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Expression analysis of recombinant *Vigna radiata* plant defensin 1 protein in transgenic tobacco plants

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ABSTRACT

Bruchid resistance is regulated by *defensin* gene. *Vigna radiata* plant defensin 1 (VrPDF1) inhibits alpha-amylase activities in insect gut; therefore, insect will die of undigestion starch. VrPDF1 content in mungbean seeds is very low. Hence an increase in content of VrPDF1 in mungbean seeds leads to enhance alpha -amylase inhibition in larvae and bruchids, which is necessary for the study to improve bruchid resistance in mungbeans. This article presents the results of *VrPDF1* gene expression in T1 generation transgenic tobacco seeds. It was confirmed that *VrPDF1* gene was attached to genome of tobacco plants and translated to synthesize VrPDF1. Recombinant VrPDF1 protein was successfully expressed in seeds of four transgenic tobacco lines (T1-7, T1-8, T1-10, T1-11), among which T1-10 line had the highest recombinant VrPDF1 content, reaching 8.57 μg mg^{-1} total protein. The extract containing recombinant *VrPDF1* from the transgenic tobacco lines effectively inhibited the activity of alpha-amylase from the intestine of larvae and weevils. However, the protein extract solution from T1-10 line had the strongest inhibitory effect, so the activity of alpha-amylase was only 18.89% compared to controls. The analysis results of *VrPDF1* gene expression in transgenic tobacco plants are fundamental to the transfer of pPhaso-dest-VrPDF1 vector into mungbean to improve bruchid resistance of mungbean and contribute to improving mungbean preservation.

1. INTRODUCTION

Mungbean is a kind of grain harvested crop. Mungbean seeds are a highly nutritious food source; however, mungbean seeds are very susceptible to bruchids. The damage caused by bruchids is very serious, up to a 60% yield [1] and even to 100% [2]; therefore, how to select or create mungbean cultivars that have high bruchid resistance is an issue of current interest. Bruchid resistance of crops is completely complex and related to activities of defensin proteins [3], [4]. Plant defensin is a group of small molecular weight proteins which are characterized by a three-dimensional structure with eight cysteine residues connected by four disulfide bridges [5] (Carvalho and Gomes 2011). Defensins were separated into 18 groups base on structure and function [6], such as enzyme inhibitory activities, antifungal activities [7], [8], [9] and antibacterial activities [10], [11], [12]. Defensin 1 in mungbean inhibits α-amylase activities, which prevents starch digestion in gut of bruchids, leading to inhibition of bruchids' growth and development and make them die [13], [14]. Meanwhile, the analysis of the *Cassia fistula* seed protease inhibitor suggested an additional function for plant defensive proteins, protease inhibitory activity [15]. VrPDF1 is a member of plant defensin family, and 0.2% of VrPDF1 in artificial seeds causes larvae and bruchids to die [3]. However, content of VrPDF1 in mungbean seeds is very low, so increasing the content of VrPDF1 in mungbean seeds to enhance alpha-amylase inhibition in larvae and bruchids is necessary for the study of improving bruchid resistance of mung beans and plants in general. Transformation has been a successfully applied technique to enhance recombinant protein expression in seeds [16], [17]. Swathi *et al*. (2008) analyzed expression of defensin gene in tobacco and peanut to enhance antifungal in transgenic plants [18].

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The analysis of *VrPDF1* gene expression in *Pichia Pastoris* markedly showed effect of antifungal and insecticidal activities [4]. In this article, we present our analysis of recombinant VrPDF1 protein in transgenic tobacco plants. In doing so, we transferred *VrDEF1* gene into tobacco plants via *Agrobacterium tumefaciens*-mediated transformation, and then the existence of VrDEF1 gene in tobacco plants were determined by PCR and Southern blot. Additionally, the experiments of Western blot, ELISA and alpha-amylase inhibitory assay were conducted to confirmed the expression of recombinant VrDEF1.

