Running Head: Plant Lipid Droplet Associated Proteins-LDAPs

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Identification of a New Class of Lipid Droplet-Associated Proteins in Plants

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One-sentence Summary: A new class of lipid droplet-associated proteins in non-seed tissues is identified by integrated omics approaches.
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ABSTRACT

Lipid droplets in plants (also known as oil bodies, lipid bodies or oleosomes) are well characterized in seeds, and oleosins, the major proteins associated with their surface, were shown to be important for stabilizing lipid droplets during seed desiccation and rehydration. However, lipid droplets occur in essentially all plant cell types, many of which may not require oleosin-mediated stabilization. The proteins associated with the surface of non-seed lipid droplets, which are likely to influence the formation, stability and turnover of this compartment, remain to be elucidated. Here, we have combined lipidomic, proteomic and transcriptomic studies of avocado (*Persea americana* L.) mesocarp to identify two new lipid droplet-associated proteins, which we named LDAP1 and LDAP2. These proteins are highly similar to each other and also to the small rubber particle proteins (SRPPs) that accumulate in rubber-producing plants. An Arabidopsis homolog to LDAP1 and LDAP2, At3g05500, was localized to the surface of lipid droplets after transient expression in tobacco cells that were induced to accumulate triacylglycerols. We propose that SRPP-like proteins are involved in the general process of binding and perhaps the stabilization of lipid-rich particles in the cytosol of plant cells and that the avocado and Arabidopsis protein members reveal a new aspect of cellular machinery that is involved in the packaging of triacylglycerols in plant tissues.
INTRODUCTION

Lipid droplets are subcellular organelles found in essentially all eukaryotic organisms (Chapman et al., 2012; Murphy, 2012). Their role in sequestering neutral lipids within the cytosol of cells has led to the concept that lipid droplet compartments serve as a stable depot for the temporary and efficient storage of high-energy carbon reserves. However, in the last decade it has become clear that while there are specialized lipid storing tissues in many multicellular organisms, including the adipose tissues of mammals and seed tissues of higher plants, essentially all cell types have the capacity to synthesize and store triacylglycerols in lipid droplets, even if they are dynamic and short-lived (Chapman and Ohlrogge, 2012; Murphy, 2012; Walther and Farese, 2012). In addition to an increasing appreciation for the prevalence of lipid droplets throughout biological systems, a broader range of functions associated with this hydrophobic compartment, beyond energy and carbon storage, has been reported, including lipid signaling (Bozza et al., 2011; van der Schoot et al., 2011; Chapman et al., 2012; Zechner et al., 2012), trafficking of intracellular components (Goodman, 2008; Murphy et al., 2009), and host-pathogen interactions (Saka and Valdivia, 2012).

To understand the diverse functions of lipid droplets, an array of proteins has been identified that are associated with lipid droplets from different cell types and organisms (Cermelli et al., 2006; Hodges and Wu, 2010; Yang et al., 2012; Jolivet et al., 2013). In higher plants, oleosins, caleosins, sterolesins, and other seed-specific lipid droplet proteins have been widely recognized for their role in lipid compartmentalization in oilseeds and some floral tissues, such as anther and pollen (Huang, 1992; Tzen and Huang, 1992; Frandsen et al., 2001; Lin et al., 2005; Huang et al., 2013). While an important function of oleosins in lipid droplets of developing seeds is to stabilize the droplets and prevent fusion during seed desiccation and rehydration (Leprince et al., 1998; Siloto et al., 2006; Schmidt and Herman, 2008; Shimada et al., 2008), there remains a general lack of information about other lipid droplet proteins in plants. For example, despite the widespread occurrence of cytosolic lipid droplets in various plant organs, the proteins associated with lipid droplets from vegetative tissues remain largely unexplored (Chapman and Ohlrogge, 2012; Murphy, 2012). We hypothesize that lipid droplets in non-seed tissues represent a compartment with protein compositions and functions different than oleosin-coated lipid droplets found in seed tissues. Indeed, oleosin isoforms are highly expressed in seed tissues during lipid accumulation and in some floral tissues (anther and
pollen), but are generally absent from most vegetative and fruit tissues of plants including the oil-rich mesocarp of oil palm, olive and other fruits (Murphy, 2012; Umate, 2012; Huang et al., 2013).

