Journal of Applied Biology & Biotechnology Vol. 10(3), pp. 7-11, May-Jun, 2022
Available online at http://www.jabonline.in
DOI: 10.7324/JABB.2022.100302

Cloning the root-specific Asy promoter and genes encoding chitinase 42 kDa of *Trichoderma asperellum* into the plant expression vector

Nguyen Hoang Tue[1], Tran Gia Cat Tuong[1], Pham Thi Huyen Trang[1], Nguyen Duc Chung[2], Phung Thi Bich Hoa[1,3], Nguyen Quang Duc Tien[1], Nguyen Hoang Loc[1]*

[1]Institute of Bioactive Compounds and Department of Biotechnology, University of Sciences, Hue University, Hue, Vietnam.
[2]Department of Postharvest Technology, University of Agriculture and Forestry, Hue University, Hue, Vietnam.
[3]Department of Biology, University of Education, Hue University, Hue, Vietnam.

ARTICLE INFO

Article history:
Received on: September 30, 2021
Accepted on: December 15, 2021
Available online: April 10, 2022

Key words:
Chi42,
Chitinase 42 kDa,
Root-specific promoter,
Synthetic chitinase gene,
Trichoderma asperellum.

ABSTRACT

The objective of the present study was to construct plant expression vectors containing chitinase genes and root-specific promoters for resistance to phytopathogenic fungi. Chitinase genes with a signal peptide sequence, such as *Chi42*, *syncodChi42-1*, and *syncodChi42-2*, were used in this work. *Chi42* is a wild-type gene of *Trichoderma asperellum* SH16, and both *syncodChi42-1* and *syncodChi42-2* genes are derived from *Chi42,* which have been optimized for the use of codon for plant expression. Methods of molecular cloning were applied in this study. The plant expression vectors pNHL20 containing chitinase genes, pNHL20.1 for *Chi42*, pNHL20.2 for *syncodChi42-1*, and pNHL20.3 for *syncodChi42-2*, were successfully constructed and transferred into *Agrobacterium tumefaciens* LBA 4404. Bacteria *A. tumefaciens* harboring the chitinase genes are ready for genetic transformation to peanuts (*Arachis hypogaea* L.) for subsequent applications.

1. INTRODUCTION

Chitinases are enzymes that break down chitin into soluble and insoluble oligosaccharides of low molecular weight [1]. Chitin is a long-chain polymer of an N-acetylglucosamine, a glucose derivative. Chitin is known as a characteristic component of the cell walls of fungi, the exoskeletons of arthropods such as crustaceans and insects [2].

Because they produce a diversity of chitinases, many fungal species of *Trichoderma* genus are effective mycoparasites and are employed for biological control of fungal infections on agricultural crop plants [3]. However, certain chitinase genes from *Trichoderma* or other species such as rice and tobacco have been incorporated into various crops to aid them against fungal infections [4-10]. To date, there has been no record of chitinase gene transfer from *Trichoderma*, especially *Trichoderma asperellum*, into peanuts (*Arachis hypogaea* L.).

Peanut is a legume that is native to Central Brazil and is now widely grown worldwide, mainly for its edible oil and seeds. However, it is also one of the susceptible crops to serious diseases such as stem rot, root rot, and pod rot caused by numerous soil-borne pathogens such as *Rhizoctonia solani*, *Aspergillus niger*, and *Sclerotium rolfsii* [11-14].

**Corresponding Author:*
Nguyen Hoang Loc,
Institute of Bioactive Compounds and Department of Biotechnology, University of Sciences, Hue University, Hue, Vietnam.
E-mail: nhloc@hueuni.edu.vn

The objective of this study was to generate pNHL20 plasmid, a plant expression vector containing the chitinase genes, and conjugate it in *Agrobacterium tumefaciens* LBA4404 to serve for gene transfer into peanuts in the next studies. Chitinase genes used for this aim include a wild-type gene *Chi42* from *T. asperellum* SH16 and two synthetic genes *syncodChi42-1 and syncodChi42-2* derived from *chi42* have been optimized for the codon usage in plant expression from our previous study [15]. The chitinase genes are controlled by the peanut Asy root-specific promoter [16], and the chitinase enzyme will be secreted extracellularly by a signal peptide fused in front of the gene. An expression cassette designed as above promises to enable chitinase transgenic peanuts to resist soil-borne phytopathogenic fungi that cause root rot.

