Detection of chitinolytic enzymes with different substrate specificity in tissues of intact sundew (*Drosera rotundifolia* L.)

Chitinases in sundew tissues

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Abstract The round-leaf sundew (*Drosera rotundifolia* L.) is a carnivorous plant expressing a wide range of chitinolytic enzymes playing role in many different processes. In this study the intact plants were analyzed for the presence of chitinase transcripts and chitinolytic activities in different organs. In situ hybridization with chitnase fragment as a probe has revealed the presence of chitinases in the mesophyll cells of leaves and vascular elements of stems of healthy, non-stressed plants. More pronounced expression was observed in cortex and stele cells of roots as well as in ovules and anthers of reproductive organs. Similarly, higher chitinase enzyme activity was typical for flowers and roots suggesting a more specific role of chitinases in these tissues. In addition to endochitinases of different substrate specificities, chitobiosidases contributed to overall chitinolytic activity of tissue extracts. The activity of chitobiosidases was again typical for flowers and roots, while their role in plant physiology remains to be elucidated.

Keywords Carnivorous plant · Chitinase expression · Chitinolytic activity · In situ hybridization

Abbreviation
PMSF Phenylmethylsulfonyl fluoride

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Introduction

Higher plants produce chitinases (EC 3.2.1.14), either constitutively or following induction, and the possible function of these enzymes within the plant have generated much interest and speculation. One of the roles attributed to them is a defence mechanism against attack by pathogens, since their expression is significantly enhanced following infection and many of them showed antimicrobial activity in vitro as well as *in planta* [1, 2]. Furthermore, some evidence exists for their developmental regulation in specific tissues and at specific stages during plant development [3].

Based on amino acid sequences, chitinases can be grouped into at least five classes [4], while different isoforms may also differ in substrate binding characteristics and specific activities. In general, chitinases are categorized into two major categories. Endochitinases (EC 3.2.1.14) which hydrolyze chitin randomly at internal sites and exochitinases acting at the ends of polysaccharide chain. The latter group beta-N-acetylhexosaminidases (EC 3.2.1.52) involves chitobiosidases that catalyze releasing of diacetylchitobiose unit from chitin chain, and chitobiases which cleave the oligomeric products of endochitinases and chitobiosidases generating monomers of GlcNAc [5]. These enzymes differ in substrate specificity that is often overlooked when measuring total tissue chitinolytic activity in plant tissue.

Here we focused on occurrence of chitinase expression as well as chitinase enzyme activity in different tissues of insectivorous sundew (*Drosera rotundifolia* L.) cultivated in vitro. Sundew is a medicinal plant that has also been studied as a potential source of antifungal compounds, including chitinases [6]. Constitutive, low level chitinase expression was detected in leaves, while expression of

some chitinases was enhanced or triggered upon induction of digestive processes in the secretory glands [7]. However, no comprehensive study on occurrence and activity of chitinases throughout the whole insectivorous sundew plant has been performed. Given that different plant tissues have different functions and priorities (physiological, metabolic etc.) they are likely to differ in presence and activity of certain chitinases. This study describes at expression as well as enzymatic levels the distribution of chitinases in leaves, stems, flowers and roots. Contribution of endo-chitinases and chitobiosidases was also studied. Activity differences detected among tissue types are discussed with respect to possible biological function.

Material and methods

Plant material

Plants of *Drosera rotundifolia* L. were cultivated in vitro on basal MS medium [8] supplemented with 2% (w/v) sucrose and 0.8% (w/v) agar [9]. The plantlets were cultivated at $20 \pm 2°C$ with a day length of 16 h under 50 μE $m^{-2} s^{-1}$ light intensity. For analyses, two months-old plants were used.

Histology

Samples from sundew leaves, stems, flowers and roots were fixed in 4% (w/v) paraformaldehyde and 0.25% (v/v) glutaraldehyde in PBS on ice, embedded in Paraplast Plus (Sigma-Aldrich, USA), sectioned and mounted on poly-L-lysine coated slides. The control sections were stained with 1% toluidine blue. The sections 6–8 μm thick were used also for in situ hybridization experiments. 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.

