

# Overexpression of *VrPDF1* gene confers resistance to weevils in transgenic mung bean plant

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Plant defensins are known for different biological functions such as insect resistance, antibacterial, antifungal and antiviral activities. The role of plant defensins against weevils is based on inhibitor of alpha-amylase activity in weevil gut, therefore, weevils can not digest starch and then die. The low content of *Vigna radiata* plant defensin 1 (VrPDF1) in mung bean seeds is prompted researches to increase the VrPDF1 content, in which leads to enhance alpha-amylase inhibition and improve bruchid resistance in mung bean. This article presents the results of overexpression of VrPDF1 gene in transgenic mung bean plants. VrPDF1 gene was successfully transferred into DX22 mung bean cultivar and expressed in T1 generation transgenic mung bean seeds. The extract containing recombinant VrPDF1 protein inhibited alpha-amylase of weevil larvae in its larval stage. The performance of alpha-amylase inhibitor of DX1-3 and DX1-7 transgenic mung bean lines increased by 166.40% and 178.19% respectively, in comparison with non-transgenic plants. The enhancement of alpha-amylase inhibitor ability of rVrPDF1 extracted from transgenic plants is scientifically fundamental to confirm the effectiveness of the application of gene technology in enhancement the ability of mung bean weevil resistance in particular and grain crops in general revenue.

# Overexpression of *VrPDF1* gene confers resistance to weevils in transgenic mung bean plants

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## ABSTRACT

Plant defensins are known for different biological functions such as insect resistance, antibacterial, antifungal and antiviral activities. The role of plant defensins against weevils is based on inhibitor of alpha-amylase activity in weevil gut, therefore, weevils can not digest starch and then die. The low content of *Vigna radiata* plant defensin 1 (*VrPDF1*) in mung bean seeds is prompted researches to increase the *VrPDF1* content, in which leads to enhance alpha-amylase inhibition and improve bruchid resistance in mung bean. This article presents the results of overexpression of *VrPDF1* gene in transgenic mung bean plants. *VrPDF1* gene was successfully transferred into DX22 mung bean cultivar and expressed in T1 generation transgenic mung bean seeds. The extract containing recombinant *VrPDF1* protein inhibited alpha-amylase of weevil larvae in its larval stage. The performance of alpha-amylase inhibitor of DX1-3 and DX1-7 transgenic mung bean lines increased by 166.40% and 178.19% respectively, in comparison with non-transgenic plants. The enhancement of alpha-amylase inhibitor ability of *rVrPDF1* extracted from transgenic plants is scientifically fundamental to confirm the effectiveness of the application of gene technology in enhancement the ability of mung bean weevil resistance in particular and grain crops in general revenue.

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33 **Subjects:** Agricultural Science, Biotechnology, Plant Science.

34 **Keywords:** alpha-amylase inhibitor, mung bean weevil, transgenic mung bean, *Vigna radiata*,  
35 *VrPDF1* gene.

## 36 INTRODUCTION

37 Mung bean (*Vigna radiata* (L.) Wilczek) belongs to the group of grain crops. Mung bean  
38 seed is a high source of protein and other nutritious components such as fiber, vitamin and  
39 minerals. However, this nutritious source of mung bean seed can be lost during the storage stage  
40 due to attack by weevil. The seed damage can reach up to 60 % - 100 % (Chainuvati et al., 1988;  
41 Bui Cong Hien, 1995); hence, it is necessary to develop weevil resistant mung bean varieties.  
42 The studies have shown that weevil resistant characteristics of crop is to complex and relate to  
43 activities of the defensin protein (Chen et al., 2002; Chen et al., 2005). Defensin are small,  
44 cysteine-rich molecules found in all vertebrate, invertebrate and plant (Ganz 2004). The first  
45 plant defensin was isolated from wheat (*Triticum aestivum*) (Colilla et al., 1990) and barley  
46 (*Hordeum vulgare*) (Mendez et al., 1990). More than 80 types of defensin have so far been  
47 identified in plant. Plant defensins have quite diverse amino acid positions and groups of the  
48 eight-cysteine residues in all kinds of plant (Terras et al., 1992). The difference in primary  
49 structure resulting in different biological activities of each defensin. The cluster of defensin was  
50 proposed based on the origin and analysis of sequences of 139 defensin genes, which were  
51 divided into 18 different functional groups, such as antifungal and antibacterial activity, inhibitor  
52 of  $\beta$ -amylase, trypsin and protein synthesis, Na<sup>+</sup> channels blocking and zinc-resistance (Monk et  
53 al., 2005; Oard et al., 2006; Nicole et al., 2013).