2. MATERIALS AND METHODS

2.1. Materials

The root-specific Asy promoter (NCBI: JQ780692) (562 bp long) was synthesized with two overhanging ends, *Hind*III and *Xba*I, and cloned in vector pUC19 by PHUSA Bio-Chemistry Co., Ltd. Plant expression binary pMYV719 vector [Figure 1] (about 12 kb long) was provided by Professor Yang Moon-Sik (Jeonbuk National University, Korea).

Genes encoding chitinase 42 kDa, including *Chi42* (NCBI: HM191683.1), *syncodChi42-1* (NCBI: MT083802.1), and *syncodChi42-2* (NCBI: MT083803.1), with two *Xba*I and *Sac*I

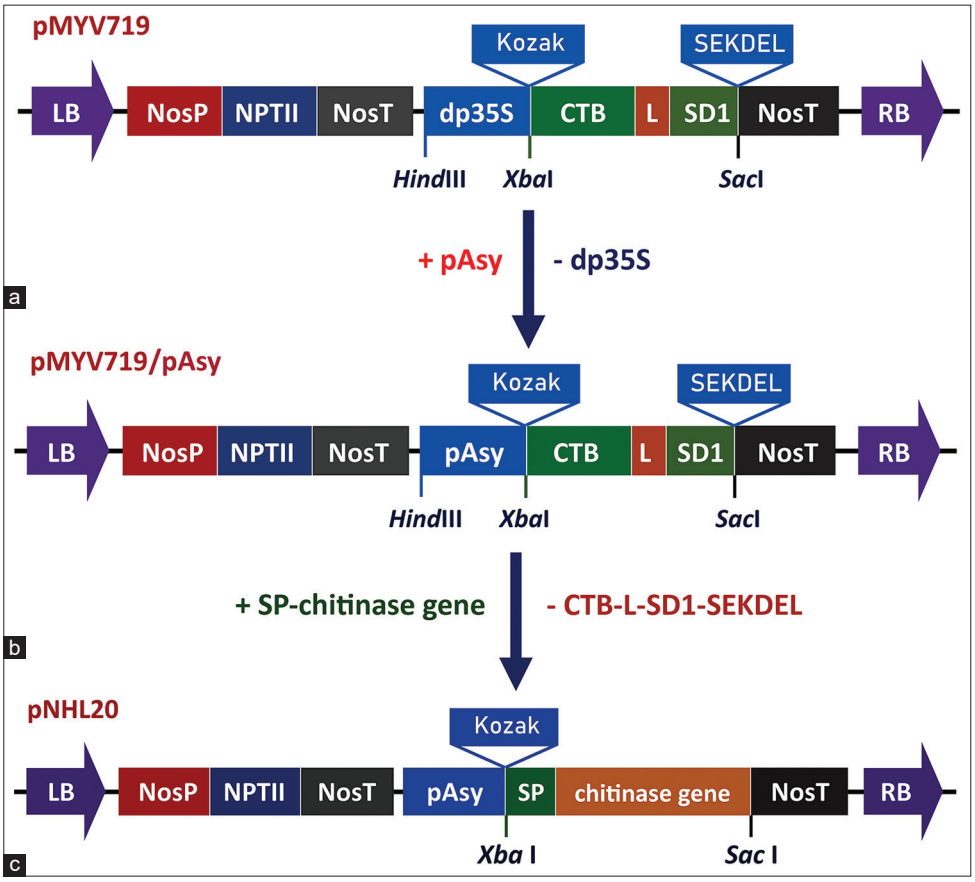

Figure 1: Construction diagram of the plant expression vector pNHL20. (a) pMYV719 vector, (b) pMYV719/pAsy vector, and (c) pNHL20 vector. LB and RB: Left and right border of the T-DNA region, NosP: Promoter of nopaline synthase gene, NPTII: Neomycin phosphotransferase II gene for kanamycin resistance, NosT: Terminator of nopaline synthase gene, pAsy: Root-specific Asy promoter, Kozak: Consensus sequence to ribosomes recognize and initiate translation, CTB: Gene encoding cholera toxin B subunit, L: Sequence Gly-Pro-Gly-Pro, S1D: Epitope region S1D, SEKDEL: Maintenance signal in the endoplasmic reticulum, dp35S: Duplicated CaMV 35S promoter, SP: Signal peptide sequence, and chitinase gene: One of three genes *Chi42*, *syncodChi42*-1, and *syncodChi42*-2.

