J Genet Resour2021;7(1): 116-124 *Homepage: http://sc.journals.umz.ac.ir/*

RESEARCH ARTICLE *DOI: 10.22080/jgr.2021.20205.1227*

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Evaluation of Antifungal Activity of Defensin (*Tfgd2*) Using Its Heterologous Expression in *E. coli*

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ARTICLE INFO

Article history:
Received 29 August 2020
Accepted 02 November 2020
Available 17 November 2020

Keywords:
Antifungal Activity
Defensin
SYTOX Green
TFGD2
Pathogenesis-related protein

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p-ISSN 2423-4257
e-ISSN 2588-2589

ABSTRACT

Defensins are a superfamily of antimicrobial peptides that can inhibit the growth of a broad spectrum of fungi. To evaluate the antifungal activity of *Tfgd2* gene of *Trigonella foenum graecum*, the coding region of this gene in the cDNA form was subcloned in the expression vector pET26b (+) and the construct was designated as pETSH1. The obtained construct expressing the recombinant protein with a hexahistidine tag at the C-terminal end transformed into the *E. coli* BL21 (DE3). Taguchi test applied for optimizing the protein expression and the expressed protein TFGD2 confirmed by SDS-PAGE and western blotting. The recombinant TFGD2 protein was purified using affinity chromatography with a Ni-NTA column. *In vitro* assay indicated a broad spectrum of antifungal activity of purified expressed recombinant TFGD2 against different fungal phytopathogens, such as *Fusarium oxysporum, Rhizoctonia solani, Sclerotinia sclerotiorum, Alternaria solani*, and *Verticillium dahliae.*

Please cite this paper as: Shiehbeiki S, Motallebi M, Zamani M, Jourabchi E. 2021. Evaluation of antifungal activity of defensin (*Tfgd2*) using its heterologous expression in *E. coli*. *J Genet Resour* 7(1): 116-124. doi: 10.22080/jgr.2021.20205.1227.

Introduction

Fungal disease is one of the major concerns of crop production. In developing countries, it has been estimated that total loss due to plant diseases reaches up to 50 % (Kirubakaran *et al.*, 2008). One-third of plant diseases are due to fungal infections (De and Vivier, 2011). Plants produce a lot of molecules that are natural preventive to pathogens, for example, carbohydrate molecules, polyanions, and several defense-related proteins such as chitinases and defensins (Pelegrini *et al.*, 2008).

Defensins belong to the superfamily of antimicrobial peptides (AMPs) which are found in various living organisms, such as vertebrates, invertebrates, plants, and fungi (Stotz *et al.*, 2009; Wilmes *et al.*, 2011; Carvalho and Gomes, 2011; De *et al.*, 2013; Karri and Bharadwaja, 2013). Plant defensins are proteins with a small molecular weight (<10 kDa) and positively charged. All parts of the plants including seeds, pods, fruits, flower parts, leaves, tubers, roots, and stems can be used to extract defensins (Carvalho and Gomes, 2009; Vriens *et al.*, 2014). Various plant defenses are known to inhibit the growth of fungi and bacteria at micro-molar concentrations (Pellegrini, 2003; Lay and Anderson, 2005). Although the mode of antimicrobial action is not well understood, earlier studies hypothesized that their toxicity might be due to interaction with the negatively charged membrane phospholipids followed by pore formation, leading to permeability enhancement and cell death (Pellegrini, 2003).

A relatively small number of defensins have also been described that inhibit the activity of serine proteases or α-amylases from various sources, such as insects, mammals, and fungi (Pellegrini, 2003; Li *et al.*, 2017; Vi *et al.*, 2017). Other biological activities like ion channel blockers (Kushmerick *et al.*, 1998; Spelbrink *et al.*, 2004; Xiang *et al.*, 2015). Protein translation inhibitor (Brook *et al.*, 2016; Chen *et al.*, 2005) and mediation of metal tolerance (Mirouze *et al.*,

2006; Mith *et al.*, 2015) have been reported from plant defensins.