54 Plant defensins contain from 45 to 54 amino acids with a molecular weight of 5 kDa. The  
55 three-dimensional folded structure of plant defensins is high stabilized by eight disulfide-linked

cysteines (Carvalho et al., 2011). Plant defensin proteins consist of three anti-parallel  $\beta$ -sheets and an  $\alpha$ -helix maintained by four disulfide bridges. The fourth disulfide bridge that links the N-C terminal produces a stable protein structure (James et al., 2015). Functionally, plant defensins act as inhibitors that strongly affect ion channels, transcription,  $\alpha$ -amylase and trypsin activities. In addition, plant defensin also inhibits the growth and development of a wide range of fungi, gram-positive and gram-negative bacteria and some kind virus in plants (Sonia et al., 2007; Viviane et al., 2015).

Ability to inhibit translation in cell-free systems of plant defensin was described by Mendez et al. (1990). The peptide isolated from barley endosperm that was designated as  $\gamma$ -hordothionin, has been shown to have inhibitory effect on protein translation in mouse liver. The other peptide was then extracted from the barley endosperm designated as  $\omega$ -hordothionin, also inhibited protein transcription in mouse liver, rabbit liver and *Escherichia coli*. Similar to other defensins, the VrD1 inhibits protein synthesis in wheat germ and translation systems in rabbit reticulocyte (Chen et al., 2002; Chen et al., 2005; Wisniewski et al., 2003).

Plant defensins have also been shown  $\alpha$ -amylase and protease inhibitory activities (Dos Santos et al., 2010; Wijaya et al., 2000). Bloch and Richardson (1991) reported biological activity of plant defensin as inhibitor of insects  $\alpha$ -amylase. Analysis of biological functional expression of *VrD1* from mung bean were identified to be resistant to weevils of azuki beans (*Callosobruchus chinensis*). In another study, the artificial seeds containing 0.2% recombinant defensin showed the lethal effect on larvae when they ate the seeds (Chen et al., 2002). The similar results were obtained by testing VrD1 recombinant expression in yeast (Chen et al., 2004). At 0.8% of concentrations of VrD1 recombinant defensin was strongly inhibited the development of mung bean bruchids in the experiment using artificial seeds (Chen et al., 2005).

Antimicrobial function of plant defensins has been interested since the early years of the 1990s by Terras et al. (1992), especially, antifungal activity (Lacerda et al., 2014). However, the studies using plant defensins to inhibit the development of fungi did not always show effective results. Therefore, plant defensins have been divided into two main groups. The first group included Rs-AFP1, Rs-AFP2 and Hs-AFP1, MsDEF1 and MtDEF4 in which MsDEF1 have been designated as non-morphogenic defensin; this group has antifungal activity (Osborn et al., 1995). The second group consists of Dm-AMP1, Dm-AMP2, Ah-AMP1 and Ct-AMP1 (Osborn et al., 1995; Olli et al., 2006). Besides, other research have also shown ability to resist gram-positive and gram-negative bacteria of plant defensins (Terras et al. 1992; Wijaya et al. 2000; Chen et al., 2005; Tavares et al., 2008). Defensin which was isolated from leaves of *Spinacia oleracea*, was capable of inhibiting *C. michiganensis* and *P. solanacearum* bacteria at concentration below 20 mM. WCBAF, other type of defensin, also inhibit *B. megaterium*, *B. subtilis*, *M. phlei* and *P. vulgaris* at concentrations of 125, 101, 86 and 68 mM, respectively (Wong et al., 2006). Thus, the most of studies on plant defensin have just focused on clarifying antibacterial and antifungal activities

