Development of an efficient tef-1α RNA hairpin structure to efficient management of lasiodiplodia theobromae

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Abstract

Lasiodiplodia theobromae is a serious worldwide-distributed plant pathogenic fungus with a wide host range in temperate and tropical climates. *L. theobromae* causes fruit rot, canker, and dieback of twigs in various woody plants. Protection of pruning wounds using fungicides is the prevalent strategy to management of the diseases caused by *L. theobromae*. Chemical control of plant diseases is not environmentally safe and the residues of fungicides are a threat to nature. Furthermore, genetic resources of resistance to plant diseases in woody plants are limited. The aim of this study was to investigate the efficiency of RNA silencing using an efficient hairpin structure based on *tef-1α* gene to management of *L. theobromae*. Hairpin structure of *tef-1α* was cloned in *pFGC*5941 binary vector and the recombinant construct was named *pFGC-TEF-d*. Transient expression of *pFGC-TEF-d* using *Agrobacterium* LBA4404 in grapevine (Bidaneh Sefid cv.) and strawberry cultivars (Camarosa and Ventana) led to reduction in disease progress. The disease reduction in grapevine was estimated by 55% and in strawberries cultivars Camarosa and Ventana by 58% and 93%, respectively. Here we introduce RNAi silencing using *pFGC-TEF-d* construct as an efficient strategy to management of *L. theobromae* for the first time.

Introduction

Members of Botryosphaeriaceae are important fungi found as endophyte, saprophyte and parasite often on woody plants. These fungi are associated with stem canker, fruit rot, dieback, and blight of blossom and leaves [32].

L. theobromae is a worldwide distributed botryosphaeriaceous species that cause important diseases in temperate and tropical regions especially on plants subjected to stress [10]. This species with a wide host range is mostly associated with woody plants including economically important fruit trees such as grapevine[1, 14]. Over the last decades in the light of global climate change, more stress on plants and changes in pathogen behavior, host-pathogen interactions and microbial communities, Botryosphaeriaceae members have become more prevalent and received much more attention [16, 41]. Moreover, *L. theobromae*, as a well-known Botryosphaeriaceae member, has been reported as a human opportunistic pathogen causing inflammation and lesion on skin [31], ocular keratitis and endophthalmitis [7, 40] and onycho-mycosis [35]. Several strategies used to manage this pathogen have mostly focused on protection of the pruning wound using chemicals and biocontrol agents, avoid wounding of plants and minimize exposure to the stress. Fungicides application and pruning of infected branches are the main methods to reduction of disease rate and severity [37]. Research findings indicated that fungicides have limited effect to protect pruning wounds from *L. theobromae* [36]. Biocontrol is an environmentally safe strategy in integrated pest management of plant diseases [27], especially using some bacteria as biocontrol agents [15]. Although some effective bacteria have been reported as biocontrol agent of *L. theobromae*, the efficacy of biological control agents are depending on climates [33]. Moreover, host trees are basically much more susceptible to Botryosphaeriaceae members under stress condition [30], which leads to the partial efficiency of biocontrol agents. Thus, to achieve an efficient management of these

fungi considering novel and alternative methods is an inevitable necessity. RNA silencing as a conserved RNA-mediated gene silencing mechanism is a new and safe strategy that recently has been widely investigated in plant disease management [9]. This is a phenomenon that reduces or stops the expression of a specific gene [49]. In eukaryotic species, gene silencing has an important role in the regulation of gene expression, DNA methylation, and cell immunity against viruses and transposons [6]. However, the RNA interference (RNAi) showing the ability for effective control of pests and diseases [28]. In RNA interference (RNAi) technology, the complementary strand of mRNA is constructed and hybridizes with the target sequence which leads to the formation of double strand RNA (dsRNA) structure. Afterwards, the dsRNA or hairpin RNA (hpRNA) is cleaved by Dicer or Dicer-like enzyme to generate 21- to 25-nt siRNAs, which are guiding RNA-induced silencing complexes (RISC) [45]. However, the translation machinery system cannot translate dsRNA and interfere with the translation of complementary mRNA [8]. Transgenic resistant plants developed based on this phenomenon have produce siRNA using hairpin gene construct containing partial inverted repeat of the gene [48]. Previous studies showed that the inserted hairpin structure confers suitable resistance to homologous sequences (more than 90% homology) [18, 48]. De novo siRNA can spreads systematically throughout the plant and interferes plant pathogen target sequence [44]. However, the use of RNAi for inhibition of plant pathogenic fungi becomes an interest for researchers, especially for filamentous fungi [23, 43]. The aim of this research was to design an effective gene construct for effective and environmentally safe management of *L. theobromae* using RNA silencing.