In situ hybridization

The RNA probes were prepared by cloning a 325 bp sundew fragment encoding conservative region of chitinase (*DrChit1*, GenBank accession No. AY622818) into the vector pGEM-T Easy (Promega, USA). 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, Germany). The in situ hybridization protocol followed the procedure described earlier [7]. The samples were observed with transmitted-light bright field Axiovert 2 microscope (Carl Zeiss, Göttingen) and photographed (KR/10M Ricoh, Japan and Sonny DXC-S500 Digital Camera System).

Protein isolation

Crude protein extracts were isolated from flowers, stems, leaves and roots of in vitro grown plants *Drosera rotundifolia* L. The extraction buffer contained 20% (v/v) glycerol, 1.5% (w/v) polyvinylpyrrolidon 40 (Sigma-Aldrich, USA), 0.1 mol l^{-1} Tris-HCl (pH 8.5), 0.001 mol l^{-1} PMSF (Serva, Germany) and 0.02% (v/v) β-mercaptoethanol. Plant tissue (0.5 g) was ground in a mortar using liquid nitrogen, transferred into extraction buffer and homogenized by vortexing. Insoluble material was removed from the homogenate by centrifugation at 14,000 rpm at 4°C for 20 min. Protein concentration was determined according to Bradford [10]. Protein extracts were directly used for enzymatic analyses.

Chitinolytic activity towards fluorogenic substrates

The fluorimetric assays were used to detect chitobiosidase and endochitinase activities in crude protein extracts using two synthetic substrates: 4-methylumbelliferyl-β-D-N, N'-diacetylchitobioside [4-MU-(GlcNAc)$_2$] and 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside [4-MU-(GlcNAc)$_3$]. The reaction mixture contained 20 μl of protein extracts mixed with 30 μl of 300 μmol l^{-1} substrate in 0.1 mol l^{-1} sodium citrate buffer (pH 3.0). The assays were carried out in 96-well black-sides assay plates. After incubation at 37°C for 1 hour, the reaction was stopped by adding 150 μl of 0.2 mol l^{-1} Na$_2$CO$_3$ and fluorescence was measured by Fluoroskan II microtiterplate reader (TITERTEK, Finland) using excitation and emission filters 355 nm/450 nm. Based on the standard curve, the chitinase activity was calculated as picomoles of methylumbelliferone (4-MU) generated per hour per microgram of soluble protein at 37°C. The experiment was carried out at least four times and in each experiment each tissue was sampled three times. Statistical analyses were performed using the program Microsoft Excel 2000 (Microsoft Office) by means of F-test for homogeneity of variances and two-sample t-test for location assuming equal variances. In case the F-test indicated that variances of the compared pairs were not equal, the two-sample t-test for location assuming unequal variances was used.

Detection of chitinolytic enzymes in activity gel

Protein samples (10 μg) were separated on a 12.5% (w/v) SDS-containing polyacrylamide slab gels [11]. 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-natured by shaking the gel in 50 mmol l^{-1} sodium acetate buffer (pH 5.0), 1% (v/v) Triton X-100 for 1 h at 8°C. The gel was then incubated in 50 mmol l^{-1} sodium acetate (pH 5.0) for

30 min. Following re-naturation of proteins, the surface of the gel was blotted with filter paper and 500 µl of 200 µmol l^{-1} of 4-MU-(GlcNAc)$_2$ or 4-MU-(GlcNAc)$_3$ was applied onto the surface gel, separately. The gel was incubated at 37°C for 20 min and the fluorescent activity bands were visualized on UV transilluminator.

Apart from fluorescent substrates, third enzyme substrate 0.01% (v/v) glycol chitin was incorporated into the polyacrylamide gel before electrophoresis. After re-naturation of separated proteins, the chitinase activity was detected by staining with 0.01% (w/v) Fluorescent Brightener 28 and UV-illumination [7].

Following enzyme detection, the gels were stained with Coomassie Brilliant Blue R 250 and the molecular weight of the re-natured chitinases was estimated by comparison with protein ladder (Mark 12 Unstained Standard, Invitrogen).

