Research Article

A Tobacco-Derived Thymosin β4 Concatemer Promotes Cell Proliferation and Wound Healing in Mice

Rylosona Janarthini,[1,2] **Xiaolei Wang,**[1,2] **Lulu Chen,**[1,2] **Lei Gao,**[1,2] **and Lingxia Zhao**[1,2]

[1]*Joint Tomato Research Institute, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China*
[2]*Plant Biotechnology Research Center, School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China*

Correspondence should be addressed to Lingxia Zhao; lxzhao@sjtu.edu.cn

Thymosin β4 (Tβ4) is a peptide that is known to play important roles in protection, regeneration, and remodeling of injured tissues in humans, and that shows great promise in a range of clinical applications. However, current strategies to Tβ4 are insufficient to meet growing demand and have a number of limitations. In this current study we investigated whether expression of recombinant Tβ4 in plants, specifically in tobacco (*Nicotiana tabacum*) leaves, represents an effective approach. To address this question, a 168 bp *Tβ4* gene optimized for tobacco codon usage bias was constitutively expressed in tobacco as a 4-unit repeat concatemer, fused to a polyhistidine tag. Quantitative polymerase chain reaction and Western blot analyses were used to verify *4×Tβ4* expression in 14 transgenic tobacco lines and enzyme-linked immunosorbent assay analysis indicated 4×Tβ4 protein concentrations as high as 3 μg/g of fresh weight in the leaves. We observed that direct administration of tobacco-derived Tβ4 was more effective than Tβ4 either obtained commercially or derived from expression in *Escherichia coli* at promoting splenocyte proliferation *in vitro* and wound healing in mice through an endothelial migration assay. This study provides new insights into the development of plant-derived therapeutic proteins and their application by direct administration.

1. Introduction

Thymosin β4 (Tβ4), a 43-amino acid peptide that is encoded by the *TMSB4X* gene on the X chromosome of mice and humans, was initially isolated from thymosin fraction 5 (TF5) as a biologically active component [1]. TF5 was originally authorized by the FDA (Food and Drug Administration) to treat the primary immunodeficiency disease, DiGeorge syndrome, in clinical trials involving young children in the US [2]. Tβ4 was initially identified as an actin monomer (G-actin) binding protein and has the capacity to sequester G-actin, thereby inhibiting intracellular actin polymerization [3–5]. In addition to regulating actin formation, extracellular Tβ4 participates in several biological processes, including blood coagulation, osteoblast differentiation, activation and degranulation of platelets, and regulation of cell migration (http://www.ncbi.nlm.nih.gov/gene/7114). Tβ4 therefore plays an important role in medical treatments, such as anti-inflammation [6], angiogenesis [7, 8], remodeling of dam-

aged tissues [9, 10], and the prevention of organic fibrogenesis [5, 11, 12].

Several studies have demonstrated that the expression of *Tβ4* is upregulated in injured tissue and differentiating cells [13, 14], and high concentrations of Tβ4 protein have been found in blood platelets, wound fluid, and a range of tissues [15, 16] and its effects appear to be widespread. It has been extensively employed to treat diabetic ulcers and bedsores [17, 18] and damaged corneas [10, 19, 20] and for cardiac cell survival and the repair of heart muscle injured during heart attacks [9, 21, 22], as well as antifibrogenesis of the liver [5], kidney [11, 23] and lung [12]. Tβ4 therefore shows great promise for numerous clinical applications [17] and the resulting increased commercial demand relies on efficient production methods. Traditionally, Tβ4 is obtained from extracts of animal thymus glands or is chemically synthesized; however, genetic engineering approaches, such as expression in *Escherichia coli*, have also been developed [24, 25]. It has also been reported that a Tβ4 protein

concatemer expressed in *E. coli* is able to promote wound healing in mice [24]. However, for clinical applications, *E. coli* derived Tβ4 protein must undergo a complex purification process to eliminate impurities and endotoxins, which represents a significant disadvantage of this expression system. Overall, the disadvantages to the aforementioned methods include high production costs associated with separation and purification of the target protein, as well as the risk of zoonosis [26, 27].

Current Tβ4 production capacity cannot meet the clinical demand, and plant-based protein/peptide expression systems represent a potentially attractive solution as they are relatively inexpensive, do not have problems associated with zoonosis, and are scalable. Accordingly, over the last decade plant-based expression systems have been extensively used to synthesize therapeutic proteins, antibodies, and vaccines [26–28]. In order to meet the rising clinical demand for Tβ4, we wanted to develop a novel expression system to produce Tβ4 protein at a lower cost, in a safe manner, and through a process that is readily scalable. To this end, a Tβ4 protein encoding gene (*Tβ4*) was designed to take into account tobacco (*Nicotiana tabacum*) codon usage bias and was integrated into the tobacco genome via *Agrobacterium*-mediated transformation. We describe here the production of tobacco-derived 4×Tβ4 protein and report that it can promote cell proliferation *in vitro* and wound healing *in vivo* in mice. Furthermore, we present evidence that the plant-derived Tβ4 protein, which can be directly applied to wounds, is more bioactive than the heterologous form derived from overexpression in *E. coli*. The study provides important information for the future development of plant expressed pharmaceutical proteins for direct administration.

2. Materials and Methods

2.1. Biological Materials. Tobacco (*N. tabacum* cv. Bairihong) seeds and DH5α (*E. coli*) and EHA105 (*A. tumefaciens*) cells were obtained from the Plant Biotechnology Research Centre, School of Agriculture and Biology, Shanghai Jiao Tong University, China. Six- to eight-week-old, healthy Balb/c mice were purchased from the Animal Centre, Shanghai Jiao Tong University, Shanghai, China.

