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Full Length Article

Transient Expression of *Chi42* Genes from *Trichoderma asperellum* in *Nicotiana benthamiana* by Agroinfiltration

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Abstract

The present study reports the transient expression of *chi42* genes encoding 42 kDa chitinase from *T. asperellum* SH16 in *N. benthamiana* via agroinfiltration. The efficacy of agroinfiltration for *chi42* genes including a wild-type gene (*Chi42*) and two synthetic genes (*syncodChi42-1* and *syncodChi42-2*) was determined. Accordingly, coinfiltration of two vectors pMYV719 carrying one of three genes *chi42* and pMYV508 carrying gene *p19* expedited the higher expression of recombinant enzymes whose genes were optimized for codon usage in plant tissues. The highest chitinolytic activity of about 290 U/mL was found in plants containing the gene *syncodChi42-2* after 7 days of injection, 1.7 and 2.6 times higher than that of genes *syncodChi42-1* and *chi42*. Recombinant chitinase has also exhibited activity against the pathogenic fungus *Sclerotium rolfsii* under *in vitro* condition. A higher expression level of *syncodChi42-2* gene in *N. benthamiana* and its antifungal activity promise potential applications in the field of transgenic crops against phytopathogenic fungi. © 2021 Friends Science Publishers

Keywords: Agroinfiltration; 42 kDa chitinase; *chi42* gene; Transient expression; *Trichoderma asperellum*

Introduction

Agroinfiltration is a simple method of gene transfer by either syringe infiltration or vacuum infiltration of *Agrobacterium tumefaciens* or plant virus harbouring the target gene to monitor transient expression of this gene in plants (Leuzinger *et al.* 2013; Del Toro *et al.* 2014). The method commonly applied in agroinfiltration is the use of a needleless syringe to inject *Agrobacterium* into the underside of leaves (Santi *et al.* 2008). Syringe infiltration has been optimized for several plant species (Wroblewski *et al.* 2005) and has demonstrated several critical advantages (Chen *et al.* 2013). This method is considered as an alternative to stable transformation for large-scale production of proteins, enzymes and biopharmaceuticals (Chen and Lai 2015; Goulet *et al.* 2019).

Agroinfiltration studies have helped to better understand some biological processes such as gene expression, role of the promoters, interactions between proteins, function of proteins and metabolisms in plants (Chen *et al.* 2013; Guy *et al.* 2016). Currently, the agroinfiltration method is being applied for model plants (*e.g.*, *N. benthamiana* Domin, *Arabidopsis thaliana* Heynh., tomato (*Lycopersicon esculentum* Mill.) and tobacco (*Nicotiana tabacum* L.)) and for crop plants (*e.g.* soybean (*Glycine max* Merr.), onion (*Allium cepa* L.), cowpea (*Vigna unguiculata* Walp.), grapevine (*Vitis vinifera* L.), rice (*Oryza sativa* L.), cacao (*Theobroma cacao* L.) and common bean (*Phaseolus vulgaris* L.)) (Shamloul *et al.* 2014; Suzaki *et al.* 2019). The agroinfiltration efficiency depends on different plant species, high levels of expression of the target gene could be obtained in *N. benthamiana* and *N. tabacum* while no such results were found in hemp and many other species (Deguchi *et al.* 2020). Agroinfiltration is usually carried out on the underside of the leaves, but in some cases, the thick epidermis limits the success of this method as *A. tumefaciens* cannot infect the leaf cells (King *et al.* 2015).

Chitinase (E.C 3.2.2.14) is enzyme that degrades chitin which is a primary constituent of fungal cell walls and exoskeletons of some animals such as insects and crustaceans (Sámi *et al.* 2001). Different chitinases were found in many fungal species of *Trichoderma* and used for biological control due to their mycolytic activity (Mohamed *et al.* 2010; González *et al.* 2012; Aoki *et al.* 2020). Besides, fungal chitinase genes were also used for improving resistance of plants to pathogenic fungi with the help of

genetic manipulations (Lorito *et al.* 1998; Emani *et al.* 2003; Limón *et al.* 2004; Khan *et al.* 2012, 2017a).