To better understand the subcellular proteins and mechanisms potentially involved in lipid droplet formation, stability and/or turnover in non-seed plant tissues, we examined the oil rich mesocarp of avocado (*Persea americana* L. cv Haas). Lipid-rich avocado fruit tissues have long been used as a model system for biochemical studies of lipid synthesis (*e.g.*, Harwood and Stumpf, 1972). Furthermore, the anatomical descriptions of the fruit tissues have emphasized the prevalence of numerous lipid droplets in parenchyma cells and specialized idioblast cells throughout the mesocarp tissue of the fruit (Cummings and Schroeder, 1942; Platt-Aloia and Thomson, 1981). Here, we used a combination of lipidomic, proteomic, and transcriptomic approaches to identify a new class of lipid droplet-associated proteins (LDAPs) in plants. Visualization of triacylglycerols in tissue prints of avocado fruit by mass spectrometry imaging was used to select mesocarp regions for lipid droplet analysis, and for comparisons of lipid compositions of isolated lipid droplets to that of whole fruit. Multidimensional protein identification technology (MudPIT) (Delahunty and Yates, 2007) was used to determine the protein composition of isolated avocado lipid droplets and the *in silico* translated transcriptome of avocado mesocarp provided a peptide database for protein identification. Transcriptional profiling of developing avocado mesocarp also revealed that the mRNAs for several of the most abundant proteins were highest during fruit maturation and lipid accumulation. Two of these proteins, which we term lipid droplet associated protein-1 and -2 (LDAP1 and LDAP2), exhibited homology to each other and to the small rubber particle proteins (SRPPs) (Oh et al., 1999) of rubber-accumulating plants. The *Arabidopsis thaliana* homolog (At3g05500) of these avocado LDAP proteins, when transiently expressed in tobacco suspension-cultured cells, was found to associate specifically with the lipid droplet surface. Collectively, using this multi-omics approach, we identified a novel class of lipid droplet proteins in non-seed plant tissues, an important step in further characterization the subcellular machinery involved in general lipid droplet ontogeny and stability in plants.
RESULTS

Imaging Lipids in Avocado Mesocarp

To characterize the lipid content and composition of avocado mesocarp tissues, the distribution of triacylglycerol (TAG) molecular species in avocado tissue slices was visualized using a novel, matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI- MSI) approach (Horn et al., 2012) (Fig. 1, Supplemental Table S1). Lipids were visualized by imprinting a slice of mature avocado fruit onto a nitrocellulose membrane, which was subsequently coated with ionization matrix. Step-wise rastering of the laser allowed reconstruction of a high-resolution, two-dimensional map of mesocarp lipids. The mesocarp is especially rich in monounsaturated fatty acids (mostly oleic acid, 18:1), and the molecular species of triacylglycerols with two oleic acids and one palmitic acid, TAG-52:2 (POO) at m/z 881.757, or with oleic acid at each position, TAG 54:3 (OOO) at m/z 907.773, were most prevalent in fruit tissue and were distributed throughout the mesocarp (Fig. 1D). Triacylglycerol species were distributed relatively uniformly throughout the fruit tissue, except for the species with oleic acid at each position, TAG-54:3 (OOO), which was relatively more abundant in certain regions of the mesocarp. Because this TAG-54:3 appeared to be the only molecular species with a marked heterogeneous distribution pattern, this heterogeneity is likely a real phenomenon, and, although the physiological relevance is unclear at this point, it suggests that lipid droplets isolated from different parts of the fruit may have a slightly different triacylglycerol composition, at least for TAG-54:3. For biochemical isolation of lipid droplets (described further below), we excised the central region of the mesocarp, avoiding the chloroplast-containing tissues at the mesocarp periphery. The MS maps of individual triacylglycerol species (Fig. 1) indicated that this region should indeed provide an abundant and representative source of lipid droplets for protein and lipid compositional analysis.

Characterization of Isolated Avocado Lipid Droplets

Lipid droplets are relatively simple to isolate from mixtures of most other subcellular components because they are the least dense of all the subcellular organelles, and unlike any other subcellular component, they float in aqueous solutions (Murphy, 2012). Lipid droplets were isolated from avocado mesocarp and enriched by flotation centrifugation. Then they were examined for relative purity by differential interference contrast (DIC) and fluorescence...
microscopy (Fig. 2; BODIPY 493/503 stains neutral lipids specifically (Listenberger et al., 2007)). By comparing DIC and BODIPY 493/503 fluorescence, we established that particles in the isolated lipid droplet fractions were BODIPY-positive and generally free from obvious visible contaminants (Fig. 2A and B). Of course purity is relative, and it was clear from the sensitive proteomics analysis (described below) that other proteins or subcellular materials were likely adhered to or trapped within this fraction as well. Nonetheless, quantitative analysis of the triacylglycerol present in various biochemical fractions confirmed that essentially all of the fractionated triacylglycerols were associated with the lipid droplet fractions and not with the cytosolic or microsomal fractions (Fig. 2C, D). The distribution of triacylglycerol content in the various fractions isolated was similar irrespective of the developmental stage of the fruit tissue used; pre-harvest fruits were obtained directly from trees (breeder, Fig. 2D) and post-harvest fruits were purchased from a local market (Fig. 2C, market). Both sources of avocados were used for most experiments, with the exception of transcriptomics analysis.

Lipid droplets have a simple structural organization; typically, they contain a triacylglycerol core covered by a monolayer of phospholipids and associated proteins (Huang, 1992; Chapman et al., 2012; Murphy, 2012). We analyzed the triacylglycerols of mesocarp lipid droplets isolated from pre- and post-harvest fruits by direct infusion-electrospray ionization mass spectrometry (ESI-MS) (Fig. 2E). The triacylglycerol composition of the postharvest tissue slices, determined by MALDI-MSI closely matched compositions quantified by electrospray ionization (ESI)-MS in total lipid extracts of the tissue homogenates and isolated lipid droplets (Fig. 2E). Differences between triacylglycerol composition of lipid droplets isolated from pre- and post-harvest samples likely reflect variation in age, growth conditions, maturity and/or post-harvest treatments (Fig. 2E). Generally, the triacylglycerols in the homogenates and lipid droplet fractions from the pre-harvest fruits were lower in triacylglycerol species with oleic acid and higher in species with linoleic acid, the two fatty acids that change markedly during fruit development and ripening (Eaks, 1990).

