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Research paper

Expression of biologically recombinant human acidic fibroblast growth factor in *Arabidopsis thaliana* seeds via oleosin fusion technology

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ABSTRACT

The potential of oleosins to act as carriers for recombinant foreign proteins in plant cells has been established. Using the oleosin fusion technology, the protein can be targeted to oil bodies in oilseeds by fusing it to the N- or C-terminus of oleosin. In this study, aFGF was expressed in *Arabidopsis thaliana* seeds via oleosin fusion technology. A plant-preferred *aFGF* gene was synthesized by optimizing codon usage and was fused to the C-terminus of the *A. thaliana* 18.5 kDa oleosin gene. The fusion gene was driven by the phaseolin promoter to confer seed-specific expression of the human acidic fibroblast growth factor in *A. thaliana*. The T-DNA region of the recombinant plasmid pKO-aFGF was introduced into the genome of *Arabidopsis thaliana* by the floral dip method. The aFGF protein expression was confirmed by SDS-PAGE and western blotting. The biological activity showed that oil bodies fused to aFGF stimulated NIH/3T3 cell proliferation activity.

1. Introduction

Human acid fibroblast growth factor (aFGF), also called FGF-1, the physiological form of which contains 154 amino acids (Zazo et al., 1992) is a heparin binding protein. It was involved in a variety of biological processes, including angiogenesis, cell proliferation, and differentiation (Kenneth and Guillermo, 1986; Jose Feito et al., 2011). The aFGF protein is an important member of the growth factor families. The aFGF were originally identified as peptides with mitogenic activity for fibroblasts. The aFGF has regenerative capabilities when administered after focal cerebral ischemia in different species (Mitani et al., 1992; Sasaki et al., 1992), so it may have broad prospects for the treatment of acute focal cerebral ischemia (Jose Feito et al., 2011). The aFGF also has fortissimo tissue-injury repair properties that might be relevant for medical applications.

Disputes about the safety of using aFGF in the human body have badly slowed down its therapeutic development. Except for the topical application of aFGF to treat burn wounds in China, no other pharmacological application of FGF has been approved (Wu et al., 2005). Lozano et al. (2000) showed that a shortened form of aFGF including amino acids 28–154 of the full-length sequence plus Met substituted for Leu27 (aFGF27–154) did not induce cell division but retained the vasoactive, neuromodulatory, and cardio- and neuro-protective characters of the full-length aFGF (Xiao ping Wu et al., 2005). In our laboratory, a shortened form of aFGF including amino acids 20–154 of the full-length sequence was high-level expressed and purified in *Escherichia coli* and the expression level was up to 150 mg/L. The biological activity showed that a shortened form of aFGF including amino acids 20–154 has no significant differences. Three groups of test results showed that the degradation problem of N-terminus was solved and dimer formation is decreased obviously. So we chose a shortened form of aFGF including amino acids 20–154. We modified the N-terminus of the aFGF gene by eliminating residues 1–19, which weakened its mitogenic properties and preserved its non-mitogenic properties.

The need for cheap production of a mass of proteins has led to a new industry for producing recombinant proteins in transgenic plants. The potential of molecular pharming, using transgenic plants as bioreactors can produce therapeutic proteins. Plants as vehicle provide an attractive expression system for many proteins (Richter et al., 2000; Petolino et al., 2000; Tacket et al., 1998). The expression of therapeutic proteins in plant has a safe and low-cost advantage, and process post-translational modifications. However, the expression level and stability of recombinant proteins in plants especially in plant seeds are influenced by several factors for example the cis-regulatory elements, mRNA stability

Abbreviations: AtOle, *Arabidopsis thaliana* oleosin gene; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pKO, The pCAMBIA1301 plasmid as a basic skeleton for transformation, containing a *Phaseolus vulgaris* seed-specific promoter, *Arabidopsis thaliana* oleosin, 35S promoter, Basta gene and nos terminator; pKO-aFGF, The construct encoding the aFGF protein.