Extract defensin (TFGD2) from *Trigonella foenum graecum* was tested *in vitro* against some fungal pathogens like *Rhizoctonia solani* and *Phaeoisariopsis personata* (Olli and Kirti, 2006). The purpose of the present study is to examine the antifungal activity of heterologous expressed TFGD2 protein in the *E. coli* system against *Fusarium oxysporum, Verticillium dahliae, Sclerotinia sclerotiorum, Alternaria solani, Rhizoctonia solani*.

Materials and Methods

Plasmids, fungal and bacterial isolates

pJETME4 construct containing *Tfgd2* cDNA was used as a template (Etebari *et al*., 2016) Expression vector pET26b (+) (Novagen) and *E. coli* BL21 (DE3) strain was used for the prokaryotic expression. The fungal plant pathogens: *F. oxysporum*, R. *solani*, *S. sclerotiorum*, *A. solani,* and *V. dahliae* were provided by H. Afshari-Azad (Iranian Research Institute of Plant Protection, Tehran, Iran). The fungi were grown on potato dextrose agar (PDA) medium (Himedia, India) and incubated at 28ºC. *E. coli* strains were grown at 37 ºC in Luria-Bertani (LB) broth medium and the media were supplemented with kanamycin and ampicillin (SIGMA) 100 and 50 mg/ml respectively.

Construction of recombinant plasmid

The coding sequence of the *Tfgd2* gene amplified using *Pfu* polymerase (Fermentas) with two specific primers of the (5'-GGAAGACAACATGAAAAACTTGTGAGAATTT GG, and 5'-CGCTCGAGACATCTTTTAGTACACCAA, which contain *Bpi*I and *Xho*I sites at their 5′ ends respectively. The amplified fragment was purified with the recovery kit (iNtRON Biotechnology) and cloned into the pET26b (+) expression vector at the *Nco*I/*Xho*I restriction site in a frame under the control of the T7 promoter and with His-tag. It was confirmed by sequencing and designated as pETSH1.

TFGD2 expression in *E. coli* BL21

E. coli BL21 transformed by pETSH1 and a single colony selected on LB medium containing 50 µg/ml of kanamycin as a selectable marker. The selected colony was cultured in 10 ml medium at 37 ºC overnight under 180 rpm as a pre-culture. One ml of this culture was used to inoculate 100 ml of LB media, containing the appropriate antibiotics, and grown at 37 °C until an optical density (OD_{600}) of 0.6.

Protein expression was induced by adding IPTG (isopropyl-1-thio-β-D-galactopyranoside 1mM) to the culture, and the cells were shaken at 28 ºC for 6h under agitation 180 rpm. Expression was visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 12 %).

Optimization of protein expression

By using Taguchi's method, the experiments were designed to achieve the best condition of expression. In this method, the effects of 3 factors that influence the expression of foreign proteins in *E. coli* for maximizing the production of foreign protein using this method (Larentis *et al.*, 2014; Papaneophytou and Kontopidis, 2014; Silaban *et al.*, 2018). Different levels for these factors have been reported by several researchers (Makrides, 1996; Volontè *et al.*, 2008; Larentis *et al.*, 2014; Papaneophytou and Kontopidis, 2014; Silaban *et al.*, 2018). In this study in 4 levels for each factor were studied on protein production, including temperature (23, 28, 33, 37ºC), IPTG concentration (0.2, 0.5, 0.7, 1mM), incubation time (2, 4, 6, 16h) (Table 2). According to the considered factors, 16 independent experiments were designed. The total protein of each experiment was extracted from the cells and visualized by SDS-PAGE. The content of the overexpressed TFGD2 protein in each fraction was quantified with a Bio-Rad GS-800 gel densitometer. The density of expressed protein was calculated relative to the reference band (total cell protein of *E. coli*). After analyzing and quantifying the data, the results were analyzed by Qualitek-4 software (version 08.10; http://www.nutek-us.com**)** (Bloomfield Hill, MI, USA) and the most important effect of different levels of each factor and the optimal expression conditions were then determined. Analysis of variance (ANOVA) was used for statistical analysis.

