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Characterization, Expression Profiling and Heterologous Function Analysis of Two Oleosin Genes *PvOle1* and *PvOle2* from Sacha Inchi (*Plukenetia volubilis*)

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Abstract

Sacha Inchi (*Plukenetia volubilis* L., Euphorbiaceae) is a potential oilseed crop for its wealth of seed oil rich in omega-3 fatty acids. The present study describes the isolation and characterization of two Sacha Inchi oleosin genes (*PvOle1* and *PvOle2*) encoding oleosin proteins. Full length cDNAs of *PvOle1* and *PvOle2* were comprised of 829 and 763 nucleotides, encoding 143 and 139 amino acids with a molecular mass of 15.2 kDa and 14.8 kDa, respectively. Comparative and bioinformatic analysis revealed the divergence of PvOle1 and PvOle2 in their amino acid sequence and hydrophobic profiles and also the L-form of both oleosin proteins. Phylogenetic analysis indicated the possible occurrence of duplication (*PvOle1* and *PvOle2*) before the speciation of Sacha Inchi. Semi-quantitative RT-PCR expression analysis revealed the high expression of *PvOle1* and *PvOle2* transcripts in the developing seeds. Further, using yeast as a heterologous expression system, we investigated the effective role of *PvOle1* and *PvOle2* in yeast lipid body accumulation, which was confirmed by SDS-PAGE and Immunoblotting analysis. Taken together, the characterization, expression profiling and heterologous functional analysis of two oleosin genes (*PvOle1* and *PvOle2*) will be helpful for further studies concerning the role of oleosin proteins in metabolic protein engineering in Sacha Inchi.

Keywords: Sacha Inchi; Oleosin; Recombinant protein; Yeast

Introduction

Sacha Inchi (*Plukenetia volubilis* L., Euphorbiaceae), also known as Inca Inchi or mountain peanut, is a perennial oleaginous woody vine indigenous to tropical Peruvian jungles at altitudes between 200 and 1500 m. This legume has a star-shaped fruit, which contains dark oval seeds, abundantly rich in oil and proteins. Sacha Inchi seeds are traditionally consumed by the Indian people of Peru to obtain flour and oil (Guillen *et al*., 2003). Recent studies have suggested that the nutritional value of Sacha Inchi seeds is highly considerable, because of its wealth of oil, fatty acids (polyunsaturated fatty acids (PUFA's) comprising about 93% of total FA's), proteins and vitamins (Hamaker *et al*., 1992; Guillen *et al*., 2003). In particular, the composition of its seed oil differs markedly in containing a large amount of α-linolenic acid (a kind of omega-3 fatty acid), known to provide protection against cardiovascular disease, rheumatoid arthritis, cancer, and possibly the severity of viral infections (Gebauer *et al*., 2006).

Seed oils are stored as triacylglycerols (TAG) in oil bodies surrounded by a monolayer of phospholipid embedding protein called oleosins. Oleosins are low molecular mass proteins (15-26 kDa), characterized by a highly conserved central hydrophobic domain, a highly variable N-terminal and a C-terminal domain (Huang, 1992; Li *et al*., 2002; Simkin *et al*., 2006). The central hydrophobic domain has a unique stretch of approximately 68-72 amino acid residues featured by a highly conserved "Proline Knot Motif" comprising of three prolines and a serine residue, which is a prominent feature of oleosin proteins among diverse plants. Earlier reports have proposed that the central hydrophobic domain is involved in forming a hairpin structure on the proline knot, which is critical for the exact targeting to lipid bodies (Lacey *et al*., 1998; Abell *et al*., 2001; Sadeghipour *et al*., 2002; Siloto *et al*., 2006). Oleosin proteins usually play a prominent role in stabilizing oil body interfaces such as TAG/cytosol preventing them from coalescence and maintaining oil accumulation in seeds (Tzen *et al*., 1990; Huang, 1992; Murphy, 1993). In general, oleosins identified in diverse plants are grouped as L-Oleosin (Low) and H-Oleosin (High) types based on their relative molecular masses (Ting *et al*., 1997). The H-Oleosin differs from the L-Oleosin in having a higher molecular mass (~2 kDa) and an 18-residue insertion in their C-Terminal domain (Tzen *et al*., 1997; Tai *et al*.,

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2002). Oleosin proteins are major storage proteins in oilseeds and genes encoding oleosin proteins are usually specifically expressed in seeds (Simkin *et al*., 2006). Yeast, as a heterologous expression system has been previously subjected for plant protein targeting (Ting *et al*., 1997; Froissard *et al*., 2009). Gene engineering using oleosin promoters in seeds to produce functional target proteins has gained great interest in producing antibiotics, enzymes, emulsifiers for the food industry as well as for pharmaceutical applications in recent years (Hou *et al*., 2003; Chiang *et al*., 2005). Recombinant human insulin fused to the oleosin promoter has, for instance, been successfully expressed and purified from transgenic plant seeds (Nykiforuk *et al*., 2006; Li *et al*., 2011).