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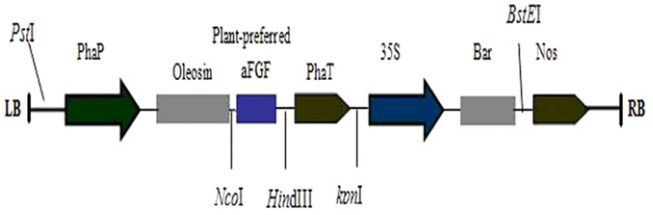

Fig. 1. Schematic map of recombinant plasmid pKO-aFGF. The T-DNA region of pCAMBIA-1301 was replaced by *pst*I and *Bst*EII between T-Border (LB) and Nos. The 35S-bar gene was inserted into the T-DNA region by *pst*I and *Bst*EI. The oleosin gene was fused with the phaseolin promoter, then fusion gene was inserted into T-DNA region by *Pst*I and *Nco*I. The aFGF gene was linked to the 3′-end of oleosin gene in T-DNA region by *Nco*I and *Hin*dIII. The recombinant plasmid was named pKO-aFGF. The T-DNA of the pKO-aFGF vector included a phaseolin promoter/terminator, an *A. thaliana* oleosin gene, *aFGF* gene, the 35S promoter, the *bar* gene and nos gene terminator. PhaP: phaseolin promoter, Oleasin: *A. thaliana* oleosin gene, aFGF: acidic fibroblast growth factor, PhaT: phaseolin terminator, 35S: CaMV35S promoter, Nos: Nopaline synthase terminator gene.

and final site of protein accumulation in plant cells or tissues (Doran, 2006). Oil bodies (OBs) are lipid-storage organelles in plant seeds that provide energy for seedlings during germination (Huang, 1996). OBs are composed of a TAG core surrounded by a phospholipid (PL) monolayer that contains oleosins, caleosins and steroleosins including several that stabilize the organelle. OBs can be easily separated from the cell components in seeds by flotation centrifugation and contain relatively few different proteins (Van Rooijen and Moloney, 1995). When used to recover oil body-associated recombinant proteins, this process greatly enriches for the target protein and can reduce purification steps.

Because of the oil body's peculiar structure, a novel technology named oil body fusion technology has been developed in which heterologous proteins are fused to the N- or C-terminus of oleosin in oil body surface and expressed under the control of an oleosin gene promoter or seed-specific promoter (Stoger et al., 2005; Boothe et al., 2010). It is one of the most popular methods because it can extend protein half-life, and allows easy transportation and storage (Bhatla et al., 2010). There have been many reports of oleosin-fusion technology being used with heterologous proteins. Recombinant human precursor insulin fused with oleosin has been expressed in *Arabidopsis* and shown biological activity (Nykiforuk, 2006). Human epidermal growth factor (hEGF) fused with oleosin has also been expressed in *Arabidopsis* and an accumulation level of 0.12% was achieved (Moloney et al., 2006; Bhatla et al., 2010). The fusion protein Oleosin-ApoA1 has been expressed in *Arabidopsis* and *Carthamus tinctorius* L. and reached commercial production standards (Moloney et al., 2004; Bhatla et al., 2010). Many heterologous proteins have been expressed in transgenic plants since the successful production of a mouse monoclonal antibody in plants (Twyman et al., 2003; Jung et al., 2010). Here, we expressed aFGF in the oil body of *Arabidopsis* through the construction of the plant expression vector pKO-aFGF. The expression of the oleosin-aFGF fusion protein was checked by SDS-PAGE and the antigenicity was detected by western blot. The aFGF protein expressed in transgenic *A. thaliana* seeds stimulated NIH/3T3 cell proliferation activity.

2. Materials and methods

2.1. Materials

Plasmids and strains: The plasmid pKO for transformation was constructed using the pCAMBIA1301 plasmid as a basic skeleton, and contained the phaseolin promoter/terminator (Patent PCT/US01/47495), the *A. thaliana* oleosin gene (*AtOLE*, GenBank accession no. X62353.1) 35S promoter (GenBank accession no. AF21 8816.1), *bar* gene (GenBank accession AF218816.1) and terminator of nos (GenBank accession AF234307), *aFGF* gene (GenBank accession no. BC03 2697.1).