Protein purification

TFGD2 protein was purified using Nickel-nitrilotriacetic acid (Ni-NTA) resin under native

conditions. Briefly, cell culture was pelleted under this condition (maximum speed, 2 min, 4 °C) in a microcentrifuge. Cell pellet (50 ml) was resuspended in 5 ml of the lysis buffer [NaH2PO4 (50 mM), pH 8.0, NaCl (300 mM), and imidazole (10 mM), 0.05% tween20].

The suspension was then lysed by sonication (12 times for 45 s at amplifier 70 with 30 s of cooling between cycles). The mixture was centrifuged at 13000 g for 30 min at 4 °C. The supernatant was mixed with 1 ml Ni-NTA (QIA-gene) and shaken for 1h 4 °C. The supernatant mixture was loaded on a column.

Unbound proteins were washed with washing buffer (20mM imidazole, 300mM NaCl, 50mM NaH_2PO_4, 0.05% tween 20) and the bound protein was eluted with elution buffer (250mM imidazole, 300mM NaCl, 50 mM NaH_2PO_4, 0.05% tween 20). All of the procedure was carried out at 4 °C and finally purified protein was monitored on a 12% SDS-PAGE. The concentration of purified protein was measured with the Bradford method.

Western blotting analysis

For immunodetection of the expressed TFGD2 by western blotting, total protein was extracted from induced *E. coli* BL21, and electrophoresed on SDS-PAGE, followed by electrotransferred to a polyvinylidene fluoride (PVDF) membrane. The immunoblots were developed with anti-His tag antibody, according to the manufacturer's instructions (Roche; http://www.roche.com). The anti-His tag antibody has been conjugated to horseradish peroxidase (HRP). As a substrate for HRP, 4-choloro-1-naphthol was used.

Antifungal activity assay

Three methods of biological assays were used for the detection of antifungal activity of expressed TFGD2: radial diffusion assay, spore germination assay, and SYTOX Green fluorescent staining.

Radial diffusion assay

For detection of antifungal activity of TFGD2, a fungal growth inhibitory assay was used with the modified method of Broglie *et al*., (1991). The zone of inhibition assay for antifungal activity was determined using 100 × 15 mm plates containing 25 ml of PDA. After the mycelia colony had developed (3 days), 5 mm holes were made around at a distance of 5 mm away from the rim of the mycelia colony. Different concentrations (5, 10, and 15 µg) of the purified TFGD2 were added to each hole. The plates were incubated at 28 °C for 24 h until the mycelia growth has covered the hole containing the negative controls (protein-free buffer and protein extraction from a host containing empty pET26b (+). The assay was performed with three replicates and the inhibition area was measured using Image Tools Software.

Spore germination assay

The fungal species were grown on a PDA plate at 28 °C for 5 days. The spores were washed with sterile water and then were counted. Based on the modified method of Nweze and *et al.,* (2010), sterile paper disks (6 mm in diameter) were placed on the PDA medium. Then 1 µl from 2 × 107 cell/ml dilution of spore suspension of selected fungal species was added to each disk.

Different concentrations of TFGD2 (2, 5, and 10 µg) were added to the disks. The Protein extraction of empty vector and protein extracting buffer was used as a negative control. Plates were incubated at 28 °C and spore germination and mycelia growth was observed. The diameter of growth zones was measured using Image Tools Software.

SYTOX Green fluorescent staining

The ability of TFGD2 protein to cause plasma membrane permeabilization was measured by the SYTOX Green (Invitrogen Corp., Carlsbad, CA, USA). The fungi were grown in PDB media for 48h at 28 °C, and the SYTOX Green staining experiment was achieved as described by Therisson *et al.,* (1999).

Statistical analysis

Statistical differences were assessed based on analysis of variance (ANOVA) using SPSS (version 15, USA). Differences were considered significant at a probability level of $p<0.05$.

