Research Article

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Expression of a functional recombinant vascular endothelial growth factor 165 ($VEGF_{165}$) in *Arabidopsis thaliana*

Arabidopsis thaliana'daki fonksiyonel bir rekombinant vasküler endotelyal büyüme faktörü 165'in ($VEGF_{165}$) ekspresyonu

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Abstract

Objective: Targeting the protein of interest to a particular tissue to achieve high-level expression is an important strategy to increase expression efficiency. The use of the plant seed oil body as a bioreactor can not only increase the amount of target protein, but also reduce the cost of downstream processing.

Methods: $VEGF_{165}$ was expressed in *Arabidopsis thaliana* seeds via oilbody fusion technology. The pKO-$VEGF_{165}$ vector was construted and transformed into *A. thaliana* seeds. T3 transgenic seeds was detected by SDS-PAGE and western blot methods. The cell activity was tested by MTT methods.

Result: The phaseolin promoter was used to drive seed-specific expression of the *$VEGF_{165}$* gene in transgenic *A. thaliana*. The coding region of *$VEGF_{165}$* was fused to the *Arabidopsis* oleosin sequence to target the protein to the oil bodies in the seeds of transgenic plants. The T-DNA region of recombinant plasmid pKO-$VEGF_{165}$ was shifted to *A. thaliana* seeds via the floral-dip method. Protein was analyzed by electrophoresis and protein hybridization analyses. Finally, MTT assays showed that the oleosin-$VEGF_{165}$ fusion protein played a part in the proliferation of HUVEC cells in vitro.

Conclusion: Oleosin-$VEGF_{165}$ was successfully expressed and it had stimulated HUVEC cell proliferation activity.

Keywords: Vascular endothelial growth factor 165; Oil body; Oleosin; *Arabidopsis thaliana*.

Öz

Amaç: Yüksek seviyeli ekspresyon elde etmek için ilgili proteinin belirli bir dokuya hedeflenmesi, ekspresyon verimliliğini artırmak için önemli bir stratejidir. Bir biyoreaktör olarak bitki tohum yağı gövdesinin kullanılması, sadece hedef protein miktarını arttırmakla kalmaz, aynı zamanda aşağı akış işleme maliyetini de düşürür.

Metod: $VEGF_{165}$, yağlı vücut füzyon teknolojisi vasıtasıyla Arabidopsis thaliana tohumlarında eksprese edildi. pKO-$VEGF_{165}$ vektörü dizilmiş ve *Arabidopsis thaliana* tohumlarına dönüştürülmüştür. T3 transjenik tohumları SDS-PAGE ve western blot yöntemleri ile tespit edildi. Hücre aktivitesi MTT yöntemleriyle test edildi. Protein, elektroforez ve protein hibridizasyon analizleri ile analiz edildi.

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Bulgular: Transazensel Arabidopsis thaliana'daki tohumdan spesifik ekspresyonunu başlatmak için fazoin yükseltici kullanılmıştır. $VEGF_{165}$, in kodlama bölgesi, proteini transjenik bitkilerin tohumlarındaki yağ gövdelerine hedeflemek için Arabidopsis oleosin dizisine kaynaştırıldı. Rekombinant plazmid pKO-$VEGF_{165}$, in T-DNA bölgesi, çiçek-daldırma yöntemi vasıtasıyla Arabidopsis thaliana tohumlarına kaydırılmıştır. Finally, MTT assays showed that the oleosin-$VEGF_{165}$ fusion protein played a part in the proliferation of HUVEC cells in vitro.
Sonuç: Oleozin-$VEGF_{165}$ başarıyla eksprese edildi ve HUVEC hücre çoğalması aktivitesini uyardı.

Anahtar Kelimeler: Vasküler endotelyal büyüme faktörü 165; Yağ gövdesi; Oleosini; Arabidopsis thaliana.

