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Research article

Identification of caleosin and oleosin in oil bodies of pine pollen☆

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ABSTRACT

Unique proteins including steroleosin, caleosin, oleosin-L, and oleosin-G have been identified in seed oil bodies of pine (*Pinus massoniana*). In this study, mature pollen grains with wing-like bladders were collected from pine (*Pinus elliottii*). Ultrastructural studies showed that oil bodies were present in pollen grains, but not the attached bladders, and the presence of oil bodies was further confirmed by fluorescent staining with BODIPY 493/503. Stable oil bodies were successfully purified from pine pollen grains, and analyzed to be mainly composed of triacylglycerols. Putative oleosin and caleosin in pine pollen oil bodies were detected by immunoassaying with antibodies against sesame seed caleosin and lily pollen oleosin. Complete cDNA fragments encoding these two pollen oil-body proteins were obtained by PCR cloning. Sequence alignment showed that pine pollen caleosin (27 kDa) was highly homologous to pine seed caleosin (28 kDa) except for the lack of an appendix of eight residues at the C-terminus in accord with the 1 kDa difference in their molecular masses. Pine pollen oleosin (15 kDa) was highly homologous to pine seed oleosin-G (14 kDa) except for an insertion of eight residues at the N-terminus in accord with the 1 kDa difference in their molecular masses.

1. Introduction

Many organisms, including plants, store a large amount of neutral lipids as nutrient source (Davis and Vance, 1996). The lipid particles are found in seeds, pollens, and tapetum of higher plants, seeds of gymnosperms, spores of ferns, fungi, and algae (Gemmrich, 1981; Murphy and Cummins, 1989; Huang et al., 2009). These storage lipids are confined to discrete spherical organelles called oil bodies, with an average size of 0.5–2.5 μm (diameter), in diverse species (Ting et al., 1996). An oil body is mainly composed of triacylglycerols and sterol esters, which are surrounded by phospholipids containing some embedded integral proteins (Lin and Tzen, 2004). During the long storage period in plants, oil bodies are stable due to the shelter of integral proteins that provide steric hindrance and electronegative repulsion (Jiang et al., 2009).

Stable oil bodies isolated from plant seeds contain proteins of 0.6–3% (Tzen et al., 1993). To date, three classes of integral proteins namely, oleosin, caleosin and steroleosin have been identified in diverse seed oil bodies (Tzen, 2012). Oleosin is a relatively small protein of 15–25 kDa, and is characterized by a conserved central domain of approximately 70 uninterrupted non-polar amino acid residues (Qu and Huang, 1990). Two distinct oleosin isoforms are present in seed oil bodies of diverse angiosperm species, i.e., oleosin-H and oleosin-L (Tai et al., 2002). A unique oleosin isoform, oleosin-G, is present in gymnosperm (Pasaribu et al., 2014), and is phylogenetically close to oleosin-P found in pollen (Jiang et al., 2007). Oleosin-like proteins are also located in the tapetum and the external surface of pollen grains (Kim et al., 2002). Caleosin contains an N-terminal hydrophilic domain with a single Ca^{2+}-binding EF hand motif, a central hydrophobic region, and a C-terminal hydrophilic domain (Chen et al., 1999). It is present in oil bodies of diverse plant species as well as in primitive species such

Abbreviations: BODIPY, boron-dipyrromethene.

☆ The nucleotide sequences reported in this paper have been submitted to the GenBank database with accession numbers KX688795 and KX688796 for caleosin and oleosin of pine pollen, respectively.

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as algae (Lin et al., 2012). Caleosin is shown to possess a peroxygenase activity, and is proposed to play a role in phytoxilipin metabolism (Hanano et al., 2006). It was also reported that caleosin was resembled in the oil bodies during germination of olive pollen tube (Zienkiewicz et al., 2010). Steroleosin comprises an N-terminal lipid anchoring segment and a sterol-binding dehydrogenase domain that belongs to a super-family of pre-signal proteins involved in signal transduction (Lin et al., 2002). It is found in seed oil bodies of gymnosperm as well as those of angiosperm, and is demonstrated to possess sterol-coupling dehydrogenase activity that may be involved in the formation or degradation of oil bodies (Pasaribu et al., 2016). In addition, different classes of oil-body associated proteins were recently reported, such as lipid droplet-associated proteins (LDAP) in avocado mesocarp (Horn et al., 2013) and major lipid droplet protein (MLDP) in unicellular green algae, including *Chlamydomonas reinhardtii*, *Haematococcus pluvialis*, and *Dunaliella* sp. (Moellering and Benning, 2010; Nguyen et al., 2011; Peled et al., 2011; Davidi et al., 2012).