Results and discussion

In our study, we have focused on detection of chitinases in different organs of intact sundew plants. As a probe for in situ hybridization was used the sundew chitinase fragment *DrChit1* [7]. The flanking regions of *DrChit1* correspond to the protein motifs SHETTGG and IWFWM that are highly conserved and present in all known plant chitinase classes [12]. We expected that under given hybridization conditions this probe will hybridize to chitinases of several classes revealing the overall distribution of homologous chitinase transcripts in sundew tissues. Most of members within the chitinase classes are considered to contribute to active or passive defence mechanisms against pathogens [3] while most of them apparently also play role in physiological and developmental processes [4] such as morphology, programmed cell death or reproduction [13–17]. We observed the presence of chitinase transcripts in leaves including tentacles (Fig. 1b), and in stem tissue (Figs. 1d), namely around the vascular elements. More pronounced accumulation of chitinase transcript was apparent in sundew roots (Fig. 1f; for comparison see sense control, Fig. 1g), especially in the cells of cortex and stele (Fig. 1f). High chitinase expression has also been observed in roots of tobacco [18], rice [19], rape [20] or sugar beet [21] and it could coincide with genetically determined role of chitinases in local defence as a consequence of permanent growth in highly microorganisms' polluted soil. Strong transcription of chitinase was also present in the floral organs (Fig. 1h–n), namely in ovules (Fig. 1i, k, l) and anthers/pollen grains (Fig. 1m, n). Despite the fact that some floral organs are largely devoid of mechanical barriers (e.g. cuticle), that can facilitate pathogen attack, the functions of chitinases in these organs

can not be explained as defence mechanism only. It was suggested that chitinases have unrecognized specific function in the sexual reproduction of higher plants [20, 22] and they are involved in plant growth of rapidly growing tissues like flowers, anthers and embryos, possibly in processes requiring cell wall disruption, including cell division [23, 24].

Total chitinase enzyme activity in different sundew tissues was also measured based on ability to hydrolyze GlcNAc oligomeric substrates and glycol chitin. The release of 4MU from 4MU-(GlcNAc)$_2$ or 4MU-(GlcNAc)$_3$ was measured fluorimetrically and indicated the activity of chitobiosidase or endochitinase hydrolyzing short oligomers, respectively. Both types of activities were studied in crude protein extracts from flowers, stems, leaves and roots. Except for flowers, in each tissue type the endochitinases revealed significant, 1.2–4.7 times higher activity than chitobiosidases (Fig. 2). The largest differences (at $P < 0.001$) were observed in roots. In contrast to our results, neither chitobiosidase activity nor short-oligomer specific endochitinases were detected in non-induced *Nepenthes* pitchers [25]. Short oligomer-specific endochitinases in sundew were of highest activity in root tissue, while in flowers reached approximately 40%, in stems 21% and in leaves 16% of activity measured in roots (Fig. 2). Chitobiosidases were shown to be involved in response of soybean root to the presence of *Trichoderma harzianum* [26] and tobacco leaves treated with an isolate of non-pathogenic *Gliocladum roseum* [27]. Since these enzymes were almost exclusively studied with respect to microbial attack (e.g. soilborn pathogens), their possible role in untreated plant physiology is unclear unlike for endochitinases.