E. coli DH5α and *Agrobacterium tumefaciens* EHA105 were obtained from the laboratory of the Engineering Research Center of Bioreactor and Pharmaceutical Development, Ministry of Education, Jilin Agricultural University, China.

Test material: Mature seeds of *A. thaliana*.

Enzymes and reagents: The restriction enzymes *Nco*I and *Hin*dIII, *pfu*DNA polymerase and T4 DNA ligase were purchased from Takara (Dalian, China). All primers were synthesized and sequenced by Sangon Bioengineering Co., Ltd. (Shanghai, China). Kanamycin (Kan), streptomycin (Str) and rifampicin (Rif) were purchased from Sigma (Hong Kong, China). Glufosinate was purchased from the Boehringer Mannheim Corporation (Mannheim, Germany). Rabbit anti-aFGF polyclonal antibody was purchased from Abcam, Inc. (Cambridge, MA, USA). Isopropyl-β-D-thiogalactoside (IPTG) and methylthiazol tetrazolium (MTT) were obtained from Gold Biotechnology (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Construction of the pKO-aFGF vector

The recombinant plasmid skeleton was derived from pCAMBIA1301 plasmid vector. The T-DNA region of pCAMBIA1301 was replaced by *pst*I and *Bst*EII between T-Border (LB) and Nos. The *35S-bar* gene was amplified by PCR from pEGAD plasmid using the forward primer with *Pst*I, *Nco*I, *Hin*dIII, *kpn*I (CCC*TGCA GCCATGGTCTAGAGGTACC*ATCCGTCAACAT GGTGG) and the reverse primer with *Bst*EII (G*GGTTACC*TCAGATCTCG GTGACGGGC). The 35S-bar gene was inserted into pCAMBIA1301-

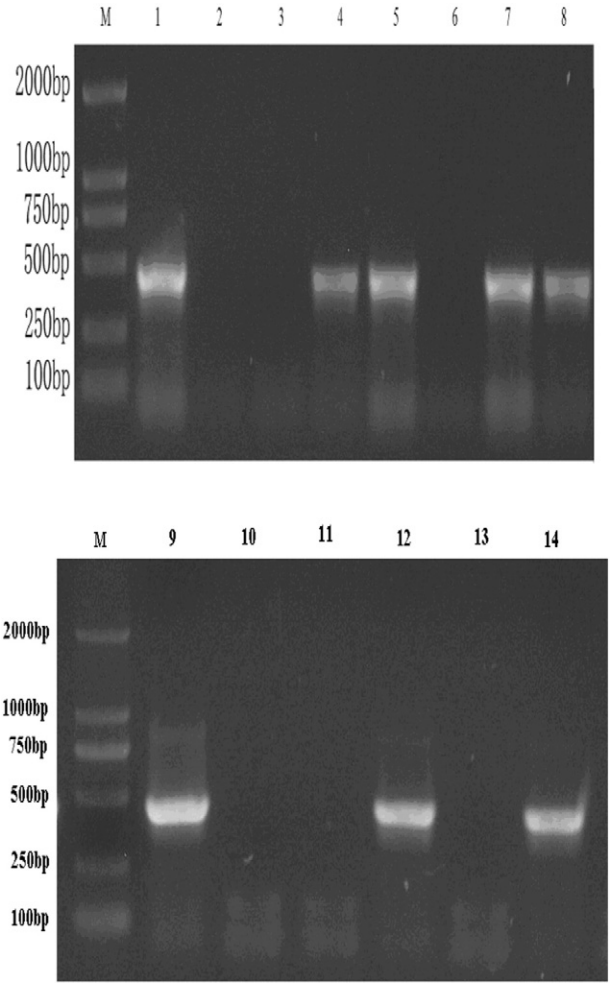

Fig. 2. Agarose gel for screening positive transgenic lines by RT-PCR amplification. Lane1 and lane9: positive control (aFGF plasmid); lane2–lane8: the transgenic *A. thaliana* T3–1,T3–2,T3–3, T3–4, T3–5, T3–6 and T3–7; lane10–lane12: the transgenic *A. thaliana* T3–8,T3–9 and T3–10; lane13: the wild-type *A. thaliana*; lane14: T3–11.

