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ORIGINAL PAPER

Expression of *Drosera rotundifolia* Chitinase in Transgenic Tobacco Plants Enhanced Their Antifungal Potential

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Abstract

In this study, a chitinase gene (*DrChit*) that plays a role in the carnivorous processes of *Drosera rotundifolia* L. was isolated from genomic DNA, linked to a double *CaMV35S* promoter and *nos* terminator in a pBinPlus plant binary vector, and used for *Agrobacterium*-mediated transformation of tobacco. RT-qPCR revealed that within 14 transgenic lines analysed in detail, 57% had *DrChit* transcript abundance comparable to or lower than level of a reference *actin* gene transcript. In contrast, the transgenic lines 9 and 14 exhibited 72 and 152 times higher expression level than *actin*. The protein extracts of these two lines exhibited five and eight times higher chitinolytic activity than non-transgenic controls when measured in a fluorimetric assay with FITC-chitin. Finally, the growth of *Trichoderma viride* was obviously suppressed when the pathogen was exposed to 100 µg of crude protein extract isolated from line 9 and line 14, with the area of mycelium growth reaching only 56.4% and 45.2%, of non-transgenic control, respectively. This is the first time a chitinase from a carnivorous plant with substrate specificity for long chitin polymers was tested in a transgenic plant with the aim of exploring its antifungal potential.

Keywords Antifungal activity · Chitinase · Chitinolytic activity · *Drosera rotundifolia* · FITC-chitin · Transgenic plants

Introduction

Chitin is the second most important biopolymer consisting of *β*-(1-4)-*N*-acetyl-D-glucosamine (GlcNAc) units and is found in the exoskeleton of arthropods and in the cell walls of fungi. In individual species of fungi, chitin accounts for a 3–60% of the total cell wall mass, and it is responsible for the rigidity, physical strength and specific shape of cell wall [1].

Chitinases (EC 3.2.1.14) are enzymes that hydrolyse the *β*-1,4-linkages in chitin. Based on the sequence similarity and different catalytic mechanisms, chitinases are grouped into glycosyl hydrolase (GH) families 18, 19, and 20 [2]. Plant chitinase genes are classified into seven classes (I–VII) [3, 4]. Class I, II, IV, VI and VII chitinases belong to the GH19 family, while class III and V chitinases are members of the GH18 family [5].

Chitinases play a role during plant growth and developmental processes, such as during pollination, senescence, germination and somatic embryogenesis [6, 7]. Although there is no conclusive information about the endogenous substrates of plant chitinases, arabinogalactan proteins and GlcNAc-containing glycoproteins in cell walls are the presumed substrates of hydrolysis [8]. Another function of

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chitinases involves abiotic stress responses. Some chitinase genes are induced by a range of abiotic stresses, including osmotic-, cold-, heavy- metal stress, and wounding [9–12]. Abiotic elicitors stimulate pathogenesis-like responses, which are vital for their survival under varied environmental condition via development of induced systematic resistance [13, 14]. Most attention has been paid to the role of chitinases in defence against invading fungal pathogens [15]. The early stage of pathogenesis is accompanied by the expression of apoplastic class II chitinases that are responsible for releasing elicitor molecules from invading fungal pathogens [16]. A range of defence compounds, including vacuolar class I chitinases, are then stimulated, and they slow the infection from spreading in the affected plant tissue. Antifungal in vitro tests have revealed higher hydrolytic and antifungal potentials in class I chitinases compared with their class II counterparts [17]. The N-terminal chitin-binding domain (CBD) that occurs in addition to the catalytic domain in plant chitinases of class I and IV are responsible for their greater hydrolytic activity. Broglie et al. [18] first generated transgenic tobacco and canola plants utilizing constitutive expression of the bean CH5B gene. The plants with obviously increased chitinolytic activity (44-fold) showed a delay of disease symptoms caused by *Rhizoctonia solani* infection. Many other studies also showed that mainly the genes for vacuolar class I chitinase genes [19, 20] or their microbial apoplastic chitinase orthologs [10, 21, 22] have the potential to enhance the resistance to fungal pathogens in a range of transgenic crops such as tomato [23], potato [24], cotton [25], wheat [26], rice [27], Indian mustard [28], peanut [29], etc. A comprehensive list of publications dealing with transgenics carrying chitinase genes is provided by review papers [14, 21, 30]. However, disease control has never been complete, while a decrease of symptoms varied with the chitinase transgene and strategy employed as well as with the characteristics of fungal pathogen [30, 31]. The experiments of Dana et al. [10] indicated that two factors were crucial to improving the tolerance of transgenic plants to fungal pathogens when overexpressing chitinase genes—the strength of chitinolytic activity of the transgenic chitinase and its targeting into the apoplast. Such transgenic chitinase(s) fulfil dual roles: inhibiting the growth of invading fungi as well as inducing other defence-related mechanisms. In most plant species, class I chitinases have vacuolar localization; however, carnivorous taxa release chitinases of various classes, including class I, into the apoplast [32, 33].

Our aim here was to investigate the contribution of a novel apoplastic sundew class I chitinase (*DrChit*) [34] as a enhancement to antifungal potential in transgenic tobacco plants. *DrChit* expression in the carnivorous species *Drosera rotundifolia* was detected in tentacles during the digestive processes. Chitinases of several classes have been found in other carnivorous species where they are involved in disrupting the outer chitinous barrier of their captured prey [35, 36].

In this study, the *DrChit* gene under the control of the *CaMV*35S promoter, as well as the gene for neomycin phosphotransferase (*npt*II) driven by the nopaline transferase promoter (*nos*-P), was introduced into the *Nicotiana tabacum* L. via *Agrobacterium*-mediated transformation. Molecular analyses confirmed successful integration and expression of sundew chitinase in regenerated transgenic plants. Subsequently, the *DrChit* expression level among transformants, their chitonolytic activity and the antifungal potential of crude protein extracts from them were evaluated.

Materials and Methods

Plant and Fungal Material

The *Nicotiana tabacum* L. (cv. Petit Havana SR1) plants used for genetic transformation were cultured on MS medium (Duchefa, Netherlands) supplemented with 2% (w/v) sucrose and 0.8% agar at 20 ± 2 °C, with a 16 h photoperiod and light intensity of 50 µmol/m^2/s.