Proteomics of Lipid Droplets and Peptide Identification from Mesocarp Transcriptome

The protein compositions of lipid droplets isolated from pre- and post-harvest avocado fruits were determined using MudPIT analysis. Specifically, individual peptide sequences were identified by comparing the obtained masses of the peptides to a reference polypeptide database
derived from Illumina- and 454-based sequencing of cDNA from developing mesocarp of avocado. Annotated lists of matched proteins were compiled and rank-ordered by relative abundance (based on spectral counts) within each subcellular fraction. Percent coverage, representing the portion of an encoded polypeptide sequence matched by independent peptide sequences, was calculated for all proteins. The Arabidopsis protein with highest sequence similarity to each avocado protein was determined using BLASTX.

Comparison of proteins identified in lipid droplet fractions isolated from either post-harvest or direct, tree-harvested avocado fruits revealed a common core list of 70 proteins (Supplemental File 1). Further comparison of these 70 proteins with proteins identified in corresponding microsomal or cytosolic fractions revealed proteins that were specifically enriched in isolated fractions (Supplemental File 1, sheet two). Proteins were considered “enriched” if they were present in more than three-fold abundance (spectral counts) in a given subcellular fraction in comparison to other subcellular fractions. Based on these criteria, 17 proteins were enriched in lipid droplet fractions relative to microsomal or cytosolic fractions. A table summarizing the results for these 17 proteins from one representative analysis is provided in Supplemental Table S2. LDAP 1 and LDAP 2 were first and fourth most abundant proteins, respectively, in lipid droplets based on spectral counts.

**Lipid Droplet-Associated Proteins 1 and 2**

Transcript expression profiles were determined for several proteins identified in the avocado lipid droplet fractions (Fig. 3). Based on RNA-Seq data from five stages of developing mesocarp (130 to 212 g fruit weight), transcript levels for the two lipid droplet-associated proteins, LDAP1 and LDAP2, were highest during the mid-stages of fruit development (~ 160 g of fresh weight) when oil accumulation was greatest (Eaks, 1990). For comparison, the gene expression profile and transcript levels for the two LDAPs were similar to that of ketoacyl-acyl carrier protein synthase II (KASII) (Fig. 3), a plastidial enzyme involved in fatty acid biosynthesis (Carlsson et al., 2002). One protein recovered in lipid droplet fractions, annotated as oxidoreductase, also was expressed at high levels in mesocarp at mid stages of development (not shown), but the closest Arabidopsis homolog At4g13010 was shown previously to be associated with inner chloroplast envelopes (Miras et al., 2002), so this protein was not investigated further as a lipid droplet protein. Other proteins were enriched in avocado lipid
droplet fractions (Supplemental Table 2; e.g., HSP60, ATCYS1 (CS1)), but their transcript levels did not appear to change much with fruit development (Fig. 3), suggesting that these proteins likely were trapped or adhered to lipid droplets during isolation and were unlikely to be bona fide lipid droplet associated proteins.

The most intriguing proteins enriched in the lipid droplet fractions of avocado were LDAP1 and LDAP2 (first and fourth in the list, respectively; Supplemental Table 2). These two proteins share 62% identity (86% similarity) at the deduced amino acid level and show extensive regions of similarity and identity with the small rubber particle protein (SRRP) of the rubber tree *Hevea brasiliensis* (Oh et al., 1999) and the SRPP-like protein At3g05500 of unknown function from Arabidopsis (Fig. 4). Given the level of polypeptide sequence identity, and the general similarity of rubber particles and lipid droplets in the storage of hydrophobic compounds in plant cells, we hypothesized that avocado LDAP1, LDAP2 and Arabidopsis At3g05500 might similarly associate with and stabilize triacylglycerol-containing lipid droplets analogous to how SRPPs bind to the surface and stabilize polyisoprenoid-containing rubber particles (Collins-Silva et al., 2012; Hillebrand et al., 2012).

To determine whether the Arabidopsis At3g05500 protein could associate with lipid droplets in plant cells, the protein was tagged at its C terminus with the mCherry fluorescent reporter protein and expressed transiently in tobacco suspension-cultured cells, which are a well-characterized model plant cell system for studying protein localization (Brandizzi et al., 2003; Miao and Jiang, 2007). Notably, these cells typically do not contain high numbers of lipid droplets, but lipid droplet formation can be induced by addition of free fatty acids to the growth media (Fig. 5). Expression of the At3g05500-mCherry fusion protein in BY-2 cells cultivated in normal growth media lacking exogenous fatty acids resulted in a predominantly cytosolic localization, with only a few BODIPY-stained lipid droplets present in these cells (Fig. 5; top row). By contrast, incubation of BY-2 cells with linoleic acid after transient transformation resulted in a significant increase in lipid droplet abundance, with the majority of At3g05500-mCherry localized to these BODIPY-stained organelles (Fig. 5, middle row). Upon closer inspection, the fluorescence attributable to the fusion protein was often torus-shaped and enclosed the punctate fluorescent signal attributable to the BODIPY-stained lipid droplets, suggesting that the fusion protein was localized to the surface of the lipid droplets (Fig. 5, bottom row).
row). These data confirmed that the SRPP-like protein of Arabidopsis, At3g05500, can indeed localize specifically to lipid droplets in non-seed plant cell types.