Alpha-amylase activity inhibitory of some plant defensins is fundamental for studies on insect - resistance using plant defensin in the seed preservation, including mung bean weevils. However, the content of *Vigna radiata* plant defensin 1 (VrPDF1) in mung bean seeds is very low, hence an increase in VrPDF1 content in mung bean seeds leads to enhance alpha -amylase inhibition and improve weevil resistance of mung bean. Transformation is considered a technological measure which enhances protein expression in transgenic plants (Dao Xuan Tan et al., 2015; Thanh Son Lo et al., 2015) and transgenic technique can also be applied to biotechnological crop improvement of the same species and potentially to other plants (Aloka

Kumari et al., 2017). In the direction of approach overexpression of defensin protein in seeds, Swathi et al. (2008) successfully expression of defensin gene in tobacco and peanut to enhance antifungal activity in transgenic plants. The analysis of *VrDI* gene expression in *Pichia Pastoris* showed that effect of antifungal and insect-resistant (Chen et al., 2004). In this study, we analyze overexpression of *VrPDF1* gene in seeds of transgenic mung bean plants and  $\alpha$ -amylase inhibitory activity of recombinant PDF1 protein extracted from transgenic mung bean seeds.

## MATERIALS AND METHODS

### Materials

Seeds of DX22 mung bean cultivar was obtained by Vietnam Academy of Agriculture Science - Field Crops Research Institute. Recombinant *Agrobacterium tumefaciens* strain CV58 with pBetaPhaso-*VrPDF1* structure was provided by Department of Genetics & modern Biology, School of Biology, Thai Nguyen University of Education, Viet Nam. Weevils (*Callosobruchus chinensis*) collected from mung bean warehouses in Bac Giang province, Viet Nam and nourished with feed is mung bean seeds in the laboratory of Faculty of Agronomy, Bac Giang Agriculture and Forestry University, Viet Nam.

### Methods

#### *Agrobacterium-mediated transformation*

*Agrobacterium tumefaciens* strains transformed with pBetaPhaso-*VrPDF1* were infiltrated into mung bean leaf explants for 30 min (Sonia et al., 2007). After infiltration, leaf explants were transferred to co-cultivation medium in the dark for 3 days. The leaf explants were then washed with sterile liquid MS medium containing 400 mg. L<sup>-1</sup> cefotaxime. The leaf explants were placed into shoot induction medium for 3 weeks. Following shoot initiation, shoots were selective on shoot elongation medium and then transferred to rooting medium. Finally, the

transformed plantlets were transplanted to pots in greenhouse and used to analyze *VrDEF1* gene expression. All mung bean tissue cultures were maintained under 16 hrs photoperiod at  $25 \pm 2^\circ\text{C}$ .

#### ***Analysis of the existence of the VrDEF1 transgene in genome of transgenic mung bean plants***

For *VrPDF1* expression analysis, total DNA was isolated from the transform mung bean leaves according to Edwards et al. (1991). PCR reaction was performed with primer pairs:

*VrPDF1-HindIII-F* forward primer: 5' CCAAGCTTGGTTAACAGTTTCTAGTGCACC 3'

*VrPDF1-SalI-R* reverse primer: 5' GCGTCGACGATGGAGAAGAAATCACTGGCC 3'

After initial  $94^\circ\text{C}$  denaturation step for 4 minutes, the reaction was carried out followed for 30 cycles at  $94^\circ\text{C}$  for 15 s, at  $58^\circ\text{C}$  for 30 s (annealing), at  $72^\circ\text{C}$  for 60 s and final extension  $72^\circ\text{C}$  for 10 minutes. PCR products were detected by 1.5% agarose gel electrophoresis.

In addition, transgenic mung bean plants were continued to analyse by Southern blot using Biotin Chromogenic Detection Kit (Themosience). Total DNA was isolated and be digested with restriction enzymes *SalI* ( $10\text{u } \mu\text{l}^{-1}$ ) overnight at  $37^\circ\text{C}$ , and then electrophorese digested DNA on 1% agarose gel. Digested DNA was transferred to the membrane, add the hybridization solution containing the biotin-labeled probe to the membrane. Finally, show and analyze results on X-ray film.