Introduction

Vascular endothelial growth factor (VEGF) is a dimeric glycoprotein that induces the production of mitogen in endothelial cells [1]. It plays a role in an basal regulator of vasculogenesis and hematopoiesis, wound healing [2–4] and pathological status such as rheumatoid arthritis, cancer, cardiovascular disease and psoriasis [5–8]. VEGF can promote vasopermeability, stimulate endothelial cell proliferation and migration [9], and drive neovascularization [10–12]. The alternative splicing of VEGF-mRNA generated five isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) [13]. A variety of VEGF isoforms are possible to carry out different effects and they synergize in the tumor angiogenesis process. $VEGF_{165}$ is the most effective angiogenic factor in the VEGF family [13]. The literature reported that increased tumor angiogenesis and poor outcomes correlate with the overexpression of $VEGF_{165}$ in colon cancer, osteosarcoma, renal cell carcinoma and non-small cell lung cancer [13–15].

Plants have been used as a source of natural products throughout history, dating back thousands of years. The development of plant transformation technology has led to the use of various plant hosts for the expression of human therapeutics [16, 17], nutraceuticals [18], antibodies [19, 20], industrial enzymes [21, 22], vaccine antigens [23, 24], and biopolymers [25, 26]. The bioactive proteins are produced in the plant bioreactor that is most safe, non-toxic, simple and lowest cost. Although they have some limitations, such as appear to be the carriers for exogenous proteins in seeds, these proteins are produced more stable in plant seeds than other tissues [27]. The purification of the exogenous protein from the wide spectrum is a major bottleneck, because it requires many tedious processes [27]. However, it is easier to extract a recombinant protein located on the surface of an oil body, since oil bodies can be separated easily from other cellular components by flotation centrifugation [27–29].

After the target protein is anchored on the surface of oil body, the oil bodies can be separated via flotation centrifugation and the oleosin-target protein can be easily extracted [27, 30]. Oil body expressed hirudin was extracted from transgenic seeds and the recombinant hirudin showed anti-thrombin activity [27, 31]. The β-glucuronidase linked to the oil body was detected to be active and remained active after it was separated from the surface of the oil body [27, 32]. Human epidermal growth factor that fused with oleosin and inserted into a chymosin cleavage factor between oleosin-hEGF has been expressed in *Arabidopsis thaliana*. Here, we expressed $VEGF_{165}$ targeted to the oil body in *A. thaliana*. The expression vector pKO-$VEGF_{165}$ was constructed and the T-DNA region of the recombinant plasmid was transformed into plant genome. The oleosin-$VEGF_{165}$ fusion protein expressed in the transgenic *A. thaliana* seeds showed good antigenicity, and it stimulated HUVEC cell proliferation.

Materials and methods

Reagents

Escherichia coli DH5α and *Agrobacterium tumefaciens* EHA105 were obtained from the team of XK Li professor in Jilin Agricultural University, China. The restriction enzymes such as *Nco*I and *Hin*d III, ExTaq DNA polymerase were purchased from TaKaRa (Dalian, China). Streptomycin (Str) and Kanamycin (Kam) were obtained from Sigma (Hong Kong, China). Glufosinate was from Boehringer Mannheim Corp [33]. (Mannheim, Germany). The rabbit anti-VEGF polyclonal antibody was acquire from Beijing Bioss Co., Ltd. Goat anti-rabbit IgG was obtained from Promega (Madison, WI, USA). Human umbilical vein endothelial (HUVEC) cells were stored in liquid nitrogen until use. Methylthiazol tetrazolium (MTT) was obtained from Gold Biotechnology (St. Louis, MO, USA) [33, 34]. The M199 medium was purchased from Gibco BRL (Grand Island, NY, USA).