Further analyses of protein extracts on SDS-PAGE revealed that different isoforms are responsible for the activities measured. Following re-naturation, the proteins were probed with both the fluorescent substrates 4MU-(GlcNAc)$_2$ (Fig. 3) and 4-MU-(GlcNAc)$_3$ (Fig. 4). A protein fraction of ~60 kDa (Chit1) was apparently responsible for the chitobiosidase activities measured. However, detection limit of the gel-technique applied identified the corresponding protein in only floral and root samples (Fig. 3). Relative high levels of chitobiosidase in these tissues might reflect to some role in reproduction or root physiology. One enzyme isoform Chit2 of ~50 kDa was also detected also for short-chain specific endochitinases (Fig. 4). In contrast to chitobiosidase, low-level activity was present in each tissue type but relatively strong signal was apparent in roots. However, gel analyses revealed also two chitinase isoforms Chit3 and Chit 4 (~25 kDa and 20 kDa, respectively) hydrolyzing a long-chain substrate glycol chitin (Fig. 5). The activities of these endochitinases were apparently similar in each tissue type but the 20 kDa isoform was missing in roots. In addition, the intensities of the two isoforms were not identical within the tissue and their
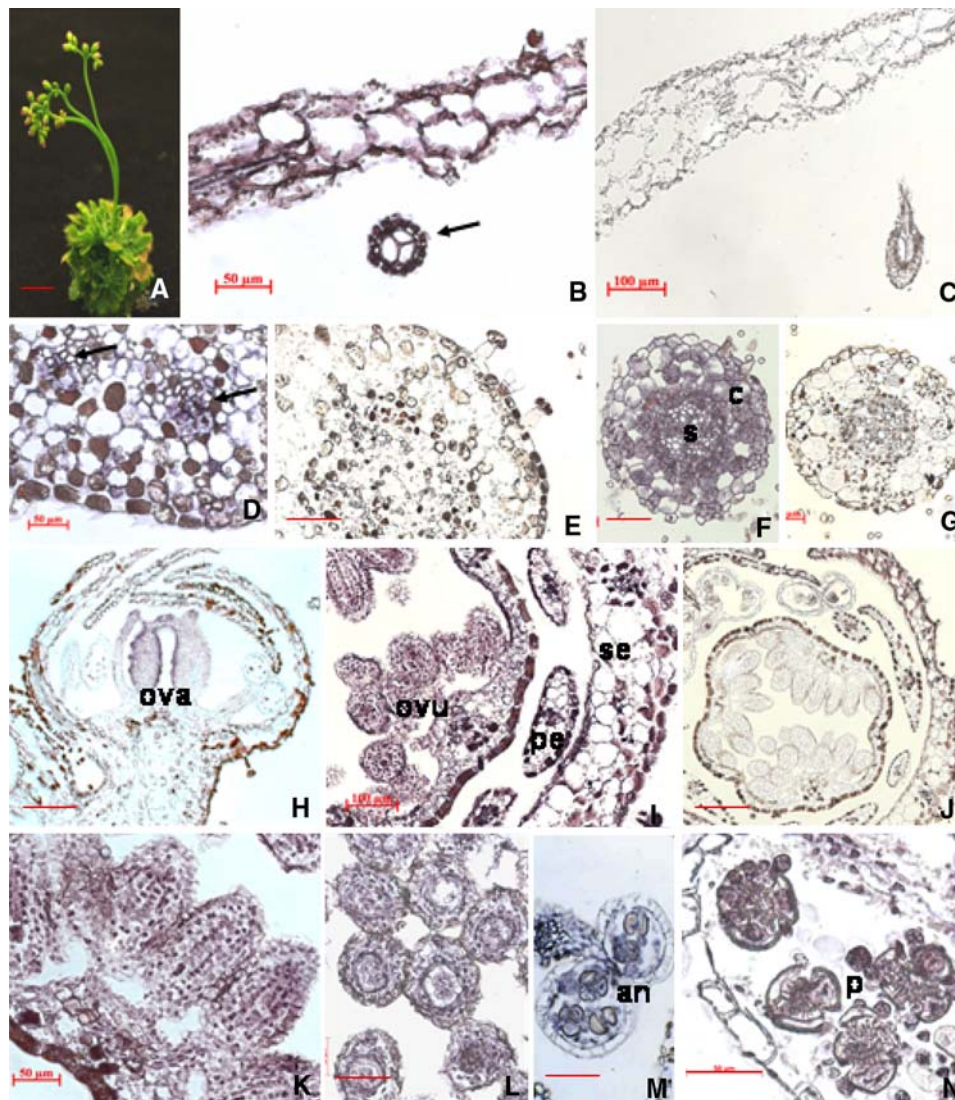
Fig. 1 In situ localization of chitinase transcripts in different organs of intact sundew plant. (**a**) Sundew plants growing aseptically in in vitro conditions. (**b**) Accumulation of chitinase (*DrChit1*) transcripts in mesophyll cells and tentacle (cross section—arrow). (**c**) Control section of leaf tissue (sense probe) with no expression of *DrChit1* in the tissues of leave and tentacle. (**d**) In stems the expression of *DrChit1* was localized preferably in and around the vascular elements (arrow). (**e**) Control cross section hybridized with sense-probe. (**f**) cross section of the sundew root—note strong accumulation of *DrChit1* transcripts in the cortex (c) and stele (s). (**g**) control section (sense probe). (**h**) Longitudinal section through sundew flower showing accumulation of *DrChit1* mainly in ovary (ova). (**i**) Cross section showing expression of *DrChit1* in placenta and ovules (ovu). Some accumulation of *DrChit1* was present also in sepals (se) and in vascular elements of petals (pe). (**j**) Control cross section (sense probe). (**k**, **l**) Detailed view on localization of *DrChit1* in ovules ((**k**) longitudinal, (**l**) cross section). (**m**, **n**) The *DrChit1* transcripts were expressed also in anthers (an) and pollen grains (p). Bars: **a**—5 mm, **b**, **d**, **k**, **l**, **n**—50 μm, **c**, **e**, **f**, **g**, **i**, **m**—100 μm, **j**—200 μm, **h**—400 μm

