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Tentacles of in vitro-grown round-leaf sundew (*Drosera rotundifolia* L.) show induction of chitinase activity upon mimicking the presence of prey

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Abstract Induction of plant-derived chitinases in the leaves of a carnivorous plant was demonstrated using aseptically grown round-leaf sundew (*Drosera rotundifolia* L.). The presence of insect prey was mimicked by placing the chemical inducers gelatine, salicylic acid and crustacean chitin on leaves. In addition, mechanical stirring of tentacles was performed. Chitinase activity was markedly increased in leaf exudates upon application of notably chitin. Application of gelatine increased the proteolytic activity of leaf exudates, indicating that the reaction of sundew leaves depends on the molecular nature of the inducer applied. In situ hybridization of sundew leaves with a Drosera chitinase probe showed chitinase gene expression in different cell types of non-treated leaves, but not in the secretory cells of the glandular heads. Upon induction, chitinase mRNA was also present in the secretory cells of the sundew leaf. The combined results indicate that chitinase is likely to be involved in the decomposition of insect prey by carnivorous plants. This adds a novel role to the already broad function of chitinases in the plant kingdom and may contribute to our understanding of the molecular mechanisms behind the ecological success of carnivorous plants in nutritionally poor environments.

Keywords Carnivory · Chitinolytic activity · In situ hybridization · Leaf exudates · Proteolytic activity

Abbreviations ID: Integrated density · SA: Salicylic acid

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Introduction

Carnivorous plants attract, capture, kill and digest mostly insects. For centuries, such plants have fascinated botanists, among which Charles Darwin (Darwin 1875) and his son (Darwin 1878). These plants also have become a valuable model system for evolutionary ecology (Ellison and Gotelli 2001). Carnivory of plants is thought to represent a special case of functional adaptation to the environment, allowing growth in nutrient-poor soils. The genus *Droseraceae* (sundews) consists of about 125 different species (Culham and Gornall 1994; Adamec 1997) that grow mostly on acidic soils or substrates that are often deficient in nitrogen and phosphorus (Chandler and Anderson 1976; Stewart and Nielsen 1992).

Once captured, insects are digested on leaves covered by stalked glands called tentacles. The glandular head of such a tentacle is usually reddish and covered with droplets of mucilage. Anatomically, the glandular head consists of external and internal layers of secretory cells, and tracheids in the centre. The tracheids are separated from glandular cells by a row of barrier cells consisting of cutinized anticlinal walls. In the basal part of the tentacle there are some outer (epidermal) and inner (parenchyma) stalk cells and a big vessel connecting the glandular head with the stalk and the leaf. All leaf cells are

photosynthetically active in addition to carnivory (Juniper et al. 1989; Muravnik and Ivanova 1999).

The mucilage from the glandular head contains several hydrolytic enzymes. The secretory cell walls of tentacles contained acid phosphatase and protease (Heslop-Harrison 1975; Clancy and Coffey 1977), as well as esterase and peroxidase activities (Heslop-Harrison 1975). Such enzymes enable the plant to decompose the insect body and to assimilate nitrogen, phosphorus and sulphur. Several studies have demonstrated that 50–76% of all nitrogen in Drosera is supplied by the insect (Dixon et al. 1980; Millett et al. 2003). The molecular mechanisms occurring during the digestion of insect prey are not known in detail. Notably the decomposition of the hard insect skeleton, that consists mainly of chitin (a β-1,4-linked homopolymer of N-acetyl-D-glucosamine), is as yet an unresolved question. Based on its composition, chitin makes a good potential source of nitrogen for the plant and part of the prey-derived nitrogen could be generated by the action of chitinases. Chitinases are members of the pathogenesis-related proteins of plants and play a role in several vital plant processes (Kasprzewska 2003), such as plant defence against pathogens (Broglie and Broglie 1993; Melchers and Stuiver 2000). Chitinase activity was found in the mucilage from several carnivorous plants, for example, in *Nepenthes mixta*, *N. maxima* and *Drosera peltata* (Amagase et al. 1972), species of the genus *Utricularia* (Sirova et al. 2003), as well as in *Drosera binata* and *D. whittakkeri* (Chandler and Anderson 1976). Robins and Juniper (1980) demonstrated chitinase activity in the mucilage of *Dionea muscipula*. However, in none of these cases microbial contamination could be ruled out (Robins and Juniper 1980; Sirova et al. 2003), so the action of plant-derived chitinases in the (possibly partial) decomposition of insect chitin is uncertain. This issue is part of a long-standing and ongoing debate on the role of insectivory and its relevance for plant nutrition, whether it is facultative or obligatory (Darwin 1878; Thum 1989; Stewart and Nielsen 1992).

By a combination of biochemical and cytological approaches we here show that sterile, in vitro-grown sundew has increased chitinase activity due to the induction of chitinase gene activity upon stimulation that mimics the presence of insect prey. This indicates that plant-derived chitinases may indeed play a role in the (partial) decomposition of the insect skeleton.

Materials and methods

Plant material

Plants of *Drosera rotundifolia* L. were cultivated in vitro as described previously (Bobák et al. 1995). Leaf pieces of ca. 3 mm in diameter and an approximate weight of 0.015 g were cut with a scalpel and used in all analyses.