In the present study, two synthetic genes (*syncodChi42-1* and *syncodChi42-2*, NCBI: MT083802 and MT083803) encoding the 42 kDa chitinase, which were optimized plant codon usage from the wild-type *chi42* gene (*chi42*, NCBI: HM191683) of *T. asperellum* SH16, were agroinfiltrated into leaf of *N. benthamiana*. This optimization was expected to result in higher expression of chitinase in transgenic plants. The high expression levels of the two synthetic *chi42* genes and the antifungal activity of the recombinant plant chitinase obtained from this study promise potential applications in the field of transgenic plants against phytopathogenic fungi.

Materials and Methods

Plant material

The leaves of six-week-old *N. benthamiana* plants raised in the plant growth chamber (JEIOTECH GC-1000TLH, Korea) at a temperature of 24ºC and under a light intensity of 3000 lux with 12 h daylight used to transiently express *chi42* genes via agroinfiltration. After *Agrobacterium* infiltration, plants were grown under the similar condition.

Binary vector

The *chi42* genes consisted of a wild-type gene (*chi42*) (Loc *et al.* 2011) and two synthetic genes (*syncodChi42-1* and *syncodChi42-2*) (Luong *et al.* 2021) containing *Xba*I and *Sac*I ends inserted into the same sites of the plant expression vector pMYV719 by removing the CTB-L-S1D segment (Fig. 1). CTB-L-S1D is a fusion protein of cholera toxin (CTB) protein and S1D epitope consisting of a marker peptide (L) employed to analyze the expression of CTB protein in another study (Huy *et al.* 2016). The pMYV508 vector harboring *p19* gene (Fig. 2) was used in combination with the pMYV719/*chi42* vector during gene transfer to enhance *chi42* expression in *N. benthamiana*. Both pMYV719 and pMYV508 vectors were provided by Prof. Yang Moon-Sik (Chonbuk National University, Republic of Korea).

Infiltration of *Agrobacterium*

Triparental mating between *A. tumefaciens* LBA4404, *Escherichia coli* containing pMYV719/*chi42* and *E. coli* containing helper plasmid pRK2013 was performed according to Van Haute *et al.* (1983). Recombinant *A. tumefacaciens* LBA4404 containing pMYV719 vector harboring one of three *chi42* genes and *A. tumefacaciens* LBA4404 containing pMYV508 vector harboring *p19* gene, a suppressor of silencing gene of tomato bushy stunt virus (TBSV), were cultured in 5 mL YEP (yeast extract peptone) medium (Muli *et al.* 2017) containing 50 μg/mL kanamycin

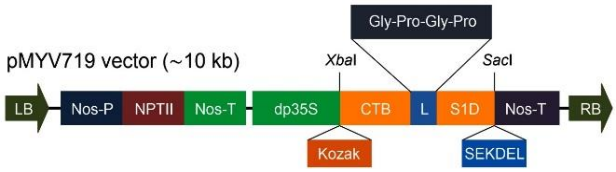

Fig. 1: Vector pMYV719. LB: left border, RB: right border, S1D: S1D epitope, CTB: cholera toxin B subunit, dp35S: duplicated CaMV 35S promoter, Nos-T: terminator of nopaline synthase gene, Nos-P: promoter of nopaline synthase gene, Kozak: consensus sequence, SEKDEL: *sequence* Ser-Glu-Lys-Asp-Glu-Leu has been shown to be a signal which leads to retention of at least two proteins in the endoplasmic reticulum, NPTII: neomycin phosphotransferase II gene, L: sequence Gly-Pro-Gly-Pro. The CTB-L-S1D sequence including the Kozak and SEKDEL sequences will be removed from the vector by *Xba*I and *Sac*I. *Chi42* genes will be then inserted into vector at the same sites

Fig. 2: pMYV508 vector. RB: right border, CaMV: cauliflower mosaic virus, Hyg^R: hygromycin resistant gene, pDu35S: duplication of CaMV 35S promoter, p19: a gene-silencing suppressor gene, LB: left border, pVS1 StaA: **s**tability protein from the plasmid pVS1 that is essential for stable plasmid segregation in *Agrobacterium*, pVS1 repA: replication protein from the plasmid pVS1 that permits replication of low-copy plasmids in *Agrobacterium*, pVS1 oriV: origin of replication from the plasmid pVS1 that permits replication of low-copy plasmids in *Agrobacterium*, Bom: basis of mobility region from pBR322, Ori: high-copy number origin of replication, Kan^R: kanamycin resistant gene

and 100 μg/mL rifampicin for 2 days at 28°C in dark with a shaking speed of 200 rpm. The culture was centrifuged at 6000 rpm for 5 min to harvest bacterial biomass, then resuspensed by MES buffer (10 m*M* 2-[N-morpholino] ethanesulfonic acid, 10 m*M* $MgSO_4$, pH 5.5) to an OD600 of 0.8−1. The suspension was then supplemented with 200 μM acetosyringone, followed by incubation in dark for 1−2 h and finally injected in the abaxial side of the leaves. After 3−7 days of injection, leaves were used for further analysis.

