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IN GEL DETECTION OF A HIS-TAGGED TRANSGENE FOLLOWING THE SEPARATION OF CRUDE PLANT PROTEIN EXTRACTS WITH SDS PAGE

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ABSTRACT

In the present study, we visualised sundew His_6-tagged chitinase protein in crude protein extracts of transgenic tobacco plants following protein separation with sodium dodecyl sulfate polyacrylamide gel electrophoresis by detecting chitinase activity as well as the His-tag. A short sequence encoding six histidines was fused downstream of the DNA sequence encoding the last amino acid of the mature protein. Following binary vector construction and plant transformation, a set of 10 transgenic plants was analysed for transgene expression. Except for one, all transgenic plants exhibited the presence of sundew chitinase protein of ~52 kDa, which was different from the calculated molecular weight of ~32 kDa. Clear identification of DrChitHis protein was performed with a Ni^{2+}:NTA complex conjugated to a fluorescent dye and visualized using light laser-based scanner. A subsequent endochitinolytic activity assay using a N-fluorescein-labelled chitin substrate confirmed that the two transgenic lines with the strongest expression of DrChitHis protein had endochitinolytic activity 6.4 and 6.7 times higher than non-transgenic control.

Keywords: Chitinase, chitinolytic activity, *Drosera rotundifolia*, FITC-chitin, His-tag detection, transgenic plants

INTRODUCTION

Plant transgenic technology has the potential to enhance the nutritional value as well as biotic and abiotic stress resistance to valuable agricultural crops **(Halpin, 2005, Soliman *et al.* 2017)**. The production of genetically modified plants involves the introduction of new-protein coding transgenes. Western blotting and enzyme-linked immunosorbent assay (ELISA) technologies are widely used to detect and quantify transgenic proteins in plants. The crucial component for both methods is an antibody with high specificity and affinity for the target molecule (**Markoulatos *et al.*, 2004**). Detection of recombinant protein is greatly simplified by engineering the DNA construct so that the encoded protein contains a C-terminal, N-terminal, and an internal small peptide tag that should interfere with the fused protein. The polyhistidine–tag (His-tag) **(Porath *et al.*, 1975)** belongs to the group of commonly used peptide tags, while His-tagged protein antibodies are directed against the His-tag itself **(Zentgraf *et al.*, 1995; Debeljak *et al.*, 2006)** or His-tag staining in gel is employed **(Bitner *et al.*, 2008)** for transgenic protein detection. The latter method implies a method where the crude protein extracts are separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and subsequent coupling of Ni^{2+} ions and an oligohistidine domain, while the Ni^{2+} ions are part of the Ni^{2+}:nitrilotriacetic acid (Ni^{2+}:NTA) complex that conjugates to a fluorescent dye.

One biotechnological strategy includes transfer of genes for chitinases with strong antifungal activity into target plant genomes to fortify plant defence against fungal diseases **(Kamo *et al.*, 2016; Khan *et al.*, 2017; Durechova, 2015)**. Chitinases (EC 3.2.2.14) are synthesised in a wide range of organisms, including higher plants, where they occur in large gene families. For example, the *Arabidopsis thaliana* genome contains 25 chitinase genes **(Passarinho and de Vries, 2002)** that perform distinct functions in plant biological processes, such as plant growth and development **(Kasprzewska *et al.*, 2003; Ohnuma *et al.*, 2015)** or responses against biotic and abiotic stresses **(Gerhardt *et al.*, 2004; Takenaka *et al.*, 2009; Albanchez *et al.*, 2018)**. In the special case of carnivorous plants, specific chitinases are involved in insect prey digestion **(Rottfold *et al.*, 2011; Jopcik *et al.*, 2017)**.

