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An oleosin-fusion protein driven by the CaMV35S promoter is accumulated in *Arabidopsis* (Brassicaceae) seeds and correctly targeted to oil bodies

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ABSTRACT. Oleosin-fusion technology is used to express desired proteins. It was developed based on the properties of oleosin; the heterologous protein gene is fused to the *oleosin* gene and the fusion gene is driven by a seed-specific promoter. We replaced the seed specific promoter with the CaMV35S promoter to dive a *gfp-oleosin* fusion gene in transformed *Arabidopsis*. The heterologous oleosin-fusion protein was mainly accumulated in the transgenic *Arabidopsis* seeds and correctly targeted to oil bodies. This provides an alternate choice of promoter in oleosin-fusion technology.

Key words: *Arabidopsis*; Oil body; Oleosin

INTRODUCTION

Oleosins are a kind of structure protein embedded on the surface of oil bodies in plant cells. Oil bodies are a type of discrete storage organelle consisting of a hydrophobic triacylglycerol core, a half-unit phospholipid layer, and oil body-related proteins such as oleosin and caleosin (Huang, 1992). Oleosins are plant-specific proteins, and some oleosin genes from various plant species have been cloned (Keddie et al., 1992; Zou et al., 1996; Chen et al., 1997). The amino acid sequences of oleosins from all plant species show high similarity, and all oleosins have three domains. A highly conserved hydrophobic central domain consists of approximately 72 residues flanked by less-conserved amphipathic N- and C-terminal domains (Huang et al., 2009). The central domain consists of two anti-parallel β-strands and a proline knot motif, which is essential for targeting to oil bodies (Abell et al., 1997), where the central part forms a hairpin-like structure and is inserted into the triacylglycerol matrix of oil bodies (Abell et al., 2002; Alexander et al., 2002; Van Rooijen and Moloney, 1995a). The less-conserved N- and C-terminal domains consist of an α-helix and a change in residues or extra ones in these areas has less effect on the targeting of oleosin to oil bodies (Van Rooijen and Moloney, 1995b).

Endogenous *oleosin* genes in various plants are driven by the *oleosin* gene promoter, which directs seed-specific expression of oleosin at early stages of seed development and throughout seed maturation (Plant et al., 1994). Due to the specific sequences in oleosin, it has several particular properties: high accumulation in plant seeds, targeting to oil bodies and the tolerance to residue change at N- and C-terminal ends. These features make oleosin an ideal vehicle for recombinant protein expression in plant-based systems. A novel technology called oleosin-fusion technology has been developed recently. According to the strategy of oleosin-fusion technology, heterologous proteins are fused to the N- or C-terminal end of oleosin, and the recombinant protein production is under the control of an *oleosin* gene promoter or other seed-specific promoter (Stoger et al., 2005; Boothe et al., 2010).

The aim of this study was to determine if a constitutive promoter can also be used in this technology. The green fluorescent protein (GFP) gene was fused to the 5'-end of an *Arabidopsis thaliana* 18.5 kDa oleosin gene, according to the method of oleosin-fusion technology, and the fusion gene driven by the widely used constitutive promoter, the cauliflower mosaic virus (CaMV) 35S promoter, was introduced into the model plant *A. thaliana*. Our results showed that the GFP-oleosin fusion protein driven by the CaMV35S promoter was also highly accumulated in *Arabidopsis* seeds and correctly targeted to the oil bodies. This reveals that oleosin has a more important role than the promoter during the process when oleosin-fusion proteins accumulate in seeds and target to oil bodies.

MATERIAL AND METHODS

Cloning of the *A. thaliana* 18.5-kDa oleosin gene

A. thaliana seedlings were grown at 22°C in a controlled-growth chamber (16-h light/8-h dark cycle). Total RNA was extracted from mature green-stage seeds using an RNAprep Pure Plant kit with on-column DNase digestion (Tiangen, Beijing, China) according to manufacturer instructions. The first-strand cDNA was synthesized using an AMV First-Strand cDNA Synthesis kit (Sangon Biotech, Shanghai, China), according to manu-

facturer instructions, in which oligo(dT) primer was used and reverse transcription was performed at 50°C for 30 min followed by 94°C for 2 min.