Detection of chitinase activity in sundew leaf exudates

The presence of insect prey on sundew leaves was mimicked with three different chemical inducers. Plugs (ca. 2×2 mm) of 8% (w/v) gelatine (Serva 22151), small pieces (30–35 mg) of crustacean chitin (Serva 16625) or 20 μl of 1 mM salicylic acid (Duchefa S 1367), all properly sterilized, were placed on leaves on plants for 24 h. Any visible remainders of the chemical inducers were removed prior to analysis. The time-course study of induction with gelatine or chitin over a period of 5–72 h was performed under similar conditions. A fourth way of induction was mechanical stimulation in which the tentacles of leaves were stirred once with a scalpel (avoiding mechanical damage of the tissue) 24 h before analysis. Leaves without any treatment were used as a control. Leaf pieces were cut and submerged into 30 μl of 0.1 M sodium acetate buffer (pH 5.0) for 5 min to collect the exudates. The exudates of 10 induced leaves were pooled (300 μl). Aliquots (20 μl) were separated on 12.5% (w/v) SDS-containing polyacrylamide slab gels (Laemmli 1970) with glycol chitin (0.01%) incorporated as enzyme substrate. Glycol chitin was obtained by acetylation of glycol chitosan (Sigma G-7753) as described by Trudel and Asselin (1989). No heat treatment of the samples was performed prior to loading. The gels were run at 8°C at a constant voltage of 120 V for 2 h. After electrophoresis, proteins were re-naturated by shaking the gel in 50 mM sodium acetate buffer (pH 5.0), 1% Triton X-100 overnight and subsequently in sodium acetate buffer for 1 h. Chitinase activity was detected by staining with 0.01% Fluorescent Brightener 28 (Pan et al. 1991) and UV-illumination. For quantification, the gels were digitally photographed (UVP Bio Doc-It System). From the photographs, background-corrected integrated density (ID) was calculated in areas of constant size using Scion Image software (http://www.scioncorp.com) according to formula: ID = N*(mean – background) where N is the number of pixels in the selected area, and background is the modal grey value (pixels). Total amount of protein was visualized by staining the gel with Coomassie Brilliant Blue R 250 (Sambrook et al. 1989).

Detection of protease activity in sundew leaf exudates

Proteolytic activity in leaf exudates was determined basically as described previously (Bohak 1970). Briefly, samples (150 μl) were incubated with 150 μl of 2% (w/v) bovine serum albumin (BSA) in 200 mM glycine-HCl (pH 3.0) at 37°C for 1 h. The reaction was stopped by the addition of 450 μl of 5% (w/v) trichloroacetic acid. Samples were incubated on ice for 10 min, and centrifuged at 20,000 g for 10 min. Absorbance of the supernatant at 280 nm was measured. One unit of proteolytic activity is defined as an increase of 0.001 per min in the absorbance at 280 nm. Total protein content

of leaf exudates was determined as described previously (Bradford 1976).

Detection of chitinase activity in sundew leaves

Sundew leaves were induced with gelatine (for 5 and 24 h) or chitin (for 2 and 48 h) as described above. Non-induced leaves were used as a control. After induction, leaves were detached from the plant, placed on medium consisting of 0.1 M sodium acetate buffer (pH 5.0), 0.01% colloidal chitin prepared from chitosan (Aldrich 41,796-3) as described by Molano et al. (1977) and 0.8% agarose and they were slightly squeezed. After incubation for 20 min at 37°C, the leaves were removed and the plates were stained with 0.5 mg.l^{-1} Congo Red (Sigma C6767) to visualize zones of clearing. Cleared zones in a red background indicate chitinase enzymatic activity (Inbar and Chet 1991). Congo Red-stained plates were digitally photographed with Canon EOS 300 and when considered necessary, the contrast was enhanced with Adobe Photoshop.

In situ hybridization of sundew tentacles

For in situ hybridization experiments, treated and control leaves were fixed for 4 h (first half hour under vacuum (~60 kPa) in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), dehydrated in ethanol series, ethanol-xylene and embedded in Paraplast Plus (Sigma). Plant tissues were handled as fast and gently as possible. All steps were performed on ice, except for final dehydration in alcohol-xylene and embedding.

The 325 bp PCR fragment *DrChit1* (GenBank accession No. AY622818), encoding for the conservative catalytic region of a putative class I chitinase from sundew (Békésiová 2000) was used as a probe for in situ hybridization. The fragment was cloned in the vector pGEM-T Easy (Promega). Digoxigenin-labelled sense (*Sst*I-T7) and antisense (*Sst*II-SP6) RNA probes were prepared by in vitro transcription using the DIG RNA (SP6/T7) Labelling Kit (Roche). The efficiency of the probe labelling was evaluated immunologically using a spot test with DIG-labelled control RNA (Roche). Concentration of the probes was measured using the Ultrospec 1000 spectrophotometer (Pharmacia Biotech).