Although genes encoding oleosin proteins have been isolated from *Jatropha* (Popluechai *et al*., 2011), rapeseed (Keddie *et al*., 1992), sunflower (Thoyts *et al*., 1995), rice (Wu *et al*., 1998), coffee (Simkin *et al*., 2006) and *Arabidopsis* (Kim *et al*., 2002), limited knowledge is available to understand the biological interest of oleosins and its evolutionary context in plants. To characterize the molecular basis for the accumulation of storage oils and proteins in Sachi Inchi seeds and to add insights in understanding the evolutionary context of oleosins in plants, we isolated and characterized two oleosin genes from the developing seeds of Sacha Inchi and inspected their phylogenetic relationships with oleosins in plants. Furthermore, we tested the functions of two oleosin genes obtained by heterologous expression in yeast (*Saccharomyces cerevisiae*) and added insights in understanding the molecular nature of oleosin genes and their potential applications in bioengineering.

Materials and Methods

Plant Material

Fresh samples of leaves, root tips, flowers and the developing seeds at three developmental stages (early, middle and late) were harvested from two-years-old individuals of Sacha Inchi, XTBG-PV003 (introduced from Peru by seeds) grown at Xishuangbanna Tropical Botanical Garden (21°56'N, 101°15'E, 600 m asl), Chinese Academy of Sciences, Yunnan, China under natural conditions. Fresh tissues were frozen immediately in liquid nitrogen and subsequently stored at -80°C until used for RNA extraction.

RNA Extraction and Cloning of Two Oleosin Genes

Total RNA was extracted from leaves, root tips, flowers and the developing seeds at three developmental stages (2, 7, 13: DAF) respectively using TRIzol reagent (Life Technologies, USA), as described previously (Xu *et al*., 2011). Extracted RNA samples were treated with DNase I (Fermentas, China) to suppress DNA contamination, and cleaned using RNA Clean Kit (Tiangen, China) following the manufacturer's protocol. RNA obtained was spectrometrically quantified (NanoDrop ND-2000) at an absorbance of 260 nm and qualitatively assessed by Goldview ™ (Bioteke, China) agarose gel electrophoresis. First strand cDNA was synthesized with the Prime Script ™ RT-PCR kit (Takara Bio. Inc.), using equal amounts of Oligo dT primer according to the manufacturer's instructions. The first strand cDNA fragment was used as template for RT-PCR reaction.

The 5' and 3' Rapid Amplification of cDNA Ends (RACE) PCR method was used to clone the full length of two oleosin genes, based on the partial sequences of two oleosin genes generated from the transcriptomic analyses of Sacha Inchi developing seeds (data unpublished). The sequence synthesized was used to design primers for amplification of the 3' and 5' ends by using the Smart™-RACE cDNA Amplification kit (Clontech, USA) as instructed. For 3'-RACE, the first-strand cDNA was primed off with the 30 CDS primer (provided in the kit) and the amplification was achieved by using in reverse the specific forward primers 5' -CGGAACTGTGATTGCCCTGGTCGT and 5'-GGTGGGCGGGGAAGGCAAGGGAGACGAA respectively. For 5'-RACE, the amplification was achieved by using the first-strand cDNA as templates with the specific primers 5'-AACCAGAGCAGGAGTTGCCACGACC and 5'-ACTCGGTGTCCAAACTGCTCAGCCCTAT respectively in combination with 30 CDS primer (provided in the kit). PCR products of both 3'-RACE and 5'-RACE were gel extracted using QIAquick Gel Extraction Kit and sub-cloned into pGEM-T Easy (Promega,China) and sequenced. The ORF region was amplified using the primer pairs 5'-ATCATGTCTCAGATGACCCGTG and 5'-TCATGATGCTTGAGTTTGAGTGG for Sacha Inchi *oleosin1* and 5'-ATCATGGCTGA CCAACAAGTGTTACG and 5'-CTAAGTCTCATGAATAGTTACTCGGT for Sacha Inchi *oleosin2*.