#### ***Western blot analysis and enzyme-linked immunosorbent assay (ELISA)***

To detect the expression of recombinant VrPDF1, protein extracted from transgenic mung bean seeds, which were confirmed to be positive by PCR and Southern blot, was electrophoresed and transferred to nitrocellulose Hybond™ (Amersham). The membrane was washed in PBS-T and blocked in blocking solution containing 3% BSA for 1 hour. After additional wash, the membrane was incubated with c-myc antibody in PBS supplemented with 0,5% BSA and 0,05% Tween 20 for 1 hour, and then, the membrane was incubated with

secondary antibody attached ALP or antimouse IgG antibody conjugated to HRP (horseradish peroxidase). Following the final wash, the membrane was incubated in NBT/BCIP to determined recombinant VrPDF1 protein.

Transform mung bean plants were analyzed for presence of recombinant VrPDF1 production by ELISA (Sun et al. 2006). Total protein extracted from mung bean transform seeds was diluted to a concentration of 200  $\mu\text{g ml}^{-1}$ . 100  $\mu\text{l}$  diluted sample was added into microplates, each sample was repeated three times. C-myc antigen, anti-mouse IgG antibody conjugated to horseradish peroxidase (HRP) and TMB solution were used to determine recombinant VrPDF1 protein. ScFv protein tagged to cmc was used as a positive control. The concentration of diluted ScFv protein was in the range of 2.5; 5.0; 10.0; 20.0; 400.0 ng per well to construct a standard curve. The amount of recombinant VrPDF1 protein could be determined by following equation  $Y = 0.0019X + 0.0562$ , where  $Y$  = amount of recombinant VrPDF1 protein and correlation coefficient (R) was also computed at 0.9993.

#### **Alpha-amylase inhibitory assay**

Mung bean weevils (*Callosobruchus chinensis* L.) in larval stage were grinded with 0.2 M phosphate buffer, pH 6.8. After centrifugation at 12000 rpm for 10 minutes, solution containing alpha-amylase was transferred to fresh micro centrifuge tubes for alpha-amylase activity assay (Bernfeld, 1955). Analysis of  $\alpha$ -amylase activity of mung bean weevils was carried out in three independent experiments, which were (1) analysis starch by only  $\alpha$ -amylase of weevils, (2) analysis starch by  $\alpha$ -amylase of weevils and protein extracted from seeds of WT, (3) analysis starch by  $\alpha$ -amylase of weevils and protein extracted from seeds of transgenic mung bean plants. After incubation for 30 minutes at 30  $^{\circ}\text{C}$ , all samples were analyzed by absorption



170 spectroscopy at 530 nm. The  $\alpha$ -amylase enzymatic activity is the amount (mg) of degradable  
171 starch for 30 min at 30 °C (U mg<sup>-1</sup>).

## 172 RESULTS AND DISCUSSION

### 173 **Creating transgenic mung bean plants containing pBetaPhaso-VrPDF1 and analysis of the** 174 **presence of the *VrPDF1* transgene in the genome of transgenic mung bean plants**

175 Based on previous evaluations of weevil resistance from different local Vietnamese mung  
176 bean cultivars, we chose the local mung bean cultivar Tam TH, which has the best resistance, as  
177 the source for *VrPDF1* gene isolation. The VrPDF1 cDNA is 228 bp in length, encoding 75  
178 amino acids. The VrPDF1 cDNA sequence from mung bean cultivar Tam TH was deposited in  
179 GenBank (NCBI) with the code LN913082.1 (Hoang et al., 2014). The *VrPDF1* cDNA was  
180 inserted into vector p201-SLHEP, which contains two alternative positions, attL1 and attL2.  
181 Vector *pDON201-SLEHP-VrPDF1* underwent the LR reaction with vector pBetaPhaso-dest to  
182 form a binary vector called pBetaPhaso-VrPDF1. Structure pBetaPhaso-dest contains a phaseolin  
183 promoter, 2S2 (a sequence to direct protein expression in the endosperms of seeds), and a c-myc  
184 tag. Inferentially, the c-myc that the recombinant protein contains allows determining the  
185 expression of recombinant protein using c-myc antibody (Fig. 1).

186 *Agrobacterium*-mediated genetic transformation of pBetaPhaso-VrPDF1 into D22 mung  
187 bean cultivar was carried out via infected leaf explants. The effect of transformation depends on  
188 many factors such as materials, cultured medium, timing of infection, gene transfer vector. In  
189 this study, 3-day-old germinated seeds are used as a source of transgenic materials, because two  
190 cotyledons is easily splitted at this stage. Transformation experiments conducted with 630  
191 samples of three independent experiments, in which 107 shoots were induced on SEM selective

medium supplemented with kanamycin 50mgL<sup>-1</sup> and 46 produced shoots could be rooted. Rooted plantlets were transferred to pots in greenhouse with 23 survived plantlets.