**DISCUSSION**

To date, proteomes of lipid droplets from plant sources (e.g., *Brassica*, Arabidopsis, sesame, *Jatropha*) have focused almost entirely on seed-derived organelles and have been dominated by oleosins, caleosins, sterolesins, and only a few other proteins (Jolivet et al., 2004; Lin et al., 2005; Katavic et al., 2006; Jolivet et al., 2009; Popluechai et al., 2011). Here we took an alternative approach to identify additional lipid droplet-associated proteins by analyzing the proteome of lipid droplets from non-seed tissues. We asked whether there are specialized proteins associated with non-seed lipid droplets or, alternatively, whether the proteins identified would overlap with those already identified in seed lipid droplets. This approach is less likely to be overwhelmed by the well-known, hyper-abundant proteins known to associate with lipid droplets in seeds (e.g., oleosins) and provides scope for identification of additional proteins. These additional proteins may represent a more universal set of proteins for lipid compartmentalization in plant cells and offer new avenues for probing the regulation of triacylglycerol accumulation in vegetative tissues of plants. Avocado fruit tissue was selected as a source for lipid droplets for this study for several reasons: 1) avocado mesocarp is readily available and well known to be rich source of lipid droplets (Cummings and Schroeder, 1942; Platt-Aloia and Thomson, 1981); 2) oleosins generally are not expressed outside of seed tissues (Chapman and Ohlrogge, 2012; Murphy, 2012; Huang et al., 2013) and thus were not expected to be a major protein in avocado mesocarp; and 3) deep sequencing analysis of the avocado mesocarp transcriptome provided a reference database for identification of proteins by peptide mass fingerprinting.

Although lipid droplets are easily fractionated from tissues by flotation centrifugation, they do have the potential to carry a variety of adventitious subcellular components with them, such as hydrophobic proteins, membrane fragments, and other lipid soluble compounds that perhaps were not associated with this compartment *in situ*. Obviously there were a number of proteins identified in isolated lipid droplet fractions that are most likely not associated with lipid droplets *in planta*. Vigorous purification techniques such as high-salt washes may be able to partially remove such ‘contaminants’, but also may remove proteins intrinsic to lipid droplets.
this study, our interest focused on discovering new lipid droplet-associated proteins. By avoiding proteins that were prevalent in other subcellular fractions, or that were not highly expressed in fruit tissues, our approach may have resulted in exclusion of some important proteins from consideration (or may have included proteins not actually found in lipid droplets). Nevertheless, we were successful in identifying a new class of lipid droplet-associated proteins not previously known to bind triacylglycerol-containing lipid droplets in plants. Specifically, we identified two homologs of SRPP-like proteins in avocado (i.e., LDAP1 and LDAP2) that were tightly associated with triacylglycerol-containing lipid droplets (Fig. 2; Supplemental Table 2), and whose gene expression patterns correlated with triacylglycerol accumulation during fruit development (Fig. 3). Similarly, in oil palm mesocarp which stores up to 90% oil by dry weight, transcript levels for an ortholog of LDAP1 and LDAP2 also were associated with the timing of oil accumulation during fruit development and were substantially higher in oil palm than in fruit tissue from date palm, which stores very little oil (Bourgis et al., 2011). Further, we demonstrated that an Arabidopsis protein (At3g05500) with closest amino acid sequence similarity to the avocado LDAP1 and LDAP2 proteins (Fig. 4) could also target to lipid droplets in non-seed plant cells (Fig. 5).

While publicly-available gene expression databases indicate that At3g05500 is expressed in all Arabidopsis tissues, expression levels for this gene are approximately eight-to-ten-fold higher in tissues with abundant lipid, including developing seeds, stamens and pollen (Schmid et al., 2005), consistent with a broad-based role for At3g05500 in lipid droplet ontogeny, structure or stability. While the three major oleosin isoforms in Arabidopsis (i.e., OLE1, OLE2, and OLE3) are expressed at levels more than 100-fold higher in developing seeds than in other tissues (Schmid et al., 2005), the broader gene expression pattern of At3g05500 suggests a more general function for At3g05500 in lipid droplet biogenesis rather than the major role proposed for oleosins in providing stability during seed desiccation/rehydration (Leprince et al., 1998; Siloto et al., 2006; Shimada et al., 2008). It is also notable that, like oleosins, the SRPP-like proteins appear to be plant specific, with the earliest SRPP-like genes present in algae, and with rapid expansion of the gene families during radiation of the angiosperms (Fig. 6 and Huang et al., 2013). No SRPP-like homologs were detected in other distantly-related organisms, such as yeast, Drosophila, mouse, or human.
The SRPP protein family is named for the association of these proteins with rubber particles in laticifer cells of plants that synthesize rubber, such as *Hevea brasiliensis*, guayule and Russian dandelion (Collins-Silva et al., 2012; Hillebrand et al., 2012). The SRPPs share homology with the rubber elongation factor and rubber allergen protein and there is evidence that SRPP isoforms may play a role in rubber synthesis (Collins-Silva et al., 2012). For instance, RNAi suppression of SRPPs in Russian dandelion caused a significant reduction in overall rubber production and rubber synthase activity in comparison to wild-type plants (Hillebrand et al., 2012). Furthermore, rubber particles isolated from the RNAi dandelion plants appeared to be less stable than those from wild-type plants, with a heterogeneity in size suggestive of rubber particle fusion (Hillebrand et al., 2012). It is presently unclear how the SRPP and rubber elongation factor proteins assemble on the rubber particle surface and influence the biosynthesis of polyisoprenoids from isopentyl pyrophosphate, but they apparently play an important role in stabilizing the surface of the rubber particle and help promote the synthesis of the hydrophobic components stored within.