Sequence Analysis

EST sequences of diverse oleosin genes containing highly conserved regions were obtained (http://www.ncbi.nlm.nih.gov/dbEST/). These EST's were selected from libraries of oleosins of seed specific localization. DNA and amino acid sequence analysis of the two oleosin genes were assessed using BLASTn and BLASTp (http://www.ncbi.nlm.nih.gov/BLAST/), respectively and aligned using ClustalX program with default settings. The aligned sequences were used to develop a phylogenetic tree using Neighbour Joining (NJ) criteria as directed in the MEGA package v4.1. Physico-chemical properties of the deduced proteins were predicted by Protparam (http://www.expasy.ch/tools/protparam.html). Relative values for the hydrophobic nature of the oleosin

proteins were obtained with the help of Protscale (http://web.expasy.org/protscale.html). Transmembrane helices of the two oleosin proteins were analyzed by TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) (Sonnhammer *et al*., 1998). AlignX software was used for visualizing the aligned sequences.

Semi-quantitative RT-PCR Expression Analysis

To investigate the expressional profile of *PvOle1* and *PvOle2* among different tissues and different stages of developing seeds, semi-quantitative RT-PCR was carried out using the first strand cDNA from leaves, root tips, flowers and the developing seeds. The gene specific primer pairs 5'-GCTGCGATAATGGTTCTG and 5'-TGAGTTTGAGTGGCTTCTT were designed for *PvOle1*, and 5'-GCACTCACCACAACCACTCCAT and 5'-AGCCCTATCTTTCGTCTCCCTT for *PvOle2*. Sachi inchi *Actin* gene specific primer pairs 5'-CGAGGTGGTGAAAGAGTAA and 5'-TATGTAGCCATCCAAGCC, designed according to a partial *Actin* gene sequences from the transcriptomic analyses of developing seeds (data unpublished), was used as an endogenous control. PCR reactions (20µl total volume) were done using ExTaq polymerase (Takara, Japan). The same amount of template cDNA and cycles were determined by preliminary experiments to ensure that amplification allowed comparison of the amplified products among different tissues. The amplification program consisted of an initial 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, 53°C for 30 s, 72°C for 50 s, and final extension at 72°C for 5 min. Negative PCR controls were performed using the purified total RNA to ensure that RNA samples were free of DNA contamination. The PCR products were electrophoresed on 1% (w/v) agarose gel stained with ethidium bromide and visualized by Genesnap image analysis software (version 4.0) for subsequent analysis. Experiments were repeated independently for three times to ensure that similar results were obtained.

Heterologous Expression in Yeast and Recombinant Protein Purification

To test the possibility of producing oleosins through a protein engineering approach using heterologous expression of oleosin genes in yeast (*Saccharomyces cerevisiae*), a wild yeast strain INVSc1 (Invitrogen,USA) was used. Both *PvOle1* and *PvOle2* (ORF region) were fused with *GAL1* inducible yeast expression vector pYES2.1/V5-His tagged-TOPO (Invitrogen, USA). The ligation products were transformed individually into one-shot chemically competent *E. coli* cells (Invitrogen, USA). Recombinant plasmids obtained from positive clones were used to transform wild yeast strain INVSc1 according to the manufacturer's instructions. Cultures grown for 2-3 days at 28-30°C were sub-cultured overnight in synthetic liquid minimal media (uracil omitted) supplemented with a carbon source 1% raffinose (w/v) at 28–30°C. Overnight cultures were centrifuged at 1500 rpm for 5 min at 4°C. Yeast aliquots obtained were induced by 2% galactose (w/v) for the expression of the recombinant proteins (Giniger *et al*., 1985). Induced yeast cells (at the exponential phase, ~20 h after culture) were centrifuged and lysed using the acid washed glass bead method (0.4–0.6 mm; Sigma-Aldrich). Total protein extracts obtained were stored at -80°C for further analysis. Protein concentration was checked by a UV spectrometer (NanoDrop ND-2000) at an absorbance of 280 nm with BSA as a standard.