In our experiments we focused on introducing a chitinase from the carnivorous plant *Drosera rotundifolia* into tobacco (*Nicotiana tabacum*) plants to test for transgene expression and subsequent chitinolytic activity that may fortify the plants' defence arsenal against fungal pathogens containing chitin in their cell walls. Chitinase expression is detectable following crude protein extracts isolation from plants and their separation with SDS-PAGE using gels containing glycol chitin that acts as a substrate. Visualisation of bands with chitinase activity is conditioned with a renaturation step. Detection of transgenic chitinase may represent a problem if the transgenic chitinase on the gel overlaps with endogenous chitinase(s) or if the position of the transgenic chitinase on the gel does not correspond to the calculated molecular weight due to post-translational modifications. When transgenic chitinase is fused to the six-histidine tag (His_6-tag), the his-tagged protein is specifically visualized with a fluorescent dye conjugated to the Ni^{2+}:NTA complex. The fluorescent band(s) is/are subsequently detected using UV light or a laser-based scanner.

In this study, the *DrChit* gene **(Jopcik *et al.*, 2017)** driven by *CaMV*35S was introduced into *Nicotiana tabacum* L. plants *via Agrobacterium*-mediated transformation. Polymerase chain reaction (PCR) confirmed the integration of the chitinase transgene into the genome of regenerated transgenic tobacco plants. Sundew chitinase protein was detected in crude protein extracts isolated from individual transgenic plants following separation with SDS-PAGE and was subsequently visualised using the InVision™ His-tag In-gel Stain. Finally, the chitinolytic activity in individual transgenic lines and in a non-transgenic control was evaluated.

MATERIAL AND METHODS

Vector construct preparation and plant transformation

A plant transformation vector, pDrChiHis, was constructed as a derivate of the pDD3 vector and was successfully used in previous experiments for tobacco transformation (**Durechova, 2015**). The cloning strategy with the pDrChiHis vector is based on ligation of three fragments, (1) HindIII–NsiI involving the double *CaMV*35S (d*CaMV*35S) promoter and the 5´-chitinase gene sequence; (2) NsiI–XbaI fragment carrying the 3´-chitinase sequence terminated by a His_6-tag and XbaI restriction site; and (3) XbaI–PacI fragment carrying the *35S* terminator sequence, into pBinPLUS **(Vanengelen *et al.*, 1995)** digested with HindIII–PacI restriction enzymes. The first and third fragments were isolated from the pDD3 binary vector and the second fragment was amplified on pDD3 template with primers FORNsiI (5´-TTTGTATGCATCCGAATGATAATGA-3´) and REVHisXbaI (5´-CGGACTCTAGAAGTGATGGTGATGGTGATGAAAAGGACGCTGATTATAGCAGTC-3´). The PCR product carried 3´- chitinase sequence with the His_6-

tag sequence inserted upstream of triplet encoding the last amino acid S_{325}. In addition, last triplet tct encoding S_{325} was replaced by ttc encoding Phe for the purpose of cloning (XbaI site). The presence of the His_6-tag within the amplified DNA sequence was verified by sequencing.

Subsequently the pDrChiHis binary vector was introduced into *Agrobacterium tumefaciens* LBA4404 and a bacterial suspension was used to transform leaf discs of tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) following the protocol described by **Mlynarova *et al*. (1994)**. Regenerated shoots were selected on shooting medium containing 50 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime (Duchefa, The Netherlands). Six weeks after transformation, the shoots were transferred onto the solid Murashige and Skoog (MS) medium (Duchefa, The Netherlands) supplemented with 20 g l^{-1} sucrose, 50 mg l^{-1} kanamycin, and 500 mg l^{-1} cefotaxime and cultured until roots developed.