The plant pathogen *Trichoderma viride* CCM F486 (obtained from Czech Collection of Microorganisms, Brno; http://www.sci.muni.cz/ccm/) was used for in vitro antifungal activity experiments. The fungal culture was maintained on potato dextrose agar (Sigma-Aldrich) and incubated in the dark at an ambient temperature of 27 °C. Colonies were subcultured to Sabouraud agar medium (40 g/l glucose, 10 g/l peptone, 20 g/l agar, pH 5.6) before the hyphal extension assay.

Expression of rDrchit in *Escherichia coli*, its purification and detection of enzyme and antifungal activity

For the expression of DrChit gene in bacteria, the vector pET32a-Trx [modified version of pET32a (Millipore) with removed sequence for the 109aa Trx•Tag™ thioredoxin protein] was used. Vector pET32a-Trx (5447 bp) was prepared by ligation of 340 bp SphI–NcoI, 1134 bp NcoI–ScaI and 4003 bp SphI–ScaI fragments; while SphI–NcoI fragment was amplified with FISphI(FOR)–FINcoI(REV) and NcoI–ScaI fragment with FIINcoI(FOR)–FIIScaI(REV) primers on pET32a vector template. SphI–ScaI fragment was isolated from pET32a vector following the digestion with corresponding restriction endonucleases.

Open reading frame of *DrChit* gene lacking the sequence of signal peptide was isolated NcoI and EcoRI fragment from pGEM-T Easy vector [34] and cloned into the pET32a-Trx vector digested with the same restriction enzymes and used for transformation of *E. coli* DH5α. After sequencing

of the *T7/DrChit* expression region of pET32a-TrxDrChit, this plasmid was introduced into *E. coli* BL21-CodonPlus (DE3) RIL expression strain (Agilent). The expression of rDrChit protein was induced by adding 1 mM IPTG to the bacterial culture at OD_{600} of 0.6 and followed by incubation for 3 h at 37 °C. Upon induction of DrChit protein expression by 1 mM IPTG and subsequent purification on Ni–NTA agarose as it was described by Jopcik et al. [34]. Detection of crude protein extracts and purified DrChit protein were analysed on 12% (w/v) SDS-PAGE (Fig. 1). The same protein samples, but without the heat treatment were separated on 12% SDS-PAGE containing 0.01% glycol chitin. After electrophoresis and re-naturation of separated proteins in the solution containing 50 mmol/l sodium acetate (pH 5.2), and 1% Triton, the bands with chitinase activity were detected as dark zones after staining the gel with 0.01% (w/v) Fluoresecent Brightner 28 for 15 min and UV illumination [34]. Subsequently 50 µg of purified rDrChit protein was tested to suppress the growth of *Trichoderma viride* compared to 50 µg BSA and clear buffer used using hyphal extension assay described below. The experiment was performed as a technical triplicate and fungal growth values were expressed as the average area in cm^2.

Construction of the Plant Expression Vector and *Agrobacterium* Transformation

The 1665-bp-long sundew (*Drosera rotundifolia* L.) gene encoding the extracellular chitinase (*Drchit*), which we previously isolated and characterized [34], was amplified from genomic DNA with the gene-specific P1–P2 primers (Table S1 Online resource 1). The PCR reaction mixture of 50 µl contained 200 ng of DNA template, 20 pmol of each primer, 0.2 mM dNTPs, 1 × PCR buffer, 2.5 mM $MgCl_2$, and 2 U DyNAzymeEXT polymerase (Finnzyme, Finland). The first PCR step was performed at 94 °C for 3 min and was followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s. The last step was performed at 72 °C for 10 min. The PCR product was cloned into pCR4-TOPO vector (Invitrogen, Carslad, CA) and subjected to Sanger sequencing (Microsynth, Austria), (pDD1 construct).

The DNA fragment of the *35S* terminator was amplified using the P3–P4 primers (Table S1 Online resource 1). The PCR reaction mixture of 25 µl contained 50 ng of DNA template, 20 pmol of each primer, 0.2 mM, dNTPs, 1 × PCR buffer, 2.5 mM $MgCl_2$, and 2 U DyNAzymeEXT polymerase (Finnzyme, Finland). The first PCR step was performed at 94 °C for 3 min and was followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The final extension was performed at 72 °C for 10 min. The PCR product containing *35S* terminator was digested with XbaI–HindIII and the *DrChit* gene was isolated from the pDD1 construct as a NcoI–XbaI fragment. These were ligated into pSL301 cloning vectors (Invitrogen, Carslad, CA) that were digested with HindIII–NcoI restriction enzymes (pDD2 construct).

Finally, d*CaMV*35S promoter was isolated from pCAMBIA1304 vector [37] as a HindIII–NcoI fragment and *DrChit* gene fused to *35S* terminator was isolated as a NcoI–PacI fragment from pDD2 construct. These were ligated into pBinPLUS [38] digested with HindIII–PacI restriction enzymes (pDD3 construct). Subsequently, the pDD3 construct (Fig. 2) was introduced into *Agrobacterium tumefaciens* strain LBA 4404 and its stability was verified by restriction analysis after re-transformation into *E. coli*.

Plant Transformation Experiments

Agrobacterium tumefaciens strain LBA4404 containing the pDD3 construct was used to transform leaf discs of tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) following the protocol described by Mlynarova et al. [39]. Regenerated shoots were selected on selective shooting medium containing 50 mg/l kanamycin and 500 mg/l cefotaxime (Duchefa, Netherlands). Six weeks after transformation, the shoots

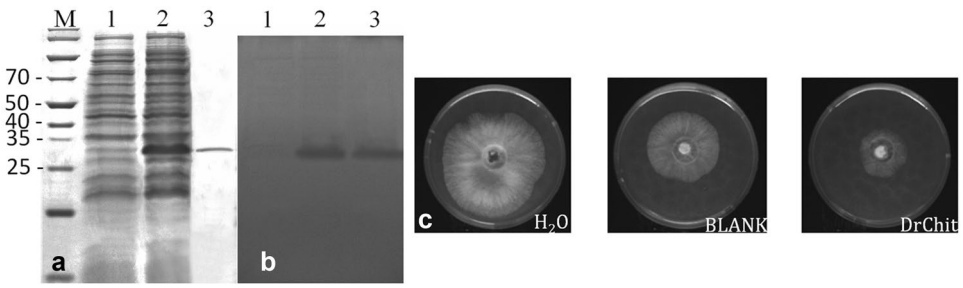

Fig. 1 **a** SDS-PAGE analysis of recombinant DrChit protein expressed in *E. coli* and isolated using His-Tag-based purification. **b** Detection of endochitinase activity in the gel containing glycol chitin as a substrate. After re-naturation and staining the gel with Fluorescent Brightener 28, the bands with chitinase activity appeared as dark zones after UV illumination. 1, total cell proteins from uninduced *E. coli* BL21-CodonPlus (DE3) RIL/pET32a-TrxDrChit; 2, total cell proteins from induced *E. coli* BL21-CodonPlus (DE3) RIL/pET32a-TrxDrChit; 3, Ni–NTA agarose-purified rDrChit protein; **c** Antifungal activity assays. Fifty µg of purified DrChit protein obviously suppressed *Trichoderma viride* growth compared to water and 0.1 M sodium acetate buffer (pH 5.1) (BLANK)