To confirm the presence of the *VrPDF1* transgene in transgenic mung bean lines, PCR reactions with the primer set VrPDF1-HinIII-F/VrPDF1-Sali-R was used to amplify the VrPDF1 transgene. According to the analysis of sequences of *VrPDF1* from genomic DNA and mRNA, the difference between them is existence or absence of intron, therefore, *VrPDF1* transgene has 220 bp and *VrPDF1* gene has 386 bp. The PCR products of amplification of *VrPDF1* from eight transgenic mung bean plants, WT and plasmid pBT-VrPDF1 in 1.5% agarose gel are shown in Figure 2. In the electrophoresis gel of PCR products to check *VrPDF1* gene, five transgenic mung bean plants at 1, 3, 4, 7, 8 positions appeared two bands with the size of approximately 0.35 kb and 0.25 kb, respectively. The positions of these 0.35 kb bands were similar to the band of non-transgenic mung bean plant (negative control) and the 0.25 kb bands in the same size as the plasmid pBT-VrPDF1 (positive control). These data indicated the predicted size of *VrPDF1* gene (isolated from DNA) and *VrPDF1* cDNA. The results also have shown that the presence of the *VrPDF1* transgene in the five transgenic lines (DX0-1, DX0-3, DX0-4, DX0-7, DX0-8) and the transformation frequency has been achieved 0.79% success rate ( $5/630 = 0.79\%$ ). As can be seen in Figure 2, the *VrPDF1* transgene could be presented in the genome of mung bean plants, however, it is necessary to further assess whether or not the *VrPDF1* transgene was integrated into the host genome. Therefore, five transgenic mung bean lines, which were positive for PCR and non-transgenic control plants, were then analysed by Southern blot (Fig. 3). The results of Southern plot analysis of five transgenic mung bean lines in Figure 3 show that all five transgenic lines have a band in hybrid membrane. Thus, it can be confirmed that pPhaso-VrPDF1 structure was moved successfully into mung bean plants and transgene *VrPDF1* was integrated

into the genome of mung bean plant DX22. Thus, the evidence for presence and integration of *VrPDF1* transgene in the transgenic plant genome are initial results that provide fundamentals for success of VrPDF1 transformation of mung bean. The five transgenic mung bean lines tested positive for PCR and Southern blot were continuing to access the growth and development and collected seeds for analysis in the next generation.

## **Analysis of expression of recombinant VrPDF1 (rVrPDF1) protein in transgenic mung bean lines**

Southern blots and PCR confirmed the presence of inserted *VrPDF1* transgene in transformants. After DNA analysis, stable expression and performance of transgene was a particularly interesting issue, therefore, Western blot and ELISA were used to determine the presence and the correct size of rVrPDF1 protein. There are many factors that govern the level of transgene expression, in which the location of the transgene integrated into the host genome plays a major role (Yin Z et al., 2003). Binding site of the transgene in the host genome expression decides gene stability (Topping et al., 1991; Yin Z et al., 1996). Thus, it is necessary to detect the integrated position for transgene in the host genome to optimize the level of transgene expression. The five transgenic mung bean lines grew and produced flowers and pods, in which DX0-3, DX0-7 and DX0-4 lines set 6, 7 and 3 pods with viable seeds respectively, DX0-1 and DX0-8 both set 2 pods without viable seeds. Seeds of DX0-3, DX0-4 and DX0-7 lines that were respectively named DX1-3, DX1-4 and DX1-7 were used for Western blot and ELISA analysis.

