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 nonseed 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) 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 that accumulate in rubber-producing plants. An Arabidopsis (Arabidopsis thaliana) homolog to LDAP1 and LDAP2, At3g05500, was localized to the surface of lipid droplets after transient expression in tobacco (Nicotiana tabacum) cells that were induced to accumulate triacylglycerols. We propose that small rubber particle protein-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 the cellular machinery that is involved in the packaging of triacylglycerols in plant tissues.

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 (TAGs) 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 have 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, steroleosins, 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; Slíto et al., 2006; Schmidt and
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 nonseed tissues represent a compartment with protein compositions and functions different from the 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 (Elaeis guineensis), olive (Olea europaea), 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 nonseed plant tissues, we examined the oil-rich mesocarp of avocado (Persea americana ‘Haas’). Lipid-rich avocado fruit tissues have long been used as a model system for biochemical studies of lipid synthesis (Harwood and Stumpf, 1972). Furthermore, 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 TAGs in tissue prints of avocado fruit by mass spectrometry imaging was used to select mesocarp regions for lipid droplet analysis and for comparisons of the lipid composition of isolated lipid droplets with that of whole fruit. Multidimensional protein identification technology (MudPIT; Delahunty et al., 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 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 (Arabidopsis thaliana) homolog (At3g05500) of these avocado LDAP proteins, when transiently expressed in tobacco (Nicotiana tabacum) suspension-cultured cells, was found to associate specifically with the lipid droplet surface. Collectively, using this multomics approach, we identified a novel class of lipid droplet proteins in nonseed plant tissues, an important step in further characterization of 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 TAG molecular species in avocado tissue slices was visualized using a novel, matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry imaging (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 TAGs with two oleic acids and one palmitic acid, TAG-52:2 at mass-to-charge ratio (m/z) 881.757, or with oleic acid at each position, TAG 54:3 at m/z 907.773, were most prevalent in fruit tissue and were distributed throughout the mesocarp (Fig. 1D). TAG species were distributed relatively uniformly throughout the fruit tissue, except for the species with oleic acid at each position, TAG-54:3, 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 TAG 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 mass spectrometry maps of individual TAG 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 and fluorescence microscopy (Fig. 2; BODIPY 493/503 stains neutral lipids specifically [Listenberger et al., 2007]). By comparing differential interference contrast 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. 2, A and B). Of course, purity is relative, and it was clear from the sensitive proteomics analysis...
(described below) that other proteins or subcellular materials likely adhered to or were trapped within this fraction as well. Nonetheless, quantitative analysis of the TAG content in the various biochemical fractions confirmed that essentially all of the fractionated TAGs were associated with the lipid droplet fractions and not with the cytosolic or microsomal fractions (Fig. 2, C and D). The distribution of TAG content in the various fractions isolated was similar irrespective of the developmental stage of the fruit tissue used; preharvest fruits were obtained directly from trees (Fig. 2D, breeder), and postharvest 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 TAG core covered by a monolayer of phospholipids and associated proteins (Huang, 1992; Chapman et al., 2012; Murphy, 2012). We analyzed the TAGs of mesocarp lipid droplets isolated from preharvest and postharvest avocado fruits by direct infusion electrospray ionization-mass spectrometry (Fig. 2E). Generally, the TAGs in the homogenates and lipid droplet fractions from the preharvest fruits were lower in TAG 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 composition of lipid droplets isolated from preharvest and postharvest avocado fruits was determined using MudPIT analysis. Specifically, individual peptide sequences were identified by comparing the obtained masses of the peptides with a reference polypeptide database derived from Illumina- and 454-based sequencing of complementary DNA (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. Percentage coverage, representing the portion of an encoded polypeptide sequence matched by independent peptide sequences, was calculated for all proteins. The Arabidopsis protein with the highest sequence similarity to each avocado protein was determined using BLASTX.

Comparison of proteins identified in lipid droplet fractions isolated from either postharvest or direct, tree-harvested avocado fruits revealed a common core list of 70 proteins (Supplemental File S1). Further comparison treatments (Fig. 2E). Generally, the TAGs in the homogenates and lipid droplet fractions from the preharvest fruits were lower in TAG 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).

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of these 70 proteins with proteins identified in corresponding microsomal or cytosolic fractions revealed proteins that were specifically enriched in isolated fractions (Supplemental File S1). Proteins were considered “enriched” if they were present in more than 3-fold abundance (spectral counts) in a given subcellular fraction in comparison with 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. LDAP1 and LDAP2 were the first and fourth most abundant proteins, respectively, in lipid droplets based on spectral counts.

LDAP1 and LDAP2

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 (130–212 g fruit weight), transcript levels for LDAP1 and LDAP2 were highest during the mid stages of fruit development (approximately 160 g fresh weight) when oil accumulation was greatest (Eaks, 1990). For comparison, the gene expression profiles and transcript levels for the two LDAPs were similar to those of ketoacyl-acyl carrier protein synthase II (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 (data 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 S2; e.g. HEAT SHOCK PROTEIN60 and ATCYS1), 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 LDAPs.