Verification of the transgenic character of regenerated plants

To verify the presence of transgenes in the putative DrChiHis transgenic plants, genomic DNA was isolated from leaf tissue of individual transgenic lines and a non-transgenic control using the DNeasy Plant Mini kit (Qiagen, Germany) and subjected to PCR. To confirm the presence of the *DrChit* expression, P1 (5´-TCGCTGATCGAATTGGTTTCTA-3´) and P2 (5´-TTTGTGAACCATCACCCAAATC-3´) primers were used. The 25 µl PCR reaction mixture contained 100–200 ng of DNA template, 10 pmol of each primer, 0.2 mmol l^{-1} dNTPs, 1× PCR buffer, 2.5 mmol l^{-1} $MgCl_2$, and 1 U FIREPol Taq DNA polymerase (Solis BioDyne, Estonia). The first PCR step at 94 ºC for 2 min was followed by 35 cycles at 94 ºC for 30 s, 58 ºC for 30 s, and 72 ºC for 40 s. The final extension step was performed at 72 ºC for 10 min. The PCR products were separated on a 1 % (w/v) agarose gel and visualized after staining with ethidium bromide.

Crude protein extract analysis with SDS PAGE

Al proteins were extracted from transgenic plants and a non-transgenic control **(Hurkman and Tanaka, 1986)**. Aliquots (30 µg) were mixed with 2x loading buffer (0.09 M Tris·Cl, pH 6.8; 20 % glycerol; 2 % SDS; 0.02 % bromophenol blue; and 0.1 M DTT), heated at 100 °C for 5 min, and separated on 1.5 mm thick 12.5 % minigels (Mini-Protean Cell apparatus, Bio-Rad, USA) according to **Laemmli (1970)**.

Chitinase activity assay on gels

The same protein samples as analysed above, but without the heat treatment were separated on 12.5 % PAGE gels containing 0.01 % glycol chitin prepared according to the protocol described by **Trudel and Asselin (1989)**. After electrophoresis and re-naturation of the separated proteins in a solution containing 50 mM sodium acetate (pH 5.2) and 1 % Triton, the bands with chitinolytic activity were detected as dark zones after staining the gel with 0.01 % (w/v) Fluorescent Brightener 28 for 15 min and illuminating with UV light **(Pan *et al*., 1991; Zur *et al*., 2013)**.

Fluorescent detection of the His_6-tag sequence of DrChitHis transgene protein

After separation of crude protein extracts with SDS-PAGE, the gels were fixed in 200 ml of solution containing 10 % acetic acid and 50 % methanol for 1 hour, washed twice with ultra pure water for 10 min, and incubated in 25 ml of the InVision™ His-tag In-gel Stain (Thermo Fisher Scientific, USA) for 1 hour. Finally, the gel was washed twice with 200 ml 20 mmol l^{-1} phosphate buffer, pH 7.8, for 10 min and analysed using the Typhoon FLA 9500 laser-based scanner (GE Healthcare, USA) with a 532 nm green laser (excitation source) and LPG filter (575 nm long pass emission filter).

Endochitinolytic activity assay

The endochitinolytic activity of crude protein extracts isolated from individual transgenic plants and a non-transgenic control was measured with a fluorometric assay using N-fluorescein-labelled chitin (FITC-chitin) **(Tikhonov *et al*., 2004)**. Briefly, leaves of the individual plants were homogenized with liquid nitrogen and extracted using 0.1 mol l^{-1} 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 (1976)** against a BSA calibration curve. The 200 µl enzymatic reaction mixtures consisted of 10 mg of FITC-chitin dissolved in the 0.1 mol l^{-1} sodium acetate buffer (pH 5.2) and 20 µg of protein extract. The reaction mixtures were incubated at 37 °C with continuous shaking for 120 min. After centrifugation for 1 min, 100 µl of supernatant (without disturbing the pellet) was transferred into new a new 1.5 ml centrifuge tube with 400 µl 0.5 mol l^{-1} 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 ten fold diluted in 0.5 mol l^{-1} Tris-HCl (pH 8.9) and transferred into 96-well black-sided assay plates and measured in a Synergy™ H1 microplate reader (BioTek, Winooski, VT, USA) using excitation (490 nm) and emission (520 nm) filters with a gain sensitivity of 120 and probe distance of 4.75 mm. Three technical replicates of each sample were analysed and chitinolytic activity values were expressed as an average of relative fluorescent units normalized to a blank.