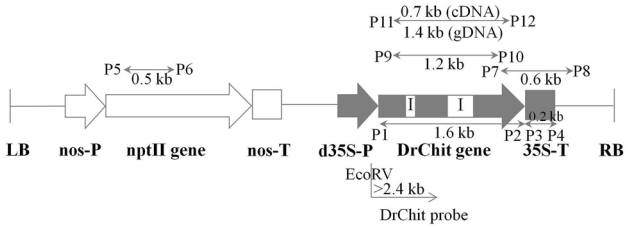

Fig. 2 The T-DNA region of the vector construct pDD3. The gene for *D. rotundifolia* chitinase (*DrChit* gene) was under the control of the double *CaMV35S* promoter (d35S-P) and terminated by the *CaMV35S* terminator (35S-T). The gene for neomycin phosphotransferase (*npt*II gene) was driven by the nopaline transferase promoter (*nos*-P) and *nos* terminator (nos-T). Grey arrows indicate the position of the primers used for PCR analyses. EcoRV denotes the restriction enzyme used for Southern blot analysis as a well as predicted fragment size

were transferred onto solid MS medium supplemented with 20 g/l sucrose, 50 mg/l kanamycin, and 500 mg/l cefotaxime and cultured until roots developed. The transformation efficiency (%) was defined as the total number of transgenic shoots that roosted on MS-20 Km^{50}/total number of explants used × 100. Individual transgenic plants/lines were subcultured onto fresh medium every 6 weeks.

PCR Analysis

To verify the presence of transgenes in the putative DD3 transgenic plants, genomic DNAs were isolated from leaf tissue of individual transgenic lines and non-transgenic controls using the protocol of Chen et al. [40] and were subjected to PCR. Primers P5–P6 and P7–P8 were used to confirm the presence of *nptII* and *DrChit* expression units, respectively (Table S1 Online resource 1, Fig. 2). The 25 µl PCR reaction mixture contained 100–200 ng of DNA template, 10 pmol of each primer, 0.2 mM dNTPs, 1 × PCR buffer, 2.5 mM $MgCl_2$, and 1 U FIREPol Taq DNA polymerase (Solis BioDyne, Estonia). The first PCR step was performed at 94 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 40 s. The last step was performed at 72 °C for 10 min. Finally, the PCR products were separated on a 1% (w/v) agarose gel and visualized with ethidium bromide staining.

Southern Blot Analysis

Genomic DNA weighing 10 microgrammes was digested with the restriction enzyme EcoRV, separated on a 1% (w/v) agarose gel, and blotted by capillary transfer with 20 × SSC on a positively charged nylon membrane (Roche Applied Science, Germany). A *DrChit*-specific probe was prepared using PCR with the P9–P10 primer set (Table S1 Online resource 1) and non-radioactively labelled using the DIG Probe Synthesis Kit (Roche, Roche Applied Science, Germany). Hybridization was performed on DIG easy Hyb hybridization solution (Roche, Applied Science, Germany) at 42 °C according to the manufacturer's instructions. Hybridization signals were visualized by DIG Nucleotic Detection Kit (Roche Applied Science, Germany).

RT-PCR

Total RNA was isolated from leaves of in vitro cultivated transgenic plants and non-transgenic controls using the RNeasy kit, (Qiagen, Germany) and was digested with DNase (Life Technologies, USA) according to manufacturer´s instructions. The integrity of RNA was checked on a 1% (w/v) agarose gel and RNA quantification was performed using a BioSpec-nano spectrophotometer (Shimadzu, Japan). First-strand cDNA was synthesized using the Maxima First strand cDNA Synthesis kit for RT-qPCR (Thermo Fischer Scientific, USA) and the DNA removal step was included. RT-PCR was performed with the primer pairs P11–P12 (sundew chitinase, KU516826) and P13–P14 (tobacco actin, XM_016618658.1) (Table S1 Online resource 1), while the programme involved one cycle at 95 °C 3 min, 35 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 40 s, and a final step at 72 °C for 7 min.

RT-qPCR

RT-qPCR was performed using the Luminaris HiGreen qPCR Master Mix (Thermo Fischer Scientific, USA) according to manufacturer´s recommendation with the primers P15–P16 (tobacco actin, XM_016618658.1) and P17–P18 (sundew chitinase, KU516826) (Table S1 Online resource 1). 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. The reaction proceeded for 40 cycles at 95 °C for 15 s and 60 °C for 60 s, and was completed with a melting curve analysis step to confirm the specificity of amplified products. A LigthCycler Nano (Roche Applied Science, Germany) was used for qPCR. Experiments were performed in biological triplicate (three independent isolations, reverse transcriptions, qPCR reactions for each plant) and technical duplicate, and the threshold Ct value was set according automatic calling methods and melting curve analysis of the associated software. The Pfaffl method was employed to process the relative gene expression data [41]. Briefly, values were expressed as a ratio between the target (transgenic *chitinase*) and a housekeeping gene (*actin*). The correct primer efficiency (*E*) was calculated using a standard curve which was obtained from qPCR reactions using 5 point tenfold diluted cDNA as a template. The target gene expression ratio was then calculated with the Pfaffl equation: $\text{ratio} = (E_{\text{target}})^{\text{Cttarget}} / (E_{\text{actin}})^{\text{Ctactin}}$.