In situ hybridization studies to 8-μm-thick sections, mounted onto poly-L-lysine coated slides, were performed as described by Cox and Goldberg (1988) with minor modifications. Briefly, sections were pre-treated with 1 μg.ml^{-1} proteinase K (Sigma) in proteinase buffer (50 mM EDTA, 100 mM TRIS-HCl, pH 7.5) for 30 min at 37°C. Blocking was performed with 0.25% (v/v) acetic anhydride in 0.1 M TEA buffer (pH 8.0) for 10 min, then washing in 2×SSC for 5 min. After rinsing in distilled water the samples were dehydrated in ethanol series and vacuum dried. Dry slides were stored under vacuum until hybridization.

Hybridization mix A was composed of 50% formamide, 5% dextran sulphate, 1x Denhardt's solution, 500 mM NaCl in hybridization buffer (100 mM TRIS-HCl, 10 mM EDTA, pH 7.5), and mix B of milli Q-water, 10 mg l^{-1} tRNA, 20 mg l^{-1} poly(A) and labelled probes. Hybridization mix B was heated at 80°C for 5 min, fast cooled and mixed with mix A. We have applied 95 μl of hybridization mix per slide with a final probe concentration of 5 ng ml^{-1}. Hybridization was carried out at 50°C in humid chamber overnight. After hybridization the samples were washed in 2×SSC (10 min at RT), 1×SSC (10 min at 37°C), and 0.5×SSC (10 min at 37°C). To remove the non-specifically bound probes washing with RNase A (final conc. 10 μg ml^{-1}) in RNase buffer (500 mM NaCl, 10 mM EDTA, 10 mM TRIS-HCl, pH 7.5) for 60 min at 37°C was performed. Sections were further washed in RNase buffer (4×15 min at 37°C), blocked in 2% BSA at RT for 30 min. RNA hybrids were detected by incubation of slides (2 h at RT) with anti-digoxigenin (1:500) coupled with alkaline phosphatase (Roche), in detection buffer (pH 7.5) and washed in the same buffer 4×10 min at RT. Alkaline phosphatase activity was revealed with NBT/BCIP (Roche) overnight.

Sections, after dehydratation and mounting in DePeX (Serva), were investigated with a transmitted-light bright field Axioplan 2 microscope (Carl Zeiss, Göttingen) and photographed (KR/10M Ricoh, Japan and Sony DXC-S500 Digital Camera System).

Results

Chitinase activity in sundew leaf exudates

To induce the molecular processes resulting in digestion of prey, leaves of sundew plants were induced with gelatine, chitin, salicylic acid, or by mechanical stimulation of the tentacles. After induction and submersion of the treated leaves in buffer, the material exudated by the leaves was analysed for total amount of protein as well as for proteolytic and chitinolytic activity. The biochemical analyses of sundew leaf exudates after 24 h of induction are shown in Fig. 1. Compared to the non-induced control (NI), there is only a slight increase in total protein content, except for the gelatine (G) induction, which obviously adds protein to the leaves (Fig. 1a). Mechanical stimulation and notably gelatine induction resulted in higher proteolytic activity (Fig. 1b), indicating an effective induction of digestive processes in sundew leaves. In all cases, chitinase activity could be detected in a polyacrylamide gel (Fig. 1c). Chitinolytic activity increased most upon inducing the leaves with chitin (CH) (Fig. 1c) and was ca. 3- to 4-fold higher than in control leaves. Stimulation with salicylic acid (SA) was neutral for all three biochemical parameters investigated. The induction of protein content and enzymatic