Preparation of the recombinant vector pKO-$VEGF_{165}$

The pKO plasmid was modifications of the pCAMBIA1301 plasmid vector. The hygromycin resistance gene in

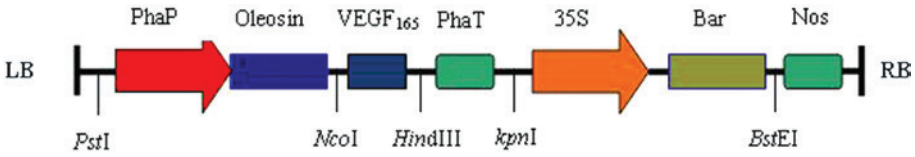

Figure 1: Structure chart of recombinant plasmid pKO-VEGF$_{165}$.
The T-DNA of the pKO-VEGF$_{165}$ vector included a phaseolin promoter/terminator, an *Arabidopsis thaliana oleosin* gene, *VEGF$_{165}$* gene, the 35S promoter, the *bar* gene and nos terminator. PhaP, phaseolin promoter; Oleasin, *Arabidopsis thaliana oleosin* gene, VEGF$_{165}$, Vascular Endothelial Growth Factor$_{165}$; PhaT, phaseolin terminator; 35S, CaMV35S promoter; Bar, the glufosinate resistance gene; Nos, Nopaline synthase terminater gene.

pCAMBIA1301 was replaced by the *basta* gene. The selectable marker *basta* gene was driven by the CaMV 35S promoter. CaMV 35S promoter (No. AF218816.1), the *basta* gene (No. AF218816.1), and the *nos* terminator (No. AF234307), phaseolin promoter/terminator (Patent PCT/US01/47495), the *A. thaliana* oleosin gene (No. X62353.1) and VEGF$_{165}$ gene (No. AF486837.1) were obtained from GenBank. The VEGF$_{165}$ gene was sticked into the pKO vector by *Nco*I and *Hin*d III (Figure 1). It was designated as pKO-VEGF$_{165}$, then was shifted into *Agrobacterium* EHA105 using the freeze-thaw method [35].

Transformation into *Arabidopsis thaliana*

Arabidopsis thaliana (Columbia ecotype) were used for infection. The floral-dip liquid medium which included 1% (w/v) B5 (200×) medium, 100 g/L sucrose, 2 mg/L 6-BA, 1 M sodium hydroxide, and 200 µL Silwet L-77 was prepared as previously described [34]. Plants' aerial parts were dipped into the medium for 5 min, and then culture dark light for 16–24 h. Then the plants were cultured in light condition. The dry seeds were (T1) were collected in a sample bag.

Protein expression analysis

Arabidopsis thaliana seeds (1 mg) were ground with 40 µL Tris-HCl (50 mM) in 1.5 mL ep tubes. The mixtures that included oil body liquid supernatant and 5×loading buffer were boiled for 10 min, and they were detected on two 12% polyacrylamide gels under reducing conditions. The polyacrylamide gels were colored overnight using Coomassie blue. In the meantime, the gels were electroblotted onto 0.45-µm polyvinylidene difluoride (PVDF) membranes. Electroblotting was carried out at 100 V for 75 min which contained 2.9 g/L glycine, 5.8 g/L Tris, 0.37 g/L SDS and 20% (v/v) methanol [36]. The PVDF membranes were incubated with a rabbit anti-VEGF polyclonal antibody antiserum (1:1000 dilution) and then washed 4 times with TBST. Then the secondary antibody which is goat anti-rabbit IgG/AP antibody (1:5000) incubated for 120 min at 25°C [36]. The PVDF membranes were colored Alkaline Phosphatase.

Activity assay of VEGF$_{165}$

Seeds (20 mg) were ground in 200 µL Tris-Cl (pH 7.5), and then the mixture was centrifuged at 10,000×*g* and 4°C for 30 min. The oil body was collected at resuspended in 200 µL Tris-Cl (pH 7.5) [33], and centrifuged again. After centrifugation, the oil body was mixed again with 10 µL Tris-Cl buffer [33]. The biological activity on the proliferation of HUVEC cells was analyzed from transgenic oil body suspension. The HUVEC cells were cultured in M199 medium containing 20% fetal bovine serum (FBS) until they achieved the logarithmic phase. Then, the cells were removed and cultured into a new 96-well plate (8×10^3/well) with 1% FBS for 24 h. The HUVEC cells were incubated with various dilutions of oleosin-VEGF$_{165}$ (250, 500, 1000, 2000, 4000, and 8000-times dilutions) or wild-type oil bodies (negative control, diluted 250, 500, 1000, 2000, 4000, and 8000 times) for 48 h. To determine the activity of the cells, 20 µL MTT was added, the cells were incubated for 4 h and 100 µL DMSO was added to each well [37]. After 10 min, the absorbance was measured at 570/630 nm in a Microplate Reader model 450.