2.2. Construction of the Plant Expression Vector. Based on the Tβ4 amino acid sequence [1], a 168 bp *Tβ4* gene optimized for tobacco codon usage bias was designed, synthesized, and subcloned into the pUC57 plasmid. The resulting pUC57-*Tβ4* plasmid was digested with two combinations of restriction endonucleases (*Spe* I/*Sac* I and *Xba* I/*Sac* I) and the released DNA fragment, with *Xba* I/*Sac* I ends, was ligated into the *Spe*I/*Sac*I sites of pUC57-*Tβ4* based on the isocaudameric properties of *Spe* I and *Xba* I to create the pUC57-*2×Tβ4* plasmid. The pUC57-*4×Tβ4* plasmid with four repeats of the *Tβ4* gene was generated using a similar approach. A DNA sequence encoding six histidines with a *Bam* HI restriction site was introduced into the 5′ end of the *4×Tβ4* construct via polymerase chain reaction (PCR) using the PCR primers *Tβ4* F1: 5′-ggggatccatgcaccaccaccaccaccggtaccatgtctagaatgtctga-3′

and *Tβ4* R1: 5′-ccgagctcttaact agtcataga-3′. The fused DNA fragment of 6×*his*-4×*Tβ4* was then digested by *Bam*HI and *Sac*I and subcloned into the 35S::*hIXF* plasmid constructed by the Plant Biotechnology Research Center, Shanghai Jiao Tong University, China, to create the plant binary expression vector, 35S::*6×his-4×Tβ4*. This plasmid was then introduced into EHA105 (*A. tumefaciens*) via the freeze thaw method [29].

2.3. Tobacco Transformation and PCR Analysis. Tobacco transformation and extraction of tobacco genomic DNA were conducted as previously described [30], with slight modifications. The concentration of genomic DNA extracted from the regenerated tobacco lines was adjusted to 50 ng/μL for PCR analysis, and the 35S::*6×his-4×Tβ4* plasmid and genomic DNA derived from untransformed tobacco were used as positive and negative controls, respectively. PCR reactions were performed in 25 μL reaction volumes containing the following components: 2 μL genomic DNA template, 2.5 μL 10x PCR buffer with MgCl$_2$, 1.5 μL 2.5 mM/L dNTPs, 1 μL 10 μM each PCR primer (P35S: 5′-ttcgtcaacatggtggagca-3′ and Noster: 5′-aagaccggcaacaggattca-3′), 0.2 μL Ex*Taq* DNA polymerase (5 unit/μL) (Takara Biotechnology, Dalian, China), and 16.8 μL deionized ddH$_2$O. The PCR program was one cycle 94°C for 3 min, followed by 35 cycles (94°C for 30 s, 54°C for 30 s, and 72°C for 1.5 min), and finally 72°C for 8 min before being held at 10°C. The PCR products were electrophoresed on a 1.0% (w/v) agarose gel containing 0.5 mg/L ethidium bromide (EB) in 1x TAE buffer (Tris base acetic acid EDTA). Gels were imaged under UV light.

2.4. qRT-PCR. Total RNA was extracted from young leaves of both transgenic and nontransgenic tobacco lines using an RNAprep pure plant kit (TIANGEN, Beijing, China) according to the manufacturer's instructions. The total RNA samples were treated with DNase (RNase-free, TaKaRa, Dalian, China) to eliminate DNA contamination. One μg total RNA was used as a template to synthesize complementary DNA (cDNA) using the Prime-ScriptTM RT Master Mix Kit (TaKaRa, Dalian, China) at 37°C for 15 min and 85°C for 5 sec. Two μL cDNA, diluted 100-fold, was used for qRT-PCR with gene specific primers (*Tβ4*F: 3′-acggtaccatgtctagaatg-5′; *Tβ4*R: 3′-ccgagctcttaactagtcatg-5′). The qRT-PCR program was initiated at 95°C for 30 sec, followed by 27 cycles of 95°C for 15 sec, 58°C for 15 sec, 72°C for 25 sec, and finally 72°C for 5 min, before being held at 4°C. The *UBIQUITIN* gene (GenBank: X58253.1) was used as a reference gene for data normalization, using the gene-specific primers UbiF: 3′-aagacctacaccaagcccaa-5′ and UbiR: 3′-aagtgagcccacacttacca-5′.

2.5. Western Blot and ELISA (Enzyme-Linked Immunosorbent Assay) Analyses. Total soluble protein (TSP) was extracted from young leaves of transgenic and nontransgenic tobacco lines (three biological replicates) using phosphate-buffered saline (PBS) extraction buffer (20 mM sodium phosphate pH 7.4, 137 mM sodium chloride, and 2.7 mM potassium chloride). Approximately 500 mg of each leaf sample was ground to a fine powder using a pestle and mortar with liquid

nitrogen and subsequently added to PBS extraction buffer in a 1 : 1 ratio (w/v). The powder and extraction buffer were mixed well using a Vortex-Genie 2 (Scientific Industries, USA) and then transferred to an ice bath for 2 hrs. The extraction mixture was then centrifuged at 12,000 g for 20 min at 4°C and the supernatant collected. The TSP concentration was determined using the Bradford method [31], using bovine serum albumin (BSA) to create a standard curve.