The SRPP proteins, however, may not always be essential for rubber production in plants, and may have other functions in addition to stabilizing rubber particles. For instance, rubber particles isolated from *Ficus carica* and *Ficus benghalensis* did not contain SRPP proteins that cross-reacted with *H. brasiliensis* SRPP antisera, despite their ability to produce significant amounts of rubber (Singh et al., 2003). Russian dandelion, on the other hand, contains five SRPP-like genes, but only three of the encoded proteins have been detected in high amounts in purified rubber particles, and one of the genes shows a distinct organ and temporal gene expression pattern that is inconsistent with a role in rubber synthesis (Schmidt et al., 2010). A recent draft genome of *H. brasiliensis* (Rahman et al., 2013) indicates that there are seven SRPP genes in this genome (Fig. 6). It is possible, therefore, that the broader family of SRPP-like proteins that includes the SRPPs in rubber producing plants, rubber elongation factor proteins, avocado LDAP1/2, and the Arabidopsis SRPP-like protein (At3g05500), plays a more general role in the stability or formation of hydrophobic lipid particles in plant cells. It is also of interest to point out the similarity in the structure of triacylglycerol-containing lipid droplets and rubber particles (Schmidt et al., 2010), since both have a monolayer of phospholipid surrounding a hydrophobic core of reduced carbon. As such, the more widespread role of SRPP-like proteins might be for modulating the compartmentalization of triacylglycerols in cytosolic lipid droplets,
which essentially all cells must do, while those species of plants that synthesize rubber might have evolved a specialized use of SRPP for this purpose. Evidence in support of this premise comes from a phylogenetic analysis of the SRPP-like proteins from distantly related rubber producing and non-rubber-producing plants (Fig. 7), which shows that the SRPPs from rubber producing plants do not form a separate clade, but rather are interspersed with other SRPP-like proteins from non-rubber producing plants, such as Arabidopsis and avocado. Notably, most of the eudicots examined contained three SRPP-like sequences (Fig. 6 and Fig. 7), and these sequences were generally grouped into one of three different clades (Fig. 7, Groups I, II, and III), suggesting early diversification and perhaps functional specialization. The monocot and bryophyte/lycophyte sequences, on the other hand, formed distinct clades that were separate from these groups (Fig. 7, Groups IV and V, respectively).

Future experiments should focus on elucidating the functional role(s) of SRPP proteins in relation to lipid droplet formation/stability. Since the SRPP-like proteins appear to be mostly hydrophilic in nature, it will also be important to determine how these proteins associate with the lipid droplet surface; although recent studies of SRPP from *Hevea* have yielded some important insight to this process (Berthelot et al., 2012). Furthermore, it will be important to identify proteins, if any, that interact with the SRPPs, as this may provide an avenue into a better understanding of the process of lipid droplet biogenesis in plant cells. By extension, this information might offer clues about the general cellular processes required for efficient packaging of reduced carbon in lipid droplets, a process that will be increasingly important for many ongoing efforts to accumulate large quantities of energy-dense lipids in vegetative tissues of plants.

**MATERIALS AND METHODS**

**MALDI-MS Imaging**

Matrix-assisted laser desorption/ionization-mass spectrometry imaging (MALDI-MSI) of avocado tissue-prints was performed on a MALDI LTQ Orbitrap-XL (Thermo Fisher Scientific, Bremen, Germany) system in a manner similar to that described for analysis of cotton seed tissue sections (Horn et al., 2012) with some modifications. Briefly, avocado purchased from a local market was sliced in half and pressed for two minutes against a 0.45 micron pore size, Protran™ nitrocellulose membrane to generate a tissue print. The tissue print was cut into five pieces and
each piece adhered to a stainless steel slide with double-sided tape. The ionization matrix 2,5-
dihydroxybenzoic acid (DHB, 99%, Acros Organics) was sprayed onto tissue prints at 20 mg/ml
in 70% methanol using a SunChrom SunCollect MALDI spotter (Verhaert et al., 2010). Raw
data were acquired using Thermo Xcalibur (v.2.1.) in positive ionization mode with automatic
gain control (AGC) on, laser energy of 14 µJ and two successive scan events ($m/z$ 400-1200 at
60k resolution and $m/z$ 50-2000 at 30k resolution). Images of individual molecular species were
generated using Metabolite Imager application as described in (Horn et al., 2012), except with
constant PPM tolerance (+/- 10 PPM) for selected peaks identified as triacylglycerol molecular
species based on direct-infusion, shotgun lipidomics of total lipid extracts (see below).