PCR amplification

Chi42 genes were amplified with specific primers (Table 1). PCR components (for a total volume of 20 μL) consist of 100 ng of DNA template, 10 pmol of each primer and 1 μL of (2×) PCR Master Mix (Thermo Fisher Scientific). PCR was performed as follows: a genomic denaturation of 95°C for 10 min; followed by 30 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min; finally, an extension of 72°C for 10 min.

Western blot analysis

Leaf samples (approx. 0.5 g) from the agroinfiltrated plants

Table 1: Nucleotide sequence of specific primers for PCR amplification of the genes *chi42*, syncodChi42-1 *and* syncodChi42-2

Primers	Nucleotide 5'- 3'	Expected size of amplicons (kb)
syncodChi42-F	GCGCTCTAGAAAAACTAAAAGTAGAAG (27 mer)	~1.3
syncodChi42-R	GCGCGAGCTCTTAATTCAAACCAGAT (26 mer)	
chi42-F	GCGCTCTAGAAAAACTAAAAGTAGAAG (27 mer)	
chi42-R	GCGCGAGCTCTTAGTTGAGACCGCTT (26 mer)	

The nucleotide sequence of primer binding regions in the genes syncodChi42-*1* and syncodChi42-*2* are the same, so they share a pair of primer syncodChi42-F and syncodChi42-R. TCTAGA and GAGCTC are recognition sites of *Xba*I and *Sac*I, respectively

were ground in liquid nitrogen and extracted with 1 mL of phosphate buffer (pH 7). An aliquot (50 μg) of total soluble protein (TSP) from the extract determined by the Bradford protein assay (1976), was denaturated at 95°C for 10 min before performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 50 V for 90 min for stacking gel and then 80 V for 120 min for separating gel. After the separation, one of the two gels was stained with Coomassie blue while the second gel was blotted onto nitrocellulose membranes (Novex™ - Thermo Fisher Scientific) using Western Blot Transfer Buffer at 145 mA for 3 h on mini-transblot (Bio-Rad, USA).

The blots were firstly treated with 5% skim milk in Tris buffered saline with Tween 20 (TBST) solution (Sigma-Aldrich) at 37ºC for 1 h with a gentle shaking for blocking non-specific linking. The blots were then washed three times with TBST for 15 min each and incubated with first antibody (mouse anti-Ta-CHI42 polyclonal antibody) (Luong *et al.* 2021) which was diluted 1:2000 in TBST at 37°C for 2 h with gentle shaking.

The blots were washed three times with TBST for 15 min each, and then incubated again with second antibody (goat anti-mouse IgG antibody conjugated with alkaline phosphatase, AbD Serotec - currently Bio-Rad Antibodies) which was diluted 1:5000 at 37°C for 2 h with gentle shaking. After washing three times with TBST and once with Tris-$MgCl_2$-NaCl buffer (TMN: 100 m*M* Tris base pH 9.5, 5 m*M* $MgCl_2$ and 100 m*M* NaCl), the blots were developed with 5-bromo, 4-chloro, 3-indolylphosphate (BCIP)/nitro-blue tetrazolium (NBT) solution (Sigma-Aldrich, Cat No B6404) for 3 min in the dark. The intensity of the Western blot signal was measured using ImageJ software (V 1.52v).

Chitinase assay

The chitinolytic action was preliminary evaluated by loading TSP (crude CHI42 enzyme extracted from *N. benthamiana* leaves) into a hole on 1.5% agar plate containing 1.2% colloidal chitin as substrate. The agar plate was then kept at 4ºC for 8 h to diffuse the enzyme, followed at 28ºC for 6 h to hydrolyze chitin, and finally stained with 0.1% Lugol's solution (Calissendorff and Falhammar 2017) for detection of hydrolysis. Colloidal chitin was prepared according to Murthy and Bleakley (2012).