The Endochitinolytic Activity Assay

The endochitinolytic activity of crude protein extracts isolated from individual transgenic plants and non-transgenic controls was measured by fluorometric assay with *N*-fluorescein-labelled chitin (FITC-chitin) [42]. Briefly, leaves of individual plants were homogenized with the aid of liquid nitrogen and extracted using 0.1 M sodium acetate buffer (pH 5.2). Following a 10 min centrifugation at 4 °C, the proteins present in the cleared protein lysates were quantified according to Bradford [43] against a BSA calibration curve. The enzymatic reaction mixtures were 200 µl and consisted of 10 mg of FITC-chitin dissolved in the 0.1 M sodium acetate buffer (pH 5.2) and 10 µg of the individual protein extracts. The reaction mixtures were incubated at 37 °C and continuously shaken for 90 min. After centrifugation for 1 min, 100 µl of supernatant (without the sedimented pellet) was transferred into a new microcentrifuge tube with 400 µl 0.5 M Tris–HCl (pH 8.9) mixed and centrifuged for 15 min at 15,000 rpm. Next, three 10 µl aliquots of the upper phase—as technical replicates—were tenfold diluted in 0.5 M Tris–HCl (pH 8.9) and transferred into 96-well black-sided assay plates and measured on a Synergy™ H1 microplate reader (BioTek, Winooski, VT, USA) using 490/520 nm excitation and emission filters, gain sensitivity of 120, and probe distance of 4.75 mm. As in the RT-qPCR, three biological replicates of each sample were analysed. Chitinolytic activity values were expressed as an average of the relative fluorescent units normalized to the blank (reaction without enzyme).

Hyphal Extension Assay

Using a hole drill, a 9.8-mm-diameter hole was pierced in the centre of Petri dishes that were filled with 20 ml Sabouraud agar medium. The bottoms of the holes were sealed with melted medium to create central cistern. A 2.8-mm disc of 4 day-old *Trichoderma viride* mycelium (previously grown on the same medium) and 0.1 M sodium acetate buffer (pH 5.1) containing 100 µg of analysed protein extract were added to the cisterns. Clear buffer or 100 µg BSA, respectively, were added as controls. The Petri dishes were incubated for 36 h at 27 °C. Fungal growth was documented, and subsequently, photographs were analysed with ImageJ [44] by manually marking the mycelial area. The experiment was performed as a technical triplicate, and fungal growth values were expressed as the average area in cm^2.

Statistical Analysis

For the statistical comparison of RT-qPCR, chitinolytic activity and fungal growth area among the individually analysed plants, the online Kruskal–Wallis rank sum test was used (http://astatsa.com/KruskalWallisTest/). For post hoc pairwise comparison of individual's pairs, the Connover method was used with *p* values adjustment by family-wide error rate (FWER) method of Holm and the superior false discovery rate (FDR) method of Benjamini-Hochberg.

Results

Expression of rDrChit in *E. coli* Protein Against *Trichoderma viride* Detected in In Vitro Test

To detect the antifungal activity of the DrChit protein, the open reading frame of *DrChit* gene without the putative signal peptid was PCR-amplified, cloned into a pET32a-Trx vector and introduced into *E. coli* BL21-CodonPlus (DD3) RIL strain. SDS-PAGE analysis of protein extract from IPTG-induced bacterial culture revealed the presence of a predominant band of approximately ~ 32 kDa corresponding to recombinant DrChit protein (Fig. 1a, lane 2). Following the purification on Ni–NTA agarose and the purified fraction was detected at the position corresponding to the overexpressed protein (Fig. 1a, lane 3). Moreover, the samples of total *E. coli* protein extracts containing rDrChit protein as well as purified rDrChit protein alone showed chitinolytic activity in PAGE containing glycol chitin as a substrate (Fig. 1b lanes 2, 3).

In hyphal growth inhibition assay, 50 µg of purified rDrChit protein effectively inhibited the growth *T. viride* compared to control used (Fig. 1c). These results showed that purified sundew chitinase protein of ~ 32 kDa with detected chitinolytic activity exhibits obvious antifungal potential.

Generation of Transgenic Tobacco Plants

The vector construct pDD3 was prepared by inserting of the *CaMV 35S* promoter with a double enhancer fused to the 1.6 kb genomic clone of sundew chitinase and the *CaMV35S* terminator into the binary vector pBinPLUS [38]. To facilitate selection of plant transformants the T-DNA contains kanamycin resistance gene (*npt*II) (Fig. 2).

Tobacco leaf explants were successfully transformed using *A. tumefaciens* strain LBA 4404 harbouring the pDD3 construct, producing kanamycin resistant shoots 4–6 weeks after cocultivation. All putative transgenic shoots developed roots in the presence of 50 mg/l kanamycin, while the morphological appearance did not differ from non-transformed control plants. In total, we generated 55 transgenic plants in two sets of transformation experiments, separated by 18 months' time. Transformation efficiency was similar and ranged from 59 to 61%. In total, 14 plants, 1–7 and 8–14 from first and second transformation experiments,

respectively, were subjected to detailed molecular, biochemical and antifungal analyses.

Molecular Characterization of Transgenic Plants

The transgenic character of all regenerated plants was verified on genomic DNA template by PCR. The expected PCR products of 500 bp and 630 bp length were identified with the P5–P6 and P7–P8 primers and corresponded to the *nptII* and *DrChit–35S-T* sequences, respectively (Figs. 2, 3).

Following transgene verification, the transgenic plants (1–14) and non-transgenic controls were analysed for copy number determination. EcoRV digestion of plant DNA and probing with the *Drchit* probe resulted in the hybridization of the RB junction fragments longer than 2.4 kb (Figs. 2, 4). Except for transgenic plant 8, all analysed plants contained one or two copies of the *DrChit* gene (Fig. 3). However, in case of the transgenic plant 7, one incomplete T-DNA copy presented problems with *DrChit* transgene expression.

The expression of the *DrChit* transgene was investigated using RT-PCR. The P11–P12 primers enabled amplification of a 725 bp stretch of cDNA that ensured detection of nearly the full chitinase transcript, while the same primers amplified 1413 bp fragment on the genomic DNA template. The cDNA quality of individual samples was assessed using the P13–P14 primers that yielded 510 bp fragment of the actin reference gene. Similarly, *DrChit* RT-PCR products of the expected size were amplified from all of the analysed lines, except for line 7 (Fig. 5). The lack of a RT-PCR product from transgenic line 7 was a consequence of this line lacking a complete copy of the *DrChit* gene. Sequencing revealed that line 7 contained a deletion at the 5´end of *DrChit* gene, including the sequence annealing location of the P11 primer (data not shown).

Quantification of Transgene Gene Expression Level

The expression level of the *DrChit* transgene driven by the double *CaMV35S* promoter was conducted using RT-qPCR analysis on mRNA isolated from leaves of fourteen transgenic lines (coming from both sets of transformants). *DrChit* expression varied substantially among individual transgenic

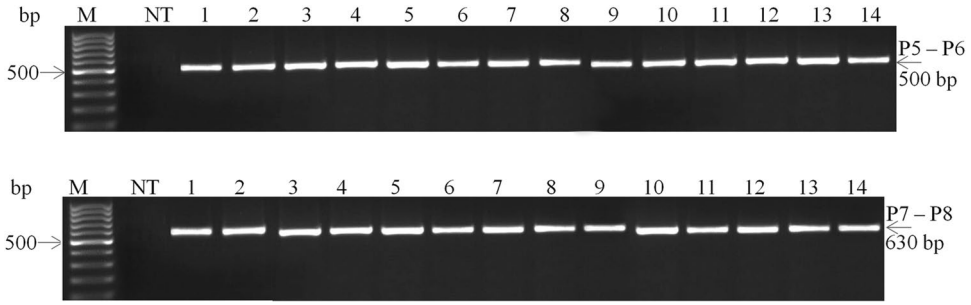

Fig. 3 PCR verification of transgenes in DD3 plants. PCR reaction was carried out on genomic DNA with P5–P6 and P7–P8 primers and yielded the 500 bp *nptII* and the 630 bp *DrChit–35S-T* amplicons, respectively. *Lane M*—100 bp GeneRuler (Thermo Fisher Scientific, USA); *NT* non-transformed plant; *lanes* 1–14 represent the PCR products of individual transgenic plants

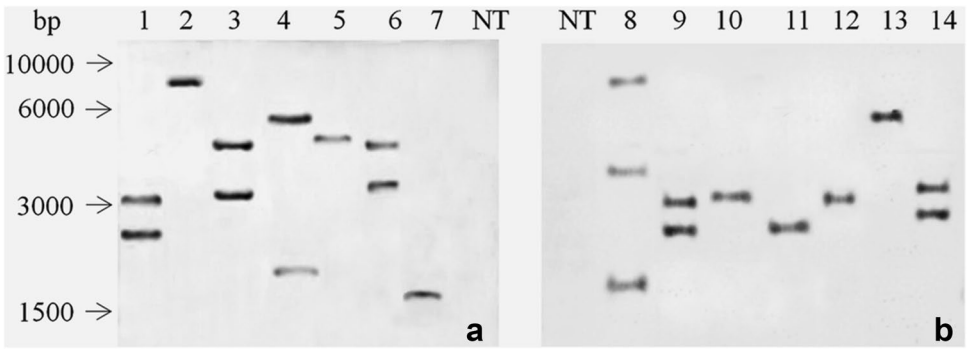

Fig. 4 Southern blot analysis with EcoRV-digested DNA from transgenic DD3 tobacco plants. The blot was hybridized with a 1.2 kb DIG-labelled fragment containing the *DrChit* gene as a probe. All bands (> 2.4 kb correspond to right border fragments and indicate number of independent transgene copies. Lanes labelled 1–7 and 8–14 represented transgenic plants from the first (A) and second (B) transformation experiments, respectively. Lane NT was the non-transgenic control

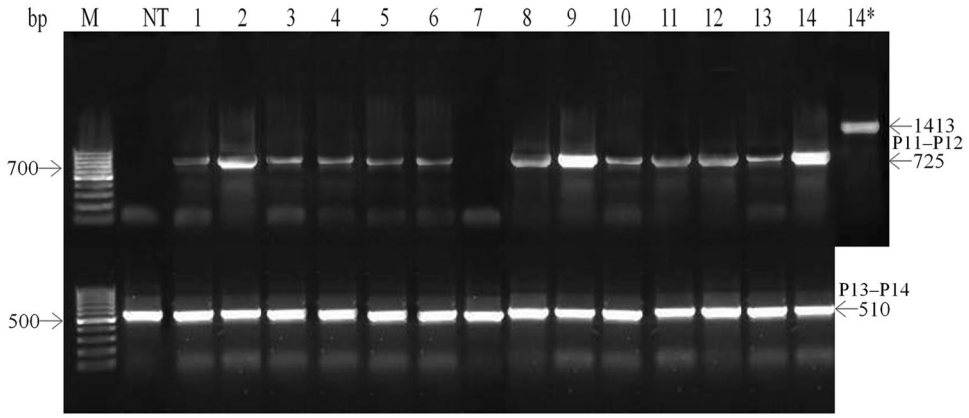

Fig. 5 RT-PCR product analysis from tobacco lines (1–14) carrying *DrChit* transgene. Expression of the *DrChit* transgene was assessed with the P11–P12 primers yielding 725 bp fragments from cDNA and 1413 bp from genomic DNA (lane 14*). Tobacco *actin* was used as an endogenous control, and it exhibited the expected size of 510 bp with the P13–P14 primers. Lane M was the 100 bp Gene Ruler (Thermo Fisher Scientific, USA) and lane NT was the non-transgenic control

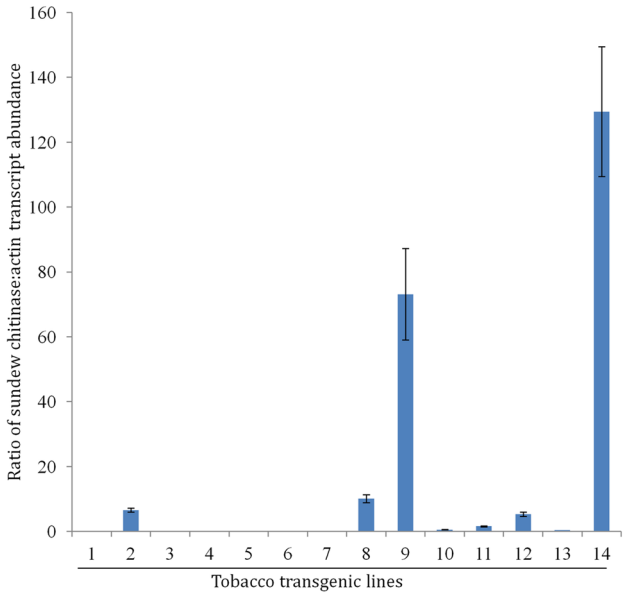

Fig. 6 Relative expression levels of the *DrChit* gene in transgenic tobacco lines (1–14). The relative expression values were calculated with the Pfaffl method as the ratio between transcript abundance of the target *DrChit* transgene and the endogenous *actin* gene control. Normalization was performed-based on amplification efficiency results from a 5 point 10 fold dilution standard curve (actin: E-1.9300, $R^2 = 0.9993$; chitinase: E-1.8870, $R^2 = 0.9984$). Error bars are standard deviation calculated from three independent biological samples, each with technical duplicates (Table S2 Online resource 1). Similarity matrix between the individual plants is available in Table S5 Online resource 1