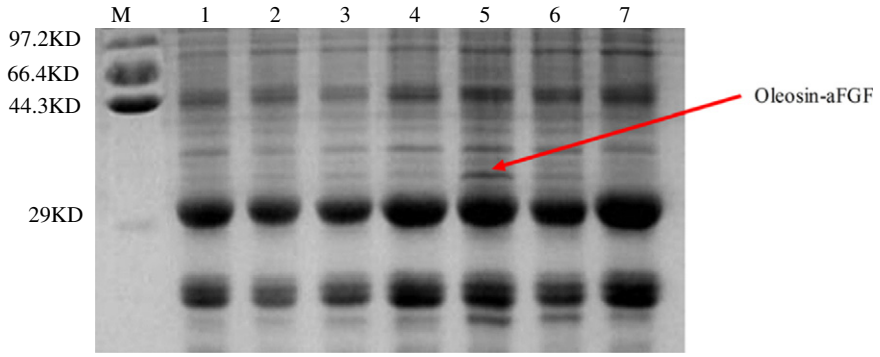

Fig. 3. Identification of SDS-PAGE of crude oil bodies in transgenic *Arabidopsis thaliana*. M: Protein marker; lane1–lane6: the crude oil body from T3 transgenic *A. thaliana* T3–3, T3–4, T3–6, T3–7, T3–10 and T3–11; lane7: the crude oil body from wild-type (WT) *A. thaliana*.

revised by *pst*I and *Bst*EI and resulted in pCAMBIA1301-35 S-bar. The phaseolin promoter was amplified by PCR from *Phaseolus vulgaris* genome (the forward primer: CG*CTGCAG*GAATTCATTGTACTCCCAG, the reverse primer: CCTCTAGCTGTATCCGCCATAGTAGAGTAGTATTGAATAT GAG). The RNA from *Arabidopsis thaliana* seeds was extracted and reversed transcription into cDNA. The *A. thaliana* oleosin gene was amplified by PCR from *A. thaliana* cDNA (the forward primer: CTCATATTCAAT ACTACTCTACTATGGCGGATACAGCTAGA GGAAC, the reverse primer: CATG*CCATGG*TAGTAGTGTGCTGGCCACCACG). The *A. thaliana* oleosin gene was fused with the phaseolin promoter by fusion PCR method (the forward primer: CG*CTGCAG*GAATTCATTGTACTCCCAG,the reverse primer: CATG*CCATGG*TAGTAGTGTGCTGGCCACCACG). The fusion gene was inserted into pCAMBIA1301-35S-bar vector by *pst*I and *Nco*I, named pCAMBIA 1301-F-B. The phaseolin terminator was amplified by PCR from *Phaseolus vulgaris* genome. It was inserted into pCAMBIA1301-F-B vector by *Hin*dIII and *kpn*I, named pKO vector (Fig. 1).