A pair of primers, AtOleF2 (5'-GCTAGCCGGATACAGCTAGAG-3', underlined is the *Nhe*I site) and AtOleR2 (5'-GGTCACCTTAAGTAGTGTGCTGGCCA-3', underlined is the *Bst*EII site), designed according to the published sequence of *A. thaliana* 18.5-kDa *oleosin* gene (GenBank accession No. X62353), was synthesized by Sangon Biotech. By using the primers and the cDNA mentioned above as a template, PCR was performed using the *pfu* DNA polymerase (Sangon Biotech) according to the following protocol: 94°C for 2 min, followed by 32 cycles of amplification (94°C for 30 s, 58°C for 30 s, 68°C for 50 s) on a thermal cycler (Applied Biosystems, USA). The PCR amplifications were extended for 8 min at 72°C after the final cycle with the addition of 1 µL *rTaq* DNA polymerase (Takara Bio, Dalian, China) for an extension of "A" at the 3'-ends (for TA-cloning). The PCR products were analyzed on a 1% agarose gel, and the bands at corresponding sizes were purified using the EZ Spin Column DNA Gel Extraction kit (Sangon Biotech) and subsequently cloned into pMD18-T vector (Takara Bio) followed by sequencing. Sequencing verified the plasmid, which was designated pMD18-T-AtOle (containing the 530-bp *oleosin* gene fragment).

Construction of p1302-GFP-AtOle plant expression vector

The plant transformation binary vector pCAMBIA1302 (Cambia, Australia) was chosen as a starting vector. There are 2 expression cassettes in the T-DNA region of pCAMBIA1302. One is the selection marker, a hygromycin-resistant gene driven by a CaMV35S promoter and terminated by a 35S-polyA terminator for transformation selection. Another is the GFP expression cassette containing an mGFP5-ER variant driven by a CaMV35S promoter and terminated by a nopaline synthase (NOS) terminator.

After cutting with *Nhe*I and *Bst*EII, a 539-bp *A. thaliana oleosin* gene fragment was released from the pMD18-T-AtOle plasmid, and the fragment was cloned into pCAMBIA1302 predigested with *Nhe*I and *Bst*EII, generating the plasmid p1302-GFP-AtOle. In the vector, the *A. thaliana oleosin* gene was fused to the 3'-end of the *gfp* gene in the vector, forming a 1251-bp *fusion* gene, which was driven by a CaMV35S promoter and terminated by an NOS terminator.

Arabidopsis growth and transformation

A. thaliana, ecotype Columbia (Col-0), was chosen as the transformation host. Seeds were surface-sterilized with 70% ethanol and then sown onto half-strength Murashige-Skoog (MS) (Murashige and Skoog, 1962) medium (Sigma-Aldrich, Inc., USA) plate containing 1% (w/v) agar and 15% (w/v) sucrose. The plates containing seeds were incubated at 4°C for 3 days to break seed dormancy and were then germinated at 22°C under a 16-h light/8-h dark cycle at 100 $\mu mol \cdot s^{-1} \cdot m^{-2}$ of white light produced by cool-white fluorescent lamps. Seven days after germination, the seedlings were transferred to soil for subsequent growth under the same growth conditions.

Vector p1302-GFP-AtOle with the *gfp-oleosin* fused gene or the vector p1302 alone (as a control) was introduced into *Agrobacterium tumefaciens* GV3101 (pMP90) using the freeze-thaw method (Hofgen and Willmitzer, 1988). Transformation of *Arabidopsis* (Col-0) was performed as previously described (Clough and Bent, 1998). Primary transformants (T0) seeds were screened on 15-cm MS basal plates supplemented with 50 µg/mL hygromycin.

Next, 30 to 50 independent hygromycin-resistant lines were transferred to soil and were further confirmed by polymerase chain reaction (PCR) using genomic DNA as template and *gfp-oleosin fusion* gene primers for amplification.

Genomic DNA was isolated from rosette leaves of *A. thaliana* using a CTAB method following the protocol of Allen et al. (2006). The quality and concentration of genomic DNA were measured by agarose gel electrophoresis and spectrophotometer analysis before later steps. The integration of the *gfp-oleosin* gene in the chromosome was detected by PCR using a pair of primers to amplify a 376-bp fragment in the *gfp-oleosin fusion* gene (Primer fusion F1/fusion R1, fusion F1: 5'-gtccacacaatctgcccttt-3', fusion R1: 5'-gacaaggattgggctgaaga-3'. The fusion F1 and fusion R1 were located in the coding sequence region of *gfp* and *oleosin* gene, respectively). In a 200-µL thin-wall PCR tube, the 50 µL reaction mixture contained 1 µL of each PCR primer (10 µM), 1 µL 2.5 mM dNTPs, 5 µL 10X PCR buffer (Mg^{2+} free), 3 µL 1.5 mM $MgCl_2$ and 0.5 U *rTaq* DNA polymerase (Takara) with 200 ng genomic DNA as template. PCR was carried out on a Thermo Hybaid cycler using the following protocol: 94°C for 2 min followed by 33 amplification cycles (94°C for 40 s, 58°C for 40 s, 72°C for 40 s). The amplified samples were analyzed on a 1% agarose gel followed by sequencing verification.