proportion was varying among tissue type. However, exact quantification could only confirm the differences observed. Nevertheless, in total of three endochitinase isoforms were detected in sundew tissues (except for roots) that differ in their substrate (long chain–short chain) specificity.

Summarizing all the results obtained in this study, chitinase expression and activity is present in sundew plants. The hybridization probe used was supposed to detect chitinase transcripts comprising several chitinase classes (if present).

These transcripts confirm that chitinases do play role in basic physiological processes e.g. development and morphology (Fig. 1). Although the plants studied were grown in vitro hence no defence was induced, the defensive role of some of detected chitinases also cannot be excluded. Several authors have shown that expression/activity of some chitinases increased upon environmental (including microbial) challenge [28]. Therefore, a pre-existing defence mechanism is likely to be present in tissues [29]. The latter authors
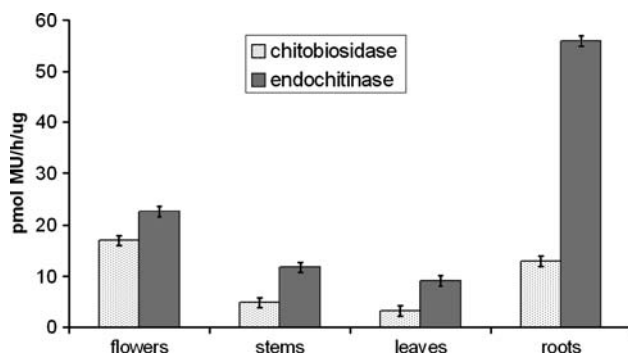
Fig. 2 Chitinolytic activities in different sundew tissues revealed by assaying of fluorescent 4-MU released from two chitin analogues: [4-MU-(GlcNAc)$_2$] and [4-MU-(GlcNAc)$_3$] representing chitobiosidase and endochitinase (hydrolyzing short oligomers) activities, respectively. The chitinase activity was calculated as picomoles of methylumbelliferone (4-MU) generated per hour per microgram of soluble protein at 37°C. Significant differences between chitinolytic activities were observed in leaves and stems ($P < 0.05$) and in roots ($P < 0.001$)
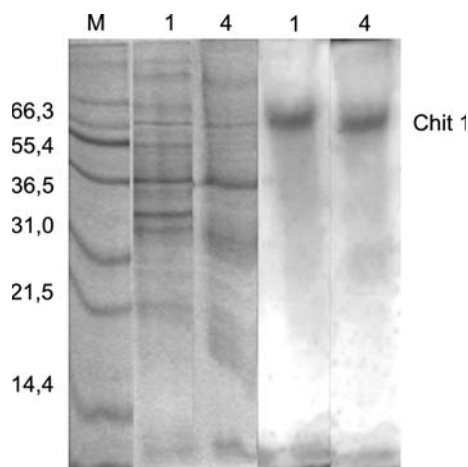