SDS-PAGE and Immunoblotting

To test the oleosin targeting (*PvOle1* and *PvOle2*) in transformed yeast cells, SDS-PAGE and immunobloting experiments were performed. For SDS-PAGE, total protein extracts were solubilized in SDS-PAGE loading buffer (50 mM Tris-Hcl with pH = 6.8, 2% SDS, 10% Glycerol, 1% B-mercaptoethanol, 12.5 mM EDTA, 0.02% Bromo blue) and resolved by 12% SDS PAGE comprising 12% (w/v) polyacrylamide in the separating gel and 4% polyacrylamide in the stacking gel. The gel was then stained with Coomasive brilliant blue stain (G-250) for 2-3 h and destained (20% methanol, 10% acetic acid) until a clear background appeared. For immunoblotting, proteins resolved were transferred to a PVDF membrane (Amersham, USA) under efficient transfer conditions using a Trans-blot system (Bio-Rad, CA, USA) following the manufacturer's instructions. Immunodetection of the transformed membrane was done using a mouse anti-His (C-term) monoclonal antibody (Invitrogen, USA) conjugated with horseradish peroxidase (HRP) (Lindner *et al*., 1997). Anti-His (C-term)-HRP conjugated antibody allows one-step detection of recombinant proteins containing a C-terminal polyhistidine tag with a carboxyl group. Immunoblotted proteins were visualized with chemiluminescent (Thermo scientific, USA) reagent according to the manufacturer's instructions.

Results

Isolation of Oleosin Genes from Sacha Inchi

Full length cDNAs of the two oleosin genes were obtained and subsequently named as *PvOle1* and *PvOle2*. The full length cDNA of *PvOle1* was comprised of 829 nucleotides with a coding ORF region of 429 nucleotides encoding 143 amino acids, 156 nucleotides at 5' untranslated region (UTR) and 244 nucleotides at 3'UTR region, where as the full length cDNA of *PvOle2* contains 763 nucleotides was comprised of 420 nucleotides in ORF region encoding 139 amino acids, 51 nucleotides in 5'UTR and 292 nucleotides in 3'UTR. The two oleosin genes (*PvOle1* and *PvOle2*) had a predicted AATAAA and ATTAAA (One base variant) polyadenylation signal sequence, along with polyA tail at their 3'UTR end (data not shown). BLAST results with the amino acid sequences of *PvOle1* and *PvOle2* showed a

high homology for the predicted protein sequences of *PvOle1* with *Ricinus communis* (80%; XP002516493), *Populus trichocarpa* (72%; XP002309527) and *Arachis hypogea* (62%; AAU21501), and that of *PvOle2* with *R. communis* (71%; XP002511014), *Ficus pumila* (70%; ABQ57397) and *Jatropha curcas* (69%; ABW90150). Sequences of *PvOle1* and *PvOle2* were deposited in GenBank under the accession number JN788777 and JN788779, respectively.

Sequence Analysis

ClustalX multiple sequence alignment indicated a Proline Knot Motif (PX_5-SP-X_3P) characterized in the central hydrophobic domain of the two oleosin amino acid residues similar to oleosin proteins of various plants (Fig. 1). The amino acid sequences of both PvOle1 and PvOle2 were variable in their N and C-terminal domains. Even within the Proline Knot Motif region, there were five divergent residues (i.e. A/L, L/M, V/I, C/F, V/A) between PvOle1 and PvOle2 sequences, suggesting that *PvOle1* and *PvOle2* are divergent in their gene structure. The molecular mass of the deduced oleosin proteins (PvOle1 and PvOle2) were 15.2 kDa and 14.8 kDa with an isoelectric point of 9.0 p*I* and 10.0 p*I*, respectively. Both PvOle1 and PvOle2 were grouped as L-form oleosins due to the absence of the 18 amino acid insertional residues at the C-terminal domain (Fig. 1). TMHMM prediction showed that the deduced amino acid sequences of both *PvOle1* and *PvOle2* had a cytosolic N-terminal and C-terminal domains with two transmembrane helices. Hydrophobicity prediction plots indicated a wide region of positive values for both PvOle1 and PvOle2 (Fig. 2) and the hydrophobicity plots of PvOle1 and PvOle1 both resembled a typical oleosin protein for having a central long hydrophobic region of 75 and 73 amino acids respectively. These results indicated that the oleosins encoded by *PvOle1* and *PvOle2* presented the typical hydrophobic nature of oleosin proteins found in several diverse plants. Phylogenetic analysis revealed a close evolutionary relationship among plant oleosins (Fig. 3). The PvOle1 was nested with the *Populus trichocarpa* oleosin (PtOle-L), and the PvOle2 was nested with the castor bean oleosin (RcOle-L), indicating that the duplication of *PvOle1* and *PvOle2* might have occurred before the speciation of Sacha Inchi. In particular, two clades L-form and H-form were identified with the L-form clade clustering all L-oleosins tested, and the H-form clade clustering all H-oleosins.