The TSP concentration was adjusted to 0.5 μg/μL with PBS buffer. After boiling for 5 min, 10 μL of the adjusted TSP was mixed with loading buffer (120 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 3% β-mercaptoethanol, and 0.02% bromophenol blue) in equal volumes (v : v = 1 : 1) and separated via sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, electrophoretically separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (filter pore size 0.22 μm, Bio-Red, USA) for Western blot analysis [24]. The target protein was detected via incubation of the PVDF membrane with a mouse anti-His-tag monoclonal primary antibody (1 : 5,000 dilutions) purchased from Generon Ltd. (Maidenhead, The United Kingdom) and an alkaline phosphatase- (AP-) conjugated goat anti-mouse IgG secondary antibody (1 : 2,000 dilution) (Shanghai ImmunoGen Biological Technology, Shanghai, China). The immunoreactive proteins were visualized by BCIP/NBT (5-bromo-4-chloroindol-3-yl phosphate/nitro blue tetrazolium) staining [30].

ELISA assays were carried out as previously described [32]. The 4×Tβ4 protein derived from *E. coli* was used as a positive control and diluted to concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625, and 0.03125 ng/μL in PBS buffer [24]. The 50 μL serial dilutions of the *E. coli*-derived 4×Tβ4 and the 4×Tβ4 containing TSP extracted from transgenic tobacco (adjusted to 1 μg/μL) were added to the wells of a 96-well plate in triplicate, and the TSP from nontransgenic tobacco leaves was used as a negative control. The same primary and secondary antibodies were used as for the Western blot analysis. BCIP/NBT was used as substrate for the color reaction and A_{410} was measured with a microtiter plate reader (BioTek Instruments, Winooski, VT, USA). 4×Tβ4 protein concentrations were calculated using the previously established standard curve.

2.6. Cell Proliferation Assay (In Vitro).
Chemically synthesized standard Tβ4 protein was diluted to 10 ng/μL and employed as a positive control. The 4×Tβ4 containing TSP extracted from the young transgenic tobacco leaves was filtered through a 0.45 μm pore size membrane (Millipore Millex, Shanghai Jinxin Bio. Shanghai, China) and the 4×Tβ4 protein concentration adjusted to 10 ng/μL with PBS extraction buffer. TSP (amount equal to the tested sample) derived from nontransgenic tobacco leaves were used as negative control.

The *in vitro* bioactivity of Tβ4 was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay [33]. Spleen cells isolated from 6–8-week-old Balb/c mice were collected via centrifugation at 1,000 ×g for 10 min at room temperature [24]. Pellets of spleen cells were subsequently resuspended and diluted to

1×10^5 cells/mL in RPMI 1640 medium (Sigma-Aldrich, Shanghai, China). A 100 μL spleen cell aliquot was added to each well of a 96-well plate and then 100 μL of the diluted standard Tβ4 protein (1 μg), and the TSP extracted from the transgenic and nontransgenic tobacco leaves and a PBS vehicle control were added separately to triplicate wells. The 96-well plate was incubated at 37°C, 5% (v/v) CO$_2$, for 24 h in a cell-culture incubator, and then 10 μL of MTT reagent purchased from Shanghai Hushi Medical Technology Co., Ltd. (Shanghai, China) was added to each well and incubated for an additional 4 h. The plate was periodically observed using a CKX31 inverted microscope (Olympus, Watford, Herts., U.K.) and 150 μL of dimethyl sulphoxide (DMSO) was added to each well when purple precipitate was observed. After swirling gently, the plate was kept in the dark at room temperature for 15 min. Subsequently, the absorbance of each reaction at wavelength of 570 nm was measured with a microtiter plate reader (BioTek, USA).

Spleen cell proliferation was calculated using the following equation:

$$\text{Proliferation (\%)} = \frac{(D_{\text{trial}} - D_{\text{control}})}{D_{\text{trial}}} \times 100. \quad (1)$$

2.7. Wound Healing Experiment (In Vivo).
The 4×Tβ4 containing TSP extracted from young transgenic tobacco leaves were filtered through a 0.45 μm pore size membrane (Millipore Millex, Merck KGaA, Darmstadt, Germany) and freeze-dried (Thermo Fisher Scientific ISS110, USA), before being diluted to 100 ng 4×Tβ4/μL and 50 ng 4×Tβ4/μL with PBS extraction buffer. Nontransgenic tobacco-derived TSP were used as a negative control.

Three, full-thickness, 5 mm punch wounds were inflicted on the dorsal surfaces of each 6–8-week-old Balb/c mouse as previously described [13]. Punch wounds were made on eighteen Balb/c mice and twelve healthy mice were chosen for the experiments. Fifty μL samples of six treatments were then applied at 24 and 48 h after wounding. The six treatments included tobacco-derived 4×Tβ4 proteins (5 μg and 2.5 μg), Tβ4 protein (Purchased from GL Biochem, 5 μg), *E. coli*-derived 4×Tβ4 protein (5 μg), nontransgenic tobacco TSP (5 μg and 2.5 μg), and PBS as a vehicle control. Three biological replicates were carried out for each experimental treatment.