RESULTS

Vector construction, tobacco transformation, and molecular characterisation of transgenic tobacco plants

The vector construct pDrChiHis was prepared by inserting a 1978 bp HindIII–NsiI fragment including the d*CaMV*35S promoter and 5´-sundew chitinase gene sequence, a 465 bp NsiI–XbaI fragment carrying the 3´-sundew chitinase sequence terminated by a His_6-tag, and a 224 bp XbaI–PacI fragment carrying the *35S* terminator sequence into a pBinPLUS binary vector (Figure 1A). The recombinant plasmid with the correct restriction profile, tested using Acc65I–XbaI, HindIII–XbaI, and PacI restriction enzymes (Figure 1B), was introduced into *A. tumefaciens* LBA 4404 strain and used for plant transformation.

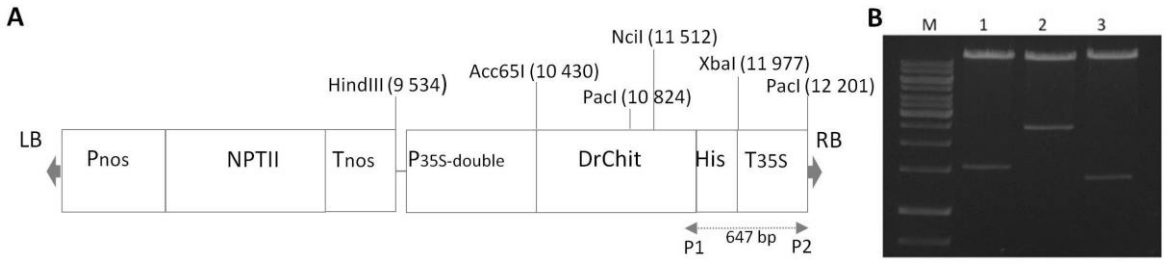

Figure 1 A) Schematic diagram of the T-DNA region of the binary vector pDrChiHis used for tobacco transformation. Abbreviations are RB – right border, LB – left border, Pnos – nopaline synthase promoter, NPTII – neomycin phosphotransferase gene, Tnos – nopaline synthase polyadenylation signal, P35S-double – double enhanced cauliflower mosaic virus (*CaMV*35S) promoter, His – His_6-tag sequence, and T35S – *CaMV*35S polyadenylation signal ***B.*** Restriction analysis of pDrChiHis plasmid. The pDrChiHis vector construct was cut in lane 1 with Acc65I–XbaI, in lane 2 with HindIII–XbaI, and in lane 3 with PacI to produce fragments of length 1547, 2443, and 1377 bp, respectively. Lane M contains a 1 kb DNA ladder (Fermentas).

Following the transformation experiment, thirty tobacco plants were regenerated on media containing growth regulators and kanamycin as a selection agent. The individual transgenic plants were maintained under *in vitro* conditions and ten were subjected to further analyses.

The presence or absence of the sundew chitinase gene in individual transgenic plants and a non-transgenic control was evaluated with PCR using the primers P1 and P2. PCR analysis amplified the predicted 647 bp DNA *DrChitHistag-35S-T* fragment encoding 3´- chitinase sequence with the His_6-tag, 35S terminator, and the 5´-sequence of the right border (RB) (Figure 2). Neither fragment was amplified in the control plant.