overhang ends, and a signal peptide located at 5' end (about 1.3 kb in total length) were also synthesized and cloned in pUC19 vector by PHUSA Bio-Chemistry Co., Ltd. *Chi42* is a wild-type gene from *T. asperellum* SH16 [17], while *syncodChi42-1* and *syncodChi42-2* are synthetic genes generated from *Chi42* that were optimized for codon usage for plant expression in our previous study [15].

2.2. Construction of Plant Expression Binary Vector

The approach for constructing a plant expression vector is described in Figure 1. The Asy promoter (pAsy) was excised from the pUC19 vector using *Hind*III and *Xba*I and ligated into the pMYV719 vector at the location of the dp35S promoter (pdp35S), which was removed from the pMYV719 vector using the same enzymes, using T4 DNA ligase (Thermo Scientific).

*Xba*I and *Sac*I were used to isolate chitinase genes from the pUC19 vector. Plant expression vectors pNHL20 were generated by removing CTB-L-SD1-SEKDEL sequences of around 1 kb from the recombinant pMYV719/pAsy vector with the same enzymes and replacing them with one of the chitinase genes.

The ligation reaction mixtures, pMYV719/pAsy and pNHL20 vectors, were transformed into *Escherichia coli* TOP10 (Thermo Scientific) by the heat-shock method [18]. The transformants were then selected on LB medium supplemented with 50 µg/mL kanamycin. The cloning efficiency was checked by restriction digestion of recombinant vector and electrophoresis on 0.8% agarose gel.

2.3. Triparental Mating

Three bacterial strains were conjugated (triparental mating) on LB medium, including *A. tumefaciens* LBA4404, *E. coli* TOP10 carrying the plant expression vector pNHL20, and *E. coli* TOP10 carrying the helper plasmid pRK2013. They were subsequently grown in the same medium but supplemented with 100 g/mL spectinomycin and 100 g/mL kanamycin. Screening for colonies of *A. tumefaciens* on the medium confirmed that the vectors pNHL20 had been conjugated [19].

2.4. Polymerase Chain Reaction (PCR) Amplification

The colony PCR method *was* used for transformed *E. coli* cells [20]. Plasmid DNA of *A. tumefaciens* LBA 4404 from triparental mating was prepared according to Kamble and Fawade [21].

PCR amplification was performed with specific primers of chitinase genes [Table 1]. The composition of the reaction consists of template DNA (20 ng of plasmid DNA from *A. tumefaciens* or *E. coli* colony),

Table 1: Nucleotide sequence of specific primers for chitinase genes [14]