Result

Expression Oleosin-VEGF$_{165}$ in *Arabidopsis thaliana* and screening for transgenic lines

We constructed the pKO vector cassette linking to the *VEGF$_{165}$* gene to express VEGF$_{165}$ and *VEGF$_{165}$* gene was inserted into pKO vector (Figure 1). The pKO-VEGF$_{165}$

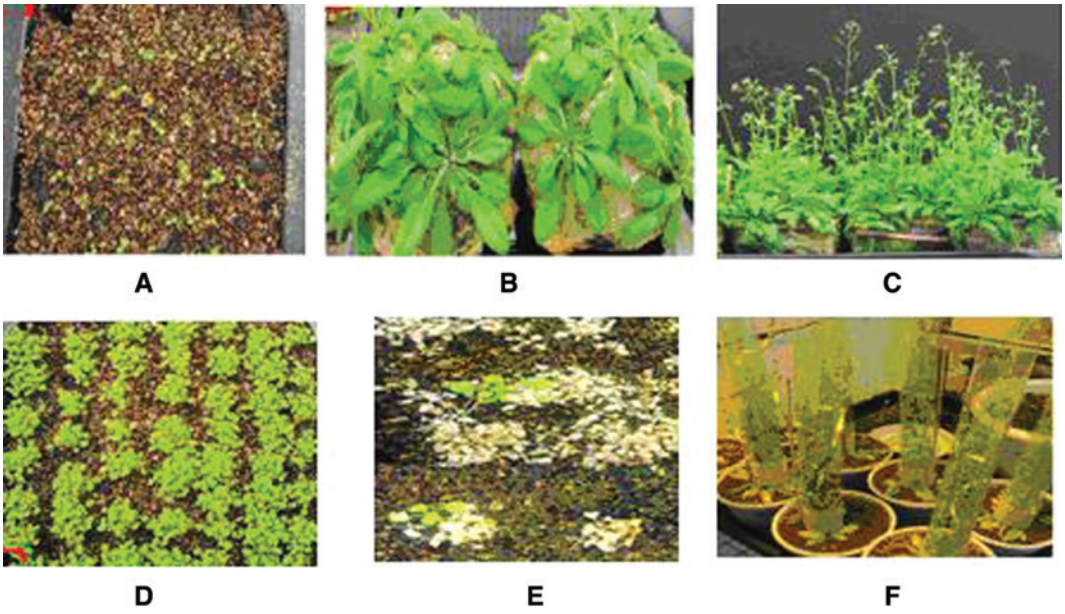

Figure 2: The infection and culture process of *Arabidopsis thaliana*.
(A) *Arabidopsis thaliana* seeds germination. (B) *Arabidopsis thaliana* culture after transplant. (C) *Arabidopsis thaliana* plants after infection. (D) T1 *Arabidopsis thaliana seeds* germination. (E) Screening for transgenic T2 *Arabidopsis thaliana* plants. (F) Single plant harvesting of T2 *Arabidopsis thaliana*.

plasmid was detected using PCR method and transformed successfully into *Agrobacterium* EHA105 competent cells by the freeze-thaw method. The VEGF$_{165}$ gene was anchored on the surface of oil body and it was specifically expressed in *A. thaliana* seeds [34]. The pKO-VEGF$_{165}$ plasmid was transformed into *A. thaliana* by floral-dip method. The infection process was as shown in Figure 2. The T1 transgenic seeds were sown until the plants grew 6–8 leaves. Afterwards the transformants were chosen by 1% (w/v) glufosinate. The cotyledons of non-transformed plants became bleached, while transgenic seedings grew normally. Positively transformed lines were grown and ultimately homozygous seeds were obtained [36]. Four lines (T3-1, T3-2, T3-3, T3-4) were confirmed as positive transformants and were used for further analysis.

