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Expression of bioactive recombinant human fibroblast growth factor 9 in oil bodies of *Arabidopsis thaliana*

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ABSTRACT

Fibroblast growth factor 9 (FGF9) has autocrine and paracrine functions in chondrogenesis osteogenesis, hair growth, and gonadal differentiation. We have expressed recombinant human FGF9 (rhFGF9) in the oil bodies of *Arabidopsis thaliana* via the floral dip method. The expression vector pOTB–rhFGF9 contained an *oleosin–rhFGF9* fusion gene and a glufosinate resistance gene for selection. This plasmid was transformed into *A. thaliana* and expression of the fusion protein oleosin–rhFGF9 confirmed by SDS–PAGE and Western blotting. Furthermore, MTT assays demonstrated that the oil bodies expressed oleosin–rhFGF9 from the transgenic *A. thaliana* had a remarkable proliferation effect on NIH/3T3 cells.

1. Introduction

Fibroblast growth factor 9 (FGF9) is present in various human body tissues where it effectively promotes mitosis and cell growth. FGF9 is involved in bone development, angiogenesis, embryonic development, damage repair, cell apoptosis, nerve regeneration, hair growth, and other physiological and pathological processes. The *hFGF9* gene is tightly on clustered chromosome 13q11–q12, whereas ox *FGF9* is tightly clustered on chromosome 12 and mouse *FGF9* is tightly clustered on chromosome 14 [1]. The coding sequence of *hFGF9* is well conserved, exhibiting 94.9% and 88.7% identity to the ox and mouse variants, respectively. There is a potential polyadenylation site in the 3′-untranslated region of *hFGF9* mRNA, and a high G/C area in the 5′-untranslated region [2].

FGF9 adopts a pyramid-like structure, with the N- and C-terminal regions lying outside the trefoil core region being ordered and forming 2 α-helices and 12 β-strands in the crystallographic dimer [3,4]. FGF9, FGF16, and FGF20 form a subfamily based on similarities in sequence and phylogeny [5]. Proteins within this subfamily do not possess the amino-terminal export sequence typical of FGFs, and are secreted through the traditional endoplasmic reticulum (ER)–golgi secretory pathway [6,7].

Diverse functions of the FGF ligands have been identified by binding and activating the FGFR family of tyrosine kinase receptors in a dependent manner [8]. There are four *FGFR* genes (*FGFR1–4*) that encode receptors comprising three extracellular immunoglobulin domains (D1–3), a single-pass transmembrane domain, and a cytoplasmic tyrosine kinase domain [9]. Given the biological role of FGF9 in important processes including ovarian cancer progression, bone development, nerve regeneration, and gonadal differentiation, studies have focused on identifying mechanisms underlying these effects. Enhanced understanding of the role of FGF9 may aid the diagnosis of ovarian cancer, treatment of cartilage disorders, and study of gonadal differentiation.

Plants have been studied extensively for their utility as an inexpensive and scalable alternative to common expression systems for the production of exogenous proteins. Plant-based expression systems offer multiple advantages over microbial or mammalian host systems, including cost effectiveness, storage and transportation convenience, genetic stability, and high yield potential [10]. Furthermore, plants possess all of the cellular machinery required for post-translational modifications of proteins, and they are intrinsically safe.

Abbreviations: rhFGF9, recombinant human fibroblast growth factor 9; pOTB, p1301-promoter-oleosin–terminator-35S-bar-Nos; PBS, sodium phosphate buffer; WT, wild *Arabidopsis thaliana* seeds; 6-BA, 6-benzylaminopurine.

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Oil bodies are maintained as small individual units with diameters of 0.5–2.0 μm, and are surrounded by a phospholipid monolayer containing multiple proteins, most of which are oleosins [11]. Low-molecular-weight oleosins are hydrophobic plant membrane proteins that cover the entire surface of oil bodies [12]. Oil body size is determined by oleosin accumulation [13]. Oleosins adopt a unique structural configuration that comprises poorly conserved amphipathic N- and C-terminal domains, and a highly conserved central hydrophobic domain [14,15]. Moreover, oleosins appear to act as a natural emulsifying and stabilizing agents at oil–water interfaces [16]. Therefore, oleosins possess potential biotechnological applications as emulsion stabilizers in various cosmetic and cosmeceutical products [17,18].

Oleosins have been employed as carrier molecules in the expression and purification of recombinant pharmaceutical peptides and industrial enzymes [19,20]. The process of oleosin fusion involves fusion of heterologous proteins to the N- or C-terminus of oleosin and subsequent expression of the recombinant protein under the control of an *oleosin* gene promoter or other seed-specific promoter [21,22]. This fusion technology extends the protein half-life, and allows easy transportation and storage [23]. Moreover, oleosin fusion has been widely used to express foreign proteins. The SemBioSys biotechnology company constructed a plant expression vector pSBS4405 and transformed it into *Arabidopsis thaliana* for production of human insulin [24]. Additionally, SemBioSys has successfully used the safflower oil body to express human insulin and achieved commercial production standards. Human epidermal growth factor fused to oleosin has also been expressed in *A. thaliana* and an accumulation level of 0.12% was achieved [25]. Our laboratory has produced functional recombinant oleosin–human hyaluronidase and biologically active, recombinant human acidic fibroblast growth factor in *A. thaliana* seeds [26,27].