2. MATERIALS AND METHODS

2.1 Materials

VrPDF1 gene was isolated from seedlings of Tam TH mungbean cultivar provided by Vietnam Academy of Agriculture Science - Field Crops Research Institute (Lien Hong ward, Gia Loc district, Hai Duong province, Vietnam). Tam TH was estimated to be the best at bruchid resistance. VrPDF1 gene sequence was registered in GenBank with LN913082 code [8]. *Nicotiana tabacum*, K326 cultivar was used as the expression host, p201-SLHEP vector, pPhaso-dest vector, *Escherichia coli* DH5 alpha, *Agrobacterium tumefaciens* CV58 *(A. tumefaciens)* are provided by Plant Cell Biotechnology Laboratory, Institute of Biotechnology Vietnam.

2.2 Design of transgenic vector and create the A.tumefacients recombinant

VrPDF1 gene was purified from pBT-VrPDF1 cloning vector by a pair of *Sal*I/*hind*III restriction enzymes and inserted into p201-SLHEP vector. pBetaPhaso-VrPDF1 transgenic vector was designed by transferring VrPDF1-cmyc-KDEL structure into pBetaPhaso vector as described by Karimi *et al*. (2002) [19].

The pPhaso-dest-VrPDF1 vectors were introduced into *A. tumefaciens* CV58 by electroporation (2.5kV, 25µF, 200Ω). Transformants were selected on Luria-Bertani medium containing kanamycin and confirmed by colony – PCR.

2.3 Agrobacterium-mediated transformation

Tobacco plants were transformed by *A. tumefaciens* following the method of Topping (1998) [20]. Tobacco leaf explants were soaked in culture of *Agrobacterium* solution (OD_{600nm} = 0.8). Transformed tobacco leaf explants were cultured on Murashige and Skoog medium with kanamycin. The regeneration tobacco plants were transplanted to greenhouse, which were called T0 generation transgenic tobacco plants. The seeds collected from T0 generation transgenic tobacco plants were T1 generation, and they were used to analyze *VrDEF1* gene expression.

2.4 Analysis of the existence of the VrDEF1 transgene in genome of transgenic tobacco plants

The investigation into the existence of the *VrDEF1* transgene in genome of transgenic tobacco plants was carried out by PCR method and Southern blot technique. Total DNA was isolated from the transgenic tobacco leaves, which was based on Edwards *et al*'s study [21]. PCR and Southern blot were used to confirm the presence of VrPDF1 transgene in T0 generation transgenic tobacco plants. PCR conditions were as follows: an initial 94 ^{o}C denaturation step for 4 minutes followed by denaturation for 15 s at 94^{o}C, annealing for 30 s at 58 ^{o}C, and extension for 60 s at 72^{o}C for 30 cycles, final extension 72°C for 10 minutes. Withdraw a sample of the amplified DNA from the reaction mixture and analyze it by 1.5% agarose gel electrophoresis. PCR reactions used the specific primer pairs, namely VrPDF1HindIII-F/VrPDF1SalI–R.

VrPDF1-HindIII-F:5'
CCAAGCTTGGTTAACAGTTTCTAGTGCACC 3' (forward primer)

VrPDF1-SalI-R: 5'
GCGTCGACGATGGAGAAGAAATCACTGGCC 3' (reverse primer)

For Southern blot analysis, the digested plant DNA was electrophoresed and transferred to hybondN+ membrane. Hybridization was performed at 42°C for 4 hours using Biotin DecaLabel DNA from *VrDEF1*gene as probes. Transgenic tobacco plants, which were confirmed to be positive by PCR and Southern blot, were subjected for the analysis of expression of the recombinant VrDEF1 protein by Western blot and Enzyme-Linked ImmunoSorbent Assay (ELISA).