lines. A large group of transgenic lines (1, 3, 4, 5, 6, 7, 10, 13) had *DrChit* transcript levels comparable or lower than the levels of the *actin* transcript (Fig. 6). A relatively low expression of the *DrChit* gene, but higher than *actin* expression, was observed in transgenic lines 2, 8, 11, and 12. In contrast, transgenic tobacco lines 9 and 14 had high *DrChit* transcript abundance, equalling 72 and 152 times higher than their *actin* transcript abundance, respectively. Lines 9 and 14 were suitable candidates for analysing the effect of *DrChit* gene expression in tobacco with respect to its antifungal potential.

Chitinolytic Activity of Crude Protein Extracts

Chitinolytic activity assays were used to investigate the contribution of the DrChit enzyme to the chitinolytic activity of crude protein extracts from transgenic lines and non-transgenic controls. Crude protein extracts of each tested plant (10 µg) were incubated with FITC-chitin, and the fluorescence of soluble FITC-labelled chitooligosaccharides was detected at 90 min. Transgenic lines (1, 3, 4, 5, 6, 7, 10, 13) with extremely low *DrChit* expression exhibited chitinolytic activity comparable with non-transgenic controls (CP1–CP3) or only slightly higher than the controls (line 11) (Fig. 7). Lines 2, 8 and 13 had chitinolytic activity of 2.5–2.9 times higher than that of the control. Transgenic line 9 and line 14 had chitinolytic activities nearly five and eight times greater, respectively, than that of the non-transgenic controls. Statistical analysis with Kruskal–Wallis test confirmed differences of lines 2, 8, 9, 12 and 14 from each of non-controls at significance of $p < 0.01$ (Table S3, Table S6 Online resource 1).

In Vitro Antifungal Activity Assay

Antifungal activity assays were used to determine whether the overexpressed *DrChit* gene in transgenic tobacco plants had the ability to increase their antifungal potential.

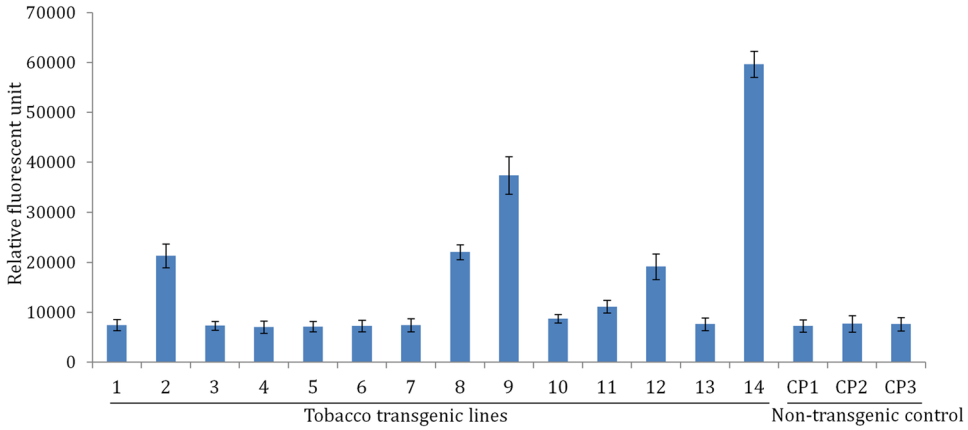

Fig. 7 The chitinolytic activity assays of crude protein extracts from transgenic tobacco lines (1–14) expressing the *DrChit* gene, and of non-transgenic controls (CP1–CP3) against FITC chitin. The measurements were performed with three biological and the *small bars* represent standard deviation (Table S3 Online resource 1). The tobacco lines 2, 8, 9, 12 and 14 significantly differed from each of non-transgenic controls at $p < 0.01$. Similarity matrix between the individual plants is available in Table S6 Online resource 1

Crude protein extracts (100 µg) from transgenic tobacco plants were dissolved in sodium acetate buffer and tested against *Trichoderma viride*. Protein extracts from non-transgenic plants (CP1–CP3) in sodium acetate buffer, and 100 µg of BSA in sodium acetate buffer were used as controls. *Trichoderma viride* had the greatest growth in the presence of BSA and protein extracts from transgenic and non-transgenic control plants retarded fungal growth compared to BSA. Mycelium size varied when we evaluated the influence of protein extracts from individual transgenic plants and non-transgenic controls on the growth of *T. viride*. Chitinolytic activity was not the only factor influencing antifungal potential of crude protein extracts.

Protein extracts from transgenic plants (2, 8, 9, 12, 14) with the greatest *DrChit* gene expression had the greatest inhibitory potential among the transgenic plants and significantly differed ($p < 0.01$) from non-transgenic controls. The greatest ability to suppress *T. viride* growth was with the protein extract from line 14 (Figs. 8, 9) and mycelium growth with this extract only reached 45.2% of the area of non-transgenic control. Transgenic lines 9, 8, and 2 followed, with 56.4%, 63.8%, and 65.5% of the area of the control, respectively. All of these lines (2, 8, 9, and 14) exhibited obvious *DrChit* gene expression and increased chitinolytic activity. Extracts of remaining transgenic lines did not exhibit statistically different growth inhibition relative to the non-transgenic controls ($p < 0.01$).