Many exogenous proteins have been expressed in transgenic plants since the first successful production of a mouse monoclonal antibody in plants [28]. We report the construction of the expression vector pOTB–rhFGF9 and subsequent expression of rhFGF9 in the *A. thaliana* oil bodies. Expression of the oleosin–rhFGF9 fusion protein was confirmed by SDS–PAGE and Western blotting. Moreover, the oil bodies expressed oleosin–rhFGF9 from transgenic *A. thaliana* seeds could stimulate NIH/3T3 cell proliferation.

2. Materials and methods

2.1. Materials

Plasmids and strains: the plasmid pOTB possesses a basic skeleton, a phaseolin promoter/terminator, the *A. thaliana oleosin* gene, a 35S promoter, the *bar* gene, and a Nos terminator. *Escherichia coli* DH5α and *Agrobacterium tumefaciens* EHA105 cells were obtained from the Ministry of Education Engineering Research Center of Bioreactor and Pharmaceutical Development, Jilin Agricultural University. The coding sequence of rhFGF9 was sourced from GenBank (Gene ID: 2254) and modified by codon optimization according to the codon use table of *A. thaliana*. The gene was then synthesized by Genewiz (Jiang Su, China).

Test material: Mature *A. thaliana* seeds.

Enzymes and reagents: Ex Taq, polymerase chain reaction (PCR) purification kit, gel extraction kit, and a plasmid miniprep kit were all purchased from Takara (Dalian, China). Restriction enzymes *Nco*I and *Hin*dIII, *pfu*DNA polymerase, and T4 DNA ligase were also purchased from Takara. All DNA primers were synthesized and sequenced by Sangon Bioengineering Co. Ltd. (Shanghai, China). Kanamycin and rifampicin were purchased from Sigma (Hong Kong, China). Glufosinate was purchased from the Boehringer Mannheim Corporation (Mannheim, Germany). Dulbecco's modified Eagle medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA). Methylthiazol tetrazolium (MTT) was obtained from Gold Biotechnology (St. Louis, MO, USA).

2.2. Methods

2.2.1. Construction of the pOTB–rhFGF9 vector

The pOTB plasmid was digested with *Nco*I and *Hin*dIII. The *rhFGF9* gene was extracted from pUC-rhFGF9 (Genewiz Biotech) and by digestion with *Nco*I and *Hin*dIII. This *rhFGF9* fragment was inserted into the cleaved pOTB plasmid by incubation with T4 DNA ligase at 4 °C for 10 h. The new recombinant plasmid pOTB–rhFGF9 was transformed into DH5α competent cells and amplified. pOTB–rhFGF9 was then transformed into EHA105 competent cells using the freeze–thaw method [29]. The positive colonies were identified by RT-PCR using rhFGF9-specific primers (forward: 5′-C TACTTCGGAGTTCAGGATGC-3′; reverse: 5′-CACCT TATCAGGGTCCACAG-3′).

2.2.2. A. thaliana transformation

A. thaliana ecotype Columbia specimens were selected for floral dip when a pot of healthy plants contained approximately 20–30 inflorescences and some maturing siliques. Siliques are routinely clipped off in our lab [30]. Floral-dip liquid medium contained 100 g/l sucrose, 1% B5 (200×) basal medium, 2 mg/l 6-BA, 1 M sodium hydroxide, and 200 μl surfactant Silwet L-77, and was prepared as previously described [31]. Plants were inverted and their aerial parts dipped in EHA105-containing floral-dipping medium for 5 min, followed by wrapping in plastic film to maintain a high humidity for 16–24 h. Plastic covers were then removed and plants grown in a growth chamber until drying and harvesting of seeds (T1) with a sample bag.

2.2.3. Selection of transgenic A. thaliana

T1 seeds were grown in sterilized soil until they had grown six leaves. Then, primary transformants were selected using 0.5% glufosinate, spraying once every other day for a total of three times, but their cotyledons became chlorotic and bleached within 3–5 days. Resistant seedlings grew healthy green leaves. The selected lines were confirmed to contain the rhFGF9 sequence by PCR, using genomic DNA as a template and rhFGF9 gene-specific primers. Additionally, many homozygous T3 seeds were obtained by further reproduction. These T3 seed lines were used for protein expression analysis and activity assays.

2.2.4. Oil bodies purification

Wild-type and T3 transgenic *A. thaliana* seeds (20 mg) were fully ground in 1.5 ml centrifuge tubes with apestle in 50 μl pre-cooled sodium phosphate buffer (PBS, pH 7.5) [32]. Then, the mixtures were spun at 12,000*g* and 4 °C for 20 min to obtain the floating oil bodies fraction. Oil bodies were washed twice with 50 μl PBS and spun as before. Precipitated material and buffer under the oil bodies was then removed, the oil bodies fraction was collected, the volume was 80 μl and stored at 4 °C until further use.

2.2.5. Protein analysis of oleosin–rhFGF9

Thereafter, 20 μl loading buffer (250 mM Tris–HCl (pH 6.8), 10% (W/V) sodium dodecyl sulfate (SDS), 0.5% (W/V) bromophenol blue, 50% (V/V) glycerol, 5% (V/V) β-mercaptoethanol) and 80 μl PBS was added to the oil bodies, mixed and boiled for 10 min, and then the mixtures were analyzed via 10% SDS–PAGE (10 μl sample per lane). Gels were stained with coomassie blue overnight and destained using coomassie blue destainer. Proteins on additional gels were transferred to polyvinylidene difluoride