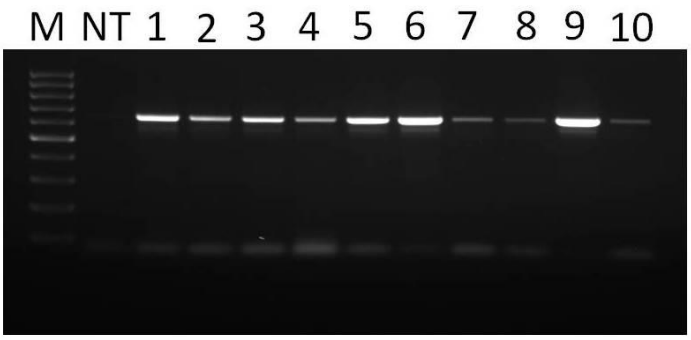

Figure 2 PCR verification of the DrChiHis plants transgenic character. PCR on genomic DNA with P1 and P2 primers yielded the 647 bp *DrChitHistag-35S-T* amplicon. Lane M contains a 100 bp GeneRuler (Thermo Fisher Scientific, USA), NT is the non-transformed plant, and lanes 1–10 contain the PCR products of individual transgenic plants.

Expression of sundew chitinase gene in tobacco

Protein separation with SDS-PAGE was performed to evaluate the protein profiles of crude protein extracts isolated from transgenic plants with integrated sundew chitinase and the non-transgenic control. The *DrChitHis* transgene open reading frame consisted of 996 nucleotides that encoded 331 amino acids. The mature protein (without the first 20 amino acids) had a predicted molecular mass of 32.7 kDa and a theoretical isoelectric point of 7.55.

As seen in Figure 3A, an ~32 kDa band solely in transgenic protein extracts was not detected with SDS-PAGE analysis. However, a specific band of ~ 52 kDa was detected in the protein extracts of transgenic lines 1, 2, 5, 9, and 10. Band of weaker intensity corresponding 52 kDa occurred in samples of remaining transgenic plants, except for line 4, where was not clearly distinguishable from the non-transgenic control.

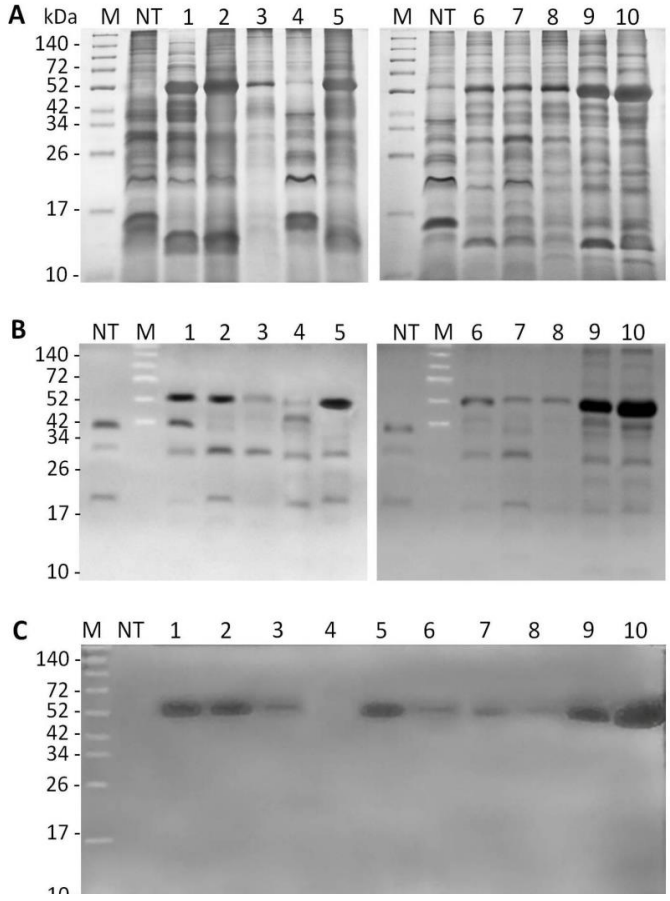

Figure 3 Analyses of crude protein extracts from DrChiHis transgenic tobacco plants and a non-transgenic control: (A) SDS-PAGE analysis, (B) SDS-PAGE with glycol chitin to detect separated proteins with chitinolytic activity, and (C) fluorescent detection of the DrChitHis transgene protein His_6-tag sequence following separation with SDS-PAGE. Lanes labelled M contain the Spectra™ Multicolor Broad Range Protein Ladder (Thermo Fisher Scientific, USA), those labelled NT contain protein extracts from non-transformed plant, and lanes 1–10 contain protein extracts from individual transgenic plants.