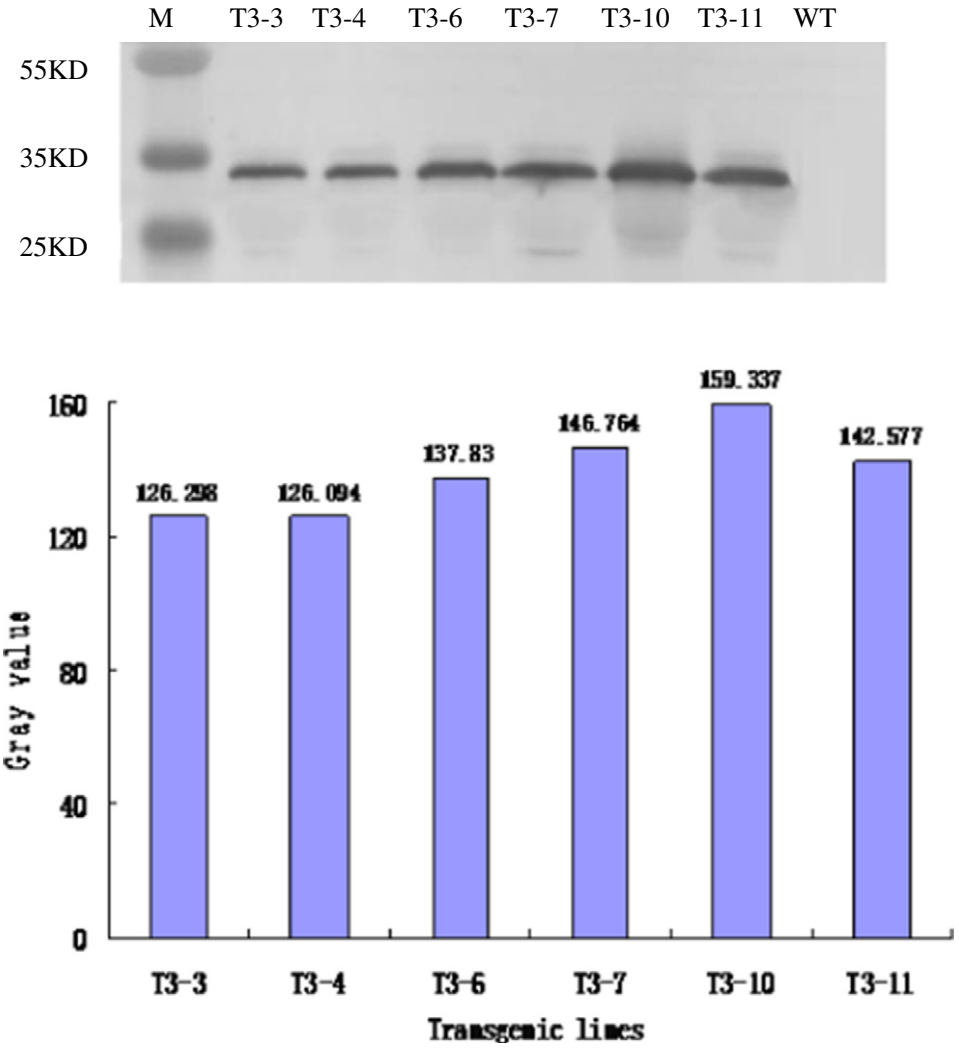

Fig. 4. Detection on aFGF protein of the transgenic *Arabidopsis thaliana* by western blot and the gray value analysis of lane1–lane6: the crude oil body from T3 transgenic *A. thaliana* T3–3, T3–4, T3–6, T3–7, T3–10, and T3–11; lane7: the crude oil body from wild-type (WT) *A. thaliana*.

The *aFGF* gene was transformed with plant-preferred codons. Then, the pUC57-aFGF vector with unique *Nco*I and *Hin*dIII sites was synthesized by Sangon Biotech. The pKO plasmid was extracted from *E. coli* and double restriction enzyme digested with *Nco*I and *Hin*dIII. The aFGF gene was inserted into the pKO plasmid. The new recombinant plasmid named pKO-aFGF was transformed into *E. coli* DH5α competent cells. The pKO-aFGF recombinant plasmid was extracted from *E. coli* DH5α and tested by PCR and digestion. Then, it was transformed into *Agrobacterium* EHA105 competent cells using the freeze–thaw method (Höfgen and Willmitzer, 1988). Positive colonies were identified by PCR with aFGF-specific primers: F1 (5 -ATGGCTAACTACAAGAAG-3 ; forward) and R1 (5 -TTAATCAGAAGAA ACTGGCAA T-3 ; reverse). ExTaq DNA polymerase (Takara Bio, Dalian, China) was used in the PCR reaction system. A total of 30 cycles were performed with a denaturation step at 94 °C for 30 s, an annealing step at 60 °C for 45 s and an elongation step at 72 °C for 90 s.