2.5 Analysis of recombinant VrDEF1 protein by Western blot and enzyme-linked immunosorbent assay (ELISA)

VrPDF1 recombinant protein expression in transgenic tobacco seeds (T1 generation) was analyzed by Western blot and protein electrophoresis according to Laemmli (1970) [22]. To extract total protein, 0.5 g of tobacco leaves were crushed in liquid nitrogen and dissolved in 1 ml of phosphate buffered saline (PBS) with 0.05% Tween 20 (PBS-T), and then centrifuged at 13000 rpm for 15 minutes. Proteins were denatured and run on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to nitrocellulose membranes using a Pierce G2 Fast Blotter (25 V, 1.3 mA for 20 min). Membranes were then blocked in blocking solution (5% skim milk in PBS-T) overnight and incubated with primary antibody (c-myc) for 3 hours by shaking at room temperature, followed by 3 washes with PBS, and then incubated with secondary antibody for 2 hours. Mouse monoclonal antibody to c-myc (Santa Cruz Biotech) was diluted in 5% milk in PBS at 1:700. For secondary antibody, antimouse IgG antibody attached to HRP (horse radish peroxidase) was diluted in 5% milk in PBS at 1:4000. Results were displayed by using TMB (3,3',5,5'-tetramethyl benzidine) or DAB (3,3'-diaminobenzidine tetrahydrochloride).

The content of recombinant VrPDF1 was determined by the ELISA method of Sun *et al*. (2006) [23]. Soluble protein was extracted from T1 generation tobacco transformation seeds and

diluted to a concentration of 200 µg ml^{-1}, and 100 µl VrPDF1 dilution was added into microplates three times. Recombinant VrPDF1 was determined using c-myc antigen, anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) and TMB solution. Recombinant VrPDF1 tagged to cmyc can be read on a spectrophotometer at 630 nm wavelength. ScFv protein, positive control, was diluted with coating buffer. A series of diluted ScFv protein tagged to cmyc were measured in 2.5; 5.0; 10.0; 20.0; 400.0 ng/well to construct a standard curve. Linear equation, Y = 0.0019X + 0.0562, was defined and correlation coefficient (R) was also computed at 0.9993.

2.6 Alpha-amylase inhibitory assay

The alpha-amylase activity of weevils was determined according to Bernfeld method [24]. *Vigna radiata* weevils (*Callosobruchus chinensis* L.) were crushed in icy 0.2 M phosphate buffer pH 6.8, centrifuged at 12000 rpm for 10 minutes, and solution containing alpha-amylase was collected. Starch (1% [w v^{-1}]) was used as substrate for alpha-amylase activity assay. Solution containing alpha-amylase from weevils and protein extracted from tobacco plants and starch were incubated for 30 minutes at 30 ^{o}C, and then continued to be analyzed by absorption spectroscopy at 530 nm. The alpha-amylase enzymatic activity is the amount (mg) of degradable starch for 30 min at 30 ^{o}C (U mg^{-1}).

3. RESULTS AND DISCUSSION

3.1 Designing vector contained VrPDF1 gene by the Gateway technique

Nucleotide sequence of *VrPDF1* gene isolated from mRNA of Tam TH mungbean cultivar has 228 nucleotides, encoding 75 amino acids [25], which was used to design transgenic vector. *VrPDF1* gene was cut by a pair of restriction enzyme *SalI*/*Hind*III and attached to p201-SLHEP vector to create p201-SLHEP-VrPDF1 recombinant vector containing cmyc and KDEL tail (Fig. 1A).

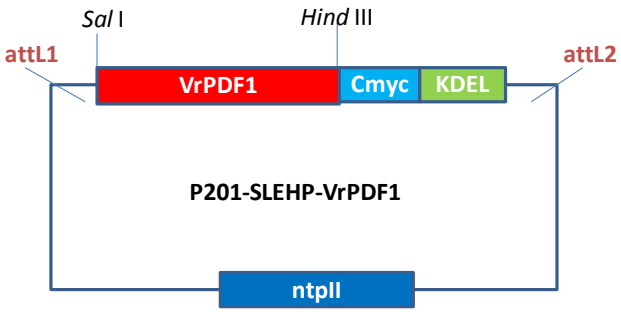

Fig. 1A:

p201-SLHEP -VrPDF1-cmyc-KDEL structure was cloned in *E. coli*. Six colony lines were randomly chosen to be checked by colony-PCR with the primer set VrPDF1-F/VrPDF1-R. PCR products were carried out on 1.5% agarose gel to analyze. The results showed that there were five colonic lines which had a DNA electrophoresis band with the size of approximately 0.25 kb. This DNA size was equivalent to the size of the *VrPDF1* gene (Fig. 1B). The colony lines positive for PCR were used to separate the recombinant plasmid p201-SLHEP-VrPDF1-cmyc-KDEL.

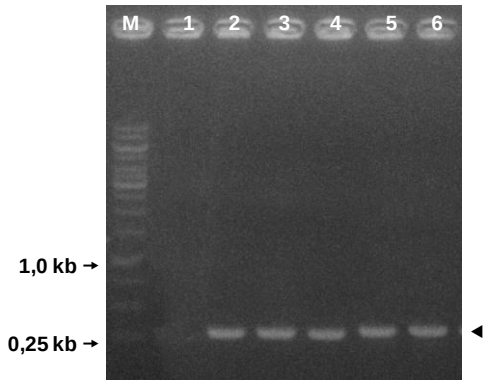

Fig. 1B:

Fig. 1: Structure of p201-SLHEP-VrPDF1 vector (A) and the result of electrophoresis colony-PCR products to test p201-SLHEP-VrPDF1 vector (B). M: DNA marker 1 kb; 1, 2, 3, 4, 5, 6: colony-PCR products of 6 colony lines.

p201-SLEHP-VrPDF1 vector which contained Phaso promoter control recombinant protein expression in seeds was formed by Gateway technique [19]. The result of recombination reaction between p201-SLEHP-VrPDF1 vector and pPhaso-dest vector was pPhaso-dest-VrPDF1 transgenic vector with cmyc and KDEL tail (Fig. 2A). pPhaso-dest-VrPDF1 vector was cloned in *E. coli* DH5α, and then was selected by medium which contained kanamycin. Results of selection lines by colony-PCR reactions with the primer set VrPDF1-F / VrPDF1-R are shown in Fig. 2B.

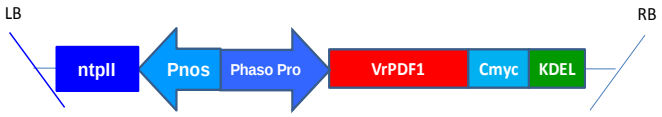

Fig. 2A:

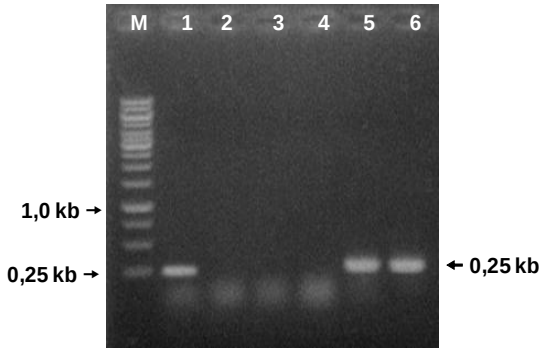

Fig. 2B:

Fig. 2: Structure of pPhaso-dest-VrPDF1 vector (A) and the result of electrophoresis colony-PCR products to test pPhaso-dest-VrPDF1 transgene vector (B). M: DNA marker 1 kb; 1, 2, 3, 4, 5, 6: colony-PCR products of 6 colony lines.

Fig 2B shows the result of the test of 6 colony lines and 3 lines (1, 5, 6) were positive with PCR. The positive lines were used to purify pPhaso-dest-VrPDF1 recombinant plasmid. pPhaso-dest-VrPDF1 vector was transformed into *A. tumefeciens* CV58 by

electrical impulses. *A. tumefaciens* bacteria was cultured on LB medium containing kanamycin, and *A. tumefaciens* recombination was checked by colony- PCR.