Chitinase activity was then determined spectrophotometrically at 420 nm (Tsujibo *et al.* 1998) with pNpGlcNAc (Merck) as a substrate. Fifty μg of TSP was added to 15 μL of 2.5 m*M* pNpGlcNAc (4-nitrophenyl N-acetyl-β-D-glucosaminide) and the enzymatic reaction was performed at 45°C for 10 min, then terminated with 1 mL of 0.2 M sodium carbonate. One unit of chitinase is defined as the amount of enzyme required to release 1 μmol *p*-nitrophenol from pNpGlcNAc per min. *p*-nitrophenol purchased from Merck was used to plot the standard curve.

In vitro assay for antifungal activity

Fungal strain *S. rolfsii* was provided by Department of Plant Protection, Hue University, Vietnam. The effect of CHI42 on the growth of *S. rolfsii* was investigated on 1/2 potato dextrose (PD) solid and liquid medium containing 10–60 U/mL of enzyme and about 10^4 fungal spores that cultured at 28°C for 36 h. After centrifuging at 4000 rpm for 5 min, the mycelium biomass from the liquid culture was washed with ddH_2O to determine fresh weight, followed by drying at 65°C to a constant mass to determine dry weight.

Statistical analysis

The experiments were designed in completely randomized design. All observations were repeated three times. The data are expressed as the means, the one-way ANOVA was conducted based on Duncan's test (*p*-value at 0.05) to compare the statistically significant difference of the means by SPSS software.

Results

Expression of *chi42* genes in agroinfiltrated *N. benthamiana*

PCR amplification of three *chi42* genes (*chi42*, *syncodChi42-1* and *syncodChi42-2*) in agroinfiltrated *N. benthamiana* plants found DNA bands of approximately 1.3 kb in size (Fig. 3). Both types of transgenic plants (infiltration and coinfiltration) displayed specific DNA bands of *chi42* genes on electrophoretic image. These results confirmed that *chi42* genes were successfully transferred into leaves.

SDS-PAGE and Western blot analysis was conducted to determine the expression of *chi42* genes in agroinfiltrated *N. benthamiana* plants. Protein bands and signals of antigen-antibody interaction with an expected molecular weight of approximately 42 kDa were found on the gels and the blots

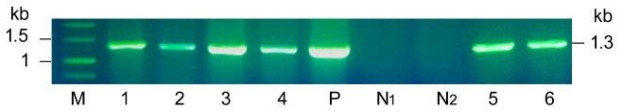

Fig. 3: PCR amplification of *chi42* genes from transgenic *N. benthamiana*. 1 and 2: infiltration and coinfiltration of *syncodChi42-1* gene, respectively. 3 and 4: infiltration and coinfiltration of *syncodChi42-2* gene, respectively. 5 and 6: infiltration and coinfiltration of *chi42* gene, respectively. P: pUC vector containing *chi42* gene as positive control. N1 and N2: wild-type *N. benthamiana* and agroinfiltrated *N. benthamiana* without *chi42* insert as negative controls, respectively

