D. rotundifolia* L. Although the occurrence of the promoter within the analysed DNA sequence was predicted with the score 0.99 by Neural Network Promoter Prediction program (Ďurechová et al. 2014) only the RACE analysis definitely confirmed the functionality of the *DrChit* promoter sequence in vivo (Ďurechová et al. 2015). The genomic clone of *DrChit* contains two introns interrupting the DNA sequence at the 5'- and 3'-ends that correspond to the amino acids SHETTG and GWPTA; QIS and YNYNY, respectively. Similar to other plant chitinase genes, their location appears to be relatively conserved and covers the motifs that are crucial for substrate binding and catalysis (Li and Greene 2010; Jiang et al. 2013; Ďurechová et al. 2015). In contrast, they are of varying length; e.g. basic chitinase of *N. khasiana* has introns 249- and 470-bp long and their length in *DrChit* gene is 115 and 572 bp. Detection of two hybridisation bands by Southern-blot analysis of genomic DNA indicates the presence of two copies of the *DrChit* gene in plant genome of sundew. It is not surprising as plant chitinases are the members of gene families and duplication of genes is a part of their evolution (Bergthorsson et al. 2007).

The open reading frame of *DrChit* gene consisted of 978 nucleotides and encoded 325 amino acid residues. In silico analysis of deduced DrChit amino acid sequence revealed that the chitinase is of class I as contains a chitin-binding

domain, PR hinge and chitinase catalytic domain. Since protein does not contain targeting signal for vacuoles (CTE), its localisation is extracellular. It is believed that extracellular chitinase(s) in carnivorous plants play a crucial role in degrading the outer barrier of the insect body and allowing other hydrolytic enzymes to fully digest the prey (Ishisaki et al. 2012a). Moreover, phylogenetic analysis of Caryophyllales chitinases performed in this study showed that the DrChit protein groups together with the extracellular Ib chitinases, the role of which probably coincides with carnivory (Renner and Specht 2012).

DrChit protein contains an auxiliary chitin-binding domain consisting of 48 amino acid residues that might coincide with the enhanced activity of the enzyme towards insoluble chitin (Vaaje-Kolstad et al. 2005; Adrangi and Faramarzi 2013). In addition, eight conserved cysteine residues are assumed to form disulfide bridges that contribute to the stability of the protein structure and allow the enzyme to possess extracellular activities (Ubhayasekera 2011).

The catalytic domain of DrChit encompasses all conserved amino acid residues essential for substrate binding and catalysis, except for the tryptophan (W) 186 that was substituted by tyrosine (Y). They both belong to the aromatic amino acid residues that are assumed to provide the necessary environment for the flexible binding and movement of the substrate through the active site (Adrangi and Faramarzi 2013). The same substitution was observed in the chitinase (AHB62682.1) of *D. muscipula* and in the chitinase (AAA32640.1) of *A. sativum*, although the significance of this substitution is unknown.

As shown in Fig. 3 the PR hinge of DrChit is relatively long, it involves 18 amino acid residues. Ubhayasekera (2011) states that the length of the linker positively contributes to the flexibility of the protein and allows the chitin-binding and catalytic domains to orientate in such a way to maximize functional efficiency. In addition, longer linkers are protected from proteolytic cleavage possibly by glycosylation, which might facilitate protein secretion and stabilise the enzyme.

Since the promoter sequence of *DrChit* contains the *cis*-regulatory elements involved in wounding and biotic or abiotic stress (Suppl. Table S2), this might reflect the origin defense role of this gene in sundew (Ďurechová et al. 2014). The environmental stimuli such as starvation and wounding presumably forced carnivorous plants to reconstruct expression mechanisms of self-defense-related genes (Schulze et al. 2012). It was supposed that transcription of genes involved in carnivory of the *Cephalotus follicularis* and *D. muscipula* is probably regulated via a transcription factor(s) that interacts with the W box or TC-rich repeat (Nishimura et al. 2013). In some cases, the employment of the same *cis*-regulatory elements during the defense and

carnivory led to a dual function of the corresponding gene(s) in Caryophyllales. This was recently postulated for class III chitinase in *Nepenthes alata*, when the presence of prey in pitcher up-regulated this gene not only in digestive glands, but at an even higher level, in the glands surrounding tissue (Rottloff et al. 2011). Interaction between defense and carnivory mechanisms was observed also by Eilenberg et al. (2010), when the chitin applied to the pitchers of *N. khasiana* induced the synthesis of endochitinase isoenzymes as well as antifungal naphthoquinones that may avoid the occurrence of competitors consuming organic compounds during the prey decay.