Glycol chitin was incorporated into an SDS-PAGE gel in order to identify bands that had chitinolytic activity. The protein extracts isolated from transgenic plants and the non-transgenic control exhibited a spectrum of proteins with chitinolytic activity (Figure 3B). However, the protein detected as an ~52 kDa band with obvious expression in lines 1, 2, 5, 9, and 10 with standard SDS-PAGE (Figure 3A) exhibited very strong chitinolytic activity on the gel that contained glycol chitin (Figure 3B). This band was not found in the non-transgenic control or transgenic line 4. With varying intensity, the remaining transgenic lines each exhibited a band at this position.

DrChitHis expression was definitively confirmed in transgenic tobacco plants by detecting the His_6-tag sequence found at the 3′-end of the DrChit recombinant protein with the InVision™ His-tag In-gel Stain kit. We elected for C-terminal His-tagging since the N-terminus of DrChit contains a 20 amino acid signal peptide that is removed during protein maturation while the His-tag remains as part of the mature protein. Fluorescent bands were detected in all transgenic lines except for line 4 and the non-transgenic sample at the ~52 kDa position using a laser-based scanner (Figure 3C).

Chitinolytic activity of crude protein extracts

Chitinolytic activity assays were used to investigate the contribution of the DrChit enzyme to the overall chitinolytic activity of crude protein extracts from DrChiHis transgenic lines. Crude protein extracts of each tested plant (20 μg) were incubated with FITC-chitin and the fluorescence of soluble FITC-labelled chitooligosaccharides was measured after 120 min incubation (Figure 4). Several transgenic lines (3, 4, 6, 7, 8) exhibited chitinolytic activity comparable or only slightly greater than the non-transgenic control. Transgenic lines 1, 2, and 5 had chitinolytic activity 2.4–2.7 times higher that the non-transgenic control. Transgenic line 9 and line 10 had chitinolytic activity nearly 6.4 and 6.7 times greater, respectively, than the non-transgenic control.

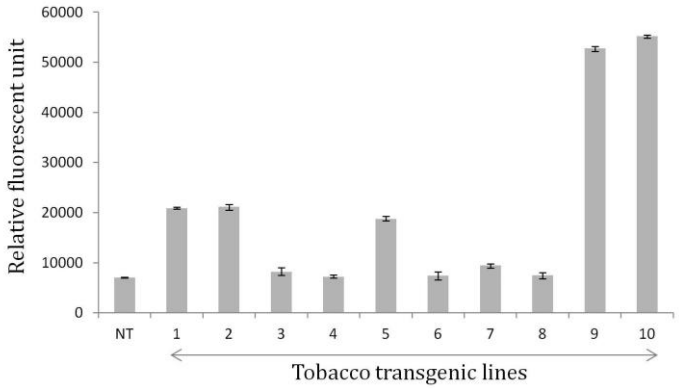

Figure 4 The chitinolytic activity of crude protein extracts from DrChiHis transgenic tobacco plants (1–10) expressing a sundew chitinase gene and a non-transgenic control (NT) was assayed against FITC chitin. The error bars represent standard deviation of measurements performed on three technical replicates.

Signals from chitinolytic activity on SDS-PAGE with glycol chitin (Figure 3B) and His_6-tag signals with SDS-PAGE (Figure 3C) were roughly correlated with the chitinolytic activity of individual transgenic lines (Figure 4). In addition, the transgenic lines 1, 2, 5, 9 and 10 with relatively strong band intensities had high chitinolytic activity. In contrast, half of transgenic tobacco plants had low (lines 3, 6, 7, and 8) or silenced (line 4) transgene expression.

