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Research article

Identification of caleosin and two oleosin isoforms in oil bodies of pine megagametophytes☆

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ABSTRACT

Numerous oil bodies of 0.2–2 μm occupied approximately 80% of intracellular space in mature pine (*Pinus massoniana*) megagametophytes. They were stably isolated and found to comprise mostly triacylglycerols as examined by thin layer chromatography analysis and confirmed by both Nile red and BODIPY stainings. Fatty acids released from the triacylglycerols of pine oil bodies were mainly unsaturated, including linoleic acid (60%), adrenic acid (12.3%) and vaccenic acid (9.7%). Proteins extracted from pine oil bodies were subjected to immunological cross-recognition, and the results showed that three proteins of 28, 16 and 14 kDa were detected by antibodies against sesame seed caleosin, sesame oleosin-L and lily pollen oleosin-P, respectively. Complete cDNA fragments encoding these three pine oil-body proteins, tentatively named caleosin, oleosin-L and oleosin-G, were obtained by PCR cloning and further confirmed by mass spectrometric analysis. Consistently, phylogenetic tree analyses showed that pine caleosin was closely-related to the caleosin of cycad megagametophyte among known caleosin sequences. While pine oleosin-L was found clustered with seed oleosin isoforms of angiosperm species, oleosin-G was distinctively grouped with the oleosin-P of lily pollen. The oleosin-G identified in pine megagametophytes seems to represent a new class of seed oleosin isoform evolutionarily close to the pollen oleosin-P.

1. Introduction

The stored energy in plant tissues is occasionally preserved in the form of proteins, yet much more commonly in the form of carbohydrates or lipids. Plant cells deposit storage resources of carbohydrates, proteins and neutral lipids in subcellular particles termed starch granules, protein bodies and oil bodies, respectively. Oil bodies are intracellular organelles for storing neutral lipids, mainly triacylglycerols and sterol esters (Murphy, 2012). These lipid particles can be found in seeds (Murphy and Cummins, 1989; Huang et al., 2009) and pollens (Stanley and Linskens, 1974; Murphy et al., 2001) of angiosperms as well as in the tissues of more primitive plants, such as seeds of gymnosperms (Ching, 1970) and spores of ferns (Gemmrich, 1981). The average sizes of seed oil bodies in diverse species are 0.5–2.5 μm in diameter, and probably affected by nutritional status and environmental factors (Ting et al., 1996). An oil body is proposed to comprise a triacylglycerol matrix covered by a layer of phospholipids embedded with some unique proteins (Huang, 1996). The stability of oil bodies is a consequence of the steric hindrance and electronegative repulsion provided by their surface phospholipids and proteins (Tzen and Huang, 1992).

Abbreviations: BODIPY, boron-dipyrromethene; GC-MS, gas chromatography-mass spectrometry; TLC, thin layer chromatography.

☆ The nucleotide sequences reported in this paper have been submitted to the GenBank database with accession numbers KJ415240, KJ415241 and KJ415242 for pine caleosin oleosin-L and oleosin-G, respectively.

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Three classes of proteins termed oleosin, caleosin and steroleosin have been identified in oil bodies of angiosperm seeds (Tzen, 2012). Oleosin is an alkaline protein of relatively low molecular weight within the range of 10–24 kDa, and is characterized by a unique central domain of approximately 70 non-polar amino acid residues (Qu and Huang, 1990). Two distinct oleosin isoforms are present in seed oil bodies of diverse angiosperms, i.e., oleosin-H and oleosin-L, according to their relative molecular masses in each species (Tai et al., 2002). Moreover, a unique isoform, oleosin-P was found in pollen grains (Jiang et al., 2007), and a distinct class of oleosin-like proteins called oleo-pollenins were found in the tapetum and the external surfaces of pollen grains (Kim et al., 2002). Caleosin, with a calcium-binding motif and several potential phosphorylation sites, may be involved in the regulation of some biological functions related to the synthesis or degradation of oil bodies (Chen et al., 1999). It has been found in oil bodies of angiosperms and gymnosperms as well as in more primitive species such as algae and fungi (Næsted et al., 2000; Lin et al., 2012; Pasaribu et al., 2014). Steroleosin, firstly identified as a minor protein in sesame oil bodies, comprises an N-terminal lipid anchoring domain and a sterol-binding dehydrogenase that belongs to a super-family of pre-signal proteins involved in signal transduction (Lin et al., 2002). Two steroleosin isoforms with distinct sterol-binding sites were found and proposed to be regulated by distinct sterols to conduct different biological functions possibly related to the formation or degradation of seed oil bodies (Lin and Tzen, 2004).

While two oleosin isoforms (oleosin-H and oleosin-L) were found in oil bodies of angiosperm seeds, only oleosin-L was detected via immunological cross-recognition in the oil bodies isolated from megagametophytes of two gymnosperm species, pine (*Pinus koraiensis*) and ginkgo (*Ginkgo biloba*) (Wu et al., 1999). It has not been addressed whether any other class of oleosin isoform is present in oil bodies of pine or ginkgo besides the oleosin-L. In contrast, no oleosin was detected in the megagametophyte of a more primitive gymnosperm species, cycad (*Cycas revoluta*); however, caleosin of 27 kDa was identified as the major protein in cycad oil bodies (Jiang et al., 2009). Whether caleosin is also present in oil bodies of pine or ginkgo remains to be clarified.