**Lipid Droplet Isolation**

Lipid droplets were isolated from mesocarp tissues of avocado (*Persea americana* L., cv
Haas) purchased from a local market or harvested from the orchards at the University of
California, Riverside (kind gift of Dr. Mary Lu Arpaia). Lipid droplets were isolated from tissue
homogenates by flotation centrifugation similar to (Chapman and Trelease, 1991), with some
modifications. Excised mesocarp tissue (approximately 7.5 g) was finely chopped with a single-
edge razor blade on ice in a 1:2 (w/v) homogenization solution of 600 mM sucrose, 1 mM EDTA
and 100 mM KCl in 100 mM potassium phosphate buffer (pH 7.2). Homogenates were filtered
through four layers of Miracloth and centrifuged in a Sorvall HB-6 swinging bucket rotor at
13,000 \( \times g \) for 30 minutes at 4°C. The top 5 mL of the samples were removed using a Pasteur
pipette and stainless steel spatula and used to further purify lipid droplets. The remaining
supernatant was used as a source for cytosolic and microsomal fractions. Lipid droplets were
purified by two additional flotations through 15 mL of the homogenization solution containing
400 mM sucrose. Cytosol and microsomes were fractionated by ultracentrifugation at 150,000 \( \times g \)
(Beckman TI-75 rotor at 4°C in a Sorvall Discovery 90 ultracentrifuge). Proteins in isolated
fractions were precipitated in cold acetone (-20°C) overnight (at least 4:1, v/v). Parallel
fractionation experiments were carried out for lipidomics analysis with total lipids extracted from
each respective fraction (Horn et al., 2011).

**Lipid Analysis**
Total lipid extracts (TLE) were spiked with the internal standard tripentadecanoin (Tri15:0-triacylglycerol, Nu-Chek Prep) for quantification. Aliquots of each extract were diluted into an infusion solution of 1:1 (v/v) chloroform : methanol plus 500 mM ammonium acetate. Samples were infused at flow rates of 7.5 µL/min into an electrospray ionization (ESI) source and analyzed on a Thermo TSQuantum triple quadrupole mass spectrometer. Typical instrument conditions for full scan mode included a 4 kV spray voltage, with 3 microscans per acquired scan, sheath gas of 25 arbitrary units, capillary temperature of 270°F and capillary offset of 35 V. Acyl chains within triacylglycerols were determined using a series of neutral loss scans for abundant fatty acids.

**Multidimensional Protein Identification Technology (MudPIT) Proteomics**

Acetone-precipitated proteins were dissolved in 2X SDS-PAGE sample buffer and heated at 70°C for 15 min to resolubilize proteins before loading on freshly prepared SDS-polyacrylamide vertical slab gels (12% resolving gel). Protein samples were electrophoresed just past the stacking/resolving gel interface. Gels were stained with Coomassie blue R-250 to visualize proteins. Protein bands were excised from the separating gel and stored in 10% acetic acid until proteomics analysis at the Michigan State University Proteomics core facility.

Proteins were trypsin-digested in-gel (Shevchenko et al., 1996) and peptides were purified by solid phase extraction (Waters nanoAcquity UPLC system; Waters Symmetry C18 peptide trap; 5micron, 180micron x 20mm). Eluted peptides were sprayed directly into a Thermo Fisher LTQ-FT Ultra MS and survey scans were taken in the FT mode (25000 resolution determined at m/z 400) with the top ten ions in each survey scan subjected to collision induced dissociation (CID). MS/MS spectra were analyzed by BioWorks Browser v3.3.1 (Thermo Fisher) and compared to the avocado clc4 protein sequence database using Mascot v 2.3. Scaffold v3.3.3 software was used to analyze and validate protein identifications with the ProteinProphet (Nesvizhskii et al., 2003) algorithm.

**Avocado Deep Sequencing and Informatics**

Avocado fruits (cv. Hass) were harvested monthly, from October, 2009 to February, 2010, from a tree (44-15-11 Hass Scion on D7 Clonal rootstock) located at University of California South Coast Research and Extension Center in Irvine, CA. Fruits were shipped
overnight at 4°C to Michigan State University. Fruits were weighed and mesocarp was isolated and flash frozen in liquid N2 and stored at -80°C until used.

Total RNA was extracted from 3 g mesocarp tissue that had been ground finely in liquid N2 and incubated for 10 min in 30 mL of TRIZol® reagent (Life technologies) and for an additional 5 min with 6 ml of CHCl3. After centrifugation at 12,000 X g for 15 min at 4°C, the aqueous phase was transferred to a fresh tube and incubated overnight with 1/3 volume of 8 M LiCl. Samples were then centrifuged at 12,000 X g for 30 min at 4°C and the pellet was resuspended in 1000 μL of RLT buffer of RNEasy kit and RNA was eluted following the manufacturer's protocol (Qiagen).