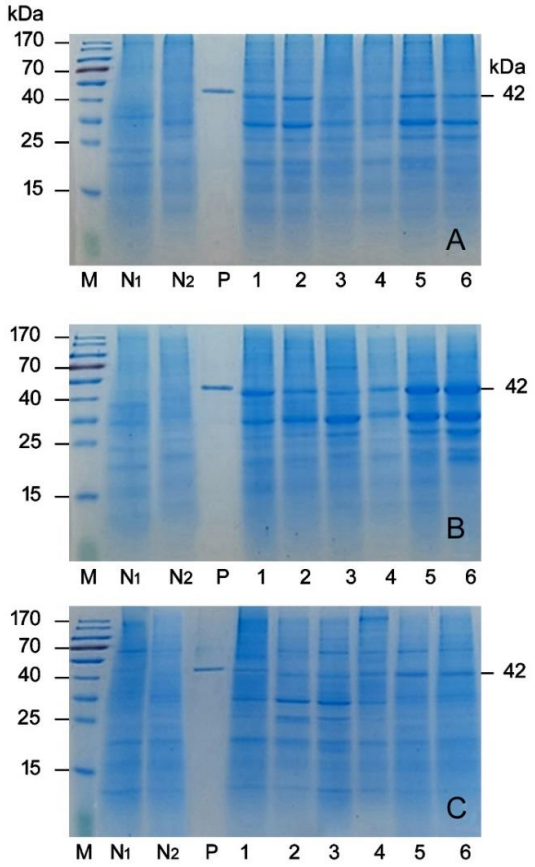

Fig. 4: SDS-PAGE analysis for *chi42* genes were agroinfiltrated in *N. benthamiana*. (A): *syncodChi42-1*, (B): *syncodChi42-2* and (C): *chi42*. M: Protein molecular weight marker (Thermo Scientific). P: purified bacterial CHI42 enzyme as positive control. N1 and N2: wild-type *N. benthamiana* and agroinfiltrated *N. benthamiana* without *chi42* insert as negative controls, respectively. 1-3: after 3, 5 and 7 days of infiltration (pMYV719/*chi42*), 4-6: after 3, 5 and 7 days of coinfiltration (pMYV719/*chi42* and pMYV508)

in positive control and transgenic plants (Fig. 4–5). Analysis of signal intensities from Western blot showed that the expression levels of *chi42* genes decreased over time from day 3 to day 7 after agroinfiltration with the single vector. In using the vector mixture, the expression levels of synthetic

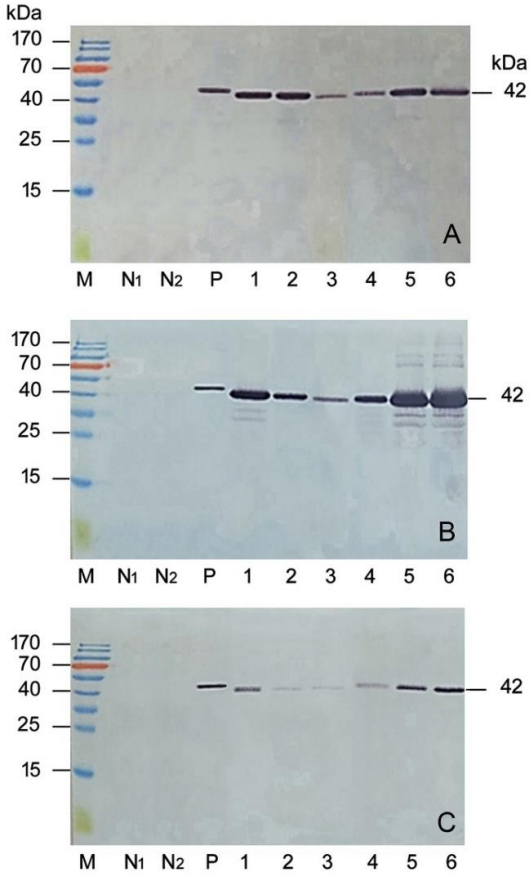

Fig. 5: Analysis of Western blot for *chi42* genes were agroinfiltrated in *N. benthamiana*. (A): *syncodChi42-1*, (B): *syncodChi42-2* and (C): *chi42*. M: Protein molecular weight marker (Thermo Scientific). P: purified bacterial CHI42 enzyme as positive control. N1 and N2: wild-type *N. benthamiana* and agroinfiltrated *N. benthamiana* without *chi42* insert as negative controls, respectively. 1-3: after 3, 5 and 7 days of infiltration (pMYV719/*chi42*), 4-6: after 3, 5 and 7 days of coinfiltration (pMYV719/*chi42* and pMYV508)

chi42 genes increased from day 3 to day 7 (*chi42* and *syncodChi42-2*) or day 5 (*syncodChi42-1*), later descending in the following days. In general, the use of vector mixture only increased the expression of *chi42* and *syncodChi42-2* genes in *N. benthamiana*. The highest intensities of the Western signals for *syncodChi42-1* gene in *N. benthamiana* plants agroinfiltrated with single vector and vector mixture were insignificantly different (Fig. 6). Generally, the two synthetic *chi42* genes were suitable for plant expression, especially the *syncodChi42-2* gene which showed significantly higher levels of expression in Western blot.