3.2 Creating transgenic tobacco plants containing pPhaso-dest structure -VrPDF1

pPhaso-dest -VrPDF1 vector was transformed into tobacco leaf tissue via infection by *A. Tumefaciens*. The results shows there are 28 transgenic tobacco plants and 10 control tobacco plants which survived in the greenhouse. Selecting 12 transgenic tobacco plants in T0, namely T0-1 to T0-12 respectively, and non-transgenic tobacco plants (WT) aimed at analyzing the presence of the transgene VrPDF1 by PCR. Also, the positive control of recombinant vector with *VrPDF1* gene was used in PCR reactions. PCR reactions with the primer set VrPDF1HindIII-F/VrPDF1SalI–R resulted in amplification of *VrPDF1* from DNA of 12 transgenic tobacco plants; WT and plasmid pBT-VrPDF1 showed in Fig 3. Nine transgenic tobacco plants were positive to PCR, and they were named number 1, 2, 4, 6, 7, 8, 10, 11, 12 on the electrophoresis gel.The products were all about 0.25 kb in length, similar to the size of the transgene. The *VrPDF1* were not determined in the three trangenic lines (T0-3, T0-5, T0-9) and WT plants.

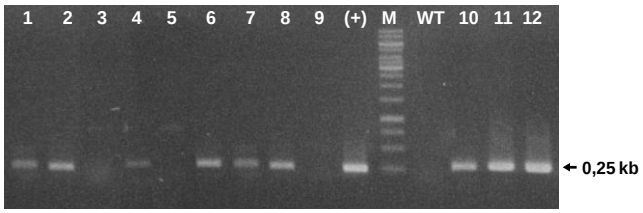

Fig. 3: Genomic PCR amplification of *VrPDF1* in T0 generation transgenic tobacco lines M: DNA marker 1 kb; (+): Positive control (plasmid pBT-VrPDF1); WT: the wild type tobacco plants (non-transgenic plants); 1-12: transgenic plants (named: T0-1, T0-2, T0-3, T0-4, T0-5, T0-6, T0-7, T0-8, T0-9, T0-10, T0-11, T0-12).

To further assess the performance of *VrPDF1* gene in transgenic tobacco plants, the 9 transgenic tobacco lines (T0-1, T0-2, T0-4, T0-6, T0-7, T0-8, T0-10, T0-11, T0 -12) positive for PCR were analysed by Southern blot technique. As shown in Fig 5, the *VrPDF1* transcripts were detected in 7 lines T0-4, T0-6, T0-7, T0-8, T0-10, T0-11, T012 and not in T0-1, T0-2 lines. Thus, it can be confirmed that pPhaso-dest -VrPDF1 structure was successfully moved into tobacco plants, and transgene VrPDF1 was attached to the tobacco genome. The transgenic tobacco lines tested positive for Southern blot continued being assessed in terms of the growth and development; therefore, their seeds were collected for analysis in their next generation.

3.3 Analyzing the expression of recombinant VrPDF1 protein in T1 generation transgenic tobacco plants

The expression of recombinant protein is an important destination in transgenic research in plants. Among 7 transgenic tobacco lines positive for Southern blot, 5 lines produced seeds, including T1-6, T1-8, T1-10, T1-12 and T1-13. The seeds of the T0 plants are the T1 generation transgenic plants. Five T1 transgenic tobacco lines and non-transgenic control plants were used to analyze the expression of VrPDF1 recombinant protein (rVrPDF1) in seeds.