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

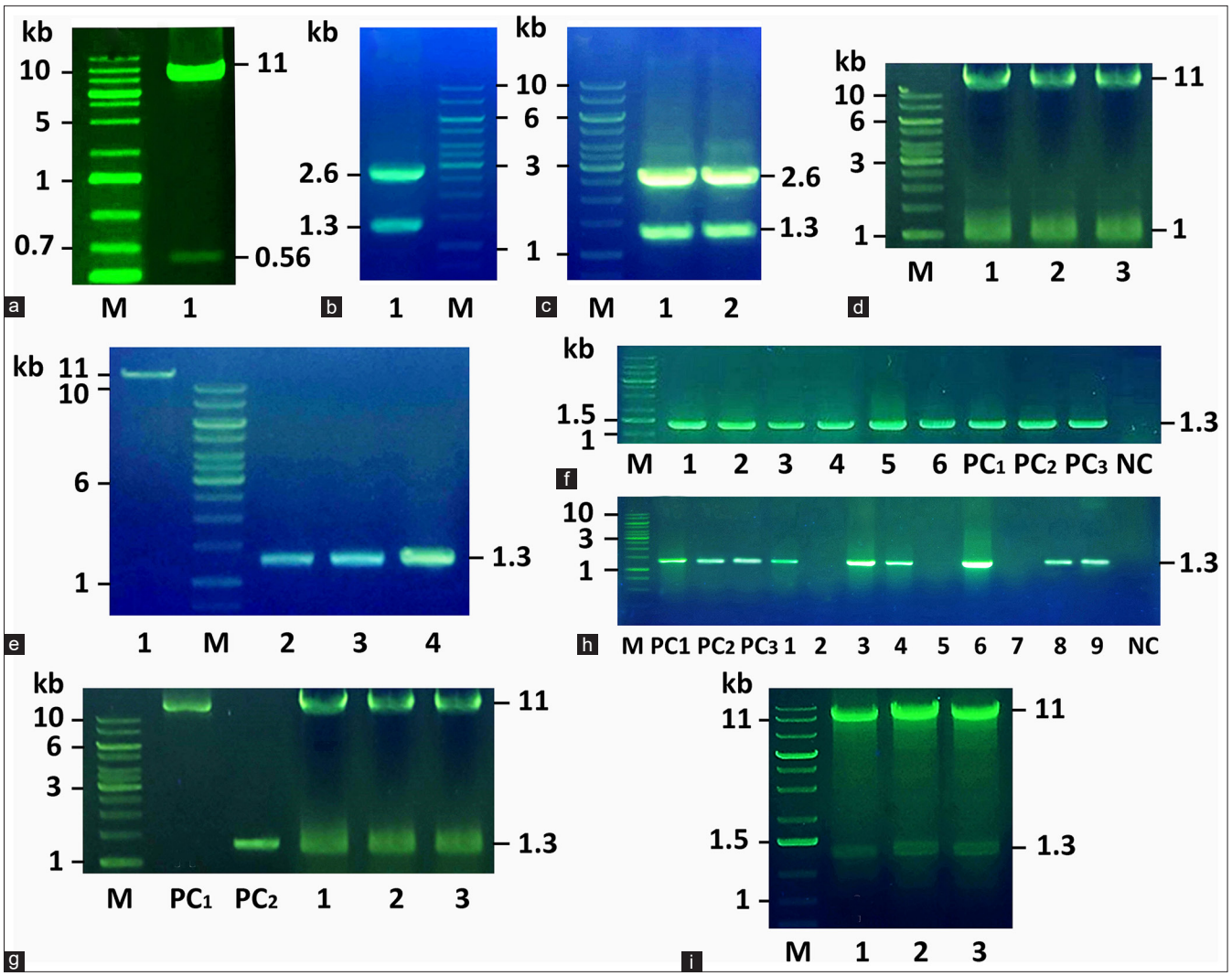

Figure 2: Cloning of the Asy promoter and chitinase genes in plant expression vector pHNL20. (a) Restriction digestion of the recombinant pMYV719/pAsy vector by *Xba*I and *Hind*III. M: GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific); 1: pMYV719 vector is about 11 kb long and pAsy promoter is about 0.56 kb long. (b and c) Restriction digestion of pUC19/chitinase vectors by *Xba*I and *Sac*I. B1: *chi42*, C1: *syncodChi42*-1, and C2: *syncodChi42-2*. (d1–d3) Restriction digestion of pMYV719/Asy vectors by *Xba*I and *Sac*I. (e) Purification of pMYV719/Asy vector and chitinase genes. 1: pMYV719/Asy vector and 2–4: *Chi42*, *syncodChi42-1*, and *syncodChi42-2*, respectively. (f) PCR amplification of chitinase genes with specific primers from transformed *E. coli* colonies. M: GeneRuler 1 kb DNA Ladder (Thermo Scientific), 1–2: *Chi42*, 3–4: *syncodChi42-1*, 5–6: *syncodChi42–2*, PC1: pUC19/*Chi42* vector, PC2: pUC19/*syncodChi42-1* vector, PC3: pUC19/*syncodChi42-2* vector, and NC: Nontransformed *E. coli*. (g) Restriction digestion of derivatives of pNHL20 vector by *Xba*I và *Sac*I. M: GeneRuler 1 kb DNA Ladder (Thermo Scientific), PC1: linearized pMYV719/Asy, PC2: *Chi42* gene, and 1–3: pNHL20.1 for *Chi42*, pNHL20.2 for *syncodChi42-1*, and pNHL20.3 for *syncodChi42-2*. (h) PCR amplification of chitinase genes with specific primers from *A. tumefaciens* containing the derivatives of pNHL20 vector. M: GeneRuler 1 kb DNA Ladder (Thermo Scientific), 1–3: *Chi42*, 4–6: *syncodChi42-1*, 7–9: *syncodChi42-2*, PC1: pUC19/*Chi42* vector, PC2: pUC19/*syncodChi42-1* vector, PC3: pUC19/*syncodChi42-2* vector, and NC: wild-type *A. tumefaciens*. (i) Restriction digestion of derivatives of pNHL20 vector by *Xba*I and *Sac*I. M: GeneRuler 1 kb DNA Ladder (Thermo Scientific), 1: *Chi42*, 2: *syncodChi42-1*, and 3: *syncodChi42-2*. Chitinase gene is 1.3 kb long, and pNHL20 vector is 11 kb long.