DISCUSSION

In a previous study we introduced *DrChit* into *E. coli*, BL21-CodonPlus (DE3) RIL expression strain, and the over-expressed chitinase protein was detected on a SDS-PAGE gel as a band of the calculated molecular weight ~32 kDa **(Jopcik *et al.*, 2017)**. However, in this study the *DrChit* gene fused to a short His-tag sequence and introduced into tobacco plants was detected as a transgenic protein of much higher molecular weight (Figure 3).

Vincenzi and Curioni (2005) demonstrated that glycol chitin incorporated into SDS-PAGE gels had a retarding effect on the migration of chitinases, but only when the samples did not contain a reducing agent. They were of the opinion that un-reduced chitinase may interact *via* a chitin-binding domain with the substrate resulting in retarded migration on SDS-PAGE gels. However, the protein samples in this study were treated with 0.05 M dithiotreithol (DTT) as a reducing agent in the loading buffer.

Most of the genes for plant chitinases encode proteins with theoretical molecular weights of 25–35 kDa. Despite this, plant crude protein extracts separated on SDS-PAGE gels and analysed for chitinolytic activity also contain chitinolytic enzymes of greater molecular mass **(Mészáros *et al*., 2013; Zur *et al*., 2013)**. In the case of the sundew chitinase, differences between its theoretical and in-gel-detected molecular weight can be attributed to post-translational modifications or (hetero)dimer formation with another protein. Our previous study revealed potential phosphorylation and glycosylation sites within the DrChit amino acid sequence **(Jopcik *et al*., 2017)**. However, post-translational modification of chitinases typically results in minor differences from the predicted molecular weight **(Zha *et al*., 2016)**. When a band of 95 kDa with chitinolytic activity was analysed by mass spectrometry, the dimeric structure consisted of endo- and exo-chitinase **(Shoresh and Harman 2010)**. Nevertheless, further analyses that include mass spectrometry are needed to fully understand the migrational shift of transgenic DrChitHis protein on SDS-PAGE gels.

Clear identification of the DrChitHis protein *via* chitinolytic activity as well as His-tag detection on SDS-PAGE gels indicated obvious transgene variability in the set of transgenic plants analysed. Considerable variation of transgene expression is frequently observed within populations of transgenic plants transformed with the same transgene construct **(Butaye, *et al*., 2005)**. In addition, the double *CaMV*35S promoter has the tendency to increase the variability in gene expression between individual transgenic plants more the other constitutive promoters **(Benyon *et al*., 2013; Erpen *et al*., 2018)**.

For final confirmation of DrChitHis protein presence we used in-gel staining of the His-tagged fusion protein with a fluorescent dye. Following crude protein separation with SDS-PAGE, DrChitHis was easily distinguishable from the weak background corresponding to non-specific proteins. Staining of DrChitHis protein with the InVision™ His-tag In-gel Stain was considered sufficiently conclusive and quick, and eliminated the need for conventional Western blotting.

CONCLUSION

In this study, we identified the transgenic DrChitHis protein in crude protein extracts from tobacco plants with two different methods. Following the separation of protein extracts with SDS-PAGE gels containing glycol chitin, the sundew chitinase protein of ~52 kDa was detected in all transgenic plants except for one. The same pattern of transgene visualisation was observed when the proteins separated with SDS-PAGE were analysed using the InVision™ His-tag In-gel Stain that detected the His_6-tag sequence. Fluorometric assays of endochitinolytic activity measured with N-fluorescein-labeled chitin confirmed that the protein samples from transgenic plants with strong fluorescent signals on SDS-PAGE gels exhibited much higher endochitinolytic activity that the non-transgenic control. Transgenic plants with strong chitinolytic activity will be included in future studies testing for resistance against fungal pathogens containing chitin in their cell wall.

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