Our expression analyses showed that in non-treated plants *DrChit* gene was highly expressed in tentacles, whereas the level of its mRNA in leaves without tentacles and other tested organs (stem and root) was ~70 and ~1000 lower, of that in secretory glands. Very low or no gene expression in most tissues of non-stressed plants was detected for some chitinase genes that play a role in various environmental stresses (Takenaka et al. 2009; Guo et al. 2013) as well as for S-like RNases involved in carnivory. Nishimura et al. (2013) state that the genes *da-I* of *Drosera adelae* and *cf-I* of *C. follicularis* were almost exclusively expressed in each trap/digestion organ of non-treated plants. As shown in Fig. 5, we also observed extremely high level of *DrChit* mRNA in tentacles.

Highly expressed plant genes contain two tandemly repeated TATA elements and C, C and G at the -3, -1, and +9 positions, respectively, in the TATA region (CACTATATATAG) of the promoter (Sawant et al. 1999). Besides the C at the -1 position, the TATA motif of the *DrChit* promoter meets these requirements. Characteristics of the transcription initiation site (CACCAAGTTACA) for *DrChit* gene only slightly differ from the consensus sequence (CAN(A/C)(A/C)(C/A)C(C/A)N₂A(C/A) of the highly expressed plant genes.

When the leaves of *D. rotundifolia* were treated with one of elicitors, we have observed an increase of *DrChit* mRNA level in tentacles. These results are in agreement with our previous study when an increase in chitinase(s) transcripts in secretory cells of tentacles upon mimicking insect prey digestion was demonstrated by in situ hybridization (Matusikova et al. 2005).

A mutual comparison of the elicitors used in this study revealed that the effect of chitin was significantly different from that of sand and gelatine; however, the difference between gelatine and sand was not significant ($P = 0.287$) (Suppl. Table S5). High expression of *DrChit* gene upon chitin induction is not surprising, as its role in the processes of carnivory is supposed. On the other hand, a mild expression of this gene as a result of non-specific substrate presence (gelatine and sand) points out on the readiness of the genes involved in the prey decay machinery. As the

carnivorous plants need to maximize a cost:benefit profit, such non-specific gene induction was observed as a temporary phenomenon (Gallie and Chang 1997; Hatano and Hamada 2012; Michalko et al. 2013). Clear accumulation of *DrChit* apoplastic chitinase in tentacles of non-treated as well as of treated sundew plants supports the hypothesis that the class I apoplastic chitinases play a crucial role in the digestion of Caryophyllales (Mithöfer 2011; Renner and Specht 2012). Although the participation of various functional hydrolytic enzymes, such as proteases, esterases, acidic phosphatases, glucanases is necessary for digestion (Heslop-Harrison 1975; Clancy and Coffey 1977; Mithöfer 2011; Michalko et al. 2013), chitinases with different substrate specificities (Ishisaki et al. 2012b, Paszota et al. 2014) appear to be key players in degrading the chitinous exoskeleton of captured insect prey. The recombinant sundew chitinase exhibited hydrolysing activity only towards long chitin polymers (FITC-chitin and glycol chitin) but not short *N*-acetylchitooligomers [4MU-(GlcNAc)₃ and 4MU-(GlcNAc)₂]. The same substrate specificity was reported for *N. khasiana* AAT40732.1 chitinase that was characterised by long PR region and had an extracellular localisation (Eilenberg et al. 2006) as well as in *N. rafflesiana* (Rottloff et al. 2011).

Although constitutive expression of chitinolytic enzymes with substrate specificity for short *N*-acetylchitooligomers in non-induced leaves of *D. rotundifolia* postulates their participation mainly in plant physiology (Libantova et al. 2009), their involvement in chitin exoskeleton degradation cannot be excluded. Detection of constitutively expressed endochitinases for short *N*-acetylchitooligomers in the liquid of closed traps of *N. khasiana* supports this hypothesis (Eilenberg et al. 2006).

In conclusion, here we present the isolation and characterisation of the genomic and corresponding coding sequence (CDS) clone for chitinase from *D. rotundifolia* L. The 1665 bp genomic clone contains two introns, whose splicing was confirmed by sequencing of the corresponding CDS clone. The adjacent promoter contained a functional transcription initiation start located 41 bp upstream of the translation ATG codon, and numerous *cis*-acting regulatory elements potentially involved in *DrChit* expression in leaves, roots and upon induction by wounding and stress. The open reading frame of the *DrChit* coding sequence consists of 978 nucleotides and encodes 325 amino acid residues. Sequence analysis indicated that *DrChit* belongs to the class I group of plant chitinases and among the Caryophyllales clusters with clade Ib, which includes the extracellular orthologues that play a role in carnivory. In sundew, the *DrChit* gene is expressed predominantly in tentacles, where it is up-regulated following treatment by insect prey mimicking inducers. Chitinolytic activity of rDrChit protein expressed in *E. coli* was confirmed and its

substrate specificity for a long chitin polymers was demonstrated.

Author contribution statement IM and JL conceived and designed research. JM, MJ, MR conducted the experiments. MB helped in research design. JL and MB wrote the manuscript. All authors read and approved the final version of the manuscript.

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