After selection for 3 generations, homozygous T3 lines were screened by PCR, and seeds from three independent homozygous T3 lines (T-5, T-6, T-13) were used for protein expression analysis and GFP analysis. Wild-type (denoted as WT lines) or the control lines transformed using a p1302 vector only (denoted as CK lines) were used as the control.

Analysis of the expression of GFP-oleosin fusion

Total seed protein extraction and expression level measurement of GFP-oleosin fusion were performed as previously described with minor modifications (Nykiforuk et al., 2006), 5 mg wild-type (Col-0) or transgenic *A. thaliana* mature seeds was ground in a centrifuge tube with a pestle in 200 µL 50 mM Tris-HCl, pH 8.0. An aliquot of 50 µL 10% SDS was added to the slurry and mixed by vortex briefly. After boiling for 10 min followed by centrifugation, the supernatant was taken for protein determination and electrophoresis. The total protein content was determined by the BCA (bicinchoninic acid) protein assay using the BCA Protein Assay kit (Pierce, Rockford, IL, USA) according to the manufacturer instruction. For negative controls, total proteins from wild-type Col-0 *Arabidopsis* seeds were prepared in the same manner. Samples containing equal protein content (20 µg) were then mixed with 1/5 volume of 6X SDS sample buffer (0.35 M Tris-HCl, pH 6.8, 30% glycerol, 4% SDS, 0.02% bromophenol blue, 300 mM DTT), boiled for 4 min and subjected to discontinuous 15% SDS-PAGE gels. Gels were subsequently stained with Coomassie blue or transferred to 0.45-µm polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) for Western blotting analysis.

Seed oil bodies were prepared according to Nykiforuk et al. (2006), where transgenic and wild-type seeds (100 mg) were ground in 1000 µL oil body extraction buffer I (0.6 M sucrose, 0.5 M NaCl, 50 mM Tris-HCl, pH 8.0). Samples were centrifuged at 10,000 *g* for 10 min. The fat pad fraction was collected in a clean microfuge tube and resuspended in 500 µL buffer II (0.5 M NaCl, 20 mM Tris-HCl, pH 8.0), followed by centrifugation again at 10,000 *g* for 10 min. The procedure was repeated three times followed by two more washes with buffer III (20 mM Tris-HCl, pH 8.0). The final fat pad was resuspended in 200 µL buffer

III. A 5-µL aliquot (10 µg) was taken for protein measurement, SDS-PAGE analysis, Western blotting, and microscope analysis.

For Western blotting, the membranes were washed twice in TBS buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% (v/v) Tween-20] and then incubated in blocking buffer (3% BSA in TBS buffer) for 1 h at 25°C, followed by incubation with the primary antibody, rabbit anti-GFP polyclonal IgG (dilution, 1:1000), for 1 h combined with two washes in TBST [50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.1% (v/v) Tween-20, 0.1% (v/v) Triton X-100] and one with TBS. Next, the secondary antibody, alkaline phosphatase-conjugated goat anti-rabbit IgG (dilution, 1:2000), was added, followed by incubation for 1 h and two washes with TBST and one with TBS before detection. All antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and antibody detection was performed using Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega, Madison, WI, USA).

Fluorescence microscopy

Mature embryos were prepared using the squeezing and pressing method. Fresh green seeds were harvested at the early mature stage, placed on the surface of a clean glass slide and covered with another slide; after pressing by hand, a drop of PBS was added and a coverslip was placed on top to prevent embryos from drying out. Oil bodies were prepared as mentioned above, and a small drop of oil bodies was placed on the surface of glass slide and coverslipped.