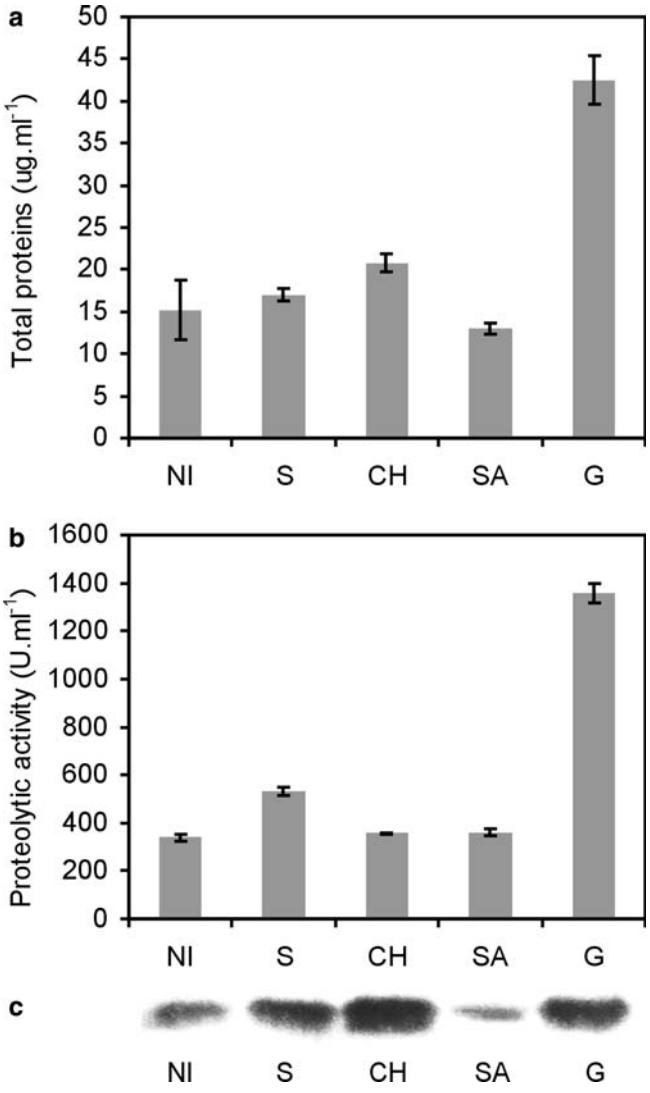


Fig. 1 Biochemical analysis of sundew leaf exudates upon induction of digestion. Leaves were stimulated with mechanical stirring (S) of tentacles 24 h before analysis. Crustacean chitin (CH), salicylic acid (SA) or gelatine (G) were applied on the leaves of plants for 24 h. Visible remains of the inducer were removed prior to analysis. Non-induced plants (NI) were used as control. Data are mean values and standard error based on three replicates. **a** Total protein amount (μg ml^{-1}) in leaf exudates. **b** Proteolytic activity (U ml^{-1}) in leaf exudates. **c** Chitinase activity in leaf exudates. Shown is a digital photograph of electrophoretically separated leaf exudates in an UV-illuminated, fluorescence brightener 28-stained, glycol chitin-containing polyacrylamide gel, demonstrating chitinase activity in all leaf exudates

activities were analysed in more detail using a time course of induction of digestion with either gelatine or chitin.

Figure 2 shows the biochemical analyses of leaf exudates from plants, in which digestion was induced with gelatine or chitin for 5, 24, 48 and 72 h, respectively. Compared to non-induced control, total protein content in exudates from gelatine-induced plants increased till 48 h before it declined. In contrast, chitin induction evoked a much lower accumulation of protein (Fig. 2a). Proteolytic activity increased rapidly between 5 h and 24 h of gelatine induction, and declined, although the proteolytic activity at 48 h and 72 h is still markedly higher than in non-induced leaves. No such proteolytic activity was apparent upon induction by chitin (Fig. 2b). The chitinolytic activity of exudates increased with induction time. After 48 h of induction, the chitinolytic activity reached its peak compared to control leaves (Fig. 2c).

As an alternative method to demonstrate chitinolytic activity in sundew tentacles (Fig. 3a) upon stimulation, a plate-clearing assay was used (Figs. 3b–f). Almost no clearing was observed in the non-induced leaves (Fig. 3b). Gelatine-induced (Figs. 3c, d) and chitin-induced (Figs. 3e, f) sundew leaves covered with glandular hairs were gently squeezed on 0.8% agarose plates containing colloidal chitin and incubated for 20 min. After removal of the leaf material, plates were stained with Congo Red. Cleared zones indicated the presence of chitinase activity after 5 h (Fig. 3c) and 24 h (Fig. 3d) of induction with gelatine, and after 2 h (Fig. 3e) and 48 h (Fig. 3f) of induction with chitin. The clear halos around the glandular head of the tentacles indicate the presence of increased chitinase activity in induced leaves. The plate clearing is more prominent after 24 h of induction with gelatine (Fig. 3d) and after 48 h (Fig. 3f) of induction with chitin.

In situ hybridization shows the presence of chitinase mRNA in sundew leaves

To confirm the presence and investigate the localization of chitinase mRNA after induction with gelatine, in situ hybridization was used (Figs. 3g–p). The sundew chitinase fragment *DrChit1* was used as a hybridization probe. In comparison with sense probe-treated sections (Figs. 3g, k), chitinase transcripts (blue colour) were detected in mesophyll cells and in cells surrounding the vascular bundles of control leaves (Figs. 3h, l). In tentacles, such induction-independent chitinase transcripts were confined to the inner (parenchyma) stalk cells and to the tracheids in the centre of the gland head (Fig. 3l). The secretory cells of the glandular head have no hybridization signal. Induction of sundew leaves with gelatine for 24 h (Figs. 3i, j, m) resulted in a strong hybridization signal in the secretory cells of the tentacle head (Figs. 3i, m). Increased amounts of chitinase RNA were also observed in the mesophyll cells (Fig. 3j). Additional cross sections demonstrated the presence of chitinase transcripts in both external (Fig. 3n) and internal (Fig. 3o) secretory cells. Such a signal was absent from the corresponding gland cells of non-induced leaves (Fig. 3l) or when a sense probe was used (Fig. 3k). The epidermal cells and sessile glands on the leaf surface (Fig. 3p) as well as the sessile glands on the