From days 2 to 10 after treatment, keratinocyte migration from six mice was examined by measuring the distance between epidermal tongues of the wound edges with a Vernier caliper (Endura-Greenlee Tools, E0531, Shanghai, China). Wound closure was calculated using the formula described by Li et al. (2007) [25]:

$$\text{Wound closure (\%)}$$
$$= \frac{\text{distance of migrated keratinocytes from the wound edge}}{\text{total wound width}} \quad (2)$$
$$\times 100.$$

To examine reepithelialization and vessel counts of the wound, six additional mice (treated as above) were euthanized on day 8 after treatment and tissues from the healing
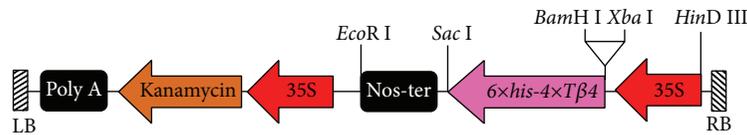
FIGURE 1

FIGURE 2: Diagram of the plant binary vector, 35S::*6×his-4×Tβ4*. LB: T-DNA border (left), poly A: CaMV 35S poly A, 35S: CaMV 35S gene promoter, Kanamycin: CDS (coding sequence) of *NPTII* (neomycin phosphotransferase II) gene, Nos-ter: *NOS* (nopaline synthetase) gene terminator, *6×his-4×Tβ4*: the *4×Tβ4* gene with DNA sequence encoding *6×his* at the 5′ end, and RB: T-DNA border (right).

wounds collected and fixed in 4% (v/v) formalin buffer. The fixed tissues were then embedded in paraffin after dehydration in a series of ethanol concentrations (75%, 85%, 95%, and 100%) and 5 μm sections from the middle of the wounds were made using a microtome (Leica, RM2200) [24]. Sections were then mounted on glass slides and stained with hematoxylin and eosin (Shanghai Dingjie Biotechnology Company, Shanghai, China) after paraffin removal. Vessel counts in the wound beds were determined by identifying vascular spaces distinguished by their endothelial lining, including those at the junction of the dermis and the hypodermis, as angiogenesis within wounds occurs to a great extent from these vessels. Counts were averaged as vessel counts per 10 high-powered fields (40x).

3. Results

3.1. Design, Transformation, and Molecular Examination of the Tβ4 Transgene.
Based on the Tβ4 amino acid sequence (Genpept accession, P62326.2), an 168 bp *Tβ4* gene optimized for tobacco codon usage bias was designed and synthesized, including the restriction endonuclease sites (colored below) *Kpn* I/*Xba* I and *Spe* I/*Sac* I at the 5′ and 3′ ends, respectively, protection bases, and both an initiation codon (**ATG**) and a termination codon (**TAA**) (bold). The sequence was as shown in Figure 1.

$4×Tβ4$ and $35S::6×his-4×Tβ4$ constructs were subsequently created (Figure 2) and transformed into tobacco and the resulting putative transgenic tobacco lines were screened by PCR. This revealed an 1,140 bp DNA band, corresponding to the *4×Tβ4* gene, in the positive control and in 14 of the putative tobacco lines, while this DNA band was not present in nontransformed tobacco samples (Supplementary Appendices 1-d to f and Appendix 2 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1973413). We concluded that the *4×Tβ4* gene had been successfully integrated into the tobacco genome of the positive lines.

3.2. Expression of the 4×Tβ4 Gene.
Expression of the *4×Tβ4* gene was evaluated in eight of the positive transgenic tobacco lines by qRT-PCR. The expression levels varied substantially, between lines, with lines 3, 5, and 13 having high
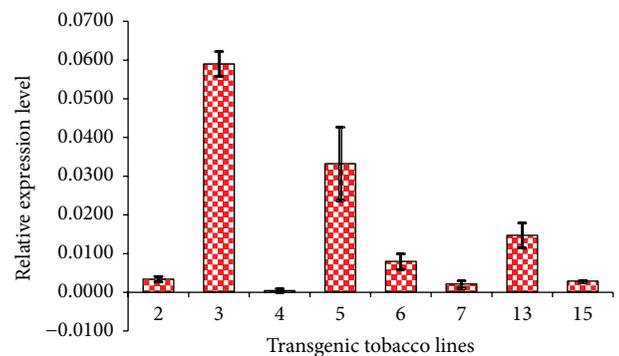
FIGURE 3: The differences in relative expression levels between the transgenic tobacco lines. Bars represent the expression levels of different transformed tobacco lines ($n = 3$).

levels of *4×Tβ4* transcript accumulation, and line 3 showing particularly high expression. The expression level in other lines ranged from relatively low (lines 2, 6, 7, and 15) to nondetectable (line 4) (Figure 3). These differences may be attributed to the copy number of the integrated *4×Tβ4* gene, as well as their sites of integration in the tobacco genome [34, 35].

3.3. Verification of Successful Expression of the Recombinant 4×Tβ4 Protein in Transgenic Tobacco.
The TSP extracted from young leaves of both transgenic and nontransgenic tobacco (negative control) was examined by Western blot and ELISA analyses using anti-His-tag monoclonal primary antibody. The TSP content derived from young tobacco leaves ranged from 4.92 mg/g fresh weight (FW) (line 2) to 6.18 mg/g FW (line 13). Western blot analysis showed a 23.2 kDa band in transgenic tobacco lines, which corresponded to the predicted size of the recombinant protein and which was absent from the nontransgenic tobacco extracts (Supplementary Appendix 4).

The *4×Tβ4* protein content in the transgenic young tobacco leaves was determined by ELISA. The concentrations of *4×Tβ4* protein in transgenic tobacco extracts ranged from 0.492 μg/g FW in line 6 to 2.946 μg/g FW in line 5 (Supplementary Appendix 3). Differences in the expression
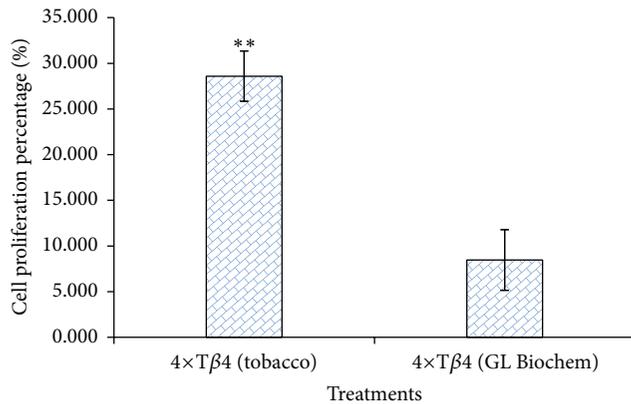
FIGURE 4: Assay of tobacco-dervied 4×Tβ4 protein promoting mice splenic lymphocyte proliferation using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. Column 4×Tβ4 (tobacco) indicates 4×Tβ4 extracted from transgenic tobacco leaves; column Tβ4 (GL Biochem) indicates commercially purchased Tβ4; ∗∗ indicates that tobacco-derived 4×Tβ4 was significantly more effective than commercial Tβ4 at promoting cell proliferation ($n = 6$, $p < 0.01$).

level of the 4×Tβ4 recombinant protein between the lines may be attributed to the number of the 4×Tβ4 copies and position of integration, as well as to posttranscriptional processes [36, 37].

3.4. Tobacco-Derived 4×Tβ4 Protein Promotes Cell Proliferation (In Vitro). A mouse splenic lymphocyte proliferation assay (MTT) was used to determine the biological activity of the tobacco-derived 4×Tβ4 protein. Application of the 4×Tβ4 protein (1 μg) derived from transgenic tobacco leaves (line 5) caused a 28.59±4.97% increase in splenic lymphocyte proliferation, which was significantly higher than the effects of applying either 1 μg of the commercial Tβ4 protein (8.49 ± 3.32%) (Figure 4) or the *E. coli*-derived 4×Tβ4 protein (18.12%; [24]).

3.5. Tobacco-Derived 4×Tβ4 Protein Promotes Healing Wound in Balb/c Mice (In Vivo). The efficiency with which tobacco-derived 4×Tβ4 protein healed wounds and promoted keratinocyte migration was examined using a full thickness cutaneous mouse wound model. The lengths of the epidermal tongues from the wound edges were measured, and we observed that reepithelialization rates were higher in all of the treatment groups (transgenic tobacco-derived 4×Tβ4, commercial Tβ4, and recombinant *E. coli*-derived 4×Tβ4) than in the negative controls (nontransgenic tobacco crude protein and PBS buffer) and that the rate of reepithelialization sharply increased during days 6–8 after treatment. Moreover, on day 8, the rate of keratinocyte migration on the wound bed treated with transgenic tobacco-derived 4×Tβ4 (5 μg) was the highest of all six treatments (Figure 5(a)). The reepithelialization rate in the transgenic tobacco-derived 4×Tβ4 (2.5 μg) treatment group was slightly lower than both commercial Tβ4 and *E. coli*-derived 4×Tβ4 during days 2–8 after treatment. However, by day 10 the keratinocyte

migration rate in tobacco-derived 4×Tβ4 (2.5 μg) exceeded that of the positive controls (commercial Tβ4 and *E. coli*-derived 4×Tβ4) (Figure 5(a)).

Histological examination of tissue sections collected at day 8 after application revealed that tobacco-derived 4×Tβ4 protein promoted an increase in blood vessels in the wound bed. We observed that its angiogenic effects significantly exceeded those of both the positive (*E. coli*-derived 4×Tβ4 and commercial Tβ4 protein) and negative controls (nontransformed tobacco-derived crude protein and PBS) (Figure 5(b)).

4. Discussion

Tβ4, which has been described as the second most biologically active peptide in thymosin fraction 5, after thymosin α1 [1, 17], is a type of actin regulating protein that forms a complex with the actin monomer in a 1:1 ratio. This complexation prevents polymerization and so inhibits the formation of actin filaments. Actin monomers released from the Tβ4/actin complex, however, can drive polymerization reactions as a normal function of the cytoskeleton in cell scaffolding and motility [38, 39]. The sequence LKKTET of the 43-amino acid Tβ4 protein, which is strongly conserved between all β-thymosins, represents the "actin-binding motif" and is similar to the sequence of WH2 domains (Wasp Homology Domain 2, a name derived from the Wiskott-Aldrich syndrome protein) [40]. Previous research has suggested that Tβ4 may be useful for treating hard-to-heal wounds, including diabetic ulcers, bedsores and damaged corneas, and heart muscle injured by heart attacks and tumor biomarkers, as well as for curing various skin, central nervous system, and lung diseases [5, 10, 14, 17, 18, 21–23]. Consequently, there is great interest in the use of Tβ4 in clinical applications.

Genetically engineered Tβ4 proteins have been produced in prokaryotic expression systems, and *E. coli*-derived 4×Tβ4 proteins have been reported to promote wound healing *in vivo* [24]. However, high production costs and difficulties in extraction and purification of the protein still limit its practical application. Plant based expression systems provide an attractive alternative as they are typically less expensive and capable of yielding high protein expression levels, as well as providing a system in which protein folding and modification are more similar to equivalent processes in humans than in prokaryotes [22, 26–28, 34, 37]. In this study, we expressed recombinant 4×Tβ4 protein in transgenic tobacco lines and observed that it was more effective in healing wounds in Blab/c mice than were either commercial or *E. coli* derived Tβ4. Additionally, the tobacco-derived 4×Tβ4 protein was more effective at increasing the number of blood vessel counts in wound beds than were the other Tβ4 proteins tested (Figure 5(b)). Another prominent feature of the tobacco-derived 4×Tβ4 is that it can be applied directly to the wound, while 4×Tβ4 derived from *E. coli* expression systems requires extraction and purification prior to any clinical applications. We propose that plant-derived 4×Tβ4 may be more effective when treating acute injuries, such as burns, diabetic complications, bedsores, and corneal transplantations, than Tβ4 derived from other expression systems.