2.3. Transformation into A. thaliana

A. thaliana Columbia ecotype (4–5 weeks old, containing numerous unopened floral buds) was chosen as the transformation host. Preparation of liquid medium containing *A. tumefaciens*, 5% sucrose, 0.1% B5, 0.001% 6-BA, 0.009% 1 M sodium hydroxide and 400 μL/L of the surfactant Silwet L-77 was performed according to the *Arabidopsis* test manual (Weigel and Glazebook, 2004). Unopened *A. thaliana* floral buds were infiltrated for 7 min, and the plants were laid flat under a plastic dome and cultured in the dark for 24–48 h after inoculation. Then, the infected *A. thaliana* plants were transferred to normal lighting conditions and grown until seeds were collected (T1).

2.4. Selection of transgenic A. thaliana

The T1 transgenic seeds were stratificated at 4 °C for 2 days, Primary transformant (T1) seeds were screened on 1/2 Murashige and Skoog (MS) basal plates supplemented with 11 mg/mL Basta. The independent Basta-resistant lines were transferred to soil after screening on culture medium and were further screened in the soil [vermiculite: soil (V:V) 7:3] with 1% Basta. *A. thaliana* plants were further grown until collected T2 seeds. The T2 seeds were screened by Basta and then collected T3 seeds. The seeds from independent homozygous T3 lines were used for protein expression analysis and activity assay.

2.5. Oil body purification

Oil bodies were purified using the method described by Tzen et al. (1997). Twenty milligrams of seeds were ground in 200 μL sodium phosphate buffer (pH 7.5). The suspension was spun at 10,000 × g and 4 °C for 30 min. The floating oil body fraction was collected, resuspended in 200 μL sodium phosphate buffer (pH 7.5), and spun as before. After centrifugation and removal of the upper buffer, the oil body fraction was collected and stored at 4 °C until further use.

2.6. Analysis of aFGF protein linked to oil body

Twenty milligrams of seeds were ground in 200 μL sodium phosphate buffer (pH 7.5). The loading quantity of sample was 10 μL crude oil body and it was separated by 12% acrylamide in SDS-PAGE. The proteins were transferred onto Immobilon-P polyvinylidene difluoride (PVDF) membranes (PerkinElmer, Boston, MA, USA) using a SEMI-DRY Transfer Cell (Bio-Rad Laboratories, Hercules, CA, USA), and immunoblotted with a Rabbit anti-aFGF polyclonal antibody (Abcam) according to the manufacturer's protocol. Immunoreactive bands were checked with western blotting luminal reagents.

2.7. Activity assay of aFGF

To verify that the aFGF produced and purified from *A. thaliana* had a stimulatory effect on the proliferation of NIH/3T3 cells, an MTT assay was performed. NIH/3T3 cells were seeded in flat-bottomed, 96-well plates at an initial density of 5×10^4 cells per mL (100 μL per well)