Qualitek-4 software (Bloomfield Hills, MI, USA) for automatic design and analysis of Taguchi experiment was used to determine the optimum recombinant protein expression conditions.

Results

cDNA cloning of *Tfgd2*

In this research, for testing the antifungal activity of the *Tfgd2* gene from *Trigonella foenum graecum* the coding region of this gene in the cDNA form was amplified from the previously reported construct (pJETME4) (Etebari *et al.*, 2016) using appropriate primers. The confirmed amplified fragment (by sequencing analysis) was subcloned into pET26b (+) containing an inbuilt His6-tag at the C-terminal end to facilitate its purification. The new construct was designated as pETSH1.The heterologous protein was overproduced in the prokaryotic expression system (*E. coli* BL21-DE3). The results of the SDS-PAGE pattern and western blot analysis confirmed the right expression of the recombinant protein (Fig. 1).

Expression of TFGD2 protein

For optimization of the expression of TFGD2 protein, 16 different experimental conditions were prepared using the Taguchi method. Three factors including temperature (23, 28, 33, and 37°C) induction time (2, 4, 6, and 16h), and concentration of IPTG (0.2, 0.5, 0.7, and 1mM) were tested to find the optimum conditions for this heterologous expression. The results of the estimation of the amount of expressed protein were calculated using densitometry of the SDS-PAGE pattern of these 16 experiments. The ratio of the density of expressed protein to the total protein density (as described in materials and methods) was used as the expression index (Fig. 2). The obtaining results showed that 37°C, 6 hours of incubation, and 0.5 mM of IPTG concentration were the optimum conditions for protein expression. The effect of each factor on the TFGD2 protein expression was shown in Table 1. ANOVA analysis of the data indicated that temperature was the most important factor in protein expression and the incubation time and IPTG concentration were placed in second and third (Table 2).

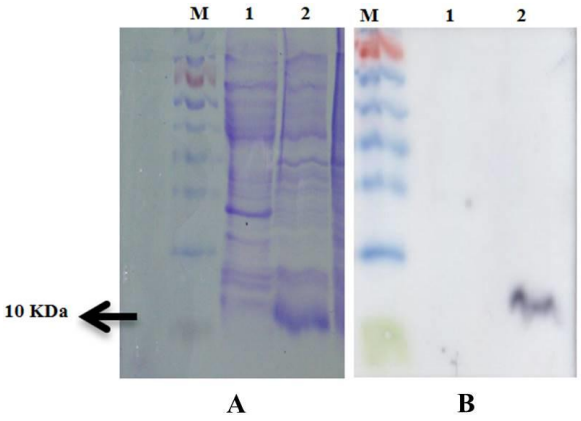

Fig. 1. SDS-PAGE and western blot showing induction of recombinant protein: (A) BL21 (DE3) cells transformed with pET26b (+) vector containing *tfgd2* gene were grown up to 0.6-0.8 OD and induced with IPTG. After harvesting cells total protein was extracted and analyzed on 15% SDS-PAGE., 1= Non-induced protein extraction, 2= Induced protein expression, M= Protein marker; (B) Western blot of recombinant protein with anti-His., 1= Non-induced protein extraction, 2= Induced protein expression, M= Protein marker.

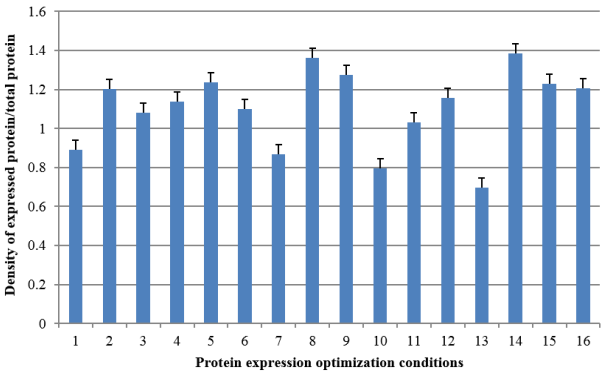

Fig. 2. Quantitative analysis of protein expression optimization conditions: 1-16= Different trials used in M16 orthogonal experimental design.