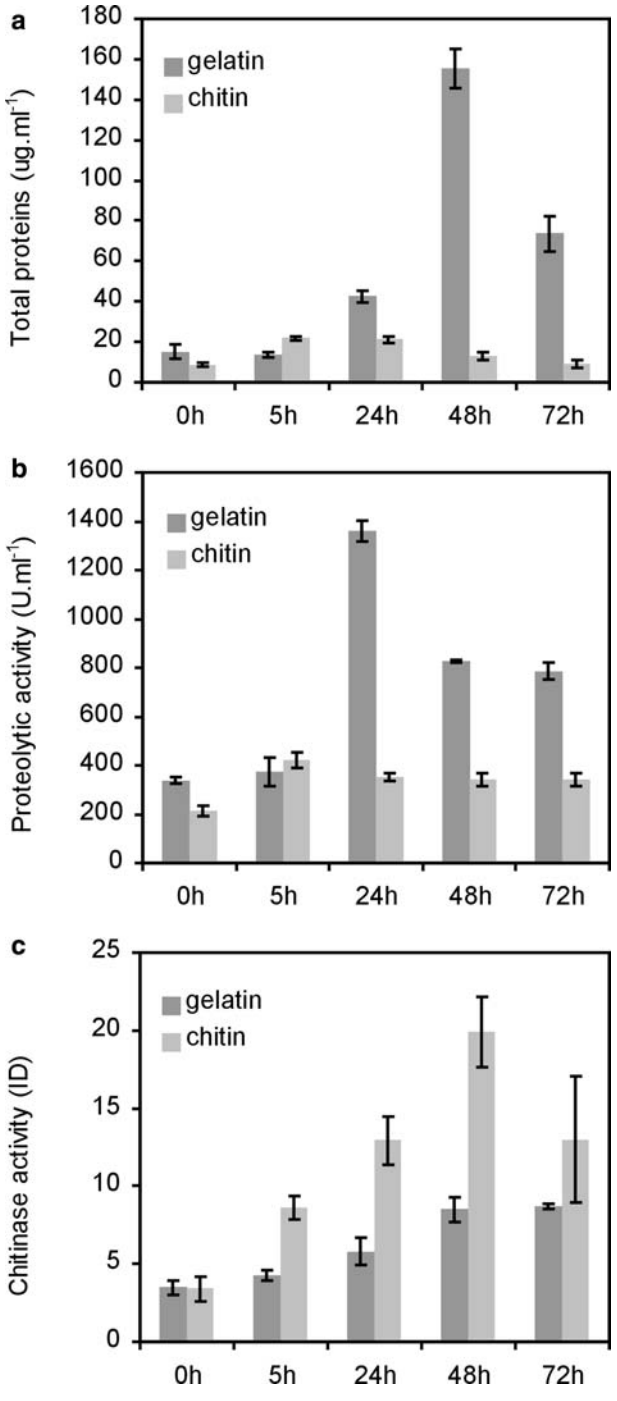


Fig. 2 Time-course study of sundew leaf exudates upon the induction of leaves with gelatine and chitin. Leaves were induced for 5, 24, 48 or 72 h. Data are mean values and standard error based on three replicates. Exudates of non-induced plants were used as control. **a** Total protein (μg ml^{-1}). **b** Proteolytic activity (U ml^{-1}). **c** Chitinase activity (ID)

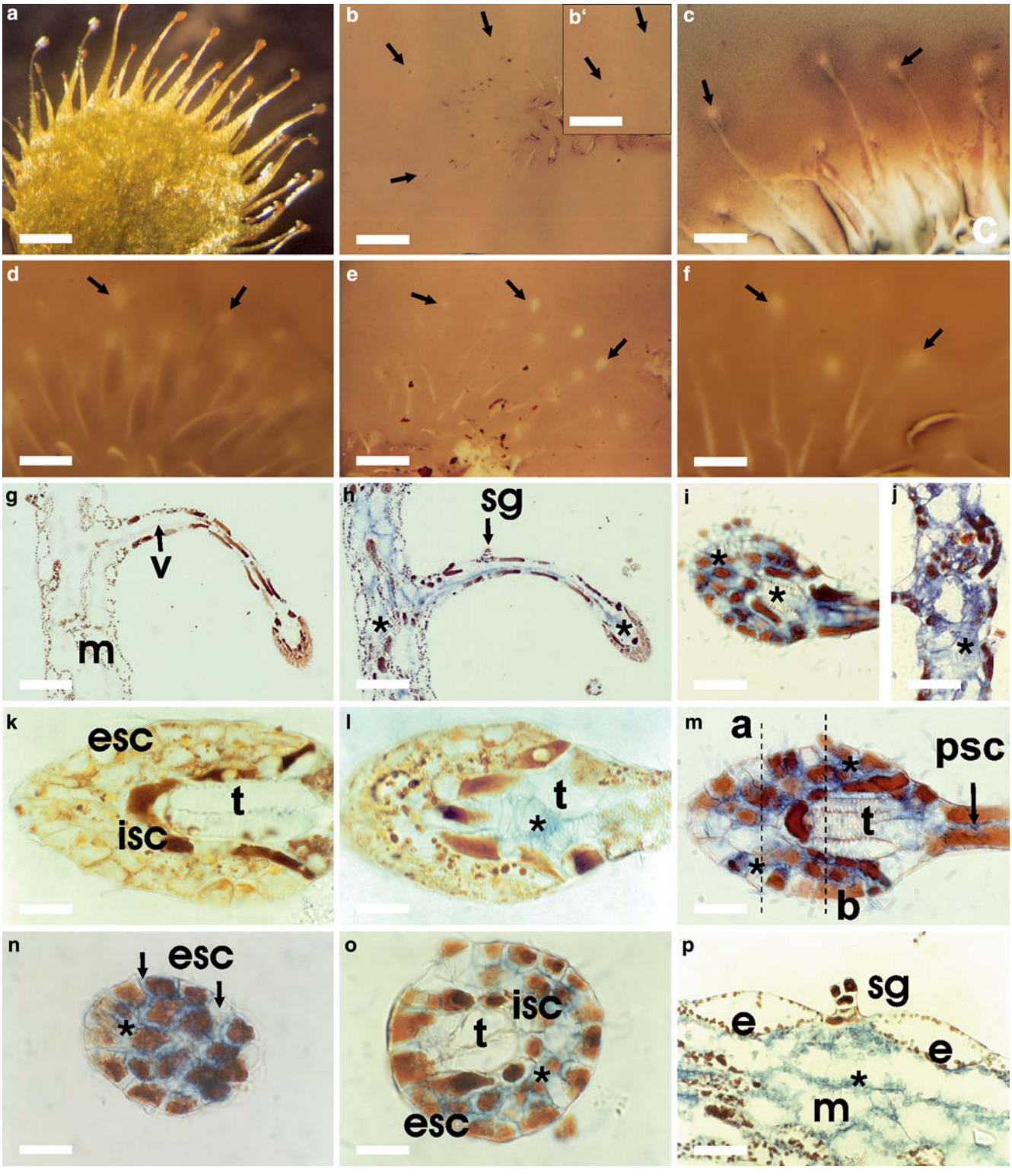
stalks (Fig. 3h) are always devoid of a hybridization signal. These results show that the amount of chitinase transcripts has detectably increased in sundew leaves upon induction of the digestive processes that mimics the presence of prey.