10 pmol of each primer, 1 µL of Master Mix (Thermo Scientific), and water to a final volume of 12 µL. The PCR conditions were as follows: a genomic denaturation at 95°C for 15 min, followed by 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min and a final extension at 72°C for 10 min. PCR products were then analyzed on 0.8% agarose gel.

3. RESULTS AND DISCUSSION

3.1. Vector pMYV719 Harboring pAsy

Promoter dp35S was removed from the pMYV719 vector using *Xba*I and *Hind*III. Promoter Asy was recovered from the pUC19/pAsy vector by the same two enzymes as above. The pMYV719 vector without the dp35S promoter was then ligated with the Asy promoter to transform into *E. coli* TOP10. The transformants were grown to produce the recombinant vector pMYV719/pAsy, which was then verified by restriction enzymes *Xba*I and *Hind*III. As a result, two DNA bands of approximately 11 kb and 560 bp were obtained, which corresponded to the vector and the pAsy sequence sizes [Figure 2a].

3.2. Vector pNHL20

Chitinase genes (*Chi42*, *syncodChi42-1*, and *syncodChi42-2*) with a signal peptide at the 5' end were isolated from the pUC19/chitinase vectors to replace the CTB-L1-SD1-SEKDEL sequence in the pMYV719/pAsy plant expression vector. Figure 2b-d show restriction digestion of two vectors, pUC19/chitinase, and pMYV719/pAsy, using the same enzymes, *Xba*I and *Sac*I. Chitinase genes are around 1.3 kb in length, while the pMYV719/pAsy vector lacking the CTB-L1-SD1-SEKDEL sequence is approximately 11 kb in length. The chitinase genes and the pMYV719/pAsy vector were purified with the GeneJET PCR Purification Kit (Thermo Fisher Scientific) before being bound to generate the vectors pNHL20.1/*Chi42*, pNHL20.2/*syncodChi42-1*, and pNHL20.3/*syncodChi42-2* [Figure 2e]. The ligation mixture of pMYV719/pAsy vector and each chitinase gene was transformed into *E. coli* cells and the presence of chitinase genes was determined by PCR amplification and restriction digestion of putative pNHL20 vectors from colonies that grew in the selection medium [Figure 2f and g]. The current findings show plant expression vectors were successfully transformed into *E. coli* cells.

3.3. Triparental Mating

A. tumefaciens harboring variants of the pNHL20 plant expression vector, pNHL20.1, pNHL20.2, and pNHL20.3 were screened using PCR amplification and restriction digestion.

As shown in Figure 2h, PCR amplification revealed that these vectors were conjugated in some *Agrobacterium* colonies. Restriction digestion using *Xba*I and *Sac*I confirmed these findings yet again; two DNA bands with predicted sizes of around 11 kb and 1.3 kb were detected on an agarose gel, which are compatible with pNHL20 vectors and chitinase genes, respectively [Figure 2i]. These findings indicate that *A. tumefaciens* carrying the binary vector is ready for chitinase gene transformation into peanuts in the subsequent investigation.