In this study, oil bodies of pine megagametophytes were isolated for further investigation. For a better resolution, proteins extracted from pine oil bodies were resolved by Tricine-SDS-PAGE and subjected to immunological cross-recognition using antibodies against caleosin, oleosin-H, oleosin-L, and oleosin-P. One putative caleosin and two distinct oleosin isoforms were detected, and their corresponding cDNA fragments were cloned to deduce protein sequences for phylogenetic tree analyses with other known caleosin and oleosin sequences of diverse species.

2. Materials and methods

2.1. Plant materials

Mature and maturing megagametophytes of three pine species (*Pinus massoniana*, *Pinus morrisonicola* and *Pinus tabuliformis*) were supplied by local farmers. They were frozen immediately in liquid nitrogen and stored at −80 °C. Mature seeds of sesame (*Sesamum indicum* L.) were gifts from the Crop Improvement Department, Tainan District Agricultural Improvement Station. They were air dried and stored at 23 °C.

2.2. Tissue preparation for transmission electron microscopy

To examine the abundance of intracellular oil bodies, megagametophytes of pine (*P. massoniana*) were fixed in 100 mM sodium phosphate, pH 7.3, containing 2.5% glutaraldehyde, 2% paraformaldehyde and 5% sucrose at 4 °C for 2.5 h. After rinsed with100 mM sodium phosphate at 4 °C, samples were post-fixed with 1% OsO_4 in 50 mM sodium phosphate, pH 7.3 at 4 °C for 1 h, and then washed with 100 mM sodium phosphate buffer for three times (15 min for each wash). The samples were dehydrated by a graded ethanol series (50, 70, 80, 90, 95 and 100%) before embedding in LR white Resin. Thin sections (70 nm) cut by a Leica Reichert Ultracut R were collected on nickel grids, post-stained with 2.5% uranyl acetate and 0.4% lead citrate, rinsed three times with water, and viewed on a JEM-1400 transmission electron microscope (JEOL, Japan).

2.3. Purification of oil bodies

Sesame oil bodies were extracted from mature seeds, and then subjected to further purification including two-layer flotation by centrifugation, detergent washing, ionic elution, treatment of chaotropic agent, and integrity testing with hexane (Tzen et al., 1997). Pollen oil bodies were isolated from lily (*L. longiflorum Thunb.* cv. Snow Queen) as described previously (Qu and Huang, 1990). Pine oil bodies were purified in a simplified protocol without treatment of chaotropic agent. In this protocol, non-specifically associated proteins were mostly washed away from pine oil bodies by detergent (0.1% Tween 20) and ionic elution with high salt concentration (2 M NaCl). Oil bodies on the top were collected and re-suspended in the grinding medium containing 0.6 M sucrose and 10 mM sodium phosphate buffer, pH 7.5 to give a concentration of about 100 mg lipid/mL.

2.4. Analysis of neutral lipids in oil bodies

Neutral lipids in the oil bodies isolated from pine megagametophytes and sesame seeds were extracted and subjected to the analysis of thin layer chromatography (TLC) according to the method described previously (Jiang et al., 2007). TLC was first developed to the $R_f = 1$ position in hexane. The plate was air-dried and then developed to the top ($R_f = 1$) in benzene. The plate was air-dried and then developed to the top ($R_f = 0.5$) in hexane: diethyl ether: acetic acid (70:30:1 v/v/v). The lipid visualization on TLC plates was performed by staining with 0.03% Coomassie blue R 250 (Sigma, USA) dissolved in 20% methanol containing 0.5% acetic acid (Abe, 1998).

2.5. Fluorescent microscopy

Stock solutions of 1.57 mM Nile red (Sigma, USA) and 3.82 mM BODIPY 493/503 (Molecular Probes, USA) were prepared in acetone and ethanol, respectively. Purified oil bodies were stained in 7.85 μM Nile red solution, or 38.2 μM BODIPY 493/503, both diluted from the stock solution with the grinding buffer, in the dark for 20 min at room temperature. The stained oil bodies were visualized by Axioskop 2 Plus microscope (Zeiss, Germany) equipped with a charge-coupled device (CCD) camera (Coolsnap-Prock, Photometrics Ltd., USA).

2.6. Fatty acid analysis

Triacylglycerols of pine oil bodies resolved on TLC plates were first identified by 0.001% primuline spraying (in 80% acetone), and then extracted according to the protocol described by Bligh and Dyer (Bligh and Dyer, 1959). Isolated triacylglycerols were saponified in 1 N sodium hydroxide-methanol solution for 15 min at 80 °C. The fatty acids were esterified in 14% boron trifluoride-methanol solution for 15 min at 100 °C. After hexane extraction, the fatty