The results of the interactions of different factors demonstrated that the interaction of 1 mM IPTG and 4 hours incubation time with 33.33% severity index exhibited the highest interaction (Table 3).

Table 1. Main factors (average effects of factors and interactions).

Factors	Level 1	Level 2	Level 3	Level 4
IPTG (mM)	1.072	1.12	1.059	1.125
Time (Hour)	1.017	1.115	1.035	1.21
Temperature(°C)	1.049	1.202	1.272	0.852

Table 2. ANOVA analysis.

Col#/Factor	DOF(f)	Sum of Square (SS)	Variance (V)	F.Ratio (F)	Pure Sum (S′)	Percent P (%)
IPTG (mM)	3	0.013	0.004	0.22	0	0
Time(Hour)	3	0.092	0.03	1.573	0.033	5.296
Temperature(°C)	3	0.415	0.138	7.038	0.356	55.742
Other Error	6	117	0.019			38.962
Total	15	639				100%

Analysis of variance; Factor: The variability due to the factor of interest; Error: The variability within the groups or unexplained random error; Total**:** The total variation in the data from the grand mean; DOF: The degrees of freedom in the source; SS: The sum of squares due to the source; F: The *F*-statistic; P**:** The *P*-value.

Table 3. Quantlitek-4 analysis.

Interacting Factor Pairs (Order based on SI)	Columns	SI (%)	OPT
IPTG ×Time	1×2	33.33	[4,2]
Time × Temperature	2×3	17.39	[2,3]
IPTG ×Temperature	1×3	13.04	[4,3]

Estimation of severity index for different factors; Column: Represent the column location to which the interacting factors are assigned; SI: Interaction severity index (100% for degrees angle between the lines, 0% for parallel lines); OPT: Indicates factor levels desirable for the optimum condition.

Purification of TFGD2 protein

The recombinant protein was overexpressed by IPTG in the bacterial system. The heterologous protein was purified by affinity chromatography using the Ni-NTA column. The expressed protein-containing His-tag was eluted with 300 mM of imidazole. The results of the SDS-PAGE confirmed the expected purified protein band (about 10KDa) (Fig. 3A). Further confirmation of the expressed protein was achieved by western blot analysis using an anti-his tag antibody (Fig. 3B).

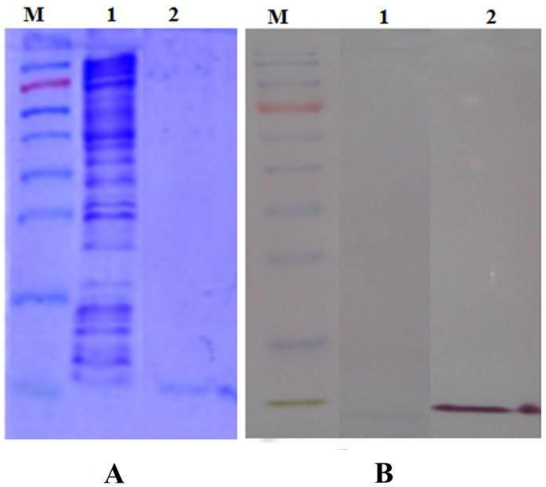

Fig. 3. SDS-PAGE and Western blot for purification of expressed protein with Ni-NTA column: (A) Purification of the expressed protein (1= Flow-through, 2= Elution, M= Protein marker); (B) Western blot showing the purified protein (1= Flow-through, 2= Elution, M= Protein marker).

Antifungal activity assay

The antifungal activity of the prokaryotic expressed TFGD2 protein was investigated using three different assays. The results of the radial diffusion assay demonstrated the fungal growth inhibition of *S. sclerotiorum, V. dahliae*, and *F. oxysporum*. The hyphal growth was decreased by increasing the concentration of the expressed protein (5, 10, and 15 µg) (Fig. 4).