Discussion

Carnivorous plants have developed different morphological and anatomical adaptations for insectivory. Their traps consist of a great number of specific glands that exude a variety of hydrolytic enzymes. Till now, mainly proteases have been described, as proteins are believed to be the main source of nitrogen absorbed from the prey by the plant. Insect-derived nitrogen constitutes a major part of total nitrogen intake of carnivorous plants (Dixon et al. 1980; Juniper 1989; Schulze et al. 1997; Millett et al. 2003). The chitin cuticle of insect prey was suggested to be a source of nitrogen (Juniper et al. 1989; Gooday 1999), but more on a speculative than on an experimental basis. Previous analyses kept the possibility open that the chitinases observed were derived from microbial sources (Robins and Juniper 1980; Sirova et al. 2003). Therefore, the analyses were performed with a representative from the sundew family that is successfully cultured aseptically in vitro, round-leaf sundew. The results presented here provide molecular support for the suggestion that chitinase activity is indeed part of the sundew digestive processes. In situ hybridization with a chitinase probe performed on gelatine-induced and non-induced sundew leaves demonstrated the absence of chitinase transcripts in the secretory cells of the sundew leaf, despite a low amount of chitinase transcripts in other cells, such as mesophyll cells. Upon induction with gelatine, a chitinase transcript could be easily detected in both the external and internal secretory cells of tentacles. This indicates the notable induction of chitinase gene expression, presumably in the digestive glands of sundew tentacles. More experiments will be necessary to determine whether the chitinase mRNA is actually transcribed in the head cells, or is transported from other cells.

The chitinase transcript data were supported by biochemical data. Chitinase activity was found in gelatine- or chitin-induced leaves and in a 20 kDa protein present in leaf exudates. Some enzyme activity in non-induced plants indicated that active chitinases are constitutively present in sundew mucilage already prior to insect digestion. They may provide other functions, such as prevention against microbial invasions from the environment, and/or be responsible for the first digestive process upon contact with the source of nitrogenous (chitinaceous) compounds (Heslop-Harrison 1975).

Upon application of different stimuli on sundew leaves, the activity of chitinase(s) in leaf exudates increased considerably. The strongest inducer of sundew chitinase in sundew leaves was chitin itself. Gelatine increased chitinase activity to a lesser extent. Induction with salicylic acid did not affect chitinase activity significantly. Salicylic acid is known as a potent inducer of many chitinases during pathogenesis (Linthorst 1991). Mechanical stimulation had detectable effects. Even gentle placement of solid or liquid inducers on the leaf



surface will cause some mechanical stimulation of tentacles that could result in weak chitinase activity. The minor effect obtained for salicylic acid is likely to be due to such an experimental background. The induction of chitinase activity evoked by mechanical stimulation of tentacles suggests that the enzyme is involved immediately after the perception of detection of prey. Such a phenomenon was previously shown in *Sarracenia* (Gallie and Chang 1997), *Pinquicula* (Heslop-Harrison and Heslop-Harrison 1981) and *Dionaea* (Juniper 1989). Inducers of chitinolytic and/or proteolytic activity, such as chitin oligomers or oligopeptides, may be released due to