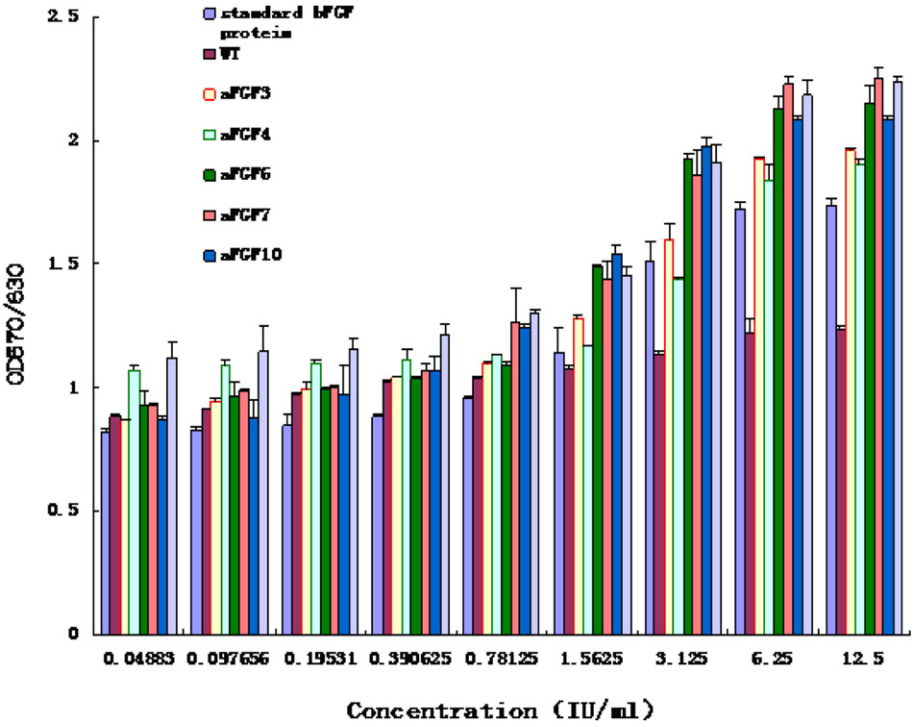

Fig. 5. The activities assay of aFGF protein in the transgenic *Arabidopsis thaliana* seeds. Dose–response curves for proliferation activity. Increase of NIH/3T3 fibroblast cells were expressed as the percentage increases in absorbance (570/630 nm). The test sample included the standard bFGF protein (◆), oil body from wild-type *A. thaliana* (■), aFGF3 (■), aFGF4 (▲), aFGF6 (✱), aFGF7 (●), aFGF10 (+), aFGF11 (–).

and cultured in DMEM low-sugar medium for 24 h at 37 °C. After 3 days of incubation with aFGF, 20 μL MTT solution was added to each well. The cells were then incubated for a further 4 h at 37 °C, the culture medium included the MTT solution in the wells was removed, and 150 μL DMSO was added to each well and mixed thoroughly to dissolve the crystals. The plates were read at 570/630 nm in a Microplate Reader model 450 to obtain absorbance values.

3. Results and discussion

3.1. Construction of a plant oil body expression vector

The T-DNA region of the pKO plasmid contained a phaseolin promoter, oleosin gene, phaseolin terminator, 35S promoter, bar gene and nos gene. The phaseolin promoter controls tissue-specific transgene expression during seed development. For the pKO-aFGF plasmid, we constructed an *aFGF* gene expression cassette (Fig. 1) containing the *aFGF* gene under the control of the phaseolin seed-specific promoter (phaP)/terminator (phaT). This construct enabled specific expression in *A. thaliana* seeds. The oleosin gene was as signal peptide to carry aFGF anchoring in oil body surface.

3.2. RT-PCR detection of recombinant aFGF transcripts in transgenic Arabidopsis

The pKO-aFGF plasmid was transformed into *A. thaliana* by a floral dip method. Primary transformant seeds were screened on 1/2 MS basal plates supplemented with 11 mg/mL Basta. The independent Basta-resistant lines were transferred into soil to continue to screen and were further confirmed using the *aFGF* cDNA as a template. The aFGF transcripts were detected by RT-PCR. Of the eleven analyzed transgenic plants, six exhibited clear bands. A specific 423 bp PCR product was generated. The six lines showed the target bands among twelve independent Basta-resistant lines and the positions of the target bands were expected at 423 bp (Fig. 2). Therefore, the aFGF proteins were expressed at the transcriptional level.

3.3. Expression of recombinant aFGF protein in transgenic A. thaliana

To evaluate the accumulation of the oleosin-aFGF fusion protein in pKO-aFGF transformants, the crude oil body was extracted and oil body-associated proteins of transgenic (T3–3, T3–4, T3–6, T3–7, T3–10, T3–11) and wild-type *A. thaliana* plants were analyzed by SDS-PAGE and western blotting. The aFGF gene was predicted to encode 135 amino acids. Determination of the crude oil body extracts from the pKO-aFGF-transformed plants showed an immunoreactive protein with a molecular weight of 33.5 kD, which is identical size to oleosin-aFGF fusion protein, suggesting that the oleosin precursor was properly assembled in the transgenic *A. thaliana* oil body. The six pKO-aFGF-transformed lines also exhibited positive signals in seeds.