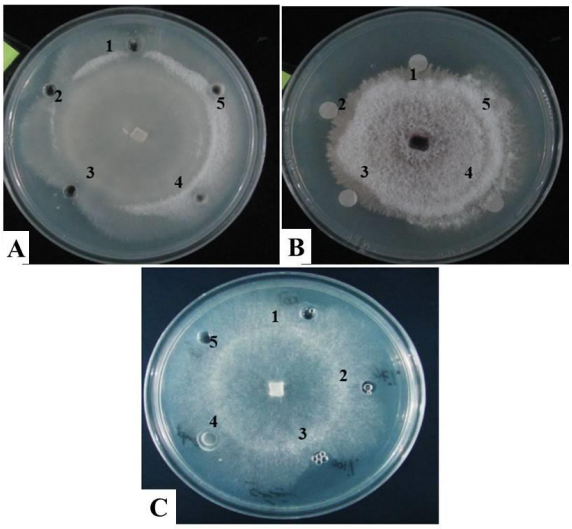

Fig. 4. *In vitro* antifungal assay of TFGD2 showing inhibition of the mycelial growth of different plant pathogenic fungi: (A) *F. oxysporum*; (B*) V. dahlia* and (C) *S. sclerotiorum* (1, 2 and 3= Different concentrations 5, 10 and 15 µg of purified protein respectively; 4= Protein extract of empty vector; 5= Elution buffer).

The inhibitory effect of the purified expressed protein on spore germination of *F. oxysporum, V. dahliae,* and *A. solani* was increased by increasing the concentration of the purified expressed protein (2, 5, and 10 µg) (Fig. 5). Furthermore, the fungi tested in spore germination assay appeared to be more sensitive than radial diffusion assay.

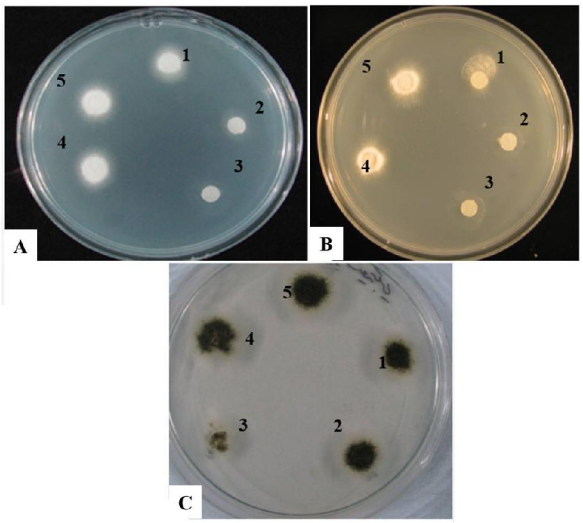

Fig. 5. *In vitro* antifungal spore germination showing inhibition of the spore germination of different plant pathogenic fungi: (A) *F. oxysporum*; (B) *V. dahliae* and (C) *A. solani* (1, 2, and 3= Different concentrations of 2, 5 and 10 µg of purified protein; 4= Protein extract of empty vector; 5= Elution buffer).

The ability of the purified expressed TFGD2 to cause cell membrane permeability of *R. solani* was assessed by the SYTOX Green uptake assay. Because SYTOX Green is a dye complex with a high affinity to a nucleic acid that penetrates through the damaged cell membrane, it is a suitable index for the determination of permeability of the plasma membrane. The results of this study confirmed the effect of purified TFGD2 protein on the induction of the fungal mycelia membrane permeability. Morphological observations using light microscopy (slide culture assay) demonstrated that penetration of SYTOX Green has appeared within 6 hours after the addition of 5 µg of purified TFGD2 protein on *R. solani* mycelia (Fig. 6). Mycelia morphology was not affected by treatment with negative control (protein-free buffer).