(a)

(A)   (B)   (C)

(D)   (E)   (F)
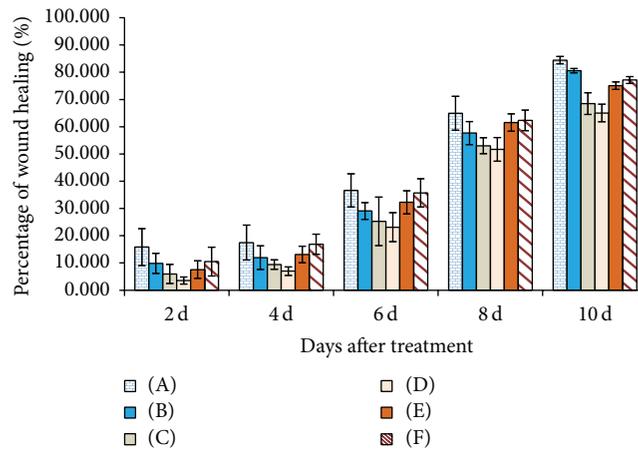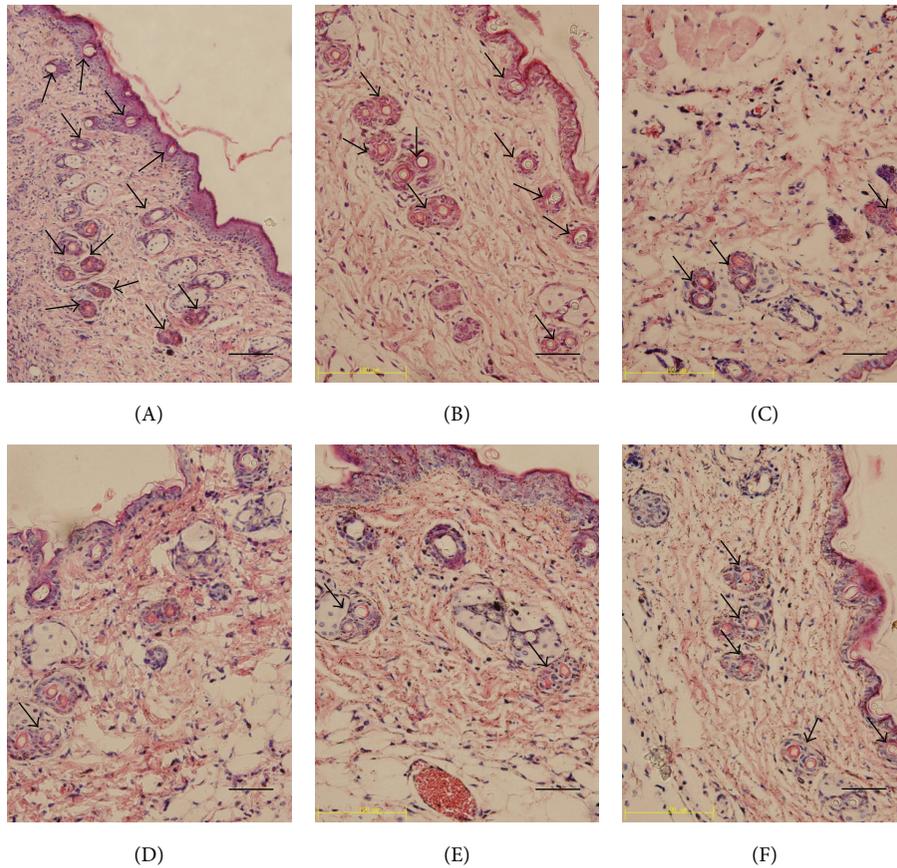
(b)

FIGURE 5: Tobacco-derived 4×Tβ4 promotes keratinocyte migration (a) and formation of blood vessels in the wound beds of mice (b). (A) Tobacco-derived 4×Tβ4 protein (5 μg), (B) tobacco-derived 4×Tβ4 protein (2.5 μg), (C) TSP (total soluble protein) (50 μL) extracted from untransformed tobacco leaves, (D) 50 μL PBS extraction buffer, (E) *E. coli*-derived 4×Tβ4 protein (5 μg), and (F) commercially purchased standard Tβ4 (5 μg). Black arrows indicate newly formed blood vessels and reepithelialization of the wound epidermis following topical treatments. Scale bars = 50 μm.

Much of the cost associated with plant expression systems comes from extraction and purification of the target protein, which is the major factor limiting the development of plant expression systems to produce therapeutic proteins. In the present study, we used plant-derived 4×Tβ4 to heal wounds in Blab/c mice via a direct application approach and showed that it was more efficient than both standard Tβ4 (commercial) and *E. coli*-derived 4×Tβ4 in promoting cell proliferation and wound healing in mice.

5. Conclusions

A 168 bp *Tβ4* gene was designed and synthesized according to tobacco codon usage, and a fused gene, comprising 4×*Tβ4* and a polyhistidine tag, was overexpressed in tobacco. Fourteen positive tobacco lines were obtained via *Agrobacterium*-mediated transformation. The successful expression of the Tβ4 protein in transgenic tobacco lines was confirmed by Western blot and ELISA analyses, and 4×Tβ4 protein concentrations as high as 3 μg/g of fresh weight were detected in the transgenic tobacco leaves. The tobacco-derived 4×Tβ4 protein was more effective than either Tβ4 derived from *E. coli* or the chemically synthesized form at promoting splenic lymphocyte proliferation and wound healing when applied directly to the wounds of mice. This research lays the foundation for the development of therapeutic proteins using plant expression systems, particularly in the context of direct delivery administration methods.

Abbreviations

BCIP/NBT: 5-Bromo-4-chloroindol-3-yl phosphate/nitro blue tetrazolium
ELISA: Enzyme-linked immunosorbent assay
MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
RPMI: Roswell park memorial institute
SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

Competing Interests

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

Authors' Contributions

Rylosona Janarthini and Xiaolei Wang contributed equally to this work, designed and cloned the transgene, performed transformation and the healing wound experiment, and wrote the paper. Lulu Chen and Lei Gao conducted the qRT-PCR and MTT assays. Lingxia Zhao designed the experiment and wrote the paper.

Acknowledgments

References

[1] T. L. K. Low, S. K. Hu, and A. L. Goldstein, "Complete amino acid sequence of bovine thymosin β4: a thymic hormone that induces terminal deoxynucleotidyl transferase activity in thymocyte populations," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 78, no. 2, pp. 1162–1166, 1981.

[2] A. L. Goldstein and H. K. Kleinman, "Advances in the basic and clinical applications of thymosin β4," *Expert Opinion on Biological Therapy*, vol. 15, supplement 1, pp. s139–s145, 2015.

[3] E. Irobi, A. H. Aguda, M. Larsson et al., "Structural basis of actin sequestration by thymosin-β4: Implications for WH2 proteins," *The EMBO Journal*, vol. 23, no. 18, pp. 3599–3608, 2004.

[4] A. L. Goldstein, E. Hannappel, and H. K. Kleinman, "Thymosin β4: actin-sequestering protein moonlights to repair injured tissues," *Trends in Molecular Medicine*, vol. 11, no. 9, pp. 421–429, 2005.

[5] J. Knop, C. App, T. Huff, F. Iavarone, M. Castagnola, and E. Hannappel, "Identification of PDGF-BB binding to thymosin β4 by chemical cross-linking," *Expert Opinion on Biological Therapy*, vol. 15, supplement 1, pp. S147–S154, 2015.

[6] N. Kumar, P. Nakagawa, B. Janic et al., "The anti-inflammatory peptide Ac-SDKP is released from thymosin-β4 by renal meprin-α and prolyl oligopeptidase," *American Journal of Physiology—Renal Physiology*, vol. 310, no. 10, pp. F1026–F1034, 2016.

[7] K. Matsuo, Y. Akasaki, K. Adachi et al., "Promoting effects of thymosin β4 on granulation tissue and new bone formation after tooth extraction in rats," *Oral Surgery, Oral Medicine, Oral Pathology and Oral Radiology*, vol. 114, no. 1, pp. 17–26, 2012.

[8] N. Smart, A. Rossdeutsch, and P. R. Riley, "Thymosin β4 and angiogenesis: modes of action and therapeutic potential," *Angiogenesis*, vol. 10, no. 4, pp. 229–241, 2007.

[9] I. Bock-Marquette, A. Saxena, M. D. White, J. M. DiMaio, and D. Srivastava, "Thymosin β4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair," *Nature*, vol. 432, no. 7016, pp. 466–472, 2004.

[10] G. Sosne and H. K. Kleinman, "Primary mechanisms of thymosin β4 repair activity in dry eye disorders and other tissue injuries," *Investigative Ophthalmology & Visual Science*, vol. 56, no. 9, pp. 5110–5117, 2015.

[11] Y. Zuo, B. Chun, S. A. Potthoff et al., "Thymosin β4 and its degradation product, Ac-SDKP, are novel reparative factors in renal fibrosis," *Kidney International*, vol. 84, no. 6, pp. 1166–1175, 2013.

[12] E. Conte, M. Iemmolo, M. Fruciano et al., "Effects of thymosin β4 and its N-terminal fragment Ac-SDKP on TGF-β-treated

human lung fibroblasts and in the mouse model of bleomycin-induced lung fibrosis," *Expert Opinion on Biological Therapy*, vol. 15, supplement 1, pp. 211–221, 2015.

[13] K. M. Malinda, G. S. Sidhu, H. Mani et al., "Thymosin β4 accelerates wound healing," *Journal of Investigative Dermatology*, vol. 113, no. 3, pp. 364–368, 1999.

[14] H.-J. Cha, M.-J. Jeong, and H. K. Kleinman, "Role of thymosin β4 in tumor metastasis and angiogenesis," *Journal of the National Cancer Institute*, vol. 95, no. 22, pp. 1674–1680, 2003.

[15] J. Caers, E. Otjacques, D. Hose, B. Klein, and K. Vanderkerken, "Thymosin β4 in multiple myeloma: friend or foe," *Annals of the New York Academy of Sciences*, vol. 1194, pp. 125–129, 2010.

[16] B. Can, F. Karagoz, L. Yildiz et al., "Thymosin β4 is a novel potential prognostic marker in gastrointestinal stromal tumors," *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, vol. 120, no. 9, pp. 689–698, 2012.

[17] J. Marx, "Thymosins: clinical promise after a decades-long search," *Science*, vol. 316, no. 5825, pp. 682–683, 2007.

[18] L. Wang, M. Chopp, L. Jia et al., "Therapeutic benefit of extended thymosin β4 treatment is independent of blood glucose level in mice with diabetic peripheral neuropathy," *Journal of Diabetes Research*, vol. 2015, Article ID 173656, 13 pages, 2015.