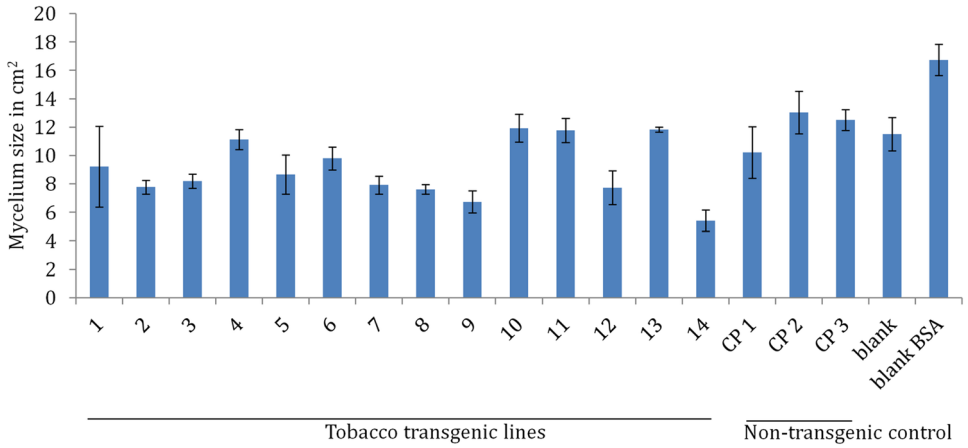

Fig. 8 The effects of 100 µg of crude protein extracts isolated from individual transgenic tobacco lines (1–14) and non-transformed controls (CP1–CP3) on the growth *Trichoderma viride* grown on Sabouraud agar in Petri dishes. As controls, sodium acetate buffer (blank) and 100 µg BSA (blank BSA) were tested. Standard deviations indicated by bars were calculated from technical triplicates (Table S4 Online resource 1). Similarity matrix between the individual plants is available in Table S7 Online resource 1

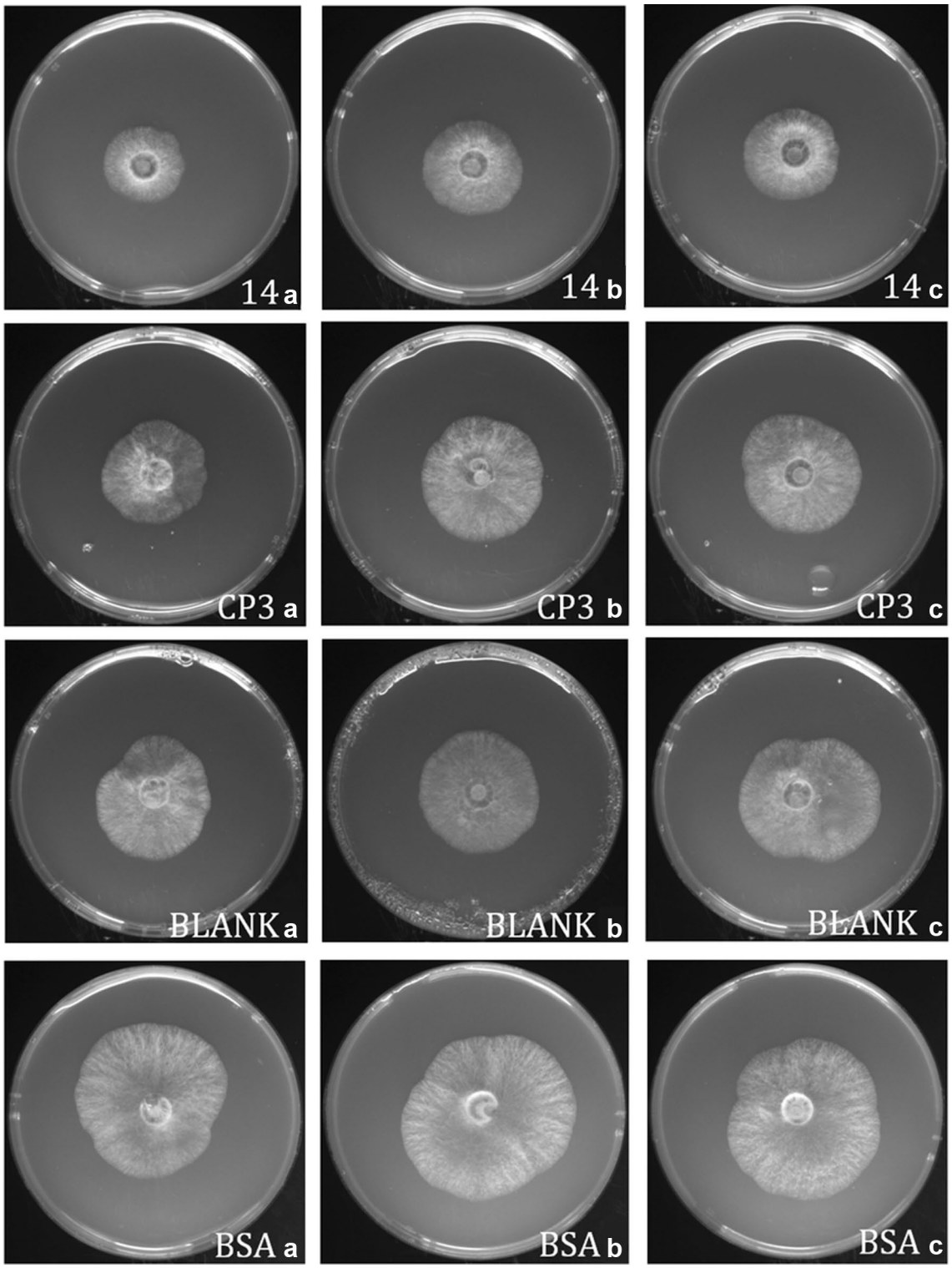

Fig. 9 Antifungal activity assays. The growth *Trichoderma viride* in the presence of 100 µg of crude protein extract from transgenic line 14, which had the highest chitinolytic activity (14 **a**–**c**), 100 µg crude protein extract from non-transgenic control plant 3 (CP3 **a**–**c**), 0.1 M sodium acetate buffer (pH 5.1) used for protein extraction (BLANK **a**–**c**), and 100 µg BSA dissolved in acetate buffer (BSA **a**–**c**). The fungal growth values were expressed as the average area size in cm^2 after 36 h-incubation at 27 °C

Discussion

Increasing plant tolerance to fungal pathogens through the expression of heterologous genes, whose products have antifungal activity, has been reported previously [21, 45]. However, there is a constant need to achieve broad-spectrum resistance to several phytopathogens, necessitating continued testing of new genes from different genetic sources.