Discussion

One of the major concerns of agriculture production is fungal diseases. It has been estimated that total losses as a consequence of plant diseases reaches estimated up to 50 % (Kirubakaran *et al.*, 2008). One-third of plant diseases are due to fungal infections (Makrides, 1996). Also, the available evidence suggests that defensins cause permeabilization and membrane destabilization (Thevissen et al., 1996). Purification of many defensins has been achieved from the plants in native conditions. To demonstrate the antifungal activity of the predicted protein from a sequence, the heterologous expression is required (Campos et al., 2008; De and Vivier, 2011).

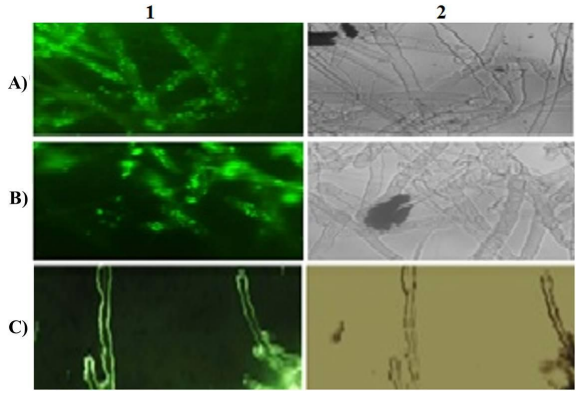

Fig. 6. Fluorescence microscopy of fungal cells in presence of SYTOX Green: R. solani cells were supplemented with 5mM MgCl2 and SYTOX green and treated for 6 h in the presence or absence of the purified protein (5µg); Right panels are light microscopic and left panels are fluorescence microscopic images; (A and B) Presence of 5µg purified protein for 6 h; (C) empty vector protein as a negative control.

Since the Tfgd2 gene contains an intron (Etebari, *et al.*, 2016), the cDNA form of Tfgd2 was used for the expression of this protein. In this study, the cDNA fragment was cloned and TFGD2 protein was successfully expressed in *E. coli* BL21 (DE3). This prokaryotic system has been used to produce several recombinant proteins, because of easy handling, inexpensive media, and large-scale production (Makrides, 1996).

It has been reported that signal peptides in the N-terminal of PR-5 preprotein affect the *E. coli* growth and failed to produce the protein (Low *et al.*, 2012; Singh *et al.*, 2013). Similarly,

heterologous expression of defensin gene from T.kirilowii with signal peptide was not induced using pET32a (+) (Hu and Reddy, 1997). In the present study to evaluate the effect of antifungal activity of an antimicrobial peptide on important fungal pathogens of crop production, a cDNA corresponding to the coding region of TFGD2 mature protein (without signal peptide) was used for heterologous expression in *E. coli*. Taguchi method for optimal expression of TFGD2 protein was successfully achieved and shown to be 6 hours of incubation, 0.5 mM of IPTG concentration, and incubation at 37 °C. Also, the western blot analysis confirmed the expected band of the expressed protein. The efficiently purified protein was used to determine its antifungal activity against several plant pathogens including *F. oxysporum*, *V. dahliae*, *S. sclerotiorum*, *A. solani*, *R. solani*. The radial diffusion and spore germination assays demonstrated that with increasing the concentration of the expressed protein the hyphal growth and spore germination of the fungi were decreased. A similar result was reported by (Karri and Bharadwaja, 2013) in a study of antifungal activity of Trigonella Foenum-graecum defensin. (Olli and Kirti, 2006) demonstrated that heterologous expressed TFGD1 in *E. coli* inhibits the mycelia growth of *R. solani*.

The SYTOX Green uptake results have also revealed that the expressed TFGD2 protein can change the fungal plasma membrane permeability. Similar results were reported by Chowdhury *et al.*, (2015) and Picart *et al.*, (2012).

In conclusion, it was shown that TFGD2 from Trigonella Poenum graecum has potential antifungal activity against some important phytopathogenic fungi. These results suggest that the *Tfgd2* gene has the potential to be efficiently used in plant biotechnology for improving and enhancing disease resistance in crop plants against fungal pathogens.

Acknowledgments

The National Institute of Genetic Engineering and Biotechnology (NIGEB) of I. R. Iran financially supported this research.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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