Expression Analysis of *PvOle1* and *PvOle2*

Semi-quantitative RT-PCR expression analysis showed that both *PvOle1* and *PvOle2* exhibited similar seed-specific expression profiles, though they were weakly expressed in leaf tissue (Fig. 4a). The expression levels of both *PvOle1* and *PvOle2* were not detectable in root and flower tissues. Based on the band intensity levels measured using

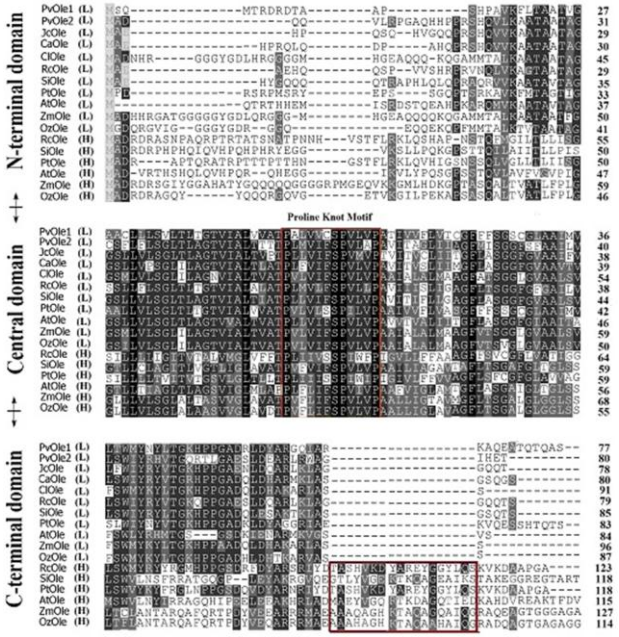

Fig. 1: Sequence alignment of PvOle1 and PvOle2 with other reported oleosin proteins. Functional "Proline Knot Motif" is marked. The amino acid number of the last residue for each oleosin sequence is denoted. Broken lines in the sequences show gaps introduced for the best alignment and conserved or semi-conserved residues are shaded. The 18-residue insertion in H-oleosins is marked. The L and H in the brackets denote L- and H-oleosins, respectively. The accession of the aligned oleosin sequences are: JcOle (*Jatropha curcas*, ABW90150), CaOle (*Corylus avellana*, AAO65960), ClOle (*Coix lacryma*, ACP27621), RcOle (*Ricinus communis*, XP002511014 for the L form and XP002511984 for the H form), SiOle (*Sesamum indicum*, AF091840 for the L form and AF302807 for H form), PtOle (*Populus trichocarpa*, XP002309527 for the L form and XP002317928 for the H form), AtOle (*Arabidopsis thaliana*, AAO22633 for the L form and AAF01542 for the H form), ZmOle (*Zea mays*, NP001105338 for the L form and P21641 for the H form), and OzOle (*Oryza sativa*, AAL40177 for the L form and AAC02240 for the H form)

Genetools 4.0 software, transcript levels of *PvOle2* were 2 fold higher than the *PvOle1*in developing seeds. Transcript levels of the two oleosins exhibited low expression during the early stage (2-DAF) of the seed development. Increased expression was observed during the mid-development stage (7-DAF). Subsequently, significant decrease in the transcript level was noticed during the late stage (13-DAF) of seed development (Fig. 4b, c) indicating their constitutive expression particularly in seeds. With full seed maturity the expression levels of both *PvOle1* and *PvOle2* further decreased. However, the decrease level was moderate when compared to the transcript level observed in the middle stage of seed development.

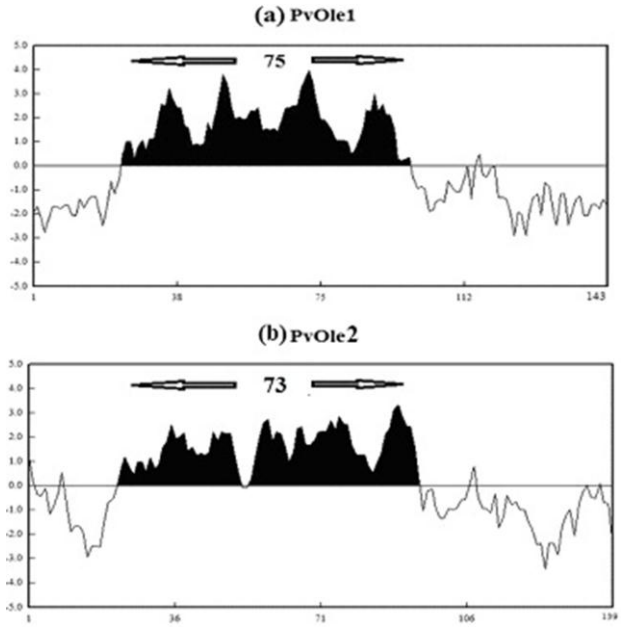

Fig. 2: Hydrophobicity analysis of PvOle1 (15.2 KDa) and PvOle2 (15.7 KDa) proteins. Hydrophobic values are indicated by the positive values (Shaded).The hydrophobicity analysis was performed using the Lasergene software (DNAMAN). The central long hydrophobic regions of 75 and 73 amino acids are shown in (a) for PvOle1 and (b) for PvOle2

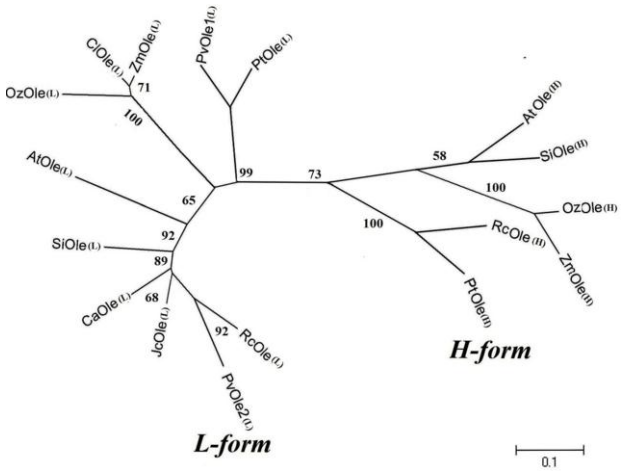

Fig. 3: A Phylogenetic tree based on Neighbour Joining criteria is shown. The numbers (>50) on the line denote the bootstrap values. The L-form and H-form represent the two major clades

Heterologous Functional Analysis in Yeast

In order to identify the recombinant proteins in transformed yeast cells SDS PAGE analysis was done. Appropriate bands for the tagged recombinant proteins of *PvOle1* with a molecular mass of 20.2 kDa (V5-epitope/His-tag [5 kDa] fused with 15.2 kDa PvOle1) and *PvOle2* with a molecular mass of 19.8 kDa (V5-epitope/His-tag [5 kDa] fused with 14.8 PvOle2) were identified on a 12% SDS PAGE gel (Fig. 5a). As for control, the protein extracts from transformed yeast with empty pYES 2.1 vector did not possess a proper band matching the exact size of the tagged recombinant proteins. Immunoblotting analysis using the anti-His-HRP conjugated antibody, further confirmed the expression of recombinant proteins of *PvOle1* and *PvOle2* (Fig. 5b) with strong signals detected, whereas no any signal was detected for the control products. These results clearly indicated the efficient expression of the two oleosin genes (*PvOle1* and *PvOle2*) encoding the oleosin proteins into the yeast lipid body complex.