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Fig. 3 Chitinase activity and transcripts in sundew leaf tissue. **a** An intact sundew leaf covered with long glandular hairs. **b** Congo Red-stained agarose plate after 20 min incubation of a sundew leaf without induction. The arrows indicate the expected position of glandular heads. (**b'** Higher magnification of two spots from 3b). **c** Congo Red-stained agarose plate after 20 min incubation of a 5 h gelatine-induced sundew leaf. **d** Congo Red-stained agarose plate after 20 min incubation of a 24 h gelatine-induced sundew leaf. **e** Congo Red-stained agarose plate after 20 min incubation of a 2 h chitin-induced sundew leaf. **f** Congo Red-stained agarose plate after 20 min incubation of a 48 h chitin-induced sundew leaf. Cleared zones (Figs. **b–d**) indicate the presence of chitinase activity, especially in glandular heads (arrows). **g–p** Localization of chitinase mRNA in the leaves of sundew. The putative class I chitinase gene fragment *DrChit1* from sundew was used as a probe in hybridizations in situ. The hybridization pattern was compared between leaves induced with gelatine for 48 hours and control (non-induced) leaves. The yellowish-brown colour in Figs. **g–p** is caused by the secondary metabolites in the cells. The blue colour also indicated by asterisks in the sections is the hybridization signal. **g–h** Longitudinal section through leaf of control plants with stalked gland (**g** sense, **h** antisense probe, *m* - mesophyll, *v* - vessel, *sg* - sessile gland). **i–j** Presence of chitinase transcripts in gland cells (**i**) and mesophyll cells (**j**) of a gelatine-induced leaf. **k** Detailed view of the glandular head (sense probe) consisting of inner tracheidal (*t*) part and two surrounding layers of secretory cells (*esc* - external secretory cells, *isc* - internal secretory cells). **l** Presence of chitinase transcripts in the tracheidal cells and parenchyma stalk cells (*psc*) of a non-induced leaf. The secretory cells lack all hybridization signal. **m** Presence of chitinase transcripts in the secretory cells of induced sundew leaves. **n–o** Two detailed cross sections (corresponding to the cuts *a* and *b*, Fig. **m**) showing the presence of chitinase transcripts in both external (**n**) and internal (**o**) secretory cells of the glandular head. **p** Detail with the sessile gland (*sg*) and epidermal cells (*e*) of a leaf. Both are devoid of a hybridization signal in contrast to surrounding mesophyll cells. Scale bars: **a** = 400 μm, **b** = 500 μm, **b'**, **c**, **d, f** = 200 μm, **e** = 300 μm, **g**, **h** = 100 μm, **i**, **j**, **p** = 50 μm, **k**,**l**, **m**, **n**, **o** = 20 μm

insect movement during its struggle to escape, and from the insect hemolymph after its death.

The difference in increase of chitinolytic activity between protein (gelatine) and chitin induction suggests that different signal transduction pathways may be operational in Drosera leaves for such compounds. Chitin and chitin-derived oligosaccharides appear to trigger more chitinase activity than gelatine. The low amount of total protein and low proteolytic activities evoked by chitin induction both suggest that sundew contains a specific sensing mechanism for chitin and/or chitin-derived compounds. As gelatine is mainly triggering proteolytic activity, it is conceivable that any extra chitinase also has to cope with the higher proteolytic environment. Protein may be a metabolically easier or cheaper source of nitrogen for a carnivorous plant than chitin. The results indicate that different stimuli activate different pathways of the plant digestive process. These pathways seem to have substrate specificity and efficacy. For *Dionaea* and *Nepenthes*, it was suggested that release of hydrolases and a viscous, acidic fluid production after mechanical stimulation are not under the same control (Robins 1976; Lichtner and Williams 1977; Juniper et al. 1989). Targeted induction after the perception of the appropriate signals may limit the cost of carnivory to the plant, optimizing the use of available resources (Gallie and Chang 1997). The efficient use of insect-derived nutrient resources may contribute to the ecological success of carnivorous plants on poor soils.

Chitinases have previously been reported to be involved in many different processes in plants, such as plant defence mechanisms against various pathogens or environmental stress, organ formation, embryogenesis, fruit ripening, leaf senescence, regulation of growth and development, programmed cell death or nodulation (for review, see Kasprzewska 2003). We here present data that establish the involvement of chitinases in the decomposition of captured insects in carnivorous sundew. Chitinase activity is induced upon stimulation that is mimicking the presence of insect prey on sundew leaves, suggesting a role in chitin digestion. However, the precise contribution of chitinases to the digestion of insect prey remains to be determined. It could be a direct contribution, providing chitin-derived nutrients to the plant. The contribution could also be indirect, for example, by facilitating the exposure of other (proteinaceous) material to enzymatic degradation and nutrient supply (Juniper et al. 1989). Detailed analyses on the biological origin of insect molecules taken up by carnivorous plants will be required to resolve such issues.

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References

Adamec L (1997) Mineral nutrition of carnivorous plants: a review. Bot Rev 63:273–299

Amagase S, Mori M, Nakayama S (1972) Digestive enzymes in insectivorous plants. IV. Enzymatic digestion of insects by *Nepenthes* secretion and *Drosera peltata* extract: proteolytic and chitinolytic activities. J Biochem 72:765–767

Békésiová I (2000) Genes for proteins with antifungal activity in *Drosera rotundifolia* L. Ph.D. Thesis, Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Nitra, pp 1–147

Bobák M, Blehová A, Krištín J, Ovečka M, Šamaj J (1995) Direct plant regeneration from leaf explants of *Drosera rotundifolia* cultured in vitro. Plant Cell Tiss Org Cult 43:43–49