Transcriptome data for developing mesocarp was generated using 454 and Illumina sequencing techniques. For 454 sequencing, mRNA was isolated from total RNA using SeraMag Oligo(dT) Magnetic Beads (Thermo Scientific) and cDNA libraries were created using the Roche cDNA Rapid Library Prep Kit (Roche Diagnostics). Sequences were obtained on the Roche 454 GS FLX sequencer using the Titanium chemistry (Roche Diagnostics). For Illumina sequencing, libraries were created using an Illumina pre-release protocol for directional mRNA-seq library prep (v1.0). A single read 75 cycle run was then performed on the Illumina GAIIx sequencer, following manufacturers’ protocols. De novo assembly of reads obtained from 454 and Illumina sequencing was accomplished with Trinity RNA-Seq software V2. Contigs obtained were identified by BLASTX annotation against Arabidopsis TAIR 10 database. Finally, the coding sequences for proteins of interest were verified against genome sequencing data provided by the avocado genome sequencing group in Laboratorio de Servicios Genómicos' of Langebio, Cinvestav, Mexico.

Lipid Droplet Targeting of At3g05500

The plasmid pRTL2/AT3g05500-mCherry, encoding the full-length open reading frame (ORF) of At3g05500 fused to the N terminus of the monomeric cherry autofluorescent protein mCherry (Shaner et al., 2004), was constructed in the following manner. First, gene-specific forward and reverse primers with NheI overhangs (Fp 5’-CCGGCCGCCCTAGCATGGCTACTCAAAACGGATC-3’ Rp 5’-CCGGCCGCCCTAGCATCAAGTGGATGGAACTCC-3’) were used to amplify (via the polymerase chain reaction) the At3g05500 ORF from a cDNA library obtained from isolated
Arabidopsis suspension-cultured cell mRNA. The resulting PCR products were digested with NheI and ligated into NheI-digested pRTL2/mCherry, a plant expression vector containing the 35S cauliflower mosaic virus promoter, followed by a multiple cloning site and the mCherry ORF (Gidda et al., 2011).

Tobacco (Nicotiana tabacum cv. Bright Yellow-2 [BY-2]) suspension cell cultures were maintained and prepared for bombardment with pRTL2/At3g05500-mCherry plasmid DNA as described previously (Lingard et al., 2008), with the exception that cells were resuspended in BY-2 growth media rather than transformation buffer prior to bombardment. Induction of lipid droplets in BY-2 cells was carried out as follows: ~30 min after bombardment, cells were transferred to a culture flask containing BY-2 growth media, the cells were then maintained at regular growth conditions for 1 hr, and thereafter linoleic acid-albumin conjugate (Sigma) was added to the cell suspension at a final concentration of 150 µM. Approximately 4 h later the cells were fixed in 4% (w/v) formaldehyde and stained with BODIPY 493/503 (Molecular Probes) at a final concentration of 0.1 µg/mL.

Confocal laser-scanning microscopy (CLSM) images of BY-2 cells were acquired using a Leica DM RBE microscope with a Leica 63x Plan Apochromat oil-immersion objective, a Leica TCS SP2 scanning head, and the Leica TCS NT software package (Leica). Fluorophore emissions were collected sequentially in double-labelling experiments; single-labeling experiments showed no detectable crossover at the settings used for data collection. Confocal images were acquired as a z-series of representative cells and single optical sections were saved as 512 x 512-pixel digital images. All fluorescence images of cells shown in individual figures are representative of >50 independent (transient) transformations from at least two independent transformation experiments. Figure compositions were generated using Adobe Photoshop CS and Illustrator CS2 (Adobe Systems).

Accession numbers

Arabidopsis thaliana (AthSRPP-like, At3g05500; Ath2, At1g67360; Ath3, At2g47780), Havea brasiliensis (HbrSRPP, AJ223389), Parthenium argentatum (ParSRPP, AAQ11374), Persea americana (PamLDAP1; PamLDAP2), Physcomitrella patens (Ppa1, EDQ55949), Prunus persica (Ppe1, EMJ06970; Ppe2, EMJ02455; Ppe3, EMJ25061), Ricinus communis (Rco1, XP_002514917; Rco2, XP_002512427; Rco3, XP_002531884), Selaginella
moellendorfii (Smo1, XP_002969776), Setaria italica (Sit1, UniProt K3Z8Z8; Sit2, UniProt K3ZVR0; Sit3, UniProt K3ZW99), Solanum lycopersicum (Sly1, XP_004239210; Sly2, XP_004230235; Sly3, XP_004247432), Zea mays (Zma1, DAA41722; Zma2, AFW82611; Zma3, AFW82612). The full-length sequences of SRRP proteins from Russian dandelion (Taraxacum brevicorniculatum) were derived from Schmidt et al. (2010) and included TbrSRPP1 (DR401025), TbrSRPP2 (DR401208), TbrSRPP3 (DR400748), TbrSRPP4 (DR403071), and TbrSRPP5 (DR401554). Accession numbers for avocado LDAP1 and LDAP2 (Fig. 4) transcripts are KF031141 and KF031142 and their nucleotide sequences will be available upon publication of this manuscript.