Chitinolytic activity of CHI42

Chitinolytic action of CHI42 enzyme from transgenic *N. benthamiana* was preliminary evaluated by agar plate containing colloidal chitin substrate. The results in Fig. 7A indicated that the difference of the diameter of the clear

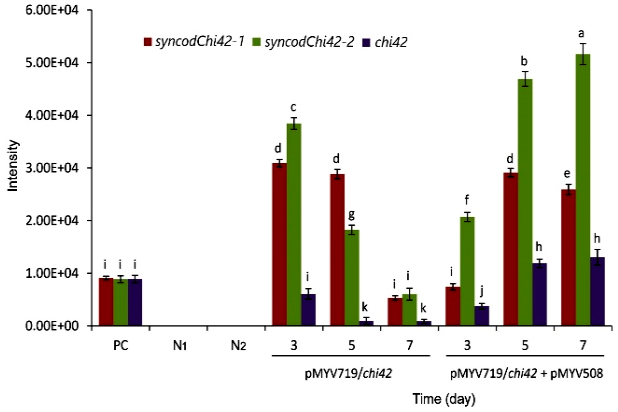

Fig. 6: Intensities of Western blot signals. PC: purified bacterial CHI42 enzyme as positive control. N1 and N2: wild-type *N. benthamiana* and agroinfiltrated *N. benthamiana* without *chi42* insert as negative controls, respectively. 3, 5 and 7: after 3, 5 and 7 days of infiltration (pMYV719/*chi42*) and coinfiltration (pMYV719/*chi42* and pMYV508). Different letters on the chart represent statistically significant differences (Duncan's test, $p<0.05$)

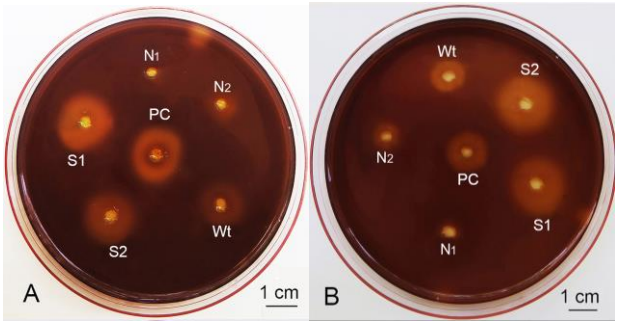

Fig. 7: Chitinolytic activity of plant CHI42 enzyme from different *chi42* genes on hydrolytic plate. (A): 3 days after infiltration (pMYV719/*chi42*), (B): 7 days after coinfiltration (pMYV719/*chi42* and pMYV508). S1: *syncodChi42-1*, S2: *syncodChi42-2*, Wt: *chi42*, PC: purified bacterial CHI42 enzyme as positive control, N1 and N2: wild-type *N. benthamiana* and agroinfiltrated *N. benthamiana* without *chi42* insert as negative controls, respectively

zone (*D*) and the diameter of the hole for loading enzyme (*d*) was about 1.5 cm (*syncodChi42-1*) and 1.4 cm (*syncodChi42-2*) after 3 days of infiltration; whilst, Fig. 7B showed the broader hydrolytic zones after 7 days of coinfiltrationl, *D-d* of CHI42 enzymes reached about 1.6 cm (*syncodChi42-1*) and 1.9 cm (*syncodChi42-2*). The hydrolytic zones of CHI42 enzyme from *N. benthamiana* agroinfiltrated without *chi42* inserts or with wild-type *chi42* gene in all treatments were weaker than CHI42 enzyme from synthetic *chi42* genes.

The chitinolytic activity of the protein extract from transgenic *N. benthamiana* peaked around 290 U/mL when coinfiltrated by two vectors, pMYV719/ *syncodChi42-2* and pMYV508, after 7 days of treatment. The *syncodChi42-1* gene also expressed quite high chitinolytic activity with more than 180 U/mL in *N. benthamiana* after 7 days of coinfiltration; whereas, the highest activity of CHI42 enzyme from *N. benthamiana* containing *chi42* gene was about 110 U/mL in the same treatment, 1.7 and 2.6 times lower than CHI42 enzyme from *syncodChi42-1* and *syncodChi42-2* genes, respectively (Fig. 8).

Generally, two synthetic *chi42* genes showed higher chitinolytic activities when agroinfiltrated into leaves of *N. benthamiana* along with pMYV508 vector. Comparison of chitinolytic activity and level of gene expression in Western blot analysis showed that they seem compatible with each other. The results on a hydrolyzed zone on an agar plate with colloidal chitin used as substrate were similar to that of chitinolytic activity.