Oleosin and caleosin have been identified in oil bodies of angiosperm pollen (Jiang et al., 2008). Whether the same or different oil-body proteins are present in oil bodies of gymnosperm pollen has not been addressed so far. A comprehensive analysis of oil-body proteins in gymnosperm pollen should scientifically advance our knowledge for the understanding of oil-body proteins in evolution. In this study, we aimed to isolate oil bodies from pollen grains of pine (*Pinus elliottii*), and to examine integral proteins in these lipid storage organelles. One caleosin and one oleosin were detected in oil bodies of pine pollen, 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. Results

2.1. Morphology of pine pollen grains

Mature pollen grains attached with two wing-like bladders were collected from microsporangia of pine (*Pinus elliottii*) (Fig. 1). The two wing-like bladders were presumably filled with air, leading to the dispersion of pollen grains over long distance via wind pollination. The presence of oil bodies in pollen grains as well as in germinating pollen tubes was confirmed by fluorescent staining with BODIPY 493/503 (Fig. 2). Moreover, the BODIPY staining showed that oil bodies were located in mature pollen grains as well as in germinating pollen tubes, but not in the attached wing-like bladders.

2.2. Two-step isolation of oil bodies from mature pine pollen grains

In the isolation of pollen oil bodies, the wing-like bladders detached from pollen grains were also inclined to float on the top after centrifugation, and the co-flotation led to severe contamination in the isolated oil body fraction. To eliminate this contamination, pollen oil bodies were isolated in a two-step process (Fig. 3). In the first step, pollen grains were gently homogenized to break the wings, and then subjected to centrifugation (Fig. 3A). After centrifugation, the pollen wings were found on the top (Fig. 3B) and the pollen grains without wings were pelleted at the bottom (Fig. 3C). In the second step, the pollen grains in the pellet were collected, broken in liquid nitrogen, and then subjected to centrifugation again (Fig. 3D). After centrifugation, putative pollen oil bodies on the top were harvested and examined in light microscopy (Fig. 3E). Similar to those in pine and sesame seed oil bodies, the neutral lipids in pine pollen oil bodies were mainly triacylglycerols as examined by the thin layer chromatography (TLC) analysis (Fig. 4A). The presence of lipids in the isolated pollen oil bodies was also confirmed by BODIPY staining (Fig. 4B and C). Fatty acids released from pine pollen grains and pollen oil bodies were analyzed by gas chromatography-mass spectrometry (GC-MS). The major fatty acid in pine pollen grains was palmitic acid (63.7%), and that in pine pollen oil bodies was stearic acid (54.7%) (Table 1).

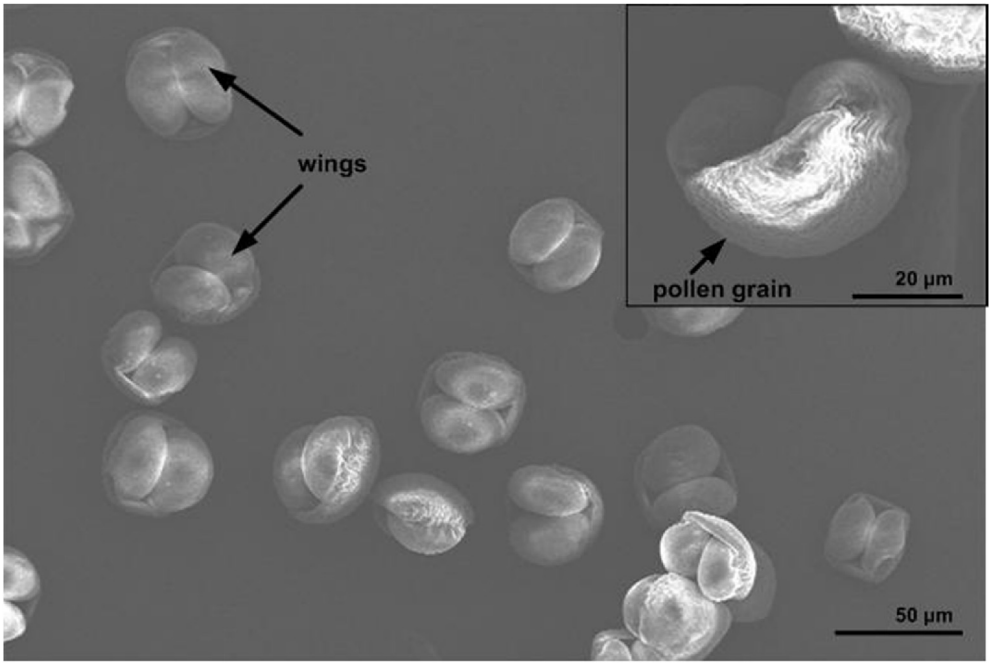

Fig. 1. Scanning electron microscopy of mature pine pollen grains. Mature pine pollen grains were observed in scanning electron microscopy.