As depicted in Figure 4, the rVrPDF1 protein in two T1 transgenic mung bean lines (DX1-3, DX1-7) were identified on hybrid membrane, the molecular weight of these protein bands was about 10 kDa. However, the band completely disappeared in DX1-4 transgenic line and non-transgenic control plants (WT). According to these results, the rVrPDF1 protein was

successfully expressed in two transgenic mung bean lines DX1-3 and DX1-7 and the heredity of *VrPDF1* gene was transferred from T0 to T1 generation via sexual reproduction. Furthermore, to assess the level of rVrPDF1 protein expression, ELISA technique was used to determine the content of rVrPDF1, the results are shown in Figure 5. The rVrPDF1 content of DX1-3 and DX1-7 were 6.23 and 9.26 ( $\mu\text{g} / \text{mg}$  total protein) respectively. In contrast, the content of rVrPDF1 in DX1-4 line and WT is 0.00 ( $\mu\text{g} \text{ mg}^{-1}$  total protein). These results proved that the expression of *VrPDF1* gene strongly enhanced in DX1-3 and DX1-7 lines.

The transformation efficiency that was evaluated based on the percentage of PCR, Southern blot and Western blot positive mung bean lines was 0.79%, 0.63% and 0.32% respectively. These results were similar to other studies on transformation of soybean (Nguyen Thu Hien et al., 2011). The transformation efficiency of mung bean and soybean was low, which was mainly caused by the high protein content in seeds and *in vitro* regenerating difficulty of soybean and mung bean. In addition, after transformation, the transgene that was attached to the genome of plant host was genetically unstable, lost or restructured. Thus, the efficiency of transgene expression is very low, or transgene can be inactivated (Srivastava et al., 1996; Chen et al., 2002).

### **Enhanced weevil $\alpha$ -amylase inhibitor of transgenic mung bean**

According to the correlation of VrPDF1 protein content from ELISA results with weevil resistance, it could be expected that the weevil resistance of DX1-7 would be higher than DX1-3. However, this prediction should be confirmed by empirical analysis of the  $\alpha$ -amylase inhibitory activity of VrPDF1 proteins between transgenic mung bean lines. Thus, protein extracts from two transgenic mung bean lines DX1-3, DX1-7 and WT were used checked  $\alpha$ -amylase inhibition ability of mung bean weevil in its larval stage.

The results in Table 1 shows that the activity of  $\alpha$ -amylase are 9.47 (IU mg<sup>-1</sup>) and 6.19 (IU mg<sup>-1</sup>) in the first experiment (using only  $\alpha$ -amylase of larvae) and the second experiment (using  $\alpha$ -amylase of larvae and protein extracted from seeds of WT) respectively. However,  $\alpha$ -amylase activity is much lower in the third experiment using  $\alpha$ -amylase of weevil larvae and protein extracts from seeds of two transgenic mung bean plants, only 2.08 (IU mg<sup>-1</sup>) with rVrPDF1 in DX1-3 line and 1.35 (IU mg<sup>-1</sup>) with rVrPDF1 in DX1-7 line. According to these results, protein extracts from WT and transgenic lines inhibit  $\alpha$ -amylase activity better than in the first experiment. The performance of  $\alpha$ -amylase decreased from 100% ( $\alpha$ -amylase of weevils and protein extracted from seeds of WT) to only 33.60% ( $\alpha$ -amylase of weevils and protein extracted from DX1-3) and 21.81% ( $\alpha$ -amylase of weevils and protein extracted from DX1-7) (Fig. 6). The rVrPDF1 in two transgenic mung bean lines DX1-3 and DX1-7 was enhanced inhibitor of  $\alpha$ -amylase from weevil larvae.

Thus, rVrPDF1 proteins of DX1-3 and DX1-7 inhibited  $\alpha$ -amylase activity of mung bean weevil larvae. As can be observed in Figure 6, the inhibitor performance of  $\alpha$ -amylase from mung bean weevil larvae of rVrPDF1 extracted from DX1-3 and DX1-7 respectively increased 166.40% and 178.19% in comparison with WT. These results have demonstrated that application of gene technology in enhancement defensin expression in seed is an effective method for improving mung bean weevil resistance.

## CONCLUSIONS

We have successfully generated transgenic mung bean plants carrying pBetaPhaso-VrPDF1 structure in the T1 generation from the DX22 mung bean cultivar. The overexpression of the recombinant VrPDF1 protein in two T1 generation transgenic mung bean lines (DX1-3 and DX1-7) with a molecular weight of around 10 kDa has been demonstrated.  $\alpha$ -amylase

inhibitory activity of rVrPDF1 from DX1-3 and DX1-7 lines respectively increased 166.40% and 178.19% compared with non-transgenic plants. The enhancement of  $\alpha$ -amylase inhibitor ability of rVrPDF1 extracted from transgenic plants is scientifically fundamental to confirm the effectiveness of the application of gene technology in enhancement the ability of mung bean weevil resistance in mung bean and grain crops.