In vitro assay for the antifungal activity of CHI42

Antifungal activity of CHI42 was measured based on its ability to inhibit the growth of mycelium in the pathogenic fungus *S. rolfsii* containing chitin in the cell wall. The antifungal activity of CHI42-1 and CHI42-2 from *N. benthamiana* coinfiltrated by two vectors, pMYV719/synthetic *chi42* and pMYV508, after seven days of treatment is shown in Fig. 9 and Table 2. The growth of *S. rolfsii* causing white mold wilt disease was inhibited on medium containing CHI42 (Fig. 9). The fresh biomass of *S. rolfsii* only achieved about 91 mg and 40 mg (about 1 mg and 0.4 mg dry biomass) when they were treated with 60 U/mL of CHI42-1 and CHI42-2, respectively. However, in the chitinase untreated control and the agroinfiltrated control without *chi42* insert, the fresh biomass of *S. rolfsii* reached about 1201 and 959 mg (nearby 11 and 9 mg dry biomass), respectively (Table 2).

Discussion

In the present study, the *chi42* genes were controlled by cauliflower mosaic virus (CaMV) 35S promoter, a promoter that can be activated in different plant tissues (Stockhaus *et al.* 1989). Transient expression of *cre* recombinase from bacteriophage P1 in leaves of *N. benthamiana* or antigen staphylococcal endotoxin B in leaves of radish (*Raphanus sativus* L.) were also driven by 35S promoter (Kopertekh and Schiemann 2005; Liu *et al.* 2018).

It was known that P19 proteins sequester small RNA duplexes, thereby preventing the induction of the silencing pathway (Danielson and Pezacki 2013). Studies have shown that transiently expressed proteins in *N. benthamiana* leaves have a higher yield when coinfiltrated with TBSV *p19* (Voinnet *et al.* 2003). *Agrobacterium* containing p35S-GSN was coinfiltrated with one of the vectors harboring a viral suppressor of silencing gene (*e.g.* p35S-TSBV.p19, p35SCMV.2b, C-terminal truncated CMV 2b (1–94), p35SPRSV.HC-Pro or p35S-TLCV.TrAP) significantly increased the transient expression of β-glucuronidase (GUS) in *N. benthamiana* (Norkunas *et al.* 2018). Yamamoto *et al.*

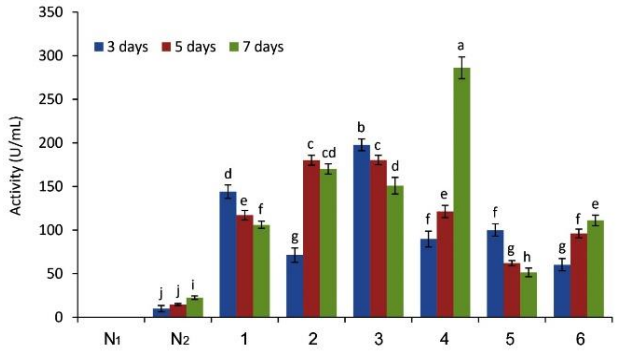

Fig. 8: Chitinase activity of plant CHI42 enzyme from different *chi42* genes after 3-7 days of agroinfiltration. N1 and N2: wild-type *N. benthamiana* and agroinfiltrated *N. benthamiana* without *chi42* inserts as negative controls, respectively. 1 and 2: infiltration and coinfiltration of *syncodChi42-1*, respectively. 3 and 4: infiltration and coinfiltration of *syncodChi42-2*, respectively. 5 and 6: infiltration and coinfiltration of *chi42*, respectively. Different letters on the chart represent statistically significant differences (Duncan's test, $p<0.05$)

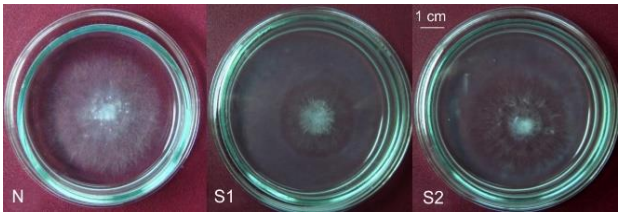

Fig. 9: Antifungal activity assay against *S. rolfsii* of plant CHI42 enzyme from *syncodChi42-1* and *syncodChi42-2* genes. N: agroinfiltrated *N. benthamiana* without *chi42* inserts as negative controls. S1: 60 U/mL of CHI42 from *syncodChi42-1* gene. S2: 60 U/mL of CHI42 from *syncodChi42-2* gene