Promoters are a critical tool in biotechnological processes to ensure that the expression of a gene of interest is effective and well-controlled. When the interest protein is not required for the entire plant, the use of the organ or tissue-specific promoters that induce and specifically control the expression of transgenes in organs or tissue may be useful to prevent wasting energy and nutrients from transgenic plants [22]. Because peanuts are sensitive to various phytopathogenic fungi containing chitin in the cell wall, such as *Rhizoctonia* and *Aspergillus*, which cause root rot or collar rot, the production of extracellular chitinase in the roots is required to protect peanuts against these fungal pathogens. To date, there were no reports of the use of a root-specific promoter in peanuts yet, the majority of transgenic plants using the CaMV 35S constitutive promoters. The usage of constitutive promoters, however, results in unnecessary gene expression, potentially interfering with other plant growth pathways [23]. As a result, the goal of this work was to boost the antifungal effectiveness of peanuts by using a root-specific promoter.

As is known, microbial genes are generally weakly expressed in plants; therefore, the expression of heterologous proteins often requires modification of the coding sequence [24]. For that reason, the *Chi42* gene encoding chitinase 42 kDa of fungus *T. asperellum* SH16 was optimized for codon usage for expression in plants to two new genes, *syncodChi42-1* and *syncodChi42-2*, as in our previous report [15]. In the present study, each derivative of plant expression pNHL20 vector harboring one of three chitinase genes controlled by root-specific promoter was successfully introduced into *A. tumefaciens* LBA4404.

4. CONCLUSION

The chitinase genes (*Chi42*, *syncodChi42-1*, and *syncodChi42-2*) from *T. asperellum* SH16 with a signal peptide at 5' end and root-specific Asy promoter were successfully cloned in plant expression vector pNHL20. This vector was transferred into *A. tumefaciens* LBA 4404 to serve the genetic transformation into peanuts for resistance to root rot disease caused by phytopathogenic fungi in further studies.

5. ACKNOWLEDGMENTS

The authors would also like to thank Hue University, Vietnam, for facilitating this study and Professor Yang Moon-Sik (Jeonbuk National University, South Korea) for providing the pMYV719 vector. Nguyen Hoang Tue and Phung Thi Bich Hoa were awarded scholarships from the Master/PhD Scholarship Program of Vingroup Innovation Fund (VINIF) and Vingroup Big Data Institute (VINBIGDATA) with corresponding codes VINIF.2021.ThS.46 and VINIF.2020.TS.111.

6. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors requirements/guidelines.

7. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

Not applicable.

9. FUNDING SOURCES

This work was supported by National Foundation for Science and Technology Development, Vietnam (Grant number 106.02-2017.346).

10. PUBLISHER'S NOTE

This journal remains neutral with regard to jurisdictional claims in published institutional affiliation.