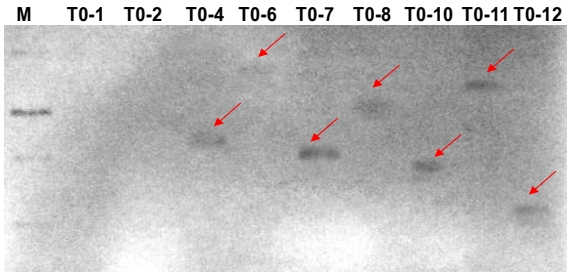

Fig. 4: Result analysis of 9 transgenic tobacco lines by Southern blot. M: DNA marker 1 kb; T0-1, T0-2, T0-4, T0-6, T0-7, T0-8, T0-10, T0-11, T012: T0 generation transgenic tobacco lines

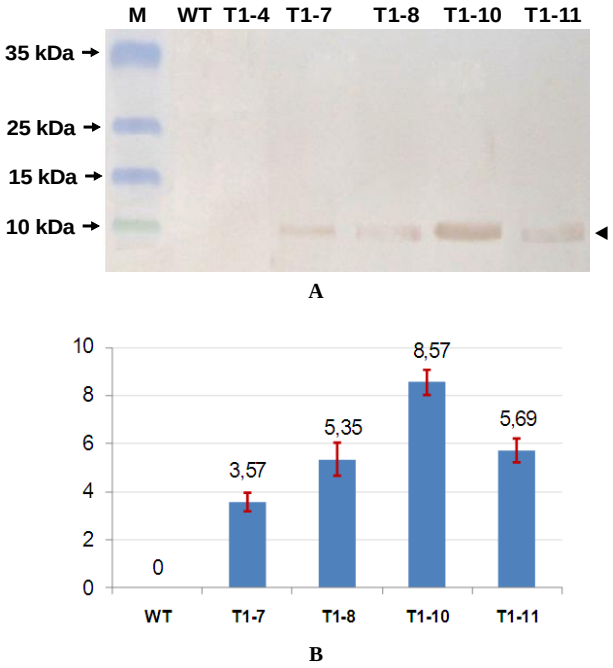

Fig. 5:A. Analysis of rVrPDF1 in WT and T1 generation transgenic tobacco plants. M: Protein marker; WT: the wild type tobacco plants (non-transgenic plants); T1-4, T1-7, T1-8, T1-10, T1-11: transgenic tobacco lines.
B- Comparison of rVrPDF1 production (µg mg^{-1} total protein) in WT and the transgenic tobacco lines. WT: the wild-type tobacco plants (non-transgenic plants), T1-7, T1-8, T1-10, T1-11: transgenic tobacco lines. Vertical bars represent standard error.

Fig 5A shows the results of Western blot analysis to detect recombinant VrPDF1 in the T1 generation transgenic tobacco lines. Four bands, product of *VrPDF1* gene in T1 generation transgenic tobacco lines (T1-7, T1- 8, T1-10, T1-11), were identified on nitrocellulose membrane; the molecular weight of these protein bands was about 10 kDa. However, the band completely disappeared in the wild type tobacco plants. Therefore, *VrPDF1* transgene which was isolated from Tam TH cultivar, a good bruchid resistant mungbean, produced VrPDF1 protein in transgenic tobacco plants. To assess the level of recombinant protein expression, ELISA technique was used to determine the

content of recombinant VrPDF1. The results are shown in Fig 5B. The rVrPDF1 content of transgenic tobacco lines were in the range of 3.57 - 8.57 (μg mg^{-1} total protein), and the rVrPDF1 content of T1-10 line was the highest (8.57 μg mg^{-1} total protein).

VrPDF1 gene that was isolated from Tam TH variety contained 356 nucleotides, including a 128 nucleotide intron between two exons. The exons of *VrPDF1* gene had 228 nucleotides, encoding 75 amino acids [25]. In VrPDF1 protein, 28 amino acids at the N-terminal constructed a signal peptide, followed by a mature peptide of 47 amino acids. Spatial structure of defensin is stabilized by four disulphide bridges (Cys31-Cys75, Cys42-Cys63, Cys48-Cys69 and Cys52-Cys71). Disulfide bridges among the cysteines in the functional region of defensin have an important role in deciding alpha- amylase inhibition in the insect gut. At the level of three-dimensional structure, defensin composes of three anti-parallel beta-sheets and one alpha-helix. The loop between the second and the third beta-strand is the binding site for inhibiting alpha-amylase activity. The link between loop 3 with operating centers of alpha- amylase in bruchids leads to preventing starch into the operating center of enzyme [14]. [26].