Bohak Z (1970) Chicken pepsinogen and chicken pepsin. Meth Enzymol 19:347–358

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Broglie R, Broglie K (1993) Chitinases and plant protection. In: Fritig B, Legrand M (eds) Mechanisms of plant defense responses. Kluwer, Dordrecht, pp 411–421

Chandler GE, Anderson JW (1976) Studies on the origin of some hydrolytic enzymes associated with the leaves and tentacles of *Drosera* species and their role in heterotrophic nutrition. New Phytol 77:51–62

Clancy FG, Coffey MD (1977) Acid phosphatase and protease release by the insectivorous plant *Drosera rotundifolia*. Can J Bot 55:480–488

Cox KH, Goldberg RB (1988) Analysis of plant gene expression. In: Shaw CH (ed), Plant molecular biology, a practical approach. IRL Press, Oxford, pp 1–34

Culham A, Gornall RJ (1994) The taxonomic significance of naphthoquinones in the *Droseraceae*. Biochem Syst Ecol 22:507–515

Darwin C (1875) Insectivorous Plants. John Murray, London

Darwin F (1878) Experiments on the nutrition of *Drosera rotundifolia*. J Linn Soc Bot 17:17–27

Dixon KW, Pate JS, Bailey WJ (1980) Nitrogen nutrition of the tuberous sundew *Dionaea erythrorhiza* Lindl. with special reference to the catch of arthropod fauna by its glandular leaves. Aust J Bot 28:283–297

Ellison AM, Gotelli NJ (2001) Evolutionary ecology of carnivorous plants. Trends Ecol Evol 16:623–628

Gallie DR, Chang SC (1997) Signal transduction in the carnivorous plant *Sarracenia purpurea*. Plant Physiol 115:1461–1471

Gooday GW (1999) Aggressive and defensive role of chitinases. In: Jolles P, Muzzarelli RAA (eds) Chitin and chitinases. Birkhäuser Verlag, Basel, pp 157–171

Heslop-Harrison Y (1975) Enzyme release in carnivorous plants. In: Dingle JT, Dean RT (eds) Lysosomes in biology and pathology. North-Holland, Amsterdam, pp 525–578

Heslop-Harrison Y, Heslop-Harrison J (1981) The digestive glands of *Pinguicula*: Structure and cytochemistry. Ann Bot 47:293–319

Inbar J, Chet I (1991) Detection of chitinolytic activity in the rhizoshepore using image analysis. Soil Biol Biochem 23:239–242

Juniper BE (1989) The path to plant carnivory. In: Juniper BE, Southwood TRE (eds) Insects and the plant surface. Edward Arnold, London, pp 195–218

Juniper BE, Robins RJ, Joel DM (1989) The carnivorous plants. Academic Press, London, pp 1–392

Kasprzewska A (2003) Plant chitinases - regulation and function. Cell Mol Biol Lett 8:809–824

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Lichtner FT, Williams SE (1977) Prey capture and factors controlling trap narrowing in *Dionaea* (Droseraceae). Am J Bot 64:881–886

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci 10:123–150

Melchers LS, Stuiver MH (2000) Novel genes for disease-resistance breeding. Curr Opin Plant Biotech 3:147–152

Millett J, Jones RI, Waldron S (2003) The contribution of insect prey to the total nitrogen content of sundew (*Drosera* spp.) determined in situ by stable isotope analysis. New Phytol 158:669–675

Molano J, Duran A, Cabib E (1977) A rapid and sensitive assay for chitinase using tritiated chitin. Anal Biochem 83:648–656

Muravnik LE, Ivanova AN (1999) Localization of Ca^{2+} and Ca^{2+}-ATPase in the secretory cells of glandular hairs in *Drosera* leaves. Tsitologiya 41:386–393

Pan QS, Ye SX, Kuć J (1991) A technique for detection of chitinase, β-1,3-glucanase, and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocusing. Phytopathology 81:970–974

Robins RJ (1976) The nature of the stimuli causing digestive juice secretion in *Dionaea muscipula* Ellis (Venus's flytrap). Planta 128:263–265

Robins RJ, Juniper BE (1980) The secretory cycle of *Dionaea muscipula* Ellis. IV. The enzymology of the secretion. New Phytol 86:413–422

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Schulze W, Schulze ED, Pate JS, Gillison AN (1997) The nitrogen supply from soils and insects during growth of the pitcher plants *Nepenthes mirabilis*, *Cephalotus follicularis* and *Darlingtonia californica*. Oecologia 112:464–471

Sirova D, Adamec L, Vrba J (2003) Enzymatic activities in traps of four aquatic species of the carnivorous genus *Utricularia*. New Phytol 159:669–675

Stewart CN, Nielsen ET (1992) *Drosera rotundifolia* growth and nutrition in a natural population with special reference to the significance of insectivory. Can J Bot 70:1409–1416

Thum M (1989) The significance of carnivory for the fitness of *Drosera* in its natural habitat: 2. The amount of captured prey and its effect on *Drosera intermedia* and *Drosera rotundifolia*. Oecologia 81:401–411

Trudel J, Asselin A (1989) Detection of chitinase activity after polyacrylamide gel electrophoresis. Anal Biochem 178:362–366