Results

Insilico analysis

Blast search of *Tef-1α* in NCBI showed more than 96% similarity between *L. theobromae* isolates. Off-target investigation using RNAi scan revealed *L. theobromae Tef-1α* gene (MG192354.1) has no fragment contain necessary homology with human, strawberry and grapevine genome requires for siRNA attack. No hit recognized in human and grapevine for 10 efficient siRNAs predicted form *pFGC-TEF-d* construct (see Supplementary Table S2 online). Sequence similarity of *Tef-1α* of *L. theobromae* and *Trichoderma atroviridae* (MT793743.1) was investigated, and it shown low sequence similarity (55%), that is not enough for induction of silencing against *T. atroviridae* as abiocontroling agent.

RNA structure software showed a high degree of predicted secondary structure in mRNA of *Tef-1α* (Supplementary Fig. S1 online). However, miRNAFold predicted two microRNAs and shows more possible role for *Tef-1α*, (Supplementary Fig. S2 online). Alignment analysis of *L. theobromae Tef-1α* [MG192354] showed low sequence similarity (72%) with *Neofusicoccum parvum Tef-1α* [JQ772082] (Supplementary Fig. S4 online), but in some regions similarity was enough to predict a hit for siRNA or microRNA produced by *L. theobromae Tef-1α*-dimer. Predicted hit regions were shown in red boxes (Supplementary Fig. S4 online).

Gene construct

PCR amplification of *Tef-1α* gene using specific primers (TEF1-α and La.TEF1-α-R) resulted to a 316 bp PCR product (Supplementary Fig. S3a online). The PCR product was digested using *Nco*I restriction enzyme and purified. Digested product was self-ligated to produce *Tef-1α* dimer (*Tef-1α-d*). After ligation, 600 bp *Tef-1α* dimer was amplified using TEF1-α-F primer (Supplementary Fig. S3b online). The amplified dimer was cloned into pTG19-T and the recombinant plasmid (pTG19-T-d) was digested using *Xba*I and *Xho*I enzymes. Moreover, the digested *Tef-1α-d* band was extracted and cloned into *pFGC*5941 binary vector. The ≈ 600 bp amplified band using clone PCR (Supplementary Fig. S3c online) and restriction digestion of recombinant binary vector using *BamH*I enzyme (Supplementary Fig. S3d online) confirmed the recombination of *pFGC*5941 and recombinant plasmid was named *pFGC-TEF-d*. Moreover, the recombinant plasmid was sequenced by Bio Magic Gene Company (Hong Kong, China). Schematic representation of different stage of *pFGC-TEF-d* construction is shown in Fig. 1.

Bioassay and evaluation of gene construct efficiency

Bioassay evaluation on strawberry leaves showed that *L. theobromae* (isolate IRAN 1499C) reacts differentially in two tested cultivars so that three and eight days post inoculation (dpi) necrosis observed in Camarosa and Ventana cultivars, respectively. Necrosis was developed on grapevine and strawberry leaves five days after inoculation with *Neofusicoccum parvum*.

To evaluate the efficacy of *pFGC-TEF-d* construct on inhibition of *L. theobromae* and *N. parvum*, Agrobacterium cell suspension containing *pFGC-TEF-d* was vacuum infiltrated into leaves and after three days, inoculation with fungal cultures was performed.