Discussion

Oleosins, apart from playing an important role in the assembly and maintenance of oil bodies in oleaginous seeds are also exogenously involved in the fusion targeting of several vital proteins (Froissard *et al*., 2009). In relation to this, two oleosin genes *PvOle1* and *PvOle2* encoding oil body oleosin proteins in Sacha Inchi developing seeds were isolated in the present study. According to our transcriptome data developed from the Sacha Inchi developing seeds (data not shown), two oleosin genes (*PvOle1* and *PvOle2*) were primarily expressed in the developing seeds of Sacha Inchi. Both oleosin proteins encoded by *PvOle1* and *PvOle2* were grouped as L-form oleosins based on their molecular mass and the absence of an 18-residue insertional motif in the C-terminal domain of oleosins (Fig. 1). This absence of an 18-residue insertional motif was a prominent feature in L-form oleosins of diverse plants. Oleosins grouped as H-forms have higher molecular mass due to the additional inclusion of these insertional motifs. As mentioned above, two immunologically distinct oleosin isoforms H-oleosin and L-oleosin have been well-known to coexist in seed oil bodies of many angiosperms (Tai *et al*., 2002). However, when inspecting the distribution of L-oleosin and H-oleosin identified in angiosperms, we found that not all plants had both L-oleosin and H-oleosin. The H-oleosins were absent in seed oil bodies of many plants like *Vernicia fordii*, *Camellia oleifera*, *Citrus sinensis*, *Cocus nucifera* and *Prunus dulcis*. In contrast, the L-oleosins were present in all angiosperms reported (data not shown). In addition the copy number of these oleosin genes seemed to be variable in different plants with a majority of plants having single copies. Interestingly, earlier investigations have shown that the oil bodies reconstituted with the L-oleosin alone were more stable than those of H-oleosin or the combination of H-and L-oleosins (Tzen *et al*., 1992; Ting *et al*., 1997). However, a detailed investigation is needed to be done to know more about the biological importance of the divergence of L-oleosin and H-oleosin in different plants.

According to our sequence analysis results, PvOle1 and PvOle2 had a distinct divergence in their amino acid sequences. In particular, the proline knot motif required for

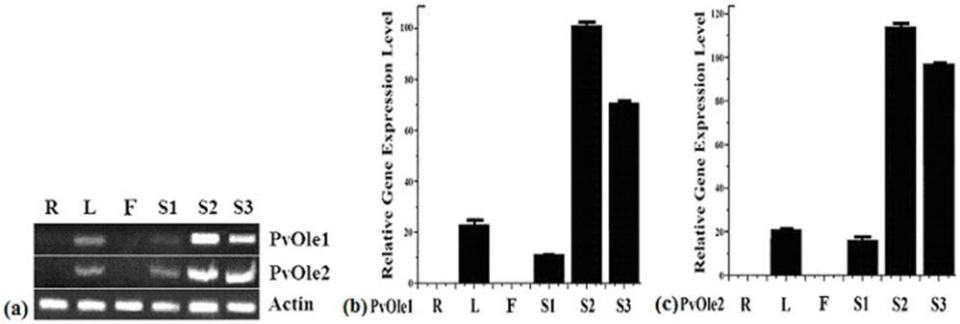

Fig. 4: RT-PCR analysis of *PvOle1* and *PvOle2* expression in various tissues and at the early, middle and late stages of the developing seeds. R denotes root, L denotes leaf, and F denotes flower. S1, S2 and S3 denote the early, middle and late stages of developing seeds. Amplification of *Actin* cDNA was used as a control. Identical results were obtained from three independent experiments. The densities of the bands were quantified by Genetools (Version 4.0) image analysis software and normalized using *Actin* expression. The error bars indicate the standard deviation (SD)

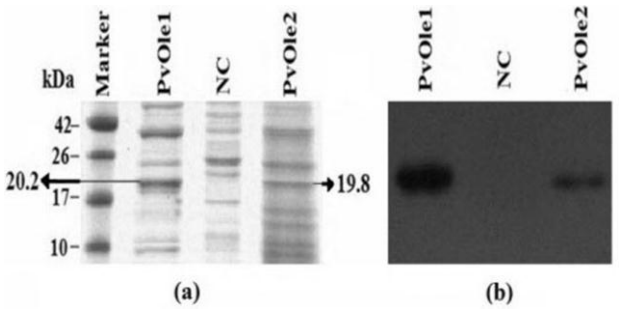

Fig. 5: SDS PAGE (a) and immunoblotting (b) of proteins of the crude extracts of yeast strains at late log phase of growth. Recombinant proteins PvOle1 and PvOle2 were identified with their molecular masses 20.2 kDa and 19.8 kDa (a) and Immunoblotting using the Anti-His (C-term)-HRP conjugated antibody. The strain transformed with the empty vector was used as a negative control (NC)