REFERENCES

1. Kramer KJ, Muthukrishnan S. Insect chitinases: Molecular biology and potential use as biopesticides. Insect Biochem Mol Biol 1997;27:887-900.
2. Tang WJ, Fernandez JG, Sohn JJ, Amemiya CT. Chitin is endogenously produced in vertebrates. Curr Biol 2015;25:897-900.
3. Ihrmark K, Asmail N, Ubhayasekera W, Melin P, Stenlid J, Karlsson M. Comparative molecular evolution of trichoderma chitinases in response to mycoparasitic interactions. Evol Bioinform Online 2010;6:1-26.
4. Nishizawa Y, Nishio Z, Nakazono K, Soma M, Nakajima E, Ugaki M, *et al*. Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase. Theor Appl Genet 1999;99:383-90.
5. Takahashi W, Fujimori M, Miura Y, Komatsu T, Nishizawa Y, Hibi T, *et al.* Increased resistance to crown rust disease in transgenic Italian ryegrass (*Lolium multiflorum* Lam.) expressing the rice chitinase gene. Plant Cell Rep 2005;23:811-8.
6. Gentile A, Deng Z, La Malfa S, Distefano G, Domina F, Vitale A, *et al.* Enhanced resistance to *Phoma tracheiphila* and *Botrytis cinerea* in transgenic lemon plants expressing a *Trichoderma harzianum* chitinase gene. Plant Breed 2007;126:146-51.
7. Baranski R, Klocke E, Nothnagel T. Chitinase CHIT36 from *Trichoderma harzianum* enhances resistance of transgenic carrot to fungal pathogens. J Phytopathol 2008;156:513-21.
8. Zarinpanjeh N, Motallebi M, Zamani MR, Ziaei M. Enhanced resistance to *Sclerotinia sclerotiorum* in *Brassica napus* by co-expression of defensin and chimeric chitinase genes. J Appl Genet 2016;57:417-25.
9. Ojaghian S, Wang L, Xie GL, Zhang JZ. Increased resistance against storage rot in transgenic carrots expressing chitinase chit42 from *Trichoderma harzianum*. Sci Hortic. 2018;234:81-6.
10. Ojaghian S, Wang L, Xie GL, Zhang JZ. Effect of introducing chitinase gene on the resistance of tuber mustard against white. Plant Pathol J 2020;36:378-83.
11. Ismail FM, Abd El-Momen SM. Effect of some soil amendments on yield and disease incidence in peanut (*Arachis hypogaea* L.). Egypt J Agric Res 2007;85:379-99.
12. Gour HN, Sharma P, Kaushal R. Pathological Aspects and Management of Root Rot of Groundnut: Root Rot of Groundnut (*Sclerotium rolfsii* Sacc.). Lap Lambert Academic Publishing; 2012.
13. Thiessen LD, Woodward JE. Diseases of peanut caused by soilborne pathogens in the Southwestern United States. Int Sch Res Notices 2012;2012:517905.
14. Xu ML, Yang JG, Wu JX, Chi YC, Xie LH. First report of *Aspergillus niger* causing root rot of peanut in China. Plant Dis 2015;99:284.
15. Luong NN, Tien NQ, Huy NX, Man LQ, Sinh DD, Tue NH, *et al*. Expression of 42 kDa chitinase (Ta-CHI42) of *Trichoderma asperellum* from a synthetic gene in *Escherichia coli*. FEMS Microbiol Lett 2021;368:fnab110.
16. Geng L, Duan X, Liang C, Shu C, Song F, Zhang J. Mining tissue-specific contigs from peanut (Arachis hypogaea L.) for promoter cloning by deep transcriptome sequencing. Plant Cell Physiol 2014;55:1793-801.
17. Loc NH, Quang HT, Hung NB, Huy ND, Phuong TT, Ha TT. *Trichoderma asperellum* Chi42 genes encode chitinase. Mycobiology 2011;39:182-6.
18. Froger A, Hall JE. Transformation of plasmid DNA into *E. coli* using the heat shock method. J Vis Exp 2007;6:253.
19. Tien NQ, Hoa PT, Tue NH, Thanh DV, Thi HA, Luong NN, *et al.* Transient expression of chi42 genes from *Trichoderma asperellum* in *Nicotiana benthamiana* by agroinfiltration. Int J Agric Biol 2021;26:177-84.
20. Bergkessel M, Guthrie C. Colony PC. Methods Enzymol 2013;529:299-309.
21. Kamble SP, Fawade MM. A rapid and inexpensive one-tube genomic DNA extraction method from *Agrobacterium tumefaciens*. 3 Biotech 2014;4:213-5.
22. Grunennvaldt RL, Degenhardt-Goldbach J. Promoters used in genetic transformation of plants. Res J Biol Sci 2015;10:1-9.
23. De Paoli LG, Camargo RL, Harakava R, Mendes BM, Filho FA. Genetic transformation of "Valencia" orange with the cecropin MB39 gene. Braz Agric Res 2008;42:1663-6.
24. Quax TE, Claassens NJ, Soll D, van der Oost J. Codon bias as a means to fine-tune gene expression. Mol Cell 2015;59:149-61.

How to cite this article:
Tue NH, Tuong NGC, Trang PTH, Chung ND, Hoa PTB, Tien NQD, Loc NH. Cloning the root-specific Asy promoter and genes encoding chitinase 42 kDa of *Trichoderma asperellum* into the plant expression vector. J App Biol Biotech. 2022;10(3):7-11. DOI: 10.7324/JABB.2022.100302

T

Type your te