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Subcloning and expression of *blf1* gene isolated from *Burkholderia pseudomallei* in hairy roots of tobacco plants

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ABSTRACT

Melioidosis is a common disease between human and animals caused by *Burkholderia pseudomallei.* There is currently no effective vaccine for it. The aim of this research was to express *blf1* gene in tobacco plants. By transforming the pBI121 vector into *Agrobacterium tumefaciens*, the *blf1* gene was transferred to the tobacco plants and the primary transgenic plants were obtained. The explants obtained from the transgenic plants were used to induce hairy roots. The presence of the *blf1* gene was investigated in the obtained hairy roots by PCR and Western blot analysis. The titer of antigen production was measured by ELISA technique. The insertion of the *blf1* gene construct into pBI121 vector containing the *ctxB* gene was confirmed by PCR. The tobacco explants inoculated with *A. tumefaciens* were cultured on the MS medium containing benzyl-aminopurine (BAP) and 1-naphthalene acetic acid (1 -NAA). After callus formation and seedling regeneration, the seedlings with active meristems were transferred onto the hormone-free medium for rooting and transgenic tobacco plants containing the blf1 gene were produced. By preparing an explant of transgenic plants by *A. rhizogenes*, inoculum and hairy roots were obtained. In hairy roots, the presence of blf1 gene was confirmed using PCR and its expression was confirmed by Western blotting. By using the ELISA technique, the titer of BLF1 antigen production in the total soluble protein of hairy roots was determined to be 0.56%.

Key words: Gene transfer, Tobacco, Melioidosis, Cloning.

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INTRODUCTION

Burkholderia pseudomallei is a gram-negative and aerobic bacterium that causes melioidosis, a severe human infectious disease (Hadpanus *et al.,* 2019). Melioidosis was first reported in 1911 by Alfred Whitmore and his assistant C.S. Krishnaswami as "a glanders-like disease" causing septicemia in morphine injectors in Rangoon, Burma. Though the bacterium was remarkably similar to *Burkholderia mallei*, the causative agent of glanders, the fact that *B. pseudomallei* was motile, distinguished it as a new species (Stanton and Fletcher, 1921). The bacterium was classified as a level B biological agent in 2002; it survives in soil, water, and under adverse environmental conditions such as low nutrient levels, low pH, and high temperatures. Melioidosis has diverse clinical presentations including pneumonia, localized cutaneous lesion, bacteremia without evident focus, osteomyelitis, septic arthritis and severe sepsis with multiple organ abscesses (Wiersinga *et al.,* 2018). It is an endemic disease in human, animals, and some plants, such as tomatoes. Historically, melioidosis was considered to be geographically restricted to countries in southeast Asia and northern Australia, because these regions have reported the highest incidence and prevalence of the disease, where annual incidence is up to 50 cases per 100 000 people (Wiersinga *et al.,* 2018). The condition can be transmitted orally, by inhalation, or through skin scratches, and bacteria can be transmitted from human to animal and from animal to human. This disease is considered a time bomb because it can remain dormant in the body for long periods and even become active after many years. This bacterium is resistant to many antibiotics, making treatment rather tricky. Conditions such as diabetes, kidney disease, lung disease, and alcohol consumption are important factors in the development of melioidosis. There are two main routes for infection with *B. pseudomallei*; inhaling contaminants or inoculating the skin. A recent modeling study estimates 2,800 deaths per year due to melioidosis in Thailand (Hinjoy *et al.,* 2018). Clinical signs of the disease can range from acute hematopoiesis to chronic infection. Most patients have sepsis, but the specific clinical manifestations and severity vary depending on the route of bacterial entry (skin penetration, inhalation, and ingestion), the host's immune function, and the extent and type of bacteria. (Wiersinga *et al.,* 2018). In animal laboratory studies, consumption of the *B. pseudomallei* spreads the bacterium to various sites, including the brain, lungs, spleen, liver, and mesenteric lymph nodes (West *et al.,* 2010). The structure of BPSL1549, a protein of unknown function from *B. pseudomallei*, reveals a similarity to *Escherichia coli* cytotoxic necrotizing factor 1. It was known that BPSL1549 acted as a potent cytotoxin against eukaryotic cells and was lethal when administered to mice. Expression levels of bpsl1549 correlate with conditions expected to promote or suppress pathogenicity. BPSL1549 promotes deamidation of glutamine-339 of the translation initiation factor eIF4A, abolishing its helicase activity and inhibiting translation. It was proposed the name BPSL1549 *Burkholderia* lethal factor 1 (BLF1) (Cruz-Migoni *et al.,* 2011). During the past two decades, we have witnessed a significant increase in the number of reports on the successful *Agrobacterium*-mediated genetic transformation of various plant species, variants and cultivars (Herrera-Estrella *et al.,* 2005). Nowadays, transgenic plants are considered as an expression system for therapeutic recombinant proteins. One of the approaches to recombinant protein production is the introduction of the relevant genes into plant cells and produce hairy roots from these cells using the plant tissue culture methods (Mohammadi *et al.,* 2020). Fundamental characteristics of hairy roots, such as their ability to proliferate without hormones, lack of geothermal, lateral branching, and genetic stability, have led to their widespread use in plant biotechnology (Yektapour *et al.,* 2022). Field tobacco (Linnaeus) is the most widely utilized plant host in molecular agriculture and for producing recombinant proteins (Sustiprijatno *et al.,* 2022). The work carried out in this article is a part of the scientific project aimed at obtaining an edible vaccine candidate against melioidosis. Edible vaccines are promisingly cheaper, easy to take in, safe and easily stored, socially justifiable, especially in poorly developed countries. These vaccines are made by inserting the necessary genes into the plants, which result in the production of the requisite encoded proteins. Many edible vaccines are currently produced for a variety of human and animal diseases (Polshettiwar Satish *et al.,* 2023). Among plant tissues, the cultivation of hairy roots from edible plants and the production of desirable antigens is a way to develop edible vaccines. Dried hairy roots that express the antigen of vaccines can be kept at a certain temperature for a long time. This simplifies their protection and transfer, and as a result, they can be used to vaccinate a large population. Advantages for processing recombinant edible vaccines in hairy roots have been mentioned, including that the production of recombinant vaccines in these roots causes the creation of a large amount of recombinant proteins. Considering that the rate of

growth of hairy roots is very high, they can be used as a permanent source for the production of valuable recombinant proteins. Hairy roots have a simple genetic manipulation due to their ability to grow a lot in successive cultures and can be easily cultivated in hormone-free environments (Qin *et al.,* 2022). For the design of edible vaccines, issues such as oral dose, stability in high stomach acidity, stability and resistance to digestive tract proteases, solubility of vaccines in pH close to neutral and permeability through the intestinal wall and basement membrane and entering the blood stream should be considered. In order to strengthen the effect of oral vaccines and to achieve the above topics, scientists pay special attention to adjuvants or helpers. One of these adjuvants is CTxB, which is considered as a strong immunoadjuvant in mucosa-dependent immunogenesis, because it is a very effective transporter protein for systemic and mucosal antibody secretion for conjugated gene antibodies (Jia *et al.,* 2020). In this research, in order to increase the stimulation of the immune system and delivery, CTxB was used along with the main target gene. Currently, there is no virtual vaccine to protect against this disease.

MATERIALS AND METHODS

Bacterial strains

Three bacterial strains used in this study included *E. coli* strain DH5α, *A. tumefaciens* strain LB4404 and *A. rhizogenes* strain A4. *E. coli* was used as a host to maintain and replicate the construct, *A. tumefaciens* was used to transfer the target gene to the tobacco plant, and *A. rhizogenes* strain produced hairy roots.

Primers

Primers for the *blf1* gene were designed according to the cleavage sites of *Bam*HI and *Xho*I enzymes in the pBI121 vector, *blf1* gene side sequences, and expression-enhancing sequences in plant systems. To design primers, Oligo 7 software was used.

blf1 forward primer:
5′TGGTACTGGTCCTGGTCCT3′

blf1 reverse primer:
5′TGAACGATCGGGGAAATTCG3′

rolB forward primer:
5′GCTCTTGCAGTGCTAGATTTG3′

rolB reverse primer:
5′GAAGGTGCAAGCTACCTCTCT3′

virG forward primer:
5′ GGTCGCTATGCGGCATC3′

virG revese primer:
5′ CCTGAGATTAAGTGTCCAGTCAG3′

The expression vector pBI121, which contains the kanamycin resistance gene for bacterial selection, *Bam*HI and *Xho*I cut-offs, CamV35S promoter, and NOS terminator sequence, was used.

Plant material and explant preparation

Tobacco plants *Nicotiana tabacum* cv. Xanthi was used (donated by the National Institute of Genetic Engineering and Biotechnology of IRAN). Sterilized tobacco seeds were grown in an MS medium without hormones and antibiotics. Stems of tobacco plants grown *in vitro* were cut into pieces of 2 to 3 cm long, each containing a bud and a leaf, and transferred to glass jars containing the solid MS culture medium without hormones and antibiotics. After two weeks, the plants, were ready for inoculation.

Construct preparation and transformation to *Agrobacterium*

The *blf1* gene was inserted inside the pUC vector, the plasmid was extracted, and the extracted plasmid was digested with *Bam*HI and *Xho*I enzymes, having cleavage sites on both sides of the *blf1* gene. Then, the pBI121 plasmid was extracted and digested with *Bam*HI and *Xho*I enzymes. Enzymatic cleavage products were separated on a 1% agarose gel, purified, and recovered (Figure 1). Finally, the *blf1* gene was cloned into the plant expression vector pBI121 (Figure 2). Bipartite constructs, including CTxB and BLF1 antigens, with plant and bacterial codon preference, were designed in the biology department of Imam Hossein University. According to the bioinformatics studies conducted using Protparam and Modeller software, it was found

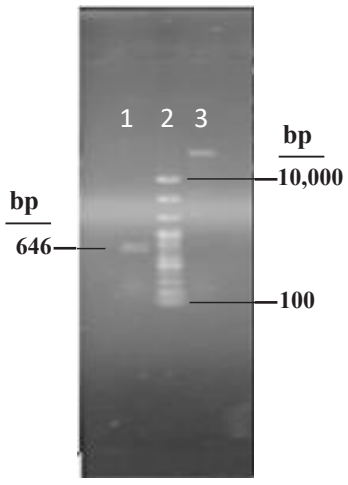

Figure 1. The quality of the recovered product. lane 1: linear *blf1*. Lane 2: 1kb ladder. lane 3: Linear pBI.

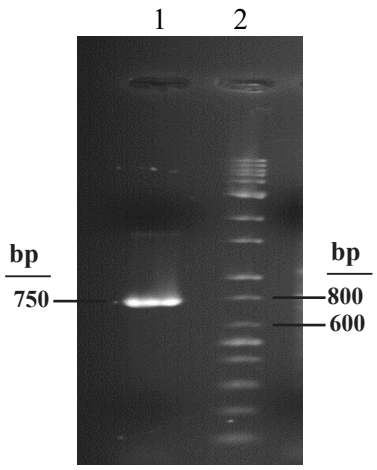

Figure 2. Confirmation of cloning using PCR technique with specific primers for *blf1* gene.

that the best placement of antigens is CTxB-BLF1. After designing the bacterial construct, this construct was expressed in pET28a expression vector and the protein was purified after gene optimization (Moshiri *et al.,* 2021). Twety micrograms of the purified recombinant protein was injected intraperitoneally to mice four times and blood sampling was done three times. After separating the serum, the antibody was stored in the freezer at -20 for the confirmation of the BLF1 protein produced in the hairy roots. In the genetic structure designed with plant codon preference, elements should be inserted in order to express this protein better. The start codon, Kozak sequence, cutting sequence and gamma zein guide peptide were placed at the beginning of the chimeric structure. Also, for the end of the gene, cutting sequence, termination codon and His tag sequence were placed using appropriate primers so that these elements were placed at the end of the gene after replication. The Kozak sequence is for the higher expression of the genetic structure in the plant and the histidine sequence (6 amino acids of histidine) is used for protein purification, and the use of the corn gamma-zein guide peptide leads to the accumulation of recombinant proteins in endoplasmic reticulum. CTxB immunoadjuvant can cause production of antigen in the form of insoluble (Inclusion body) and increase the share of recombinant protein in the total protein. It seems that the use of CTxB adjuvant, in addition to its role in stimulating the immune system and protein transport, also affects the structure of the recombinant protein and insolubilizes the protein, removes the produced protein from the reach of proteases, and as a result, the protein lasts longer and as a result, the share of recombinant protein increases. Then, *E. coli* was transformed with the prepared construct using the heat shock method (Froger and Hall, 2007). The standard freeze and thaw method using calcium chloride was used for *Agrobacterium* transformation. The transformed bacteria were cultured on plates containing kanamycin (50 mg/L) and rifampicin (30 mg/L) and incubated at 28 °C for two days.

Plant transformation and molecular analysis

To generate transgenic tobacco, young sterile leaves of the plant (2×2 cm pieces) were placed on a shaker for 2 h in the presence of inoculum (liquid MS medium containing 3% sucrose, 5% glucose, and 10 mM acetosyringone) poured on the recombinant *Agrobacterium* precipitate.

After drying on filter paper, they were placed in a co-culture medium (without hormones and antibiotics) for 72 h. Tobacco explants were then selected on a medium containing 50 mg/L of kanamycin (for selection of transgenic cells) and 100 mg/L of the meropenem (to eliminate the *Agrobacterium*) enriched with BAP (2 mg/L) and NAA (0.1 mg/L). After seedling formation, they were transferred to a hormone-free medium for rooting. In this study for the evaluation of transgenic plants, DNA was extracted by the CTAB method (Clarke, 2009) from the leaves of young plants grown on the antibiotic containing medium. PCR analysis was performed using specific primers for *blf1* gene, and the plants containing the desired gene were identified.

***Agrobacterium rhizogenes* mediated hairy root induction in transgenic plants**

To form hairy roots, the resulting transgenic plants were transformed (indirectly) by *A. rhizogenes* strain A4. Initially, a culture of *A. rhizogenes* strain A4 in the LB medium containing 30 mg/L rifampicin was incubated at 25 °C and 150 rpm. Bacterial culture was centrifuged under sterile conditions at 4 °C and 6000 rpm for 10 min. The supernatant was discarded, and a 25 ml inoculum containing 5% sterile glucose and 10 mM acetosyringone was added to the precipitate; when its optical density (at 600 nm) reached 0.5, the sample was transferred to a shaking incubator for 2 h at 28 °C and 180 rpm. Hairy roots were induced by co-cultivating leaf segments with *A. rhizogenes* strain A4 for 10 min. Then, the explants were blotted dry and transferred to MS basal medium and maintained in dark. After three days, they were transferred to the MS medium supplemented with Meropenem were transferred to a fresh medium containing kanamycin (50 mg/L) and meropenem (100 mg/L) every three weeks. Four weeks after gene transformation, hairy

roots were developed from the cut ends of the leaf segments. For further growth, the roots were transferred to a liquid MS medium in Erlenmeyer. The Erlenmeyer flasks were incubated at 25 °C at 105 rpm. After 30 to 40 days, the grown roots were removed from flasks and stored at -70 °C for further analysis (Valimehr *et al*., 2014).

Molecular analysis of transgenic hairy roots

For the extraction of genomic DNA from transgenic hairy roots, 30 mg of root tissue was frozen in liquid nitrogen and pulverized. After that, DNA extraction of plant tissues was performed using the CTAB protocol (Clarke, 2009). Agarose gel electrophoresis and spectrophotometry (260 and 280 nm) were used to control the quality and quantity of purified DNA. The presence of the *blf1* and *rolB* genes in the hairy roots was investigated by PCR.

Protein extraction

Protein extraction was carried out from 100 mg of frozen hairy roots with two volumes of 0.01 M sodium borate buffer (pH=8.6). The homogenate was centrifuged at 12000 *g* for 20 min and the supernatant was collected. This was designated as crude extract. The pellet was suspended in one volume of 0.01 M sodium borate buffer pH=8.6. The supernatant was collected after 20 min centrifuge at 12000 *g* and mixed with the first extract. All steps were carried out at 4 °C.

Protein electrophoresis (SDS–PAGE) and Western blotting

For protein electrophoresis, the Laemmli method was used (Laemmli, 1970). Proteins were analyzed on a 12.5% acrylamide gel and with chromatin prestained protein ladder (vivantis). The subunit molecular mass of the BLF1 from transgenic hairy roots was 35 kDa. The extracted proteins from hairy roots were electrophoresed on a 12.5% gel and electro transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma) for 3 h with 18 V. Nonspecific binding sites were blocked in the blocking buffer (5% non-fat skimmed milk in phosphate buffered saline (PBS), pH 7.4, with 0.05% Tween 20 (PBS/T) at 4 °C. Subsequently, the membrane was washed three times with PBS/T, and incubated with diluted horseradish peroxidase (HRP)-conjugated rabbit anti-ANS (Sigma) (1:8000) with gentle shaking for 2 h at 25 °C. The membrane was washed with PBS/T, followed by a second incubation step with horseradish peroxidase-conjugated anti-mouse total IgG from rabbit (Abcam). Immune activity was detected with diaminobenzidine (DAB) as a chromogenic substrate until a brownish patch appeared (Hirano, 2012).

Determination of protein concentration

The concentration of total protein was determined by Bradford's method using bovine serum albumin as standard (Bradford, 1976; Sedmak and Grossberg, 1977). To measure BLF1 protein in hairy roots, total soluble protein was extracted and used as the antigen on the bottom of the ELISA microplate reader . Then, the presence of the protein was evaluated using the antibody obtained from mice immunized with the recombinant BLF1 protein produced in *E. coli*, produced in the Biology Center, Imam Hossein University. In this test, the obtained intensity of absorption was compared with the standard ELISA chart drawn using different concentrations of BLF1 recombinant protein produced in *E. coli*. The anti-mouse IgG HRP was used as the secondary antibody in ELISA.

RESULTS

Transfer of pBI*blf1* to *A. tumefaciens*

Colony PCR analysis confirmed the presence of *blf1* gene in *A. tumefaciens* with the amplification of a 750 bp (Figure 3). Tobacco plants were transformed using *A. tumefaciens* LB4404 and whole plants were obtained in the selection medium (Figure 4). Evaluation of transgenic plants: Genomic DNA was extracted from the young leaves of regenerated plants. PCR analysis on the genomic DNA of these plants using specific primers showed the presence of a 750 bp fragment, while in control plants, no band was observed. (Figure 5). For further growth of hairy roots, the roots were transferred to a liquid MS medium in Erlenmeyer flasks and incubated at 25 °C at 105 rpm (Figure 6).

Molecular analysis of hairy roots

To confirm the presence of the transgene, the extracted DNA from hairy roots was used as a template in PCR reaction with specific primers for the *blf1* gene. The result of gene amplification showed the expected 750 bp fragment (Figure 7). To confirm the presence of genes responsible for developing hairy root phenotype, PCR was performed with specific primers for the *Agrobacterium rolB* gene (Figure 8).

Confirmation of the absence of *A. rhizogenes* in transgenic hairy roots

PCR was performed using the genomic DNA of hairy roots and *virG* specific primers. The absence of 435 bp fragment in PCR products of hairy roots confirmed that hairy roots were not infected with *A. rhizogenes* (Figure 9).

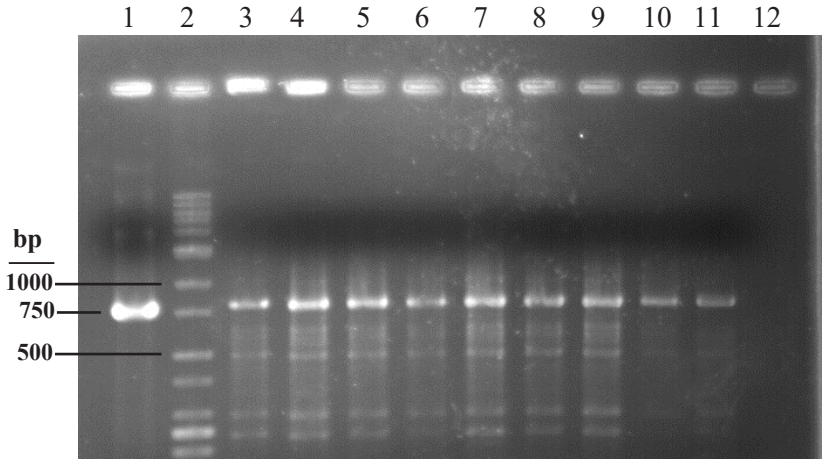

Figure 3. Confirmation of the presence of *blf1* gene (about 750 bp) in *Agrobacterium tumefaciens* LB4404 using colony PCR technique and specific primers. lane 1: Positive control (*E. coli* containing pBIblf1 construct). lane 2: Molecular size marker. lanes 3 to 11: *Agrobacterium* containing pBIblf1 construct. lane12: Negative control (*Agrobacterium* without construct).

Figure 4. A: Transgenic leaf explants on the selection medium containing antibiotics and hormones. **B & C:** The growth of seedlings from calluses created on the edge of leaves in the medium containing antibiotic and hormones. **D:** The formation of complete plants in a medium without hormones.

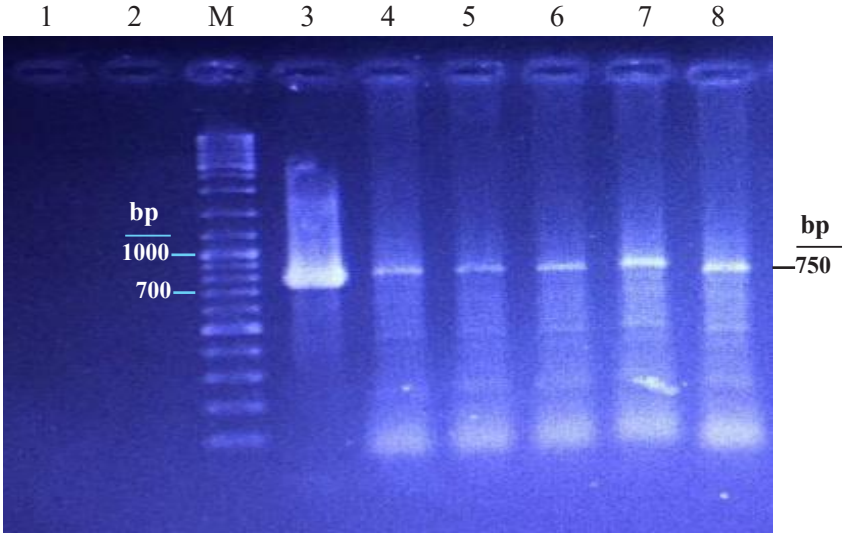

Figure 5. Confirmation of the presence of gene constructs in transgenic plants by PCR on genomic DNA using specific primers for the *blf1* gene. lane 1: Negative control (reaction with water) lane 2: Negative control (no transgenic plant). Lane M: Size marker (Mix 100 bp) DNA. lane 3: Positive control (plasmid containing the desired fragment). lanes 4 to 8 transgenic plants with the desired fragment.

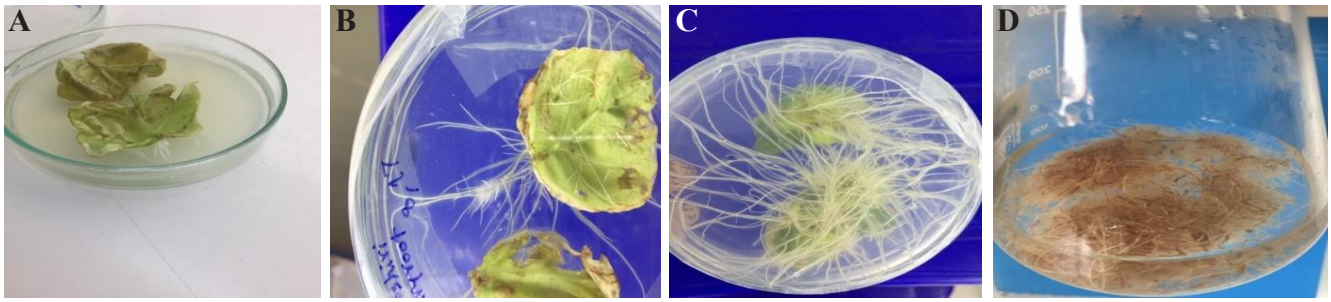

Figure 6. Regeneration and growth of hairy roots. **A:** Emergence of hairy roots from the margins of plant leaves cultured in the selection medium containing kanamycin (80 mg/liter). **B & C:** Further growth of hairy roots in the selection medium. **D:** Culturing and propagation of hairy roots in the liquid culture medium.

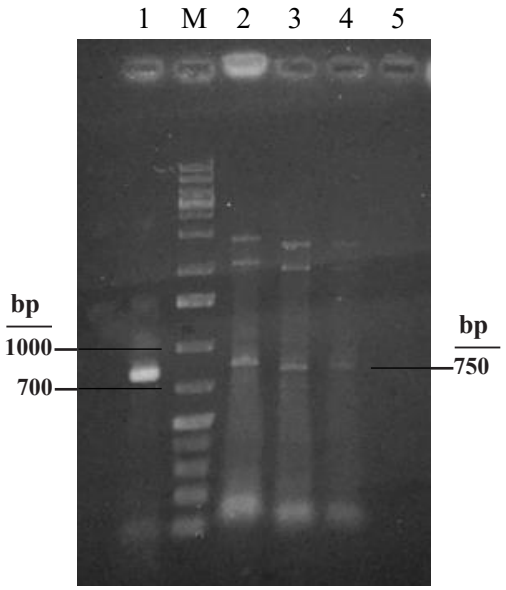

Figure 7. Confirmation of the presence of the desired gene (*blf1*) in the obtained hairy roots. lane 1: Positive control (plasmid containing the desired fragment) lane M: Size marker (Mix 100 bp) DNA. Lanes 2 to 4: hairy root samples containing the pBI*blf1* construct. Lane 5: Negative control (hairy roots of non-transgenic plant).

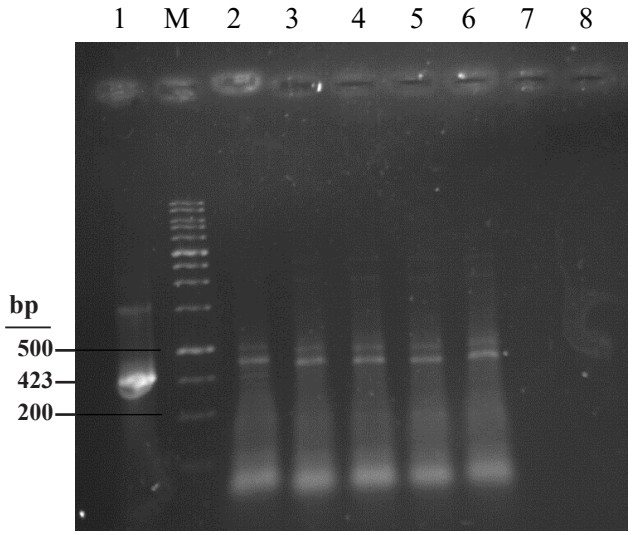

Figure 8. Confirmation of the presence of the *rolB* gene using specific primers. Lane 1: Positive control (PCR colony from *Agrobacterium rhizogenes*). Lane M; Size marker (Mix 100 bp) DNA. Lanes 2 to 6: Samples of tobacco transgenic hairy roots with pBIblf1 fragment. Lane7: Negative control (reaction with water). Lane 8: Negative control (non-hairy roots).

Recombinant protein expression

The expression rate of BLF1 recombinant protein in hairy roots was evaluated in the transgenic plants, using semi-quantitative ELISA. The expression level of BLF1 recombinant protein from total soluble protein (TSP) was equal to 0.56% of TSP. It should be noted that the protein extracted from the hairy roots of the non-transgenic plants was used as a negative control of the reaction.

SDS–PAGE analysis

The BLF1 protein presented a molecular weight of approximately 35 kDa with its relevant band on SDS-PAGE. This band was absent in non-transgenic samples (Figure 9).

Western blotting

The expressed protein was observed as the predominant band at 35 kDa (Figure 10). Also the BLF1 proteins were confirmed by Western blot assay (Figure 11). Thewse proteins were recognized by an specific antibody of anti-mouse IgG HRP.

DISCUSSION

B. pseudomallei is the causative agent of melioidosis, a severe, debilitating, and often fatal disease in human and animals (Burtnick *et al.,* 2012). A modeling study in 2016 estimated that about 165,000 people are infected with melioidosis every year worldwide, of which south Asia alone contributed upto 44% of the global burden

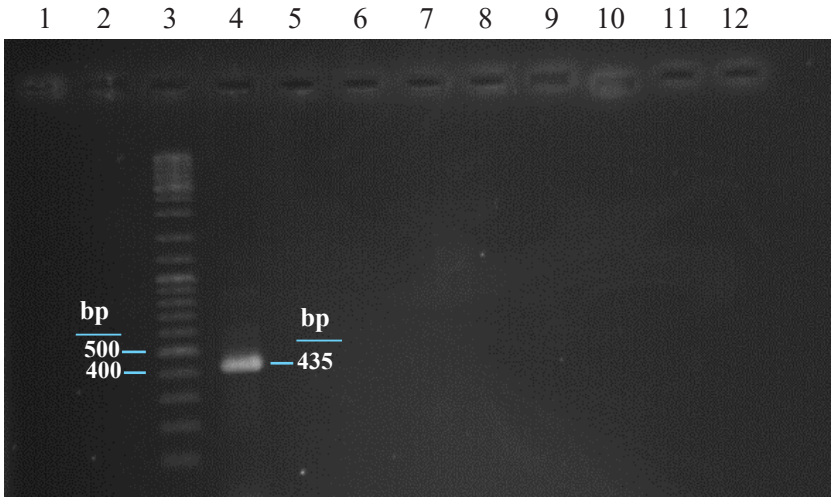

Figure 9. Ensuring that plants are not contaminated with Agrobacterium by PCR using *virG* primers. Lane1: negative control, no DNA sample; Lane2: negative reaction control, PCR genomic DNA product of non transgenic tobacco. Lane3: size marker (Mix 100bp) DNA; lane4: Positive reaction witness, *Agrobacterium* colony; lane 5 to 12: genomic PCR product of transgenic tobacco with pBI121*blf1* construct.

of melioidosis (Limmathurotsakul *et al.,* 2016). The estimated global burden of melioidosis during 2019 in the form of disability-adjusted life-years (DALYs) was 4.64 million (Birnie *et al.,* 2019). Increasing awareness among clinicians and microbiologists, early point-of care diagnosis, appropriate case management, and disease prevention are fundamental approaches for controlling melioidosis (Mohapatra and Mishra, 2022). The pathogen has the ability to remain latent in a host for decades (Ngauy *et al.,* 2005). Antibiotic treatment of melioidosis is prolonged with variable results; hence effective vaccines are urgently needed (Biryukov *et al.,* 2022). Choosing a suitable host for the production and expression of chimeric proteins is one of the considerations in subunit vaccine research. The use of plant systems to produce recombinant proteins, mainly to produce oral vaccines, has attracted much attention. This has been due to the relative advantage of plant systems in making stable proteins free from human pathogens in the last two decades. Due to concerns about biological safety, regulation, and complete control of transgenic plants, plant cell cultures or particular tissues, such as hairy roots, have been widely explored (Skarjinskaia *et al.,* 2013). Since hairy roots have a high growth rate, they can be used as a permanent source of valuable recombinant proteins. So far, there has been no attempt to produce a plant-based vaccine candidate against melioidosis in Iran, therefore we are hopeful that researchers would utilize the results of this research for candidate vaccine design. This project has considered various arrangements to increase protein expression in the plant system. CtxB

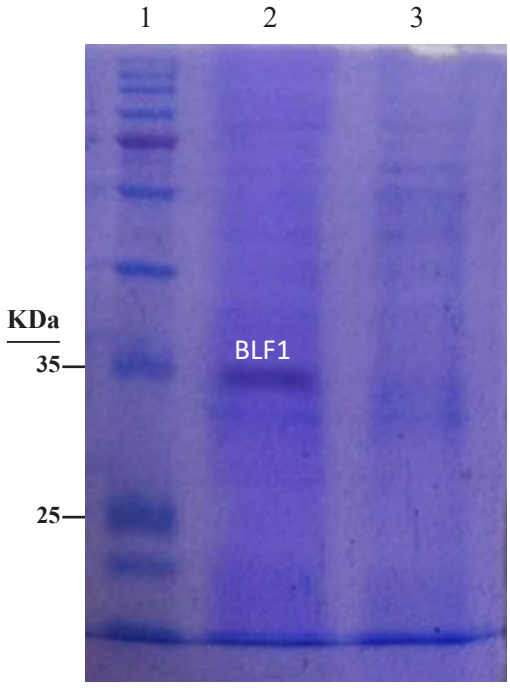

Figure 10. Results of BLF1 protein expression in hairy roots on 12% SDS-PAGE gel. Lane 1:molecular weight marker. Lane 2: protein extracted from the transgenic hairy roots. Lane 3: protein extracted from non-transgenic wild type hairy roots as negative control.

adjuvant, maize gama zein guide peptide, and *blf1* gene were subcloned in plant expression vector pBI121. The vector also encoded for Kozak and His tag sequences to increase expression and streamline purification processes. According to the functional properties of the B subunit Ctx toxin and its non-toxicity, this molecule

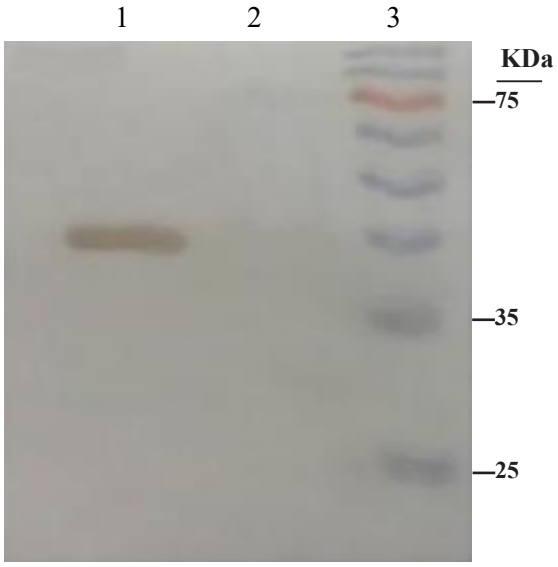

Figure 11. Western blot analysis of BLF1. Lane 1: total protein extracted from transgenic hairy roots. Lane 2: whole protein extracted from transgenic hairy root (negative control). Lane 3: molecular weight marker.

will be a suitable candidate for use in vaccines. The B subunit, by binding to various receptors on the target cell's surface, can be well accessible to the immune system. Furthermore, due to its amino acid composition, it can stimulate the immune response in the host. The B subunit of Ctx toxin has been shown to act as a natural carrier for antigens, and its viability to act as an adjuvant has been proven, and as a result of its presence in a recombinant structure, it causes a more robust response of the immune system to others parts of this recombinant protein (vaccine candidate) (Jia *et al.,* 2020). As mentioned, to produce hairy roots, we used tobacco leaf explants and an A4 strain of *A. rhizogenes*. (Bagheri *et al.,* 2015; Dehghani *et al.,* 2018; Beigmohammadi *et al.,* 2019; Yaqi *et al.,* 2020). In this research, hairy roots (creating hairy roots from the transgenic parent plant) were used; accordingly, we could compare the two direct and indirect methods simultaneously. It has been reported that the level of expression in the indirect method can be increased by more than seven times as compared to the direct method (Bazyari and Salmaniyan, 2016). Based on the obtained results, it was found that the expression level of BLF1 protein in transgenic hairy roots increased significantly compared to non-transgenic hairy roots. Considering the advantages of hairy roots for the production of recombinant proteins, this system has the potential to produce BLF1 protein on a large scale. After obtaining the expression level of BLF1 protein in transgenic hairy roots, it is possible to calculate the amount of hairy roots needed to treat the animal model by feeding. Then, by measuring the antibody titer, performing the trial, comparing the results of different treatments of mucous and edible on mice, it might be possible to determine the best type and amount of treatment and then select the most suitable form of the candidate vaccine.

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