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*Table 1.  $\alpha$ -amylase activity of mung bean weevils (IU mg<sup>-1</sup>)*

Only $\alpha$ -amylase of weevils	$\alpha$ -amylase of weevils and protein extracted from seeds of WT	$\alpha$ -amylase of weevils and protein extracted from seeds of transgenic mung bean plants	
		<i>DX1-3</i>	<i>DX1-7</i>

$$9.47 \pm 0.29$$

$$6.19 \pm 0.09$$

$$2.08 \pm 0.06$$

$$1.35 \pm 0.05$$

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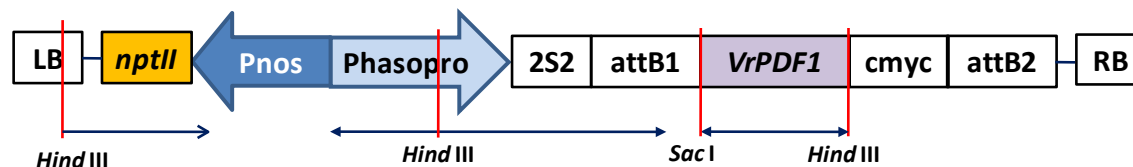
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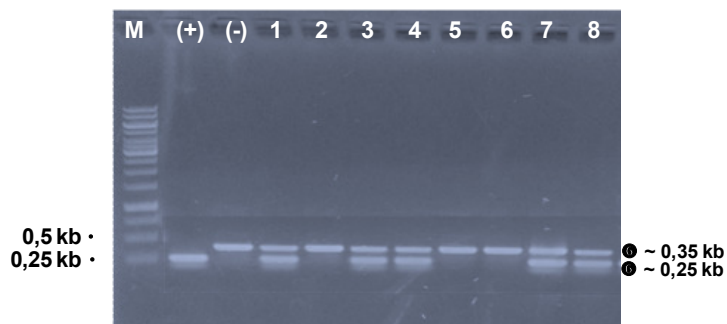
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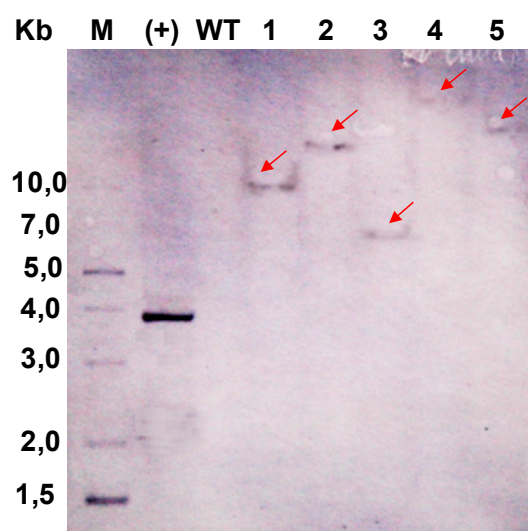
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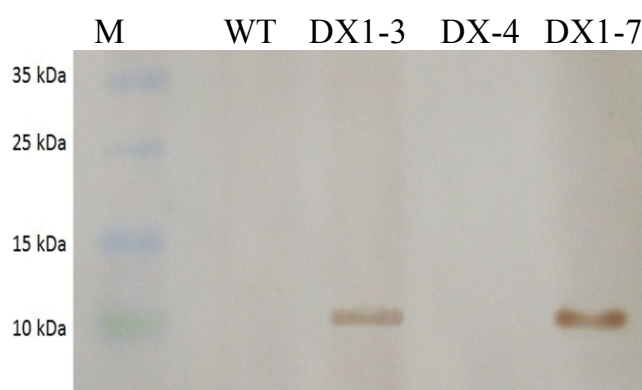
**Figure 1.** Diagram of pBetaPhaso- VrPDF1 structure. *nptII*: kanamycin resistance. *Phaso Pro*: Phaseolin promoter; *2S2*: a sequence to direct protein expression in the endosperms of seeds; *VrPDF1*: *Vigna radiata plant defensin 1* gene isolated from local mung bean cultivar; *cmc*: the sequence encodes cmc antigen; *attB1* and *attB2*: recombinant positions for the LR reaction; *LB*: left T-DNA border; *RB*: right T-DNA border;