Here, we tested a chitinase isolated from the carnivorous plant species *Drosera rotundifolia.* Our previous work found that a class I chitinase from *D. rotundifolia* with substrate specificity for long chitin polymers actively participates in cell wall degradation of captured insect prey [34]. In addition to a catalytic domain, class I chitinases contain a chitin-binding domain that enhances their antifungal potential. When gene for an antimicrobial peptide (an alfalfa defensin) was fused to the rice chitinase CBD using a helix-forming linker and introduced in tobacco, transgenic plants exhibited enhanced resistance to *Fusarium solani* [46]. Our experiments showed that DrChit protein overexpressed in *E. coli* expression system and purified on Ni–NTA agarose has obvious potential to suppress the growth of *T. viride* tested in hyphal extension assay. Following the generation of intact transgenic tobacco plants, fourteen randomly selected individuals were subjected to detailed molecular and biochemical analyses. Southern blot analysis indicated that most of these plants contained one or two copies of the sundew chitinase transgene. Except of one transgenic line, all tested plants yielded RT-PCR products with the P11–P12 primers. Although T-DNA insertion into the plant genome is often accompanied by small or large rearrangements at junction sites [47], deletions in transgenes or regulatory sequences were also reported [48, 49]. Except for one line, all contained at least one complete T-DNA copy since RT-PCR confirmed the expression of a functional *DrChit* gene.

The expression level of *DrChit* was analysed using a RT-qPCR approach. Only six out of 13 RT-PCR positive plants exhibited *DrChit* expression higher than the reference gene (*actin*). The considerable variability of transgene expression that is often observed within a population of transgenic plants transformed with the same transgene construct [29, 50, 51] is attributable to the position effect, transgene copy number, and various epigenetic silencing phenomena [52]. In addition, *CaMV 35S* promoter was reported to frequently yield a bimodal expression pattern in populations of transformants that is characterized by a limited number of plants with high transgene expression, but the majority of transformants with very low expression levels [50, 53].

Nevertheless, the transgenic lines 9 and 14 exhibited 72 and 152 times higher *DrChit* transgene expression than that of the internal control. Both high-expression transgenic lines had high chitinolytic activity, as measured 90 min after incubating their crude protein extracts with FITC-chitin. The suitability of FITC-chitin as a substrate for the DrChit enzyme was tested in a previous study [34] and the purified protein exhibited long oligomer-specific endochitinase activity on glycol chitin and FITC-chitin, but not short oligomer-specific endochitinase [substrate 4MU-$(GLCNAC)_3$] or chitobiosidase [substrate 4MU-$(GLCNAC)_2$] activities.

Enzymatic activity difference among plants with significant *DrChit* expression and non-transgenic control plants was observed only after protein extracts and substrate were incubated for 90 min. It is unclear why increase in fluorescence was not detectable after 30 or 60 min. In this fluorometric assay, the fluorescence signal is a function of soluble FITC-chitooligomers resulting from tobacco as well as sundew chitinolytic activities. Since we do not know the length of FITC-chitooligomers resulting from DrChit activity, we suppose that it was a mixture of long soluble and insoluble FITC-chitooligomers. However, increased fluorescence after 90 min of incubation may result from subsequent conversion of insoluble long FITC-chitooligomers (product of sundew chitinase) into soluble FITC-chitooligomers by tobacco endochitinases. A second explanation takes into account the different kinetic properties of sundew chitinase and tobacco chitinases. The fluorimetric method using FITC-chitin as a substrate for determining endochitinase activity [34] in plant extracts is a very rapid and more sensitive method relative to the turbidimetric and viscosimetric methods [54, 55].

Finally, crude protein extracts from transgenic and non-transgenic plants were tested for their ability to inhibit the growth of *Trichoderma viride* hyphae in in vitro assays. To evaluate correctly the contribution of DrChit endochitinase in the protein extracts, several controls were also included. As expected, *T. viride* exhibited the highest growth rate in the presence of BSA. The buffer (nutrient-free control) had a comparable influence on fungal growth than crude protein extracts from non-transgenic plants, suggesting that crude protein extracts from non-transgenic plants contained proteins of nutritional character and antifungal proteins, including chitinolytic enzymes in balance, resulting in no obvious effect on fungal growth. Transgenic plants had variable sundew chitinase expression resulting in variable fungal growth retardation. Unlike the clear association between *DrChit* expression and chitinolytic activity, mycelium size did not always correspond to high or low *DrChit* expression or chitonolytic activity. For example, one line (11) had only slightly increased *DrChit* transgene expression compared to the reference gene, slightly increased chitinolytic activity, but no increased antifungal activity, suggesting that it was insufficient for suppression of fungal growth. In contrast, a clear association between

hyphal growth inhibition and *DrChit* expression, as well as chitinolytic activity, was reported for two transgenic lines (9 and 14). These lines exhibited 72 and 152 times higher *DrChit* transcript abundance than *actin* transcript level, and their protein extracts significantly inhibited fungal growth relative to the control by 44 and 55%, respectively.

This study demonstrates that an endochitinase enzyme that is natively involved in the digestive processes of the carnivorous plant, *Drosera rotundifolia* has the potential to inhibit growth of plant phytopathogens with chitin in their cell walls. Optimal evaluation of antifungal effect of transgene protein in plants includes both, in vitro as well as in vivo assays. The former responds the question if tested hydrolytic enzyme suitably complements modes of actions of endogenous plant hydrolytic enzymes required for maximum efficacy [56], while quantification of obtained data represents the advantage of this approach. As in vitro assay, this study also confirmed the strong correlation between the fungal growth inhibition level and endochitinase activity of crude protein extracts from transgenic tobacco plants; in vivo testing of other transgenic valuable crops against significant pathogens can follow. The determination of disease symptoms in in vivo tests is performed on ordinal scale, while (hidden) factors of the environment can complicate the final evaluation. Therefore, confirming of antifungal activity in in vitro test can be helpful, when the plant–pathogen interaction is evaluated in in vivo tests.

Conclusions

This is the first time an endochitinase natively involved in the digestive processes of the carnivorous plant, *Drosera rotundifolia*, was tested in transgenic plants with the aim of exploring its antifungal potential. Our results showed that the constitutive expression of sundew extracellular class I chitinase in tobacco resulted in enhanced antifungal potential of crude protein extracts against *Trichoderma viride*. This work and further research will probably add the sundew chitinase gene to the list of chitinases that are useful in genetic manipulation strategies.

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Author contributions JL and MJ and designed research. DD, MJ, MR and JM conducted the experiments. MJ analysed data. JL wrote the manuscript.

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