However, the accumulation of oleosin-aFGF fusion protein in the oil body of transgenic lines was not accurately quantified, because ELISA method was not suited for testing fusion protein in the oil body. So the fusion protein was roughly quantified by western blot method. There was no band at this position in the wild-type seeds (Figs. 3 and 4). Then there were more oil body-associates observed in transgenic lines #7, 10, and 11 and WT than the others. Furthermore, these differences may have come from the purification efficiency during oil body preparation.

3.4. NIH/3T3 activity analysis of plant oil bodies containing the aFGF protein

The NIH/3T3 cell line is generally used for evaluation of the biological activity of aFGF. To evaluate the biological activity of different transgenic lines (T3–3, T3–4, T3–6, T3–7, T3–10, T3–11), NIH/3T3 cells were used to test the stimulation of cell proliferation. As shown in Fig. 5, the standard bFGF concentration was set as a gradient from 0.04883 to 12.5 IU/mL. The results suggested that aFGF proteins from the T3 different transgenic lines had a dose-dependent cell proliferative effect on the NIH/3T3 cells. The oil bodies with aFGF from different lines had stimulation of cell proliferation activity that was higher than the bFGF standard protein.

Oil body-based pharmaceutical formulations include therapeutic, diagnostic and delivery agents. Oil body-based emulsions can be used as adjuvants in pharmaceutical protein base for dermatological products, components for orally administered medicines, etc. In these products oil bodies may also carry an active ingredient to be delivered to host, if needed. Personal care products wherein oil body-based emulsions may be used include various cosmetic and cosmeceutical products such as creams, lotions and makeup products, hair care products, and bath products such as soaps, washes and cleansers (Deckers et al., 2001, 2003). In products like toothpaste, oil bodies may also serve as carriers of components such as flavoring agent, fluoride, silicas, chelating agents, and sweetener. The recombinant proteins expressed in the oil body can be stored for longer periods than those obtained from other sources. GUS enzyme has been reported to remain active for more than one year when expressed as oleosin fusion in seeds (Van Rooijen and Moloney, 1995). Moreover, recombinant seeds can be easily transported. Furthermore, proper refolding of proteins takes place spontaneously when expressed on oil bodies (Nykiforuk et al., 2005). Recombinant proteins can be expressed in the oilseeds themselves, or otherwise the protein can be expressed in any system of choice. It can then be targeted to oil bodies for purification and refolding. So the oil body expression system was the better option than the other different expression systems. The aFGF fused to the C-terminus of oleosin and expressed under the control of a seed-specific promoter. Thus, expensive steps needed for purification and refolding of proteins are not required. The expression of aFGF with plant organelle signaling sequences need to purify aFGF protein, thus the purification cost of products should be increased. The oil body linked to aFGF was applied in many fields. Many researches were done in SemBioSys Genetics Inc. The oil body was suitable for expressing FGF protein.

4. Conclusions

Here, an *aFGF* gene was expressed by *A. thaliana* and accumulated in the transgenic seeds. We successfully constructed the plant binary expression vector pKO-aFGF and T3 transgenic homozygotes were obtained. The oil body-associated proteins were analyzed by SDS-PAGE and western blotting, and the results showed that the T3–3 and T3–4 transgenic plants expressed the recombinant protein at relatively low levels, while the T3–10 line expressed it at relatively high levels. The results of NIH/3T3 cell analysis demonstrated that the activity of aFGF from the transgenic *A. thaliana* seeds was higher than that of bFGF standard protein. This is the first trial of aFGF production in an oil body expression system, and the recombinant protein exhibited typical activity. This result might be useful for the mass production of aFGF. We have confirmed that the oil body expression system can be used effectively for the production of aFGF.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2015.04.036.

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