**Figure 2.** Genomic PCR amplification of VrPDF1 in T0 generation transgenic mung bean lines. M: DNA marker 1 kb; (+): plasmid pBT-VrPDF1; (-): the non-transgenic mung bean plant; 1-8: transgenic plants (named: DX0-1, DX0-2, DX0-3, DX0-4, DX0-5, DX0-6, DX0-7, DX0-8).

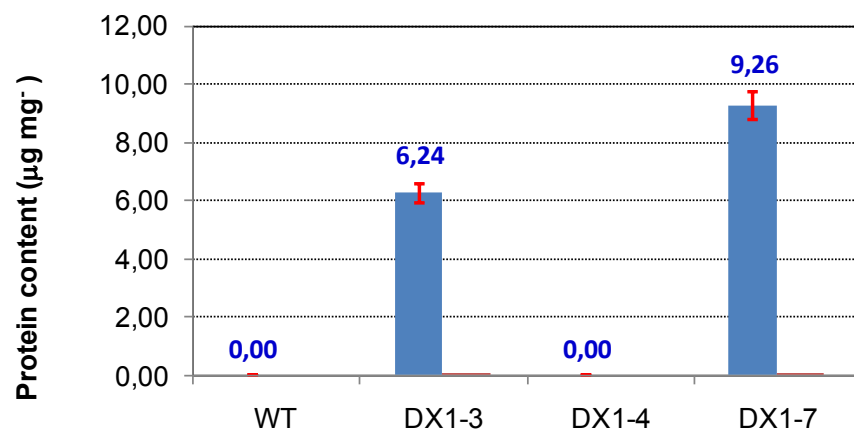


**Figure 3.** Results of Southern blot analysis of the transgenic mung bean lines. M: Marker 1kb, (+): pBetaPhaso-VrPDF1 vector; WT: non-transgenic mung bean plant DX22; 1: DX0-1; 2: DX0-3; 3: DX0-4; 4: DX0-7 and 5: DX0-8



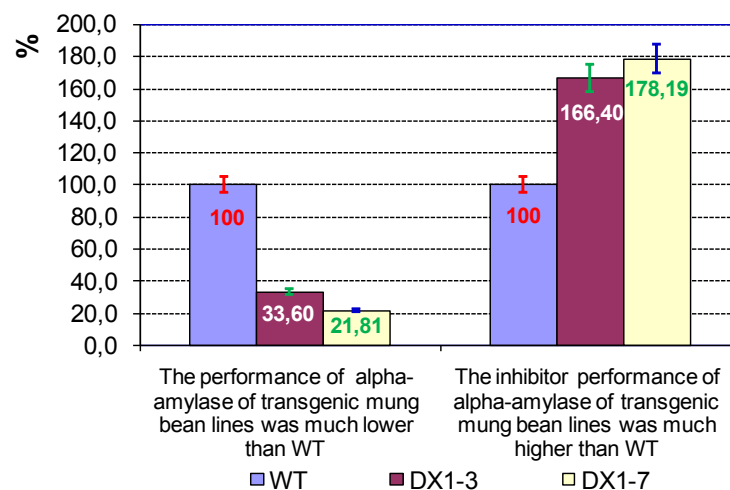
**Figure 4.** Analysis of rVrPDF1 in WT and T1 generation transgenic mung bean lines. M: Protein marker; WT: non-transgenic mung bean plant; DX1-3, DX1-4 and DX1-7: transgenic mung bean lines





Transgenic mung bean lines and non-transgenic mung bean plant

**Figure 5.** Comparison of rVrPDF1 production ( $\mu\text{g mg}^{-1}$  total protein) in WT and T1 generation transgenic mung bean lines. WT: non-transgenic mung bean plant; DX1-3, DX1-4 and DX1-7: transgenic mung bean lines



**Figure 6.** The performance of  $\alpha$ -amylase and the inhibitor performance of rVrPDF1 protein extracted from transgenic mung bean lines in comparison with WT