oil body targeting showed some conservative amino acid substitutions among the two oleosin sequences, which was relatively similar to the amino acid substitutions noted in *Theobroma cacao* (Guilloteau *et al*., 2003). Any functional significance associated with this difference is currently unknown. However, their transmembrane helices and the expression profiles among different tissues and at the developmental stages of seeds were very similar, strongly implying their functional convergence. Such functional similarities were also noted in oleosin isoforms previously isolated from coffee (Simkin *et al*., 2006) and *Theobroma cacao* (Guilloteau *et al*., 2003). The hydrophobocity plots of each PvOle1 and PvOle2 resembled a typical oleosin protein family and showed a relatively similar hydrophobic pattern except having a slight variation in the central core region of PvOle2 (Fig. 2). However, the exact significance of this variation needs to be elucidated. The expression profiles of *PvOle1* and *PvOle2* among different tissues and at the developmental stages of seeds were consistent with the accumulation of TAG in leaf and developing seeds (although only a small amount of predicted TAG was accumulated in leaf and in developing seeds at early stage) indicting their constitutive expression in developing seeds. Concurrent to our results, oleosin accumulation in non-lipid synthesizing tissues have been previously reported in *Arabidopsis*, rapeseed, tobacco and in moss *Physcomitrella patens* (Kim *et al*., 2002; Huang *et al*., 2009). However, the exact requirement of oleosin presence in leaf and their involvement in TAG accumulation needs to be studied. From our results, we conclude that the Sacha Inchi oleosin genes isolated showed a strong seed specific expression similar to oleosin expression in diverse oleaginous plants like rapeseed (Keddie *et al*., 1992), sunflower (Thyots *et al*., 1995) and sesame (Tai *et al*., 2002). Interestingly, two distinct clades of L-form and H-form were identified in the phylogenetic analysis, suggesting that the L and H-oleosins in angiosperms may have evolved independently before the speciation of several taxa. The assumption that H-oleosin derived from L-oleosin was duplicated independently within several species is unsupportive (Tai *et al*., 2002). Relatively, L-oleosin was found in both gymnosperms and angiosperms (Huang *et al*., 2009), while H-oleosin seemed to be found only in angiosperms. Thus, the duplication of H-oleosin during evolution might have occurred in an early phase of angiosperm differentiation. Due to a limited identification of oleosin genes in plants, the evolutionary history of the oleosin gene family remains uncertain.

The yeast *Saccharomyces cerevisiae*, as a heterologous eukaryotic system, has been extensively used to study the intracellular targeting of proteins to different organelles (EcKart and Bussineau, 1996; Martin and Parton, 2006). In contrast, yeast lipid body is devoid of oleosin proteins (Froissard *et al*., 2009). Previous studies have shown the effective targeting of oleosin proteins encoded by plant oleosin genes in to the oil bodies of yeast, *Saccharomyces cerevisiae* system (Ting *et al*., 1997; Beaudoin and Napier, 2002; Rajakumari *et al*., 2008; Froissard *et al*., 2009). In addition several reports have acclaimed the signal recognition particle dependent, co-translational insertion of

oleosin protein in to the ER membrane of the lipid bodies and also the importance of central hydrophobic regions in signal recognition dependent insertions (Tzen *et al*., 1992; Thoyts *et al*., 1995). Interestingly, yeast lipid complex is believed to recognize these intrinsic information present on the central hydrophobic region of plant oleosin protein and surprisingly no any structurally similar plant lipid body proteins have been reported in yeast lipid machinery till date (Rajakumari *et al*., 2008). Much importantly, production of commercially significant proteins with the usage of oleosin fusion targeting technology have been done without any biological complications (Nykiforuk *et al*., 2006; Banilas *et al*., 2011; Li *et al*., 2011). Thus, in view of the potential fusion recombinant application of these oil body proteins, our current study has elaborated the efficient targeting of the two plant oleosin genes (*PvOle1* and *PvOle2*) encoding the oleosin proteins in yeast using a V5/His tagged single-step detection methodology. This approach has allowed us to detect the recombinant proteins using antibodies recognizing the fused V5/His tagged epitope, which is comparably much easier and quicker than the normal heterologous (yeast) detection approaches. Based on these studies, it is presumed feasible to produce oleosin proteins using heterologous expression of Sacha Inchi oleosin genes in practice. However, further research is necessary to characterize the biochemical nature and optimize production of oleosins generated by transgenic *Saccharomyces cerevisiae* strains for developing yeast as an efficient biosynthesis system to generate plant oil body proteins.

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