Table 2: Effect of CHI42 enzyme from *syncodChi42-1* and *syncodChi42-2* on fresh and dry biomass of *S. rolfsii* mycelium after 36 h of culture

Enzyme	Level (U/mL)	Fresh biomass (mg)	Dry biomass (mg)
CHI42-1	10	902.12^{c}	7.33^{c}
	20	524.67^{d}	5.19^{d}
	40	372.04^{e}	3.32^{e}
	60	91.11^{g}	0.97^{g}
CHI42-2	10	873.03^{c}	7.01^{c}
	20	504.55^{d}	5.13^{d}
	40	272.82^{f}	2.40^{f}
	60	39.68^{h}	0.37^{h}
Control 1		1201.04^{a}	10.73^{a}
Control 2		958.88^{b}	8.84^{b}

CHI42-1: CHI42 from *syncodchi42-1* gene. CHI42-2: CHI42 from *syncodchi42-2* gene. Control 1: CHI42 untreated culture. Control 2: protein extract from agroinfiltrated *N. benthamiana* without *chi42* inserts. Different letters in a column represent a statistically significant difference (Duncan's test, $p<0.05$)

(2018) found replication initiator of geminivirus in combination with a double terminator improved obviously transient expression of green fluorescent protein (GFP) in *N. benthamiana* through agroinfiltration. As an alternative method, Zhao *et al.* (2017) improved the transformation efficiency of infiltration by some factors such as 5-azacytidine (AzaC), ascorbate acid (ASC), or Tween-20. These substances when added to infiltration buffer at concentrations of 20 μM for AzaC, 0.56 m*M* for ASC, or 0.03% (*v*/*v*) for Tween-20 increased GUS expression level in a leaf of *N. benthamiana*.

The genes that encode chitinase from some *Trichoderma* species such as *T. harzianum* and *T. virens* have been also transformed into tobacco, potato, cotton and apple against some pathogens such as *Alternaria alternata*, *A. solani*, *Botrytis cinerea*, and *Rhizoctonia solani* (Lorito *et al.* 1998; Emani *et al.* 2003; Schäfer *et al.* 2012). However, the expression of fungal-derived synthetic chitinase was only found in some reports such as the *NiC* gene from *Rhizopus oligosporus* for *Petunia hybrida* (Khan *et al.* 2012), tobacco and tomato (Kong *et al.* 2014) and *Brassica napus* (Khan *et al.* 2017b). This study is therefore probably the first findings of the synthetic chitinase genes derived from *T. asperellum* that has been expressed in plants and demonstrated strong antifungal activity against *S. rolfsii*. Agarwal *et al*. (2019) also optimized the codon of the bacterial bar gene to express in tobacco. Observations showed that a suitable proportion of optimum codons may be sufficient to reach a high expression of a transgene. If this ratio is exceeded, there is likely to be no significant improvement in gene expression.

Similar to our results, genes such as chitinase from barley (*Hordeum vulgare* L.) and *CeChi1* from the Australian pine (*Casuarina equisetifolia* L.) were introduced into potato and tobacco, respectively via *Agrobacterium*-mediated transformation have exhibited their antifungal activity (Veluthakkal and Dasgupta 2015). On the other hand, a study by Ibrahim *et al*. (2007) shown that the fungal elicitation increased oleandrin production in the *Agrobacterium* transformed *Nerium oleander* cell suspension culture. In the present study, *Agrobacterium* without *chi42* insertion itself also induced chitinase expression in plant cells after infiltration and plants produce enzyme as a defense response based on elicitation, so it slightly inhibited the growth of *S. rolfsii*.

Conclusion

The efficacy of agroinfiltration for transient expression of CHI42 enzyme encoded by *chi42* genes including a wild-type gene and two synthetic genes from *T. asperellum* SH16 in *N. benthamiana* was determined. Accordingly, coinfiltration expedited the higher expression of recombinant enzymes, whose genes were optimized for codon usage in plant tissues, with the peak found in *syncodChi42-2* gene. Plant CHI42 enzyme exhibited a strong antifungal activity against *S. rolfsii*.

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Author Contributions

NHL designed the experiments and analyzed tha data; NQDT, PTBH, NXH, NHT, DVT, HAT, and NNL performed the experiments; NQDT, PTBH and NXH analyzed the data; NHL prepared the manuscript. All the authors agreed on the final submission.

Conflict of Interest

All authors declare no conflicts of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable in this paper.

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