We carried out the fluorescence microscopy studies with an Olympus Model BX51TF Microscope (Olympus Beijing Technology, Beijing, China) equipped for epi-illumination using an HBO 200 high-pressure mercury light source, and with 10- and 25-X plan neofluar objective lenses. GFP was viewed using a 360-400-nm bandpass excitation filter and a 510-nm center wavelength chromatic beam splitter. Images were acquired with the same exposure settings and were rescaled according to a standard offset and gain using the GNU Image Manipulation Program.

Oil bodies were confirmed by staining using Nile red according to Greenspan et al. (1985) (data not shown). Stock solutions of Nile red (100 µg/mL) in acetone were prepared and stored in the dark. Isolated oil bodies were suspended in PBS and mixed with Nile red stock solutions at a ratio of 100:1. After incubation at room temperature for 5 min, a small drop of oil bodies with Nile red was placed on the surface of a clean glass slide and coverslipped. Nile red fluorescence was viewed using a 450-500-nm bandpass excitation filter and a 510-nm center wavelength chromatic beam splitter.

RESULTS

Cloning of the *A. thaliana* 18.5-kDa oleosin and vector construction

The construction used for *A. thaliana* transformation encoded a fusion protein comprising *gfp* fused to the 5'-end of the *A. thaliana* 18.5-kDa oleosin. The expression of the GFP-AtOle *fusion* gene was under the control of the CaMV35S promoter and the NOS polyA terminator. For positive transgenic plant selection, a hygromycin-resistant gene under the control of another CaMV35S promoter and 35S polyA terminator was established within the T-DNA. The core structure of the construction is shown in Figure 1.

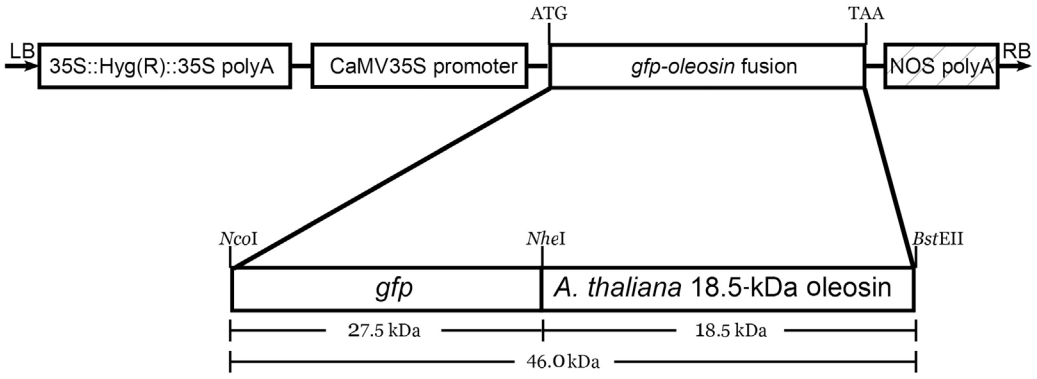

Figure 1. Construction of plant expression vector p1302-GFP-AtOle. The *Arabidops thaliana* 18.5-kDa *oleosin* gene was fused to the 3'-end of the *gfp* gene in the original vector pCAMBIA1302, generating a 1251-bp fusion gene. The predicted molecular mass of the fusion protein expressed was 46.0 kDa (27.5-kDa GFP and 18.5-kDa oleosin). CaMV = cauliflower mosaic virus; NOS = nopaline synthase.

GFP-AtOle expression analysis

Arabidopsis was transformed with the p1302-GFP-AtOle binary construct as mentioned above. The positive T0 seeds were selected on the basis of resistance to the antibiotic hygromycin and the integration of the *fusion* gene (a pair of detection primers were used, the forward primer is located in the coding sequence area of the *gfp* gene and the reverse primer is located in the coding sequence area of the *oleosin* gene) was further confirmed by PCR. Positive transformation lines were grown to the third generation (T3) when homozygotes were obtained. Three independent T3 lines (T-5, T-6, T-13) were selected for further analysis.

To evaluate the accumulation of the GFP-oleosin fusion protein, total proteins from leaves, total seed proteins or seed oil body proteins of transgenic and wild-type plants were analyzed by SDS-PAGE and Western blotting using anti-GFP antibodies. No significant bands were found in both transgenic and wild-type plants when total protein from leaves was analyzed. When total seed protein or purified oil body protein was analyzed by Western blotting using anti-GFP antibody as the primary antibody, a band appeared indicating the presence of the GFP-AtOle fusion protein at the predicted size of 46.0 kDa from transgenic seeds. No band at this position was observed in either total seed protein or oil body protein from wild-type seeds (Figure 2).

GFP-oleosin fusion was correctly targeted to oil bodies

Targeting of GFP fusion protein was analyzed using fluorescence microscopy, and the images showed that oil bodies from transgenic plant seeds exhibited bright green fluorescence while oil bodies from wild-type plant seeds were invisible under the same exposure settings (Figure 3). During the development of *Arabidopsis* seeds, endogenous oleosin starts to accumulate in the late heart embryo stage (Kroj et al., 2003). When checking the GFP expression at embryo, it was found that the GFP-oleosin fusion protein driven by the 35S promoter was also accumulated at this stage. Embryos from transgenic plants appeared bright green while embryos from wild-type plants appeared red, which was due to the autofluorescence of chlorophyll (Figure 4).

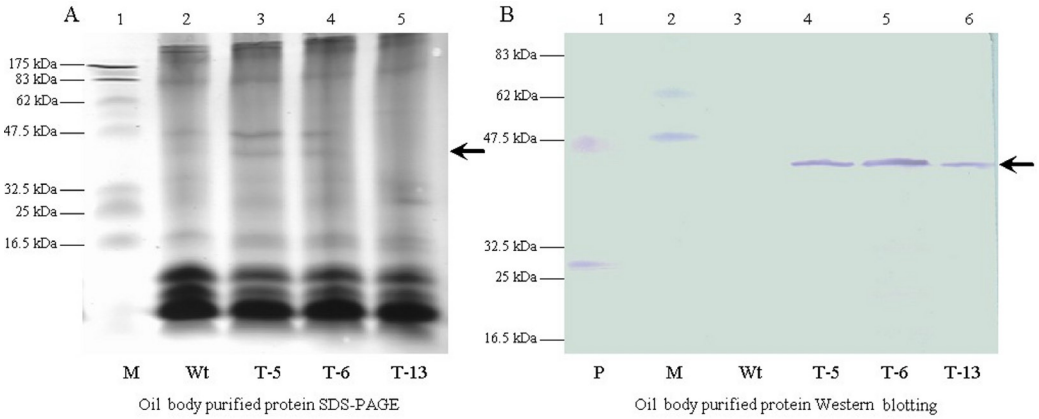

Figure 2. Expression of GFP-oleosin fusion protein in transgenic *Arabidopsis* T3 seeds. Oil body proteins from three transgenic *A. thaliana* lanes (*T-5*, *T-6* and *T-13*) and wild-type *A. thaliana* were analyzed on a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and by Western blotting. **A.** Coomassie-stained SDS-PAGE. Arrows (A and B) indicate the 46.0-kDa fusion protein. **B.** Duplicate gels of A transferred to PVDF membrane for Western blotting using antibodies against GFP as the primary antibody. *Lane Wt* = wild-type *A. thaliana* seeds. *Lane P* = native 27.5-kDa GFP (Sangon Biotech, Shanghai, China) used as a control.

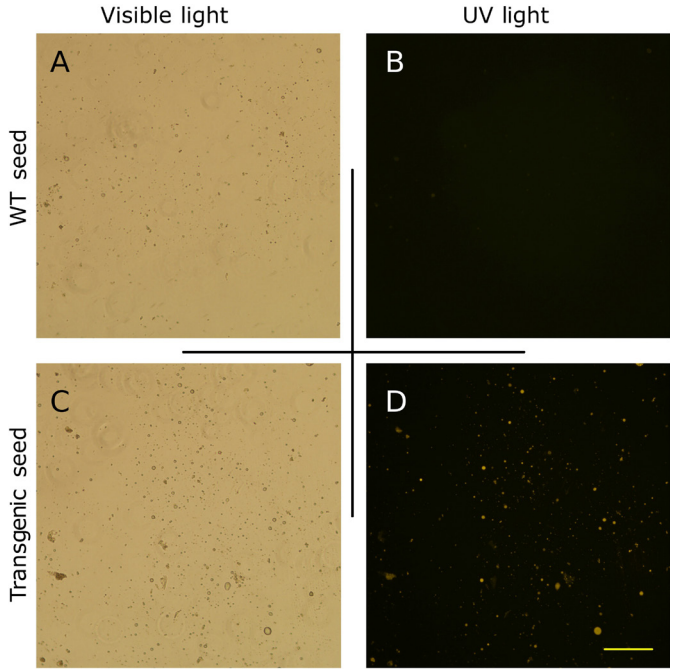

Figure 3. Fluorescence microscopy image of *Arabidopsis* oil bodies. **A.** Oil bodies extracted from wild-type (WT) plant seeds. Oil bodies are liquid drop-like spheres under visible light, which exhibit bright red fluorescence when stained with Nile red (data not shown). **B.** Same field under excitation light for GFP. Oil bodies are invisible. **C.** Oil bodies from transgenic plant seeds under visible light. There is no significant difference with wild-type oil bodies. **D.** Same field under excitation light for GFP. Oil bodies show a bright green fluorescence. Bar = 5 µm.

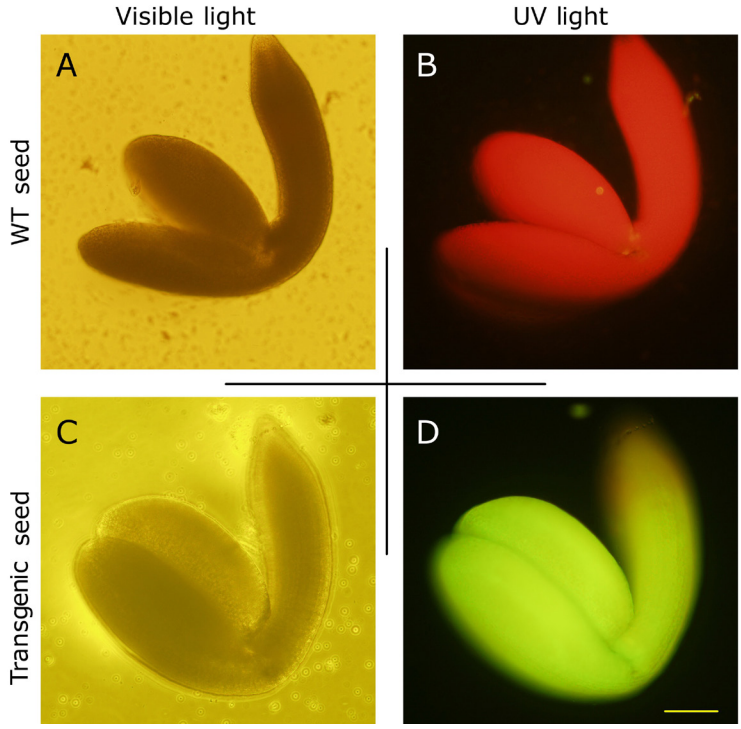

Figure 4. Fluorescence microscopy image of *Arabidopsis* embryo. **A.** Mature embryo from wild-type (WT) *Arabidopsis* plants under visible light. **B.** Same field under excitation light. The embryo appears bright red due to the presence of chlorophyll in the embryo. **C.** Mature embryo from transgenic *Arabidopsis* plants under visible light. **D.** Same field under excitation light. The embryo appears bright green indicating the accumulation of GFP-fusion protein in embryo. Bar = 25 µm.

DISCUSSION

Plant-based recombinant protein expression systems have become more and more popular as they have many benefits such as cost-efficiency, excellent scaling-up capacity, high product quality and low contamination risks (Twyman et al., 2003; Fischer et al., 2004; Boothe et al., 2010). Among the various plant-derived protein expression systems developed, there is one called the oleosin-fusion technology, which uses oleosins as a vehicle for recombinant protein production. It is one of the most attractive methods because of its high expression level, effective protein storage ability and easy purification procedure (Boothe et al., 2010). According to the oleosin-fusion technology strategy, heterogeneous proteins are covalently attached to the N- or C-terminal of oleosin, and the *fusion* gene is driven by a seed-specific promoter.

In this study, the GFP-oleosin fusion protein, driven by a constitutive CaMV35S promoter, was accumulated in transgenic *Arabidopsis* seeds, and no significant expression was detected in leaves by Western blotting using an anti-GFP antibody. According to the structure of oleosin, the central domain is highly hydrophobic. It may not be well folded without the presence of oil bodies, and the expression of oleosin-fusion protein driven by a CaMV35S

promoter is also not as high as those driven by a seed-specific promoter. This study reveals that the spatial and temporal regulation is not as important as the oleosin itself. It provides an alternative for other stronger promoters to be developed in oleosin-fusion technology.

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