Oleosin-VEGF$_{165}$ fusion protein expression analysis

To detect whether *VEGF$_{165}$* genes was expressed in the transgenic T3 *A. thaliana* seeds, oil bodies were extracted from T3 transgenic *A. thaliana* seeds. Four independent transgenic lines was evaluated by extraction of the oil bodies which expressed oleosin-VEGF$_{165}$ protein (Figure 3A and B). The accumulate of oleosin-VEGF$_{165}$ was calculated. The fusion protein were analyzed by SDS-PAGE. The SDS-PAGE results showed that the target band of oleosin-VEGF$_{165}$ fusion protein was about 36 kDa from T3 transgenic plant T3-1, T3-2, T3-3. The protein from wild type seeds had no band at this position and there was no target band at this position from T3-4 transgenic plant (Figure 3A). To further confirm the presence of 36 kDa oleosin-VEGF$_{165}$ in the oil body, the immunodetection by rabbit anti-VEGF$_{165}$ polyclonal antibody was carried out in the experiment. The Oleosin-VEGF$_{165}$ fusion protein was identified from T3 transgenic plant T3-1, T3-2, T3-3 by the anti-VEGF$_{165}$ polyclonal antibody (Figure 3B). The hybridization band appeared at 36 kDa from T3 transgenic plant T3-1, T3-2, T3-3 in accordance with the predicted oleosin-VEGF$_{165}$ fusion protein. However, there was no band at this position in the analysis of the total proteins from wild-type seeds. The *VEGF$_{165}$* gene was expressed in *A. thaliana* and its product accumulated in the oil bodies in the transgenic seeds.

Activity assay of VEGF$_{165}$

According to western blot result, transgenic line T3-1, T3-2, T3-3 were chosen to evaluate the bioactivity of the oleosin-VEGF$_{165}$ fusion proteins. Because the oil bodies expressed oleosin-VEGF$_{165}$ was obtained from T3-1, T3-2, T3-3 seeds, these seeds were used to detect the proliferative activity by the MTT method with HUVEC cells. The oil bodies extracted from Line T3-1, T3-2, T3-3 were analyzed. In Figure 4, the x- axis shows the dilution ratio of the oil

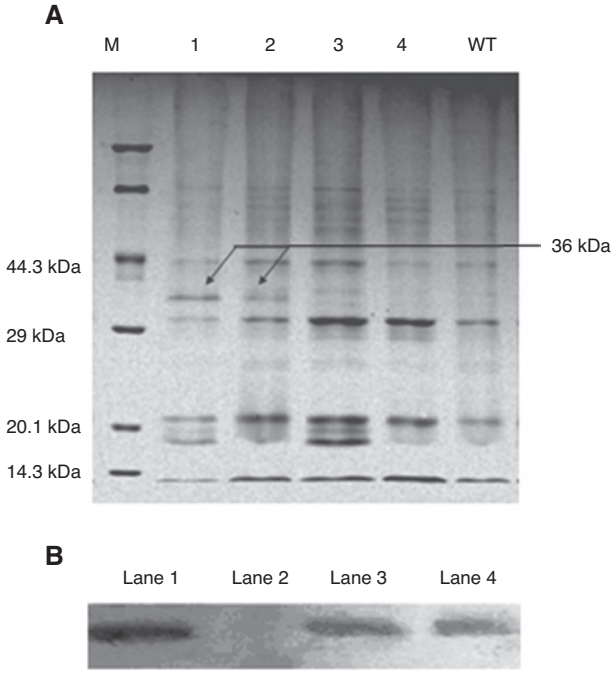

Figure 3: Identification of SDS-PAGE and western blot of oil bodies in transgenic *Arabidopsis thaliana*.
(A) M: Protein Marker; lane1–lane4: the oil body from T3 transgenic *Arabidopsis thaliana* T3-1, T3-2, T3-3, T3-4; lane5: the oil bodies from wild-type (WT) *Arabidopsis thaliana*. (B) Lane 1: oil bodies from T3-1 transgenic *Arabidopsis thaliana*; lane2: the oil bodies from wild-type *Arabidopsis thaliana*; lane3–lane4: the oil body from T3 transgenic *Arabidopsis thaliana* T3-2, T3-3.