Necrotic spot was appeared 3 dpi in both treatment and control of Camarosa strawberry cultivar transformed with recombinant (*pFGC-TEF-d*) and empty (pFGC5941) vectors, respectively. At 7 dpi, due to the spread of necrotic spot on the leave surface, control leaves expressing pFGC5941 became completely necrotic. In treatment leaves expressing *pFGC-TEF-d* disease progress rate was significantly different from control leaves at $P = 0.05$ (Table 1, T. test score was 0.012). The statistical analysis using T-test showed the average size of necrotic spot in treatment leaves (10 cm^2) reduced by 58% compare to the control leaves (24 cm^2) (Table 1 and Fig. 2).

The analysis of construct efficiency on Ventana strawberry cultivar indicated the significant difference between treatment and control ($P < 0.05$). In Ventana cultivar the necrotic spot appeared 8 and 5 dpi in treatment and control leaves, respectively. At 16 dpi, whole surface of the control leave covered by the necrotic spot, but not the treatment leaves and, significant difference was observed between the mean size of necrotic spots in control (14 cm^2) and treatment leaves (1 cm^2) at this time poit at $P = 0.05$ (Table 1, T. test score was 0.012) and necrotic spot diameter reduced by 93% in treatment leaves (Table 1 and Fig. 3).

Collected data from grapevine (Bidaneh sefid cultivar) revealed the efficiency of *pFGC-TEF-d* construct in reduction of necrotic spot diameter caused by *L. theobromae*. In grapevine, the necrosis symptom was appeared at 3 dpi. Although, the necrosis spot was appeared in all control and treatment leaves at 8 dpi,

but data analysis at this time showed the significant difference between control and treatment at P = 0.05 (Table 1). The mean size of necrotic spots in treatment leaves expressing *pFGC-TEF-d* construct (12 cm^2) was reduced by 55% compare to the control leaves (27 cm^2, Fig. 4).

Table 1
) T. test using SPSS software in Strawberry and Grapevine

Strawberry Camarosa		repeat	mean	Std. Deviation	Std. error mean	T. test
	c	6	24	3	1	**0.012**
	t	6	10	9	3	
Strawberry Ventana	c	6	14	0	0	**0.00**
	t	6	1	1	0	
Grapvine (Bidaneh sefid)	c	10	27	9.06	2	
	t	10	12	4	1	**0.00**

Efficiency of *pFGC-TEF-d* on *N. parvum* was evaluated on Camarosa strawberry cultivar. Disease symptom (necrotic spot) was appeared at 4 dpi. At 7 dpi the control leaves were completely covered by necrotic spot (Fig. 5). Statistical analysis of the mean size of necrotic spots in control (23 cm^2) and treatment leaves (7 cm^2) showed significant difference at P = 0.05. Data analysis at 7dpi confirmed reduction in disease progress in treatment leaves by 70% (Table 2 and Fig. 5). Despite low sequence similarity (72%) with *L. theobromae*, statistical analysis using T. test at 7 dpi indicated the efficiency of *pFGC-TEF-d* in control of *N. parvum*.

Table 2
) Statistical analysis of transiently expressed p*FGC-TEF-d* on necrosis spot caused by *N. parvum* in strawberry (Camarosa cultivar) using T. test.

	repeat	**mean**	**Std. Deviation**	**Std. error mean**	**T. test**
c	4	23	4	0	**0.003**
t	4	7	1	2	

Discussion

L. theobromae is an important worldwide necrotrophic phytopathogenic fungus reported from several plant species [1, 32] and it is also known as skin pathogen in human [31]. Over the years, management of the Botryosphaeriaceae members (e.g. *L. theobromae*) has been relied on chemical control, but today because of the concerns about the side effect of fungicides and chemical residues, we need to pay

significant attention to the green control of plant diseases. Among several safe alternative methods recently considered, RNA silencing has received special attention [47] and here we decided to examine the ability of this technology to management of *L. theobromae*.

According to the previous studies, the expression of complimentary hpRNA structures can efficiently repress the target sequences [46]. Thus, the aim of this study was to management of *L. theobromae* using a self-complementary hairpin structure. As a sequence region per se plays a role in efficiency of gene construct [11], we used the transient expression system using *Agrobacterium* to analysis the effectiveness of gene construct in induction of resistance against *L theobromae*. The *Agrobacterium*-mediated transient expression assay has developed as an easy and rapid method to analyze gene constructs in plants [5, 22]. Increasing evidence about the role of *Agrobacterium* strain [39], plasmid components such as promoter [39, 11] and host plants [39], led us to transiently evaluate the efficiency of designed construct before essay on transgenic plants. Given the fact that the selection of target sequences with highly conserved region is one of the preferable critical points, a conserved gene region of *L. theobromae* was selected to achieve a broad-spectrum resistance [21]. In the recent decades, researchers have made efforts to improve the resistance efficiency of RNA silencing through choosing the best target sites [3](and to achieve the best results, some protocols and critical points were published and emphasized [17, 42] .

Therefore, we searched to find an efficient target site to control of *L. theobromae*, a plant pathogen with broad host range and found *Tef-1α*, as a conserved gene in *L. theobromae* isolates with more than 96% similarity. Despite the frequent recommendations of RNAi technology to management of plant diseases, one drawback associated with this technology is generation of siRNAs that silence unintentional genes [42]. However, no high similar (or close) target site was detected through *in silico* searching for off-targets in human and some important plant hosts (e.g. grapevine and strawberry). Furthermore, significant role in fungal protein translation machine, two predicted microRNAs (Supplementary Fig. S2 online) suggesting more putative biological role, and existence of a single copy in fungal genome [29], led to the selection of *Tef-1α* as a candidate gene.

Although, previous studies suggest that regions containing high secondary structure in RNA sequences is less accessible to siRNA and targeting sequences with less structured area may shows high resistance level [21], in this research we found that *Tef-1α* with high secondary structure can induce reasonable resistance against *L. theobromae*. In this survey the candidate gene region was 316 bp in range of recommended fragment sequences 200–400 bp [38]. It is important to note that shorter fragments is not efficient for induction of resistance and off-targets chance increases with longer sequences [38]. Since, for each particular silencing construct experimental analysis requires [17], the efficiency of prepared construct was evaluated transiently in grapevine and strawberry as the important hosts.

As we can infer from the literatures resistance based on silencing can protect plants against related sequences in different pathogens [26]. Thus, we examined the induction of resistance against *Neofusicoccum* (*N. parvum*) as a closely related genus to the *Lasiodiplodia*. In spite of low sequence

similarity (72%) with *L. theobromae* (Supplementary Fig. S4 online), our results showed reduction in disease progress caused by *N. parvum* (Fig. 5 and Table 2). Sequence alignment between designed construct and *N. parvum Tef-1α* showed *pFGC-TEF-d* can produce two predictable microRNAs against *N. parvum Tef-1α*.. Therefore, the attachment of produced siRNA as microRNA to the *N. parvum Tef-1α* mRNA and also to the DNA sequence of *N. parvum Tef-1α* as a repressor of transcription may be the possible expectance for the induction of silencing against *N. parvum*.

Targeting multifunctional proteins (e.g. *Tef-1α*) as candidate gene for silencing is an advantage [20], because as previously indicated silencing of multifunctional genes can induce stable resistance against pathogens [26].

As it was observed in the previous studies that hairpin RNA worked best, compare the sense and antisense RNAs [5, 13] in this research hairpin structure was developed for control of *L. theobromae*. Our findings on a necrotrophic fungus *L. theobromae* showed RNA silencing can be a new prospect technology for control of necrotrophic pathogens as biotrophs and this finding are in line with Andrade et al., [4] who showed effective control of *Sclerotinia sclerotiorum* using gene silencing. Using hairpin structure for silencing of *chitin synthase* (*chs*) in necrotrophic fungus, *Sclerotinia sclerotiorum*,, showed 55.5–86.7% reduction in disease severity in transgenic tobacco plants 72 h post inoculation [4].

According to Tan et al, [39], who mentioned that efficiency of gene constructs may depend on the host genetic background, our results revealed that occurrence of disease symptom depends on the cultivars genetic background as disease symptoms was observed 3dpi in strawberry Camarosa cultivar compare to 8dpi in Ventana cultivar.

Time interval between agro-infiltration and pathogen inoculation was showed as important parameter for the efficiency of silencing construct [5, 12], thus in this study we inoculated leaves by fungal pathogen 3 days after agro-infiltration to allow the accumulation of siRNAs before the fungal inoculation. As the results indicated, the *pFGC-TEF-d* cannot prevent the disease initiation, but decreases the disease progress as expected based on the function of targeted gene *Tef-1α*. Based on our knowledge, this is the first study used *Tef-1α* for control of plant pathogenic fungi using RNA silencing technology. Our results revealed that transient expression of *pFGC-TEF-d* in strawberry and grapevine is able to reduce disease progress and management of *L. theobromae* and *N. parvum* from a close genus belong to same family Botryosphaeriaceae. Here in this study we introduce an efficient silencing construct against *L. theomobrae* to develop transgenic plants or for exogenous application as it was used for some other pathogens [24], especially for countries that GMO is not allowed. More research is needed to investigate the effect of RNAi technology using *pFGC-TEF-d* construct on closely related pathogens from Botryospaheriaceae on different hosts in future studies.

Material And Methods

Fungal strains and experimental plants

L. theobromae (IRAN 1499C) and *Neofusicoccum parvum* (IRAN 1535C) strains were obtained from the Mycology lab, Department of Plant Protection, University of Kurdistan. The efficiency of construct was examined on grapevine (Bidaneh Sefid cultivar) and strawberry (Camarosa and Ventana cultivars) from the Strawberry Research Center, University of Kurdistan.

DNA extraction

DNA extraction was carried out according to the modified method of Raeder and Broda (1985) as described by Abdollahzadeh et al., [2]

Selected gene of interest

The *Tef-1α* gene contain a conserved region in *L. theobromae* population was selected as a candidate gene. The conservation of gene sequence was checked in GenBank, NCBI. *In silico* analysis revealed that the selected gene shows a high intra-species but low inter-species similarity. Analysis of off-target in plants and human sequences was investigated using RNAi scan (http://bioinfo2.noble.org/cgi-bin/RNAiScan/RNAiScan.pl) and [plantgrn.noble.org/pssRNAit/]. Secondary structure of RNA was predicted using RNA structure software and predicted miRNA from this gene was revealed using Evry RNA-miRNAFold online software.

Cloning

When target sequence selected, specific primers (Supplementary table S1 online) designed for amplification of partial *Tef-1α* gene. In a primer pair, the restriction enzymes site sequences and three nucleotides as anchor were considered. PCR amplification was carried out using La.TEF1-α-F and La.TEF1-α-R primers in Biorad (T100TM) thermal cycler. PCR condition was as follows: 94˚C for 5 min; 30 cycles of 94˚C for 30 S, 54˚C for 1 min, 72˚C for 1 min; and a final extension of 72˚C for 10 min. PCR product was loaded in 1.2% agaros gel with 0.5X TBE buffer. PCR product (3 µl, 316 bp) (Supplementary Fig. S1a online) was digested using *Nco*I restriction enzyme and after digestion the product was purified using Favoregen (Taiwan) kit. The digested product was self-ligated in 10 µl reaction by T4 ligase (100 ng of DNA, 1 µl of 10X ligase buffer, and 100 u of T4 ligase) for 1 h at 22˚C and an overnight at 4˚C.

The ligation reaction was performed as a template for amplification of *Tef-1α* dimer using TEF1-α primer with the same PCR amplification condition. The amplified dimer was cloned in pTG19-T vector (Vivantis, Malaysia) using T4 ligase (100 U T4 ligase, 25 ng pTG19-T, 100 ng PCR product, and 1 µl of 10X ligase buffer) for 1 h in 22˚C and an overnight in 4˚C. The ligation reaction product transformed to *E. coli* DH5α using heat shock method [34]. Plasmid was extracted from white clone and then the recombinant plasmid was confirmed by digestion. Thereupon, the recombinant pTG19 was digested for separation of *tef1-α* dimer using *Xba*I and *Xho*I enzymes. The digestion product was loaded on 1% agaros gel and the dimer band was purified using FavorPrep™ GEL/ PCR Purification Kit (Favoregene, UK). Moreover, the *pFGC*5941 binary vector was digested using the same enzymes, and the plasmid backbone was purified on agarose gel. Insertion of *tef1-α* dimer into *pFGC*5941 was done using ligation reaction containing 1 µl 10X ligation buffer, 100 ng *tef1-α* dimer, 50 ng digested *pFGC*5941, and T4 ligase (100 U) in a 10 µl reaction volume. Again, the ligation product was transformed to *E. coli* DH5α using heat shock method and plasmid

extraction was carried out using alkaline lysis protocol [19]. Moreover, recombination of *pFGC*5941 was performed using clone PCR, endonuclease restriction digestion and sequencing. Cloning steps is shown in Fig. 1.

Agrobacterium transformation

Agrobacterium LBA 4404 strain was plated out in LB medium containing rifampicin for 48 h, at 28 ˚C/150 rpm. Then, the bacterial suspension precipitated in 2 ml tube using centrifuge (5000 rpm, 3 min). The precipitated bacterial cell was re-suspended in 250 µl TE buffer, washed, and then centrifuged. The bacterial cell pellet was re-suspended in 250 µl of LB with 0.1 concentrations, and then the tube putted in liquid nitrogen for seconds and kept on ice for 30 minutes. Then, 2 µl of binary vector (100 ng/µl) was added and the tube putted in liquid nitrogen for seconds and after that, the sample was kept in 37 ˚C for five minutes. Finally for recovery, sample tubes kept on shaker incubator in room temperature (25–28ºC, 180 rpm) for 4 h. Finally, 200 ul of the cell suspension was spread on selective LB media containing rifampicin and kanamycin.

Transient expression

Grape (Bidaneh Sefid cultivar) and strawberry leaves (Camarosa and Ventana cultivars) were infiltrated with *Agrobacterium tumefaciens* LBA4404 isolate using vacuum-based infiltration as described by Kapila et al. [25]. For infiltration, the 2-day-old Agrobacterium cell suspension (OD600 = 0.8) was centrifuged (5000 rpm, 3 min, 4˚C) and the harvested cells was re-suspended in sterile water and kept on ice for use. Plant leaves were plunged in cell suspension under vacuum pressure to penetration of cell suspension into the mesophyll cells. When the majority part of leaves was soaked, the leaves were transferred to the petri dishes containing soaked sterile paper. The petri dishes incubated in growth chamber (16/8 h light/night regime, 25˚C). Each experiment was carried out using 6–10 leaves and repeated two times. *A. tumefaciens* LBA4404 containing empty p*FGC*5941 and *pFGC-TEF-d* used as control and treatment, respectively.

Fungal inoculation

Three days after vacuum infiltration, a small disc (0.5 × 0.5 cm) of 4 day-old fungal colony on PDA was placed in the middle of each leaves incubated in growth chamber (16/8 h light/dark regime, 25˚C). Data were recorded until the end of experiments for a period of three weeks.

Evaluation of resistance

Resistance evaluation began with the development of necrotic spot on leaves until the spot covered whole leave in a control plants. Resistance evaluation analysis was performed based on the necrotic spot diameter.

Statistical analysis

Collected data subjected to T-test statistical comparison analysis using SPSS (SPSS Statistics Version 22) software.

Declarations

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Conflict of interest

The authors declare no conflicts of interest regarding the publication of this article.

Author contributions

O.N: DNA extraction, cloning and laboratoary work. A.A: design and leading the work, writing original draft,. J.A: Study monitoring, coordination, Editing the original draft.

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