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FIGURE LEGENDS

Figure 1. In-situ lipidomics by MALDI-MS imaging of avocado fruit reveals the distribution of triacylglycerol molecular species throughout the mesocarp tissue of mature fruit. Avocado was obtained from a local market and the mesocarp was sliced in half (A) and pressed/printed onto nitrocellulose membrane (B). The tissue print was coated with 2,5-dihydroxybenzoic acid matrix and subjected to MALDI-MS imaging. The laser rastered over the specimen in a 300 micron step size to generate a mass spectrum at each location (C). Images of triacylglycerol (TAG) molecular ions were reconstructed such that the tissue distribution and abundance of each molecular species could be visualized (D). The heat map for each of the six major triacylglycerol species in the avocado mesocarp is shown. The scale for relative triacylglycerol abundance is shown at the bottom (based on mol % of total TAG). Each triacylglycerol molecular species is denoted below the respective image according to the total acyl carbons and numbers of double bonds; e.g., TAG 54:3 is a triacylglycerol molecule with three 18-carbon acyl groups, each with one double bond. P, palmitic acid; O, Oleic acid; L; linoleic acid. The bottom number is m/z of the parent ion of the K+ adduct (see Supplemental Table S1 for m/z analysis).
Figure 2. Isolation of lipid droplets from avocado mesocarp. Lipid droplets were isolated by differential centrifugation and flotation through sucrose, then the “fat pad” fraction was stained with the neutral-lipid-specific fluorescent dye, BODIPY 493/503 and imaged using differential interference contrast (A) or fluorescence (B) microscopy. Scale bar represents 25 µm. The triacylglycerol content of each fraction was also quantified by mass-spectrometry (C, D), confirming the enrichment of triacylglycerols in isolated lipid droplets but not in other subcellular fractions. Similar results were obtained for lipid droplets isolated from mature fruit obtained from either a local market (C) or from the breeder, whereby the fruit was harvested directly from the tree at the orchard (D). Comparison of the molecular species profiles (in mol %) of triacylglycerols (TAG) in isolated lipid droplets (E) from avocado mesocarp (store bought versus breeder harvested) or in tissue prints of store-bought whole fruits analyzed by MALDI-mass spectrometry imaging (MSI, see Fig. 1). The lipid molecular species are indicated by total number of acyl carbons and numbers of double bonds, as is Fig. 1.

Figure 3. Transcript profile for the four most abundant proteins in lipid droplets was determined by the transcriptome analysis of developing mesocarp of avocado. Expression for genes encoding lipid droplet-associated protein (LDAP) 1 and 2 peaked at mid-stage of mesocarp development and was similar to that of ketoacyl-acyl carrier protein synthase II (KASII), a plastidial fatty acid synthesis gene. Transcripts for cysteine synthase C1 (CS1) and heat shock protein 60 (HSP60) remained about 2-fold lower than that of KASII. RPKM, reads per kilobase per million mapped reads

Figure 4. Polypeptide sequence alignment of the avocado lipid droplet-associated proteins LDAP1 and LDAP2 (Pam_LDAP1, Pam_LDAP2) with the closest homolog from Arabidopsis (Ath_SRPP-like; At3g05500) and the Hevea brasiliensis SRPP (Hbr_SRPP). Sequences were aligned using the ClustalW algorithm. Peptide regions of the two avocado proteins identified by MudPIT are highlighted gray. Percent coverage of LDAP1 is 50% and LDAP2 is 41%.

Figure 5. The SRPP-like protein from Arabidopsis (At3g05500) localizes to lipid droplets in tobacco BY-2 cells. The top row shows the predominantly cytosolic localization of transiently-expressed At3g05500-mCherry in BY-2 cells that were stained with BODIPY 493/503. The second row shows the localization of the same fusion protein in BY-2 cells that were incubated with linoleic acid (LA) after transient transformation, which induces a dramatic increase in the number and size of lipid droplets in these cells, and which reveals extensive co-localization of
the fusion protein with lipid droplets. The region of the cell denoted by the hatched box is shown in the bottom row at higher magnification, which reveals that the fusion protein exhibits a torus-shaped fluorescence pattern that encloses the BODIPY 493/503-stained lipid droplets. Bar represents 10 μm.

**Figure 6.** Comparison of gene copy number for oleosin and SRPP-like proteins in distantly related plants and algae. Oleosin and SRPP-like proteins of organisms whose genomes had been sequenced were identified using the BLASTP algorithm available at www.phytozome.net, using *Arabidopsis* SRPP-like protein (At3g05500) or oleosin 1 (At4g25140) as protein “queries”. Organisms with SRPP-like sequences that were also included in the phylogenetic analysis presented in Figure 7 are marked with an asterisk.

**Figure 7.** Phylogenetic analysis of SRRP-like proteins from rubber producing and non-producing plants. Each protein is labeled with the respective Genus and species, and *bona fide* SRPP proteins from rubber producing plants are highlighted green, while the SRPP-like proteins shown to associate with lipid droplets in this study (i.e., avocado [Pam] LDAP1 and LDAP2, and *Arabidopsis* [Ath] SRPP-like At3g05500) are highlighted in red. Other SRPP-like proteins from non-rubber accumulating plants are labeled numerically and shown in black. Note that most of the eudicots examined (e.g., *S. lycopersicum*, *R. communis*, *P. persica*, and *A. thaliana*) contained three SRPP-like proteins that were found in three distinct clades (Groups I, II, and III, circled in blue), while the sequences of the monocots (*S. italica* and *Z. mays*) and bryophyte/lycophyte (*P. patens* and *S. moellendorfii*) formed other distinct clades (Groups IV and V, respectively). The accession numbers of all sequences are provided in the MATERIALS AND METHODS section. BioEdit (v7.1.3.0) (Hall, 1999) was used for sequence alignments and reconstructions. The phlyogram was generated using the program TreeView (v1.6.6).
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