[19] G. Sosne, E. A. Szliter, R. Barrett, K. A. Kernacki, H. Kleinman, and L. D. Hazlett, "Thymosin beta 4 promotes corneal wound healing and decreases inflammation *in vivo* following alkali injury," *Experimental Eye Research*, vol. 74, no. 2, pp. 293–299, 2002.

[20] G. Sosne, P. Qiu, M. Kurpakus-Wheater, and H. Matthew, "Thymosin β4 and corneal wound healing: visions of the future," *Annals of the New York Academy of Sciences*, vol. 1194, pp. 190–198, 2010.

[21] R. Hinkel, C. El-Aouni, T. Olson et al., "Thymosin β4 is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection," *Circulation*, vol. 117, no. 17, pp. 2232–2240, 2008.

[22] C. Wei, S. Kumar, I.-K. Kim, and S. Gupta, "Thymosin beta 4 protects cardiomyocytes from oxidative stress by targeting anti-oxidative enzymes and anti-apoptotic genes," *PLoS ONE*, vol. 7, no. 8, article e42586, 2012.

[23] E. Vasilopoulou, P. J. D. Winyard, P. R. Riley, and D. A. Long, "The role of thymosin-β4 in kidney disease," *Expert Opinion on Biological Therapy*, vol. 15, supplement 1, pp. S187–S190, 2015.

[24] X. Wang, G. Yang, S. Li, M. Gao, P. Zhao, and L. Zhao, "The *Escherichia coli*-derived thymosin β4 concatemer promotes cell proliferation and healing wound in mice," *BioMed Research International*, vol. 2013, Article ID 241721, 7 pages, 2013.

[25] X. Li, L. Zheng, F. Peng et al., "Recombinant thymosin beta 4 can promote full-thickness cutaneous wound healing," *Protein Expression and Purification*, vol. 56, no. 2, pp. 229–236, 2007.

[26] R. K. Jhinjer, l. Verma, S. H. Wani, and S. S. Gosal, "Molecular farming using transgenic approaches," in *Advances in Plant Breeding Strategies: Agronomic, Abiotic and Biotic Stress Traits*, J. M. Al-Khayri, S. M. Jain, and D. V. Johnson, Eds., pp. 97–145, Springer, New York, NY, USA, 2016.

[27] J. K.-C. Ma, P. M. W. Drake, and P. Christou, "The production of recombinant pharmaceutical proteins in plants," *Nature Reviews Genetics*, vol. 4, no. 10, pp. 794–805, 2003.

[28] G. Walsh, "Biopharmaceutical benchmarks 2010," *Nature Biotechnology*, vol. 28, no. 9, pp. 917–924, 2010.

[29] G. Jyothishwaran, D. Kotresha, T. Selvaraj, S. M. Srideshikan, P. K. Rajvanshi, and C. Jayabaskaran, "A modified freeze-thaw method for efficient transformation of Agrobacterium tumefaciens," *Current Science*, vol. 93, no. 6, pp. 770–772, 2007.

[30] L. J. Cui, H. Z. Peng, R. Zhang, Y. H. Chen, L. X. Zhao, and K. X. Tang, "Recombinant hHscFv–RC-RNase protein derived from transgenic tobacco acts as a bifunctional molecular complex against hepatocellular carcinoma," *Biotechnology and Applied Biochemistry*, vol. 59, no. 5, pp. 323–329, 2012.

[31] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analytical Biochemistry*, vol. 72, no. 1-2, pp. 248–254, 1976.

[32] Y. Chen, A. Wang, L. Zhao, G. Shen, L. Cui, and K. Tang, "Expression of thymosin α1 concatemer in transgenic tomato (*Solanum lycopersicum*) fruits," *Biotechnology and Applied Biochemistry*, vol. 52, no. 4, pp. 303–312, 2009.

[33] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays," *Journal of Immunological Methods*, vol. 65, no. 1-2, pp. 55–63, 1983.

[34] A. J. M. Matzke and M. A. Matzke, "Position effects and epigenetic silencing of plant transgenes," *Current Opinion in Plant Biology*, vol. 1, no. 2, pp. 142–148, 1998.

[35] Q. Que, H.-Y. Wang, J. J. English, and R. A. Jorgensen, "The frequency and degree of cosuppression by sense chalcone synthase transgenes are dependent on transgene promoter strength and are reduced by premature nonsense codons in the transgene coding sequence," *Plant Cell*, vol. 9, no. 8, pp. 1357–1368, 1997.

[36] L. F. Maxfield, C. D. Fraize, and J. M. Coffin, "Relationship between retroviral DNA-integration-site selection and host cell transcription," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 5, pp. 1436–1441, 2005.

[37] S.-W. Wang, A. L. Stevenson, S. E. Kearsey, S. Watt, and J. Bähler, "Global role for polyadenylation-assisted nuclear RNA degradation in posttranscriptional gene silencing," *Molecular and Cellular Biology*, vol. 28, no. 2, pp. 656–665, 2008.

[38] D. Safer, M. Elzinga, and V. T. Nachmias, "Thymosin β4 and Fx, an actin-sequestering peptide, are indistinguishable," *The Journal of Biological Chemistry*, vol. 266, no. 7, pp. 4029–4032, 1991.

[39] H.-S. Moon, S. Even-Ram, H. K. Kleinman, and H.-J. Cha, "Zyxin is upregulated in the nucleus by thymosin β4 in SiHa cells," *Experimental Cell Research*, vol. 312, no. 17, pp. 3425–3431, 2006.

[40] B. Xue, A. H. Aguda, and R. C. Robinson, "Models of the actin-bound forms of the β-thymosins," *Annals of the New York Academy of Sciences*, vol. 1112, pp. 56–66, 2007.