pPhaso expression vector carrying *VrPDF1* gene and containing phaso promoter has been designed and successfully transferred into tobacco plants. *VrPDF1* transgene in the transgenic tobacco plants was determined by Southern blot. The results of Western blot and ELISA analysis demonstrated that the recombinant VrPDF1 protein was successfully expressed in transgenic tobacco seeds. The analysis of VrPDF1 gene expression in transgenic tobacco provides a highly feasible application for the transfer of pPhaso-dest-VrPDF1 vector into mungbean to increase defensin content in seeds, therefore improve the insecticidal resistance of mungbean.

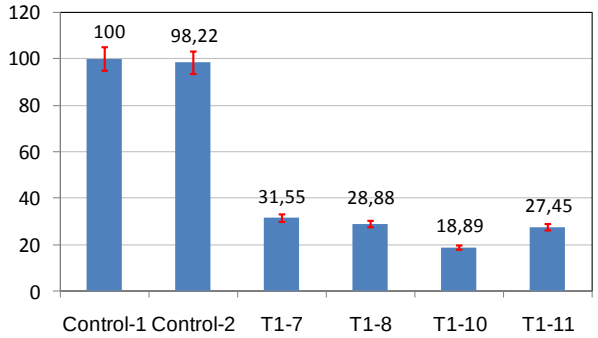

Fig. 6: Enzyme assay of alpha-amylases of *Vigna radiata* weevils in samples containing protein extracts from wild type and transgenic tobacco plants. Control-1: Only alpha-amylase of *Vigna radiata* weevils; control-2: mixture of alpha-amylase of *Vigna radiata* weevils and protein of non-transgenic plant; T1-7, T1-8, T1-10, T1-11: mixture of alpha- amylase of *Vigna radiata* weevils and protein of T1 transgenic tobacco plants. Vertical bars represent standard error.

To analyse biological function of recombinant VrDEF1 protein in the transgenic tobacco lines in T1, the solution containing alpha - amylase of of *Vigna radiata* weevil was incubated with protein extracts from transgenic and non-transgenic seed to examine the effect of VrDEF1 on alpha-amylase enzymatic activity.

The results showed the enzymatic activity when incubated alpha-amylase with protein extract from the transgenic tobacco lines T1-7, T1-8, T1-10, T1-11 were 31.55%, 28.88%, 18.89%, 27.45%, respectively. The enzymatic activity was significantly lower than incubated solution containing alpha-amylase with protein extract from non-transgenic plants (98.22%) or alpha-amylase alone (100%) (Figure 6). These results showed that weevil alpha-amylase activity was inhibited by recombinant VrDEF1 protein. Research in tobacco plants has created the basis to generate mungbean plants with resistance to weevil and will contribute to improving mungbean preservation.

4. CONCLUSIONS

pPhaso-dest-*VrPDF1* vector was completely designed, and transgenic tobacco plants were tested for recombinant VrPDF1 protein expression. *VrPDF1* transgene was attached to the genome of tobacco plants and translated to synthesis VrPDF1 protein. Recombinant VrPDF1 protein was successfully expressed in seeds of four transgenic tobacco lines (T1-7, T1-8, T1-10, T1-11). T1-10 line had the highest VrPDF1 protein content, reaching 8.57 μg mg^{-1} of protein. The extract containing recombinant VrPDF1 proteins from the transgenic tobacco lines effectively inhibit the activity of alpha-amylase from the intestin of the larvae and weevil, in which protein extract solution from T1-10 line had the strongest inhibitory effect. The activity of alpha-amylase was only 18.89% compared to controls. The results of analysis of VrPDF1 gene expression in transgenic tobacco plants are the basis of the transfer of pPhaso-dest-VrPDF1 vector into mungbean to improve bruchid resistance of mungbean and will contribute to improving mungbean preservation.

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