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 S2). These two proteins share 62% identity (86% similarity) at the deduced amino acid level and show extensive regions of similarity and identity with the 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 and LDAP2 and Arabidopsis At3g05500 might similarly associate with and stabilize TAG-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 the addition of free fatty acids to the growth medium (Fig. 5). Expression of the At3g05500-mCherry fusion protein in Bright Yellow-2 (BY-2) cells cultivated in normal growth medium 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 lipid droplets, suggesting that the fusion protein was often torus shaped and enclosed the punctate fluorescent signal attributable to the BODIPY-stained lipid droplets (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). These data confirmed that the SRPP-like protein of Arabidopsis, At3g05500, can indeed localize specifically to lipid droplets in nonseed plant cell types.

**DISCUSSION**

To date, proteomes of lipid droplets from plant sources (e.g. Brassica spp., Arabidopsis, sesame [Sesamum indicum], and Jatropha spp.) have focused almost entirely on Arabidopsis and its close relatives such as Brassica and Jatropha. However, the Arabidopsis lipid droplet proteome is likely not representative of all plants, and it remains to be determined whether the SRPP-like proteins found in Arabidopsis are present in other plant species. Further studies are needed to determine the function of these proteins in lipid droplet formation and stability and to explore their potential role in lipid metabolism and storage in different plant species.
on seed-derived organelles and have been dominated by oleosins, caleosins, steroleosins, and only a few other proteins (Jolivet et al., 2004, 2009; Lin et al., 2005; Katavic et al., 2006; Popluechai et al., 2011). Here, we took an alternative approach to identify additional LDAPs by analyzing the proteome of lipid droplets from nonseed tissues. We asked whether there are specialized proteins associated with nonseed 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, hyperabundant proteins known to associate with lipid droplets in seeds (e.g. oleosins) and provides scope for the 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 TAG 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 a 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 the 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. In this study, our interest focused on discovering new LDAPs. 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 the 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 LDAPs not previously known to bind TAG-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 TAG-containing lipid droplets (Fig. 2; Supplemental Table S2) and whose gene expression patterns correlated with TAG 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...
Phoenix dactylifera), which stores very little oil (Bourgis et al., 2011). Furthermore, 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 nonseed 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 8- to 10-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; Huang et al., 2013). No SRPP-like homologs were detected in other distantly related organisms, such as yeast (Saccharomyces cerevisiae), Drosophila melanogaster, 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 rubber tree, guayule (Parthenium argentatum), and Russian dandelion (Taraxacum brevicornucatum; 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

![Figure 6](https://www.plantphysiol.org/)

**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 OLEOSIN1 (At4g25140) as protein “queries.” Organisms with SRPP-like sequences that were also included in the phylogenetic analysis presented in Figure 7 are marked with asterisks.
play a role in rubber synthesis (Collins-Silva et al., 2012). For instance, RNA interference suppression of SRPPs in Russian dandelion caused a significant reduction in overall rubber production and rubber synthase activity in comparison with wild-type plants (Hillebrand et al., 2012). Furthermore, rubber particles isolated from the RNA interference 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 unclear at present 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 rubber tree 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 the rubber tree (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 and LDAP2, 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 TAG-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 to modulate the compartmentalization of TAGs 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-

Figure 7. Phylogenetic analysis of SRPP-like proteins from rubber-producing and non-rubber-producing plants. Each protein is labeled with the respective genus and species, and bona fide SRPP proteins from rubber-producing plants are highlighted in 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. tomato, R. communis, P. persica, and Arabidopsis) 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 maize) and bryophyte/lycophyte (P. patens and S. moellendorffii) formed other distinct clades (groups IV and V, respectively). The accession numbers of all sequences are provided in "Materials and Methods." BioEdit version 7.1.3.0 (Hall, 1999) was used for sequence alignment and reconstruction. The phylogram was generated using the program TreeView (version 1.6.6). [See online article for color version of this figure.]
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 (Figs. 6 and 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/lycorhiza 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 Hveva spp. have yielded some important insight into 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-MSI

MALDI-MSI of avocado (Persea americana) tissue prints was performed on a MALDI LTQ Orbitrap-XL (Thermo Fisher Scientific) system in a manner similar to that described for analysis of cotton (Gossypium hirsutum) seed tissue sections (Horn et al., 2012) with some modifications. Briefly, avocado purchased from a local market was sliced in half and pressed for 2 min against a 0.45-μm pore size, Protran nitrocellulose membrane to generate a tissue print. The tissue print was cut into five pieces, and each piece was adhered to a stainless steel slide with double-sided tape. The ionization matrix 2,5-dihydroxybenzoic acid (99%; Acros Organics) was sprayed onto tissue prints at 20 mg mL⁻¹ in 70% methanol using a SunChrom SunCollect MALDI spotter (Verhaert et al., 2010). Raw data were acquired using Thermo Xcalibur (version 2.1) in positive ionization mode with automatic gain control on, laser energy of 14 J, and two successive scan events (m/z 1,200 at 60,000 resolution). Images of individual molecules were generated using the Metabolite Imager application as described (Horn et al., 2012), except with constant ppm tolerance (±10 ppm) for selected peaks identified as TAG 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 (cv Haas) purchased from a local market or harvested from the orchards at the University of California, Riverside (a 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 Suc, 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 13,000g for 30 min at 4°C. The top 5 mL of the samples was 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 cyto- and microsