Theor Appl Genet (2013) 126:2289–2297
DOI 10.1007/s00122-013-2135-4

ORIGINAL PAPER

Embryo-specific expression of soybean oleosin altered oil body morphogenesis and increased lipid content in transgenic rice seeds

Wen Xian Liu · Hua Liang Liu · Le Qing Qu

Received: 5 March 2012 / Accepted: 21 May 2013 / Published online: 8 June 2013

Abstract Oleosin is the most abundant protein in the oil bodies of plant seeds, playing an important role in regulating oil body formation and lipid accumulation. To investigate whether lipid accumulation in transgenic rice seeds depends on the expression level of oleosin, we introduced two soybean oleosin genes encoding 24 kDa proteins into rice under the control of an embryo-specific rice promoter *REG-2*. Overexpression of soybean oleosin in transgenic rice leads to an increase of seed lipid content up to 36.93 and 46.06 % higher than that of the non-transgenic control, respectively, while the overall fatty acid profiles of triacylglycerols remained unchanged. The overexpression of soybean oleosin in transgenic rice seeds resulted in more numerous and smaller oil bodies compared with wild type, suggesting that an inverse relationship exists between oil body size and the total oleosin level. The increase in lipid content is accompanied by a reduction in the accumulation of total seed protein. Our results suggest that it is possible to increase rice seed oil content for food use and for use as a low-cost feedstock for biodiesel by overexpressing oleosin in rice seeds.

Communicated by H. Becker.

W. X. Liu · H. L. Liu · L. Q. Qu (✉)
Key Laboratory of Plant Molecular Physiology,
Institute of Botany, The Chinese Academy of Sciences,
Beijing 100093, China
e-mail: lqqu@ibcas.ac.cn

W. X. Liu
College of Pastoral Agriculture Science and Technology,
Lanzhou University, Lanzhou 730020, China

Introduction

Plant seeds store lipids as a food reserve for germination and seedling growth. As the predominant component of seed lipids, triacylglycerols (TAGs) are essential for human nutrition and are valuable as feedstocks for various industrial products and biofuels (Dyer and Mullen 2008). Seed TAGs are generally stored in small, spherical, discrete intracellular organelles termed as oil bodies (Hsieh and Huang 2004). Oil bodies are simple organelles with a diameter of 0.5–2.0 μm (Huang 1996; Zienkiewicz et al. 2010), comprising a TAG matrix enclosed by a monolayer of phospholipids and structural oil body membrane proteins, principally oleosins (Bhatla et al. 2010). Both TAGs and oleosins are generated in the smooth endoplasmic reticulum (ER), where oil bodies are formed through a budding process during seed maturation (Hsieh and Huang 2004; Sarmiento et al. 1997).

Oleosins are comparatively small proteins (15–24 kDa) that are embedded in the surface of oil bodies. Oleosins are proposed to comprise three domains: a hydrophilic N-terminal domain, a hydrophobic central domain and a hydrophilic C-terminal domain (Hsieh and Huang 2004; Shimada and Hara-Nishimura 2010). The central domain, which is highly conserved, is essential for targeting oleosins to oil bodies and for stabilizing these lipid storage organelles via steric hindrance and electronegative repulsion provided by the N- and C-terminal amphipathic domains (Sarmiento et al. 1997; Tzen et al. 1992; Wu et al. 2010). Oleosins allow TAGs to remain in discrete, tightly packed organelles without coalescing, which becomes important as the cells desiccate or are exposed to freezing conditions (Siloto et al. 2006; Shimada et al. 2008). The small size of oil bodies provides a large surface area per unit TAG, which would likely facilitate lipase binding and

catalysis during seed germination (Huang et al. 2009; Wu et al. 2010).

Oleosins play an important role in oil body formation and lipid accumulation. The expression level of oleosin in *Brassica napus* seeds with high oil content is greater than that of seeds with low oil content (Hu et al. 2009), while the suppression of oleosin in *Arabidopsis* seeds results in larger oil bodies and reduced TAG accumulation (Shimada et al. 2008; Siloto et al. 2006). In rice, knocking out the endogenous 16 and 18 kDa oleosins reduces the seed TAG content to either approximately 60 or 80 %, respectively, of the TAG content of wild-type seeds (Wu et al. 2010). Conversely, the overexpression of a castor oleosin gene significantly enhances the accumulation of hydroxyl fatty acids in transgenic *Arabidopsis* seeds (Lu et al. 2006).

In rice seeds, oil bodies are present mainly in the embryo and in the aleurone layer. The accumulation of TAGs in embryos accounted for approximately 34 % of the total TAG content in rice seeds (Wu et al. 1998). Rice bran, the by-product of the milling process, is a mixture of embryo and aleurone and sub-aleurone layers of brown rice. Rice bran has substantial economic importance as an animal feed and as a raw material for pharmaceuticals (Lee et al. 2005). Rice bran oil (RBO) is one of the most nutritious oils, containing a unique combination of naturally occurring biologically active and antioxidant compounds (Zullaikah et al. 2005). RBO is also an inexpensive raw material for the production of biodiesel (Zullaikah et al. 2005; Ju and Vali 2005). Therefore, increasing the oil content of rice bran is beneficial for both the food and oil industries.

To date, most efforts at improving oil quality in rice seeds have been focused on the genes involved in the biosynthesis and use of fatty acids for TAG assembly (Anai et al. 2003; Kohno-Murase et al. 2006), while less attention has been paid to the effect of oleosin on rice seed lipid content. Lu et al. (2006) reported that the overexpression of a castor oleosin gene significantly enhances hydroxyl fatty acid accumulation in transgenic *Arabidopsis* seeds, raising the possibility that higher lipid content *in planta* could be achieved by increasing the expression of oleosin in transgenic rice seeds. In this study, we introduced two soybean oleosin genes into rice under the control of an embryo-specific rice globulin *REG-2* promoter (Qu and Takaiwa 2004). Ectopic expression of soybean oleosin genes significantly increased the lipid content in transgenic rice seeds. We also examined the effects of soybean oleosin overexpression on the morphology of oil bodies and on the accumulation of seed reserves in transgenic seeds.

Materials and methods

Production of transgenic plants

Two soybean (*Glycine max*) cDNAs encoding either the 24 kDa oleosin isoform A (Gm-A, GenBank accession U09118) or isoform B (Gm-B, GenBank accession U09119) were cloned from cotyledons by RT-PCR. The cDNAs were inserted into the binary vector pGPTV-REG-2-GUS-35S-HPT (Qu and Takaiwa 2004), containing the *REG-2* promoter, by replacing the *GUS* gene (Fig. 1). The resulting constructs of Gm-A and Gm-B were designated as *REG-2:Gm-A* and *REG-2:Gm-B*, respectively. The binary vectors were introduced into rice (*Oryza sativa* cv. Kitaake) by *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation as described previously (Qu et al. 2008). Successful transformation was verified by PCR analysis using primers from the *REG-2* promoter (5′-ACCGCATTTGTTCCCATCC-3′) and from *Gm-A* and *Gm-B* (5′-TCATGCGGTTGCGGTTGTTG-3′). Transformants were grown in a greenhouse as described previously (Qu et al. 2008) and self-pollinated for two generations. Homozygous lines were selected and used for further experiments.

Southern blot analysis

Rice genomic DNA was extracted from leaves using the cetyltrimethylammonium bromide (CTAB) method. Ten micrograms of genomic DNA were digested overnight with *Eco*R I, fractionated on a 0.8 % agarose gel and blotted onto a Hybond N^+ nylon membrane (Amersham). Hybridization was carried out with a ^{32}P-labeled DNA fragment of the hygromycin phosphotransferase gene (*hpt*) at 42 °C overnight. The filter was washed with 2× SSC and 0.5 % SDS for 20 min at 37 °C and then washed with 0.1× SSC and 0.5 % SDS for 20 min at 65 °C. The resulting filter was autoradiographed at −80 °C.

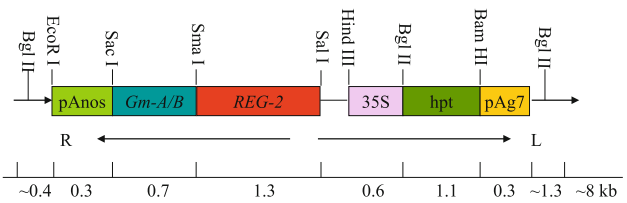

Fig. 1 Schematic diagram of the chimeric soybean oleosin gene construct. Soybean oleosin cDNA was fused to rice embryo-specific *REG-2* promoter. The chimeric genes were inserted into the pGPTV-35S-hpt binary vector between the restriction sites *Sal* I and *Sac* I

Semi-quantitative RT-PCR

Total RNA from seeds at 15 days after flowering (DAF) was extracted as described previously (Qu et al. 2005). After treatment with RNase-free DNase (Takara), 1.0 μg of total RNA was reverse transcribed using the oligo (dT) primer according to the manufacturer's instructions (RT-PCR kit, Promega, Madison, WI, USA). The following primer pairs were used to amplify cDNA: for *Gm-A*, 5′-ATGACCACACAAGTACCACCAC-3′ and 5′-TCATGC GGTTGCGGTTG TTGC-3′; for *Gm-B*, 5′-ATGACCACA GTGCCACCACAC-3′ and 5′-TCATGCGGTTGCGGTT GTTGC-3′; for *OsActin-1*, 5′-TCCATCTTGGC ATCTC TCAG-3′ and 5′-GTACCCGCATCAGGCATCTG-3′. Thermal cycling using *Taq* polymerase was as follows: denaturing at 94 °C for 3 min, followed by 25 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min. Each reaction was performed in triplicate.

Antibody preparation

Full-length cDNAs for soybean (*Gm-A* and *Gm-B*) and rice (16 and 18 kDa) (Wu et al. 1998) oleosin genes were cloned into pET28a (Novagen, Madison, WI, USA) and expressed in *E. coli* BL21 cells (TransGen Biotech, Beijing, China). Proteins were resolved by SDS-PAGE, and oleosin bands stained with Coomassie blue were cut out and extracted according to the modified gel-extraction procedure reported by Tzen et al. (1990). Purified oleosins were used for the production of antibodies in mice, as described previously (Wu et al. 2010).

Western analyses

The expression pattern and strength of soybean oleosins in mature seeds of transgenic rice were examined by in situ western analysis and western blot analysis as described previously (Qu et al. 2003, 2005).

Electron microscopy of oil bodies

Developing embryos at 15 DAF from both wild-type and oleosin-overexpression lines were cut into small pieces (approximately 1 mm^3) and fixed in 2.5 % glutaraldehyde (in 50 mM sodium phosphate buffer, pH 7.5) overnight. The samples were washed with phosphate buffer, postfixed in 2 % OsO_4, rinsed with phosphate buffer (3 × 15 min) and dehydrated by a graded series of acetone (20, 50, 70, 90, and 100 v/v). After infiltration through a graded acetone/Epon/Spurr's epoxy resin and polymerization at 60 °C for 24 h, thin sections (70 nm) were cut using an ultramicrotome (Leica MZ6) and placed onto copper grids. Samples were poststained with supersaturated uranyl acetate and 0.4 % lead citrate, rinsed for 6 × 15 s with ddH_2O and viewed under a JEOL JEM-1230 transmission electron microscope.

Five randomly selected cells were used for measuring the size and density of oil body. The diameters of six randomly selected oil bodies from each cell were measured. The density of oil bodies was determined by counting the number of oil bodies within a sub-divided 100 μm^2 area of embryo sections. Results are presented as mean values ± standard deviations. Mean comparisons were calculated by Student's *t* test with *P*-values indicated in "Results".

Lipid, protein and starch analysis

Lipid content and fatty acid composition were determined according to a method described previously (Kitta et al. 2005). Three grams of ground rice seed powder were suspended in 5 mL of ethyl alcohol, and 50 mL of 7 M HCl was added to the suspension. After digestion at 80 °C for 30 min, lipids were extracted with a mixture of diethyl ether and petroleum ether. After dehydrating the extracts with anhydrous sodium sulfate, the solvent was evaporated, and the extracted lipids were weighed. The lipids were *trans*-esterified with 5 % H_2SO_4 in methanol at 90 °C for 1 h, and the fatty acid methyl esters were extracted with hexane and separated on a Hewlett-Packard 6890 gas chromatograph, supplied with a hydrogen flame ionization detector and an HP INNOWAX capillary column (30 m; i.d. 0.25 mm). Heptacanoic acid (17:0, Sigma) was used as the internal standard. Fatty acid composition was expressed as a percentage of total fatty acid.

For protein content determination, 50 mg of rice seed was homogenized in 10 mL of protein extraction buffer (125 mM Tris–HCl, pH 6.8, 4 % SDS, 5 mM EDTA, 4 M urea, 0.5 M NaCl and 1 % Triton X-100). The homogenates were boiled for 15 min and centrifuged for 10 min at 15,000 rpm. The upper phase was collected, and the debris was extracted twice with extraction buffer. The amount of protein was measured using the BCA protein assay reagent (Pierce).

To determine total starch content, 50 mg of ground rice seed powder was washed three times with ethanol (80 % v/v) and extracted with 9.2 M and then 4.6 M perchloric acid. The supernatant was collected and brought up to 50 mL with water. An aliquot of this solution was analyzed for starch content using the anthrone method (Fu and Xue 2010).

All experiments were performed in triplicate for five biological replicates in separate experiments. The statistical analysis of the data was performed using Student's *t* test.

Results

Generation and characterization of transgenic rice plants

To determine whether lipid accumulation in transgenic rice seeds depends on the expression level of oleosin, two soybean oleosin cDNAs, *Gm-A* and *Gm-B*, were introduced into rice under the control of the embryo-specific promoter *REG-2* (Fig. 1), via *Agrobacterium*-mediated transformation. Successful transformation was determined by PCR analysis using genomic DNA extracted from leaves of T_0 plants. Transformants derived by introducing *REG-2*:*Gm-A* and *REG-2*:*Gm-B* into cultivar Kitaake were denoted as OGA (nine lines) and OGB (eight lines). Transgenic rice lines were self-pollinated through two generations to obtain homozygous lines. Four independent homozygous transgenic lines, OGA3, OGA4, OGB6 and OGB7, were selected and used for further analyses. Southern hybridization with genomic DNA from T_2 transgenic rice digested with *Eco*R I (there is no *Eco*R I site within *hpt*) showed two bands in OGA lines and one band in OGB lines, indicating that OGA lines contain two copies of *Gm-A* and that OGB lines contain a single copy of *Gm-B* (Fig. 2a). Semi-quantitative RT-PCR, using total RNA extracted from T_3 developing seeds (15 day after flowering, DAF), revealed a single band of 700 bp corresponding to the expected size of *Gm-A* and *Gm-B*, indicating that these genes are expressed in seeds of each line. No expression of *Gm-A* or *Gm-B* was detected in non-transformed rice seeds (Fig. 2b).

Accumulation and distribution of oleosin in transgenic rice seeds

The expression pattern of soybean oleosins driven by the rice embryo-specific promoter *REG-2* was determined using in situ western hybridization. In transgenic lines, the expression of both soybean Gm-A and Gm-B was limited to the embryo and aleurone layers and was not detected in the endosperm (Fig. 3a). The non-transformed rice seeds used as a control remained unstained. In situ western hybridization patterns observed in transgenic lines were consistent with previously reported tissue-specific expression of the *REG-2* promoter (Qu and Takaiwa 2004). The expression level of soybean oleosins in the OGA and OGB lines was investigated by western blot analysis of total protein extracted from mature seeds. Polyclonal antibodies directed against soybean Gm-A and Gm-B oleosins were bound to a 24 kDa protein from transgenic plants, while no binding was observed in non-transformed plant extracts (Fig. 3b). Expression of the endogenous 16 and 18 kDa rice oleosin proteins in the transgenic plants was not appreciably different from expression in non-transformed plants (Fig. 3c).

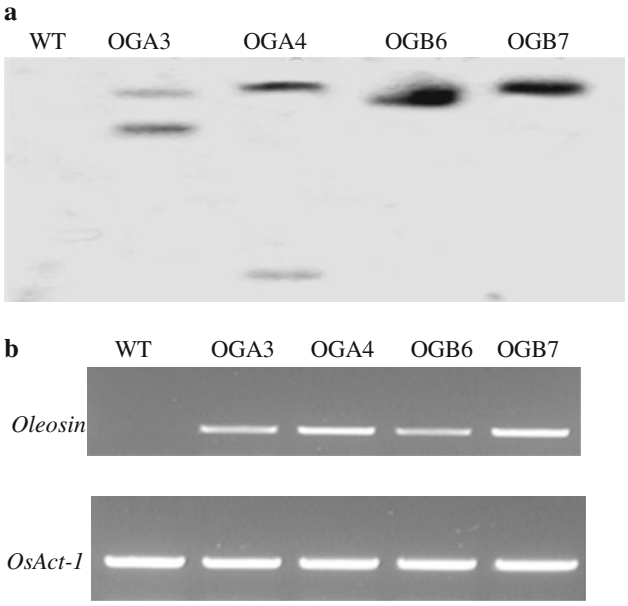

Fig. 2 Southern and semi-quantitative RT-PCR analysis of transgenic plants. **a** 10 μg genomic DNA was digested by *Eco*R I, fractioned by 0.8 % agarose gel, transferred onto a nitrocellose membrane and hybridized with cDNA of *hpt*. **b** Expression of two soybean oleosin genes in their corresponding transgenic lines, evaluated by semi-quantitative RT-PCR. The *OsActin-1* gene was used as a control

Morphology of oil bodies in transgenic seeds

Microscopy was employed to investigate the effects of oleosin accumulation on the morphology of oil bodies in embryos. In wild-type embryos, oil bodies were mostly present in the periphery of the cells and between protein bodies as discrete organelles of regular size (Fig. 4). This pattern is consistent with previous observations in rice seeds (Chuang et al. 1996). When soybean oleosin was overexpressed, the size and number of oil bodies changed markedly. In the embryos of transgenic rice, the average diameter of the oil bodies was 0.61 ± 0.11, 0.61 ± 0.12, 0.61 ± 0.11 and 0.64 ± 0.16 μm for OGA3, OGA4, OGB6 and OGB7 lines, respectively, while that of the wild type was 0.88 ± 0.14 μm (Fig. 5a). The density of oil bodies of OGA3, OGA4, OGB6 and OGB7 lines was 116.2 ± 7.1, 128.0 ± 20.1, 103.4 ± 25.1 and 140.8 ± 26.1 per 100 μm^2, respectively, comparing to that of 49.6 ± 6.2 per 100 μm^2 in the wild-type (Fig. 5b). These results suggest that the accumulation of oleosins determines the size of oil bodies. In contrast, some of the protein bodies in the OGA and OGB lines were several times bigger than those of the wild-type control (Fig. 4).

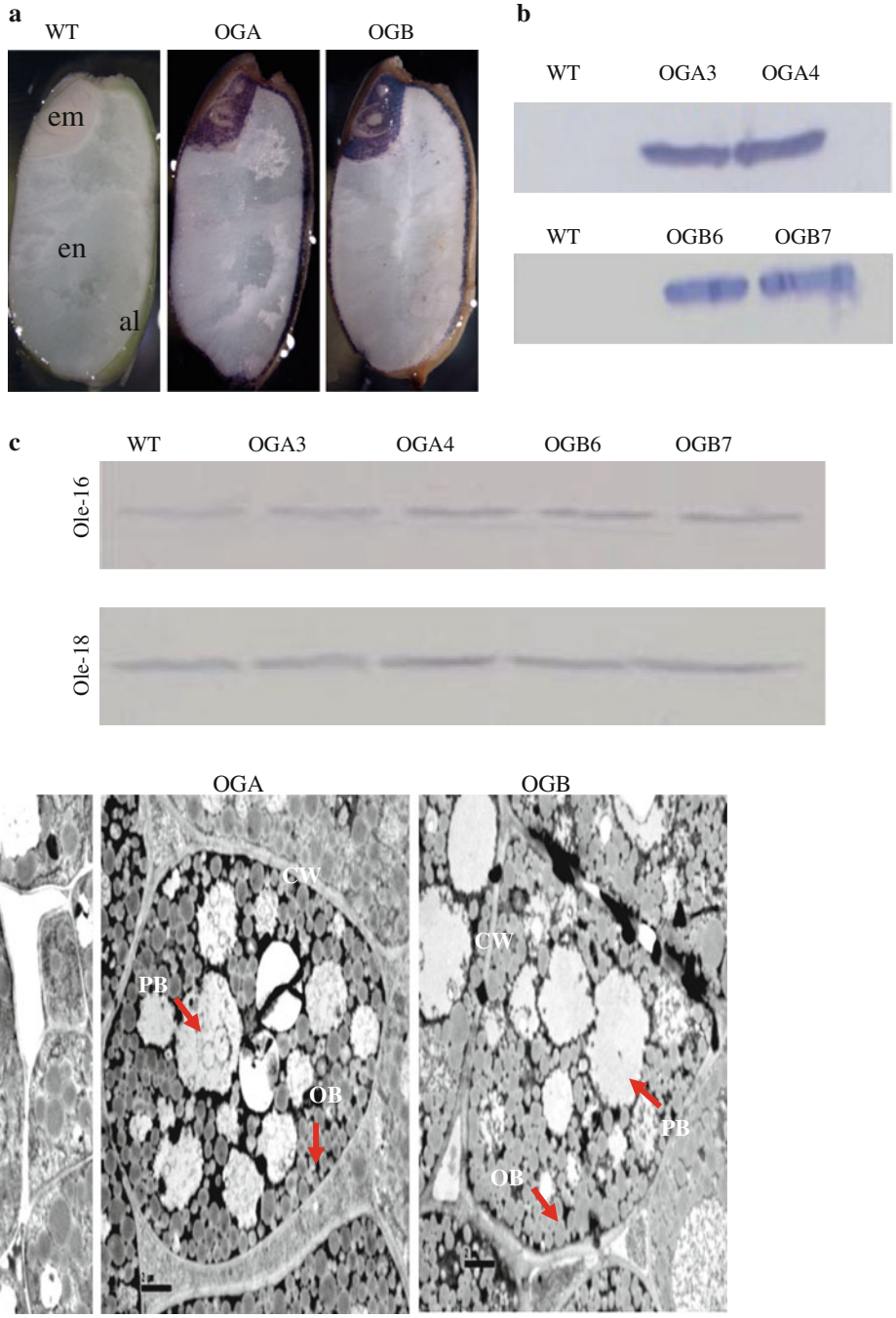

Fig. 3 Expression of soybean and rice oleosins in transgenic rice seeds. **a** in situ western localization of soybean oleosin in transgenic rice seeds. **b** Western blot analysis of soybean oleosin accumulation in mature transgenic rice seeds using anti-Gm-A or anti-Gm-B rabbit polyclonal antibodies. **c** Detection of endogenous oleosin isoforms in transgenic rice seeds. *em* embryo, *en* endosperm, *al* aleurone. Ole16 and Ole18, 16 and 18 kDa oleosins

Fig. 4 Electron microscopy of rice embryo cells from wild type and transgenic seeds. *OB* oil body, *PB* protein body, *CW* cell wall. *Scale bars* 2 μm

Accumulation of seed reserves in mature seeds of transgenic lines

To investigate whether alterations in oil body biogenesis would affect the accumulation of lipids and other seed reserves, we performed biochemical analyses of seed compositions. The lipid content in the homozygous T_3 seeds of OGA3, OGA4, OGB6 and OGB7 lines was 3.13, 3.30, 2.88 and 3.52 % (by dry weight), respectively, compared with 2.41 % in the non-transformant (Fig. 6a). The seed lipid contents were 29.88, 36.93, 19.50 and 46.06 % higher than that of the control, respectively. Concomitant with the increase in lipid content, the seed protein content was reduced by 9.46, 15.58, 4.95 and 18.20 in the OGA3, OGA4, OGB6 and OGB7 lines, respectively, compared with wild type (Fig. 6b). The starch content in

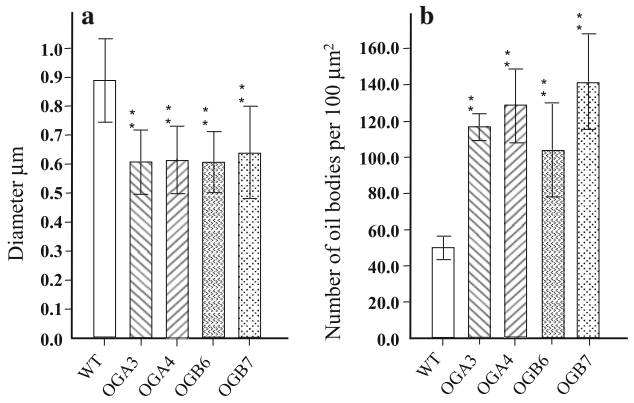

Fig. 5 Quantification of oil bodies in embryo cells from wild type and transgenic seeds. **a** Average diameter of oil bodies. Values are expressed as the mean ± standard deviation ($n = 30$). **b** Density of oil bodies. Values are expressed as the average number of oil bodies per 100 μm^2 ± standard deviation ($n = 5$) (**$P < 0.01$)

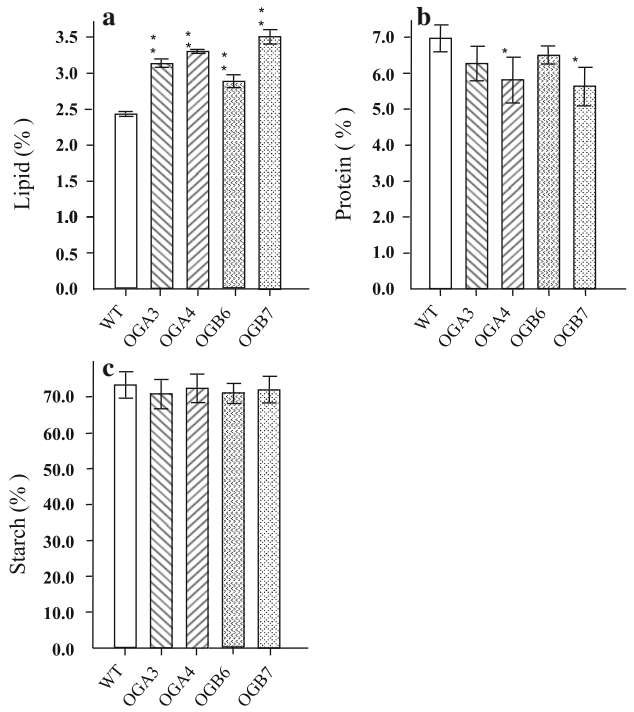

Fig. 6 Lipid, protein, and starch content of transgenic rice seeds. **a** Lipid content, **b** protein content, **c** starch content. Mean values are expressed as a percentage of seed dry weight ± standard deviation ($n = 5$) (*$P < 0.05$; **$P < 0.01$)

the seeds of transgenic lines was not significantly different compared with the wild type (Fig. 6c). We further measured fatty acid composition of TAGs in oleosin-overexpression rice lines. Compared with wild type, the proportions of C16:0 (palmitic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid) in TAG of transgenic lines were not dramatically different (Fig. 7).

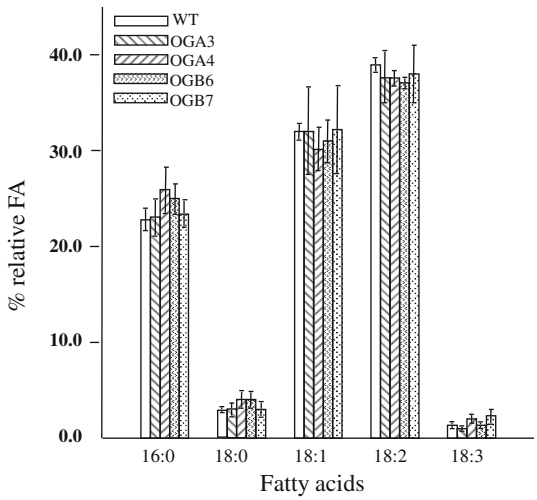

Fig. 7 Fatty acid composition of seed lipids from wild type and transgenic rice seeds. Values are expressed as the mean ± standard deviation ($n = 5$)

Discussion

Rice bran oil is one of the most nutritious oils and is rich in important phytonutrients, such as oryzanol, lecithin and vitamin E. It is also an alternative low-cost feedstock for biodiesel production (Zullaikah et al. 2005). Therefore, increasing rice oil content is nutritionally and commercially important. Conventional cereal crop breeding for high oil content has focused on selecting large embryos to improve capacity of lipid storage, since seed storage oil mainly deposited in embryo and aleurone layers. However, whether altering oil body biogenesis can improve lipid storage ability and increase oil content has less been studied.

Oleosin plays an important role in oil body formation and lipid accumulation. It has been shown that depressed oleosin in *Arabidopsis* and rice resulted in decreased seed oil content (Siloto et al. 2006; Wu et al. 2010). In contrast, the overexpression of a castor oleosin gene significantly increased hydroxyl fatty acid accumulation in transgenic *Arabidopsis* seeds (Lu et al. 2006). Hu et al. (2009) reported a positive correlation between oleosin levels and oil content in *Brassica napus* seeds. To date, most of the research on oleosin has been focused on oilseed plants, with less attention on other plants, including starchy seed plants such as rice.

It has been suggested that increasing the oil body membrane protein level may increase the oil storage ability of cells, stimulate oil biosynthesis and subsequently sequester the oil in the oil body (Froissard et al. 2009; Siloto et al. 2006). Based on this hypothesis, we successfully developed two types of oleosin-overexpression transgenic rice lines using the strong embryo-specific promoter, *REG-2* (Qu and Takaiwa 2004). As expected,

soybean oleosin was synthesized as a 24-kDa protein in transgenic rice seeds, which is the correct size for the native matured subunit. The expression and accumulation of oleosin were restricted to the embryo and aleurone layers (Fig. 3a). Overexpression of soybean oleosin in two types of transgenic rice lines led to an increase in seed lipid content up to 36.93 % (OGA lines) and 46.06 % (OGB lines) higher than that of the control (Fig. 6a). The expression level of endogenous rice 16 and 18 kDa oleosins in transgenic lines did not appear to differ from the wild type (Fig. 3c). It is notable that high expression of soybean oleosins greatly increased the lipid content in transgenic rice seeds, with no detrimental effects on growth, development, seed weight, seed germination and other yield traits (data not shown). These results indicated that the increased lipid content in transgenic plants was due to the overexpression of soybean oleosin and suggested that oleosin overexpression might be an effective way to improve lipid content. The lipid content in the transgenic rice seed increased significantly while the overall fatty acid profile of TAGs was not different from that of the control, reflecting that the content of unsaturated fatty acids increased in the transgenic rice seeds (Figs. 6a, 7). It is reasonable to deduce that the content of oryzanol, lecithin and vitamin E might also be increased, although we did not measure their contents in the transgenic rice seeds. The high lipid content transgenic rice is expected to contribute for improvement of human nutrition and for use as a low-cost feedstock for biodiesel.

Embryo-specific expression of oleosin in rice significantly increased the seed lipid content. To determine whether the overexpression of oleosin in rice endosperm could also improve lipid accumulation in rice seeds, we overexpressed *Gm-A* and *Gm-B* in rice under the control of an endosperm-specific rice glutelin *GluC* promoter (Qu et al. 2008). Although soybean oleosins were able to specifically accumulate in rice seed endosperm, there was no significant change in seed lipid content (data not shown). A previous study on the ectopic expression of a sunflower oleosin in transgenic *Arabidopsis* showed that although the oleosin protein could accumulate in non-oil-storage tissues, accumulation occurred in the microsomal membrane but not in the lipid body (Beaudoin and Napier 2000). Oil body biogenesis is a result of the coordinate synthesis of oleosins and TAGs. Without active TAG synthesis in the rice endosperm, ectopic overexpression of oleosins in this tissue may not help facilitate oil body biogenesis, which would improve the seed lipid content.

TAG in rice seeds is mainly composed of fatty acids, such as palmitic acid (PA, C16:0), stearic acid (SA, C18:0), oleic acid (OA, C18:1), linoleic acid (LA, C18:2) and α-linolenic acid (ALA, C18:3). Although the TAG content increased greatly in oleosin-overexpression rice lines, the overall fatty acid profile of TAGs did not show observable differences compared with the control (Fig. 7). These results indicated that the content of these fatty acids increased accompanied by an increase of TAG. It is noteworthy that oleosin overexpression also resulted in a reduction in the seed protein content (Fig. 6b), even though some of the protein bodies in transgenic embryo cells were much larger than in the wild-type cells (Fig. 4). The band pattern of seed storage glutelin, prolamine and globulin in the transgenic rice seeds revealed by SDS-PAGE showed no obvious difference with that of the wild-type control (data not shown), reflecting that all protein compositions, but not a special type of protein, were reduced in transgenic rice seeds. It is not surprising since the oil content is negatively correlated with protein content in seeds (Hu et al. 2009; Siloto et al. 2006) and the decreased protein content might not be caused by the alteration of protein bodies in the embryo which accounts for a very small portion of seed.

It has been reported that oleosin content correlates with the size of oil bodies in several oilseeds (Hu et al. 2009; Ross et al. 1993; Ting et al. 1996; Tzen et al. 1993). Reverse genetic analysis in *Arabidopsis* and rice revealed that lacking of oleosins may promote fusion of oil bodies in the cells during seed maturation, resulting in unusually large oil bodies (Shimada et al. 2008; Siloto et al. 2006; Wu et al. 2010). The soybean oleosin-overexpression transgenic rice seeds had more and smaller oil bodies in cells compared with wild type (Figs. 4, 5), confirming that the size of the oil bodies is negatively related to the oleosin content. Oil bodies are simple organelles comprising a matrix of TAG, coated by a phospholipid monolayer embedded by oleosins. These organelles are synthesized in the ER as nascent oil bodies and subsequently bud off from the ER, forming mature oil bodies (Hsieh and Huang 2004; Murphy and Vance 1999; Siloto et al. 2006). In oleosin-suppressed transgenic *Arabidopsis* lines, oil bodies probably coalesce until a critical surface density is reached, resulting in larger oil bodies (Siloto et al. 2006). In oleosin-overexpression rice, more oleosin is synthesized in the ER compared with wild type, providing an oleosin-saturated environment that may allow the nascent oil bodies to be covered quickly and completely by oleosins, leading to the formation of smaller oil bodies.

It has been reported that the sizes of protein bodies were reduced accompanied by enlarged oil bodies in the oleosin-deficient mutants of *Arabidopsis* (Shimada et al. 2008). Similar phenomena were observed in *DGAT2A* overexpression of transgenic soybean seeds (Lardizabal et al. 2008). In the embryo cell of oleosin-overexpression rice, the size of oil bodies was reduced, while the size of protein bodies is enlarged (Fig. 4). These results supported the hypothesis that oil bodies and protein bodies might be

formed coordinately on the ER during seed maturation by competing for the spaces in seed cells (Shimada et al. 2008).

Seed oil content is negatively related to oil body size. Generally, when a seed contains a high percentage of oils, its oil bodies are small. Hu et al. (2009) reported that low oil content correlates strongly with large oil bodies in rapeseeds. However, Ting et al. (1996) reported that maize kernels with high oil content (having a high oil-to-oleosin ratio) generated by breeding have large oil bodies, while kernels with low oil content have small oil bodies with irregularly shaped surfaces. Our results showed that in oleosin-overexpression rice lines, the seed oil content increases significantly whereas the oil body size decreases. In addition, the number of oil bodies is increased in the transgenic lines, with the shape of the oil body remaining spherical (Fig. 4). These results confirmed that soybean oleosin participates in the formation of oil bodies and enlarges oil storage capacity. These results also suggest that it is possible to further increase the oil content by co-expression of oleosin and lipid biosynthesis genes.

Acknowledgments This work was supported by the National Program of Transgenic Variety Development of China (2013ZX08001-006), National High Technology Research and Development Program (863) (2011AA100604) and the Natural Science Foundation of China (No. 31171368). The *REG-2* promoter was kindly provided by Dr. F. Takaiwa, NIAS, Japan.

References

Anai T, Koga M, Tanaka H, Kinoshita T, Rahman SM, Takagi Y (2003) Improvement of rice (*Oryza sativa* L.) seed oil quality through introduction of a soybean microsomal omega-3 fatty acid desaturase gene. Plant Cell Rep 21:988–992

Beaudoin F, Napier JA (2000) The targeting and accumulation of ectopically expressed oleosin in non-seed tissues of *Arabidopsis thaliana*. Planta 210:439–445

Bhatla SC, Kaushik V, Yadav MK (2010) Use of oil bodies and oleosins in recombinant protein production and other biotechnological applications. Biotechnol Adv 28:293–300

Chuang RL, Chen JC, Chu J, Tzen JT (1996) Characterization of seed oil bodies and their surface oleosin isoforms from rice embryos. J Biochem 120:74–81

Dyer JM, Mullen RT (2008) Engineering plant oils as high-value industrial feedstocks for biorefining: the need for underpinning cell biology research. Physiol Plantarum 132:11–22

Froissard M, D'Andrea S, Boulard C, Chardot T (2009) Heterologous expression of AtClo1, a plant oil body protein, induces lipid accumulation in yeast. FEMS Yeast Res 9:428–438

Fu FF, Xue HW (2010) Coexpression analysis identifies rice starch regulator1, a rice AP2/EREBP family transcription factor, as a novel rice starch biosynthesis regulator. Plant Physiol 154: 927–938

Hsieh K, Huang AH (2004) Endoplasmic reticulum, oleosins, and oils in seeds and tapetum cells. Plant Physiol 136:3427–3434

Hu Z, Wang X, Zhan G, Liu G, Hua W, Wang H (2009) Unusually large oilbodies are highly correlated with lower oil content in *Brassica napus*. Plant Cell Rep 28:541–549

Huang AH (1996) Oleosins and oil bodies in seeds and other organs. Plant Physiol 110:1055–1061

Huang CY, Chung CI, Lin YC, Hsing YI, Huang AH (2009) Oil bodies and oleosins in physcomitrella possess characteristics representative of early trends in evolution. Plant Physiol 150:1192–1203

Ju YH, Vali SR (2005) Rice bran oil as a potential resource for biodiesel: a review. J Sci Ind Res 64:866–882

Kitta K, Ebihara M, Iizuka T, Yoshikawa R, Isshiki K, Kawamoto S (2005) Variations in lipid content and fatty acid composition of major non-glutinous rice cultivars in Japan. J Food Compos Anal 18:269–278

Kohno-Murase J, Iwabuchi M, Endo-Kasahara S, Sugita K, Ebinuma H, Imamura J (2006) Production of *trans*-10, *cis*-12 conjugated linoleic acid in rice. Transgenic Res 15:95–100

Lardizabal K, Effertz R, Levering C, Mai J, Pedroso MC, Jury T, Aasen E, Gruys K, Bennett K (2008) Expression of *Umbelopsis ramanniana DGAT2A* in seed increases oil in soybean. Plant Physiol 148:89–96

Lee TTT, Chung MC, Kao YW, Wang CS, Chen LJ, Tzen JTC (2005) Specific expression of a sesame storage protein in transgenic rice bran. J Cereal Sci 41:23–29

Lu C, Fulda M, Wallis JG, Browse J (2006) A high-throughput screen for genes from castor that boost hydroxy fatty acid accumulation in seed oils of transgenic Arabidopsis. Plant J 45:847–856

Murphy DJ, Vance J (1999) Mechanisms of lipid-body formation. Trends Biochem Sci 24:109–115

Qu LQ, Takaiwa F (2004) Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. Plant Biotechnol J 2:113–125

Qu LQ, Tada Y, Takaiwa F (2003) In situ western hybridization: a new, highly sensitive technique to detect foreign and endogenous protein distribution in rice seeds. Plant Cell Rep 22: 282–285

Qu LQ, Yoshihara T, Ooyama A, Goto F, Takaiwa F (2005) Iron accumulation does not parallel the high expression level of ferritin in transgenic rice seeds. Planta 222:225–233

Qu LQ, Xing YP, Liu WX, Xu XP, Song YR (2008) Expression pattern and activity of six glutelin gene promoters in transgenic rice. J Exp Bot 59:2417–2424

Ross JHE, Sanchez J, Millan F, Murphy DJ (1993) Differential presence of olesoins in oleogenic seed and mesocarp tissues in olive (*Olea europea*) and avocado (*Persea americana*). Plant Sci 93:247–280

Sarmiento C, Ross JH, Herman E, Murphy DJ (1997) Expression and subcellular targeting of a soybean oleosin in transgenic rapeseed. Implications for the mechanism of oil-body formation in seeds. Plant J 11:783–796

Shimada TL, Hara-Nishimura I (2010) Oil-body-membrane proteins and their physiological functions in plants. Biol Pharm Bull 33:360–363

Shimada TL, Shimada T, Takahashi H, Fukao Y, Hara-Nishimura I (2008) A novel role for oleosins in freezing tolerance of oilseeds in *Arabidopsis thaliana*. Plant J 55:798–809

Siloto RMP, Findlay K, Lopez-Villalobos A, Yeung EC, Nykiforuk CL, Moloney MM (2006) The accumulation of oleosins determines the size of seed oilbodies in *Arabidopsis*. Plant Cell 18:1961–1974

Ting JT, Lee K, Ratnayake C, Platt KA, Balsamo RA, Huang AH (1996) Oleosin genes in maize kernels having diverse oil contents are constitutively expressed independent of oil contents. Size and shape of intracellular oil bodies are determined by the oleosins/oils ratio. Planta 199:158–165

Tzen JT, Lai YK, Chan KL, Huang AH (1990) Oleosin isoforms of high and low molecular weights are present in the oil bodies of diverse seed species. Plant Physiol 94:1282–1289

Tzen JT, Lie GC, Huang AH (1992) Characterization of the charged components and their topology on the surface of plant seed oil bodies. J Biol Chem 267:15626–15634

Tzen J, Cao Y, Laurent P, Ratnayake C, Huang A (1993) Lipids, proteins, and structure of seed oil bodies from diverse species. Plant Physiol 101:267–276

Wu LS, Wang LD, Chen PW, Chen LJ, Tzen JT (1998) Genomic cloning of 18 kDa oleosin and detection of triacylglycerols and oleosin isoforms in maturing rice and postgerminative seedlings. J Biochem 123:386–391

Wu YY, Chou YR, Wang CS, Tseng TH, Chen LJ, Tzen JT (2010) Different effects on triacylglycerol packaging to oil bodies in transgenic rice seeds by specifically eliminating one of their two oleosin isoforms. Plant Physiol Biochem 48:81–89

Zienkiewicz K, Castro AJ, Alche Jdé JD, Zienkiewicz A, Suárez C, Rodríguez-García MI (2010) Identification and localization of a caleosin in olive (*Olea europaea* L.) pollen during in vitro germination. J Exp Bot 61:1537–1546

Zullaikah S, Lai CC, Vali SR, Ju YH (2005) A two-step acid-catalyzed process for the production of biodiesel from rice bran oil. Bioresource Technol 96:1889–1896