Fig. 4 Detection of endochitinase activity (for short oligomers) in gel using 4-methylumbelliferyl-β-D-N,N',N''-triacetylchitotrioside as a substrate. Proteins were separated on 12.5% SDS-PAGE and visualized with Coomassie Brilliant Blue R 250 after detection of enzyme activity. Lane M—protein marker, lane 1—flowers, lane 2—stems, lane 3—leaves, lane 4—roots, Chit2—protein band indicating an endochitinase activity for short oligomers

Fig. 3 Detection of chitobiosidase activity in gel using 4-methylumbelliferyl- β-D-N,N'-diacetylchitobioside as a substrate. Proteins were separated on 12.5% SDS-PAGE and visualized with Coomassie Brilliant Blue R 250 after detection of enzyme activity. Lane M—protein marker, lane 1—flowers, lane 4—roots, Chit1—protein band indicating a chitobiosidase activity

Fig. 5 Detection of endochitinase activity (for long polymers) in gel using glycol chitin as a substrate. Proteins were separated on 12.5% SDS-PAGE and visualized with Coomassie Brilliant Blue R 250 after detection of enzyme activity. Lane M—protein marker, lane 1—flowers, lane 2—stems, lane 3—leaves, lane 4—roots, Chit3, Chit4—protein bands indicating an endochitinase activity for long polymers

proposed that chitinases might co-operate with phytoalexins to effectively control fungal growth and development. Nevertheless, the exact role of chitinases in this mechanism remains to be elucidated.

Our data also revealed strike differences in chitinolytic activities among different tissue parts. Up to three endochitinase isoforms revealed relatively high activity in roots and flowers but much less in stems and leaves. This phenomenon coincides with some theories that explain non-linear distribution of defence throughout the plant [30]. For example, according to optimal defence theory, most defended are tissues of highest fitness value (e.g. reproductive organs) or tissues most probably defended (e.g. roots exposed to
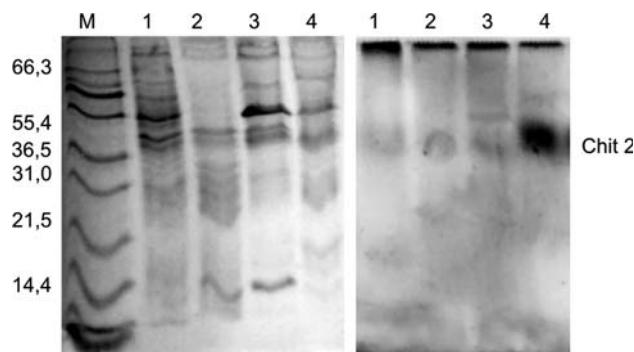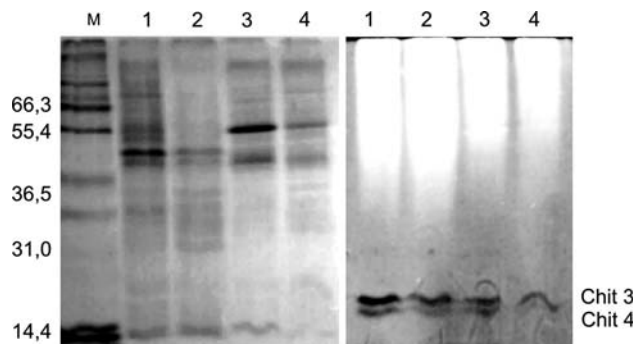
microorganisms in soil). Detected activity of chitobiosidase in these tissue types also evokes questions on the role of these enzymes in plant physiology. Thus, to address the physiological roles of chitinases in sundew, several important factors should also be considered such as substrate specificity of different chitinase types as well as the role of development and/or tissue type at the whole plant level, rather than at the meristem or leaf level.

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