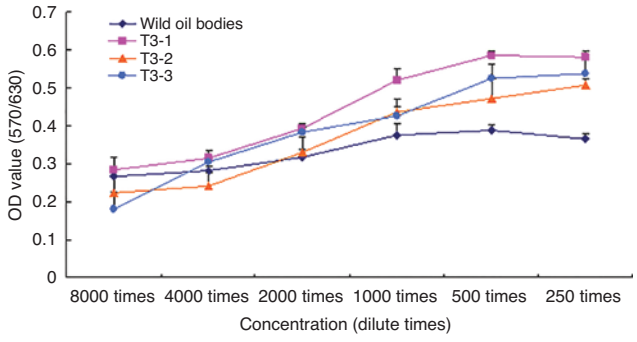

Figure 4: The activity assay of oil body expressed *oleosin-VEGF*$_{165}$ in the transgenic *Arabidopsis thaliana* seeds.
Dose-response curves for proliferation activity. The OD value was changed in HUVEC cells in absorbance (570/630 nm). The test sample included, oil body from wild-type *A. thaliana* (◆), oil body expressed oleosin-VEGF$_{165}$ from T3-1(■), oil body expressed oleosin-VEGF$_{165}$ from T3-2(▲), oil body expressed oleosin-VEGF$_{165}$ from T3-3(●).

bodies and the y-axis shows the OD value (Figure 4). A gradient of VEGF$_{165}$ dilution ratio was used and the dilution ratio was set as a gradient from 250 to 8000 times (Figure 4). The results showed that the oil bodies that expressed oleosin-VEGF$_{165}$ fusion protein from the T3-1, T3-2, T3-3 had a dose-dependent proliferative effect on HUVEC cells. The wild-type oil bodies used as the negative control did not obviously show this dose-response relationship with cell proliferation.

Discussion

The plant expression systems can produce active exogenous proteins in many studies [36]. Such systems show great advantages in terms of cost-efficiency, product quality and safety [36]. Oil bodies can also serve as an efficient system for the expression of cellulolytic enzymes [27], because the enzyme is restricted to the cell surface, it is prevented from contacting its substrates (cell wall constituents) in plants [38]. The target protein is expressed in the seed after being linked with the oleosin and oleosin accounts for 2%–10% of the total protein in the seeds. So the expression level of target protein is lower. But the advantage of oil body system is that the target protein need not be purified and the oil body is directly applied to the skin surface. So the oil body expression system represents a perfect system for the production of therapeutic proteins. The oleosin-haFGF protein was expressed in *A. thaliana* seeds via oleosin fusion technologyand it had biological activity which stimulated NIH/3T3 cell proliferation activity [33]. Human fibroblast growth factor 9 was expressed in *A. thaliana* oil bodies [34]. Fibroblast growth factor 10 was expressed in *Carthamus tinctorius* L. seeds [39]. The process of purifying VEGF$_{165}$ from *E. coli* cells was more complicated than purifying the protein from the oil body. VEGF$_{165}$ was targeted to the C-terminus of oleosin and the process of purification was easier. The purification process was simple and refolding of proteins was not required [34, 40]. Oleosins can act as a natural emulsifying and stabilizing agent at oil/water interfaces; therefore, there are many potential applications [34]. It is important to have an economical, reliable, and up-scalable method to synthesize bioactive oleosin-VEGF$_{165}$ to evaluate such applications. The oil body expression system represents a perfect system for the production of therapeutic proteins. This system has the potential to produce safe, biologically active proteins with low purification costs. When the dilution ratio of oil bodies was 250–4000 times, the absorbance of oil bodies harboring VEGF$_{165}$ was higher than that of wild-type oil bodies. When the dilution ratio was 8000 times, the absorbance of the transgenic oil bodies began to decline slowly. The oil bodies harboring VEGF$_{165}$ promoted

the proliferation of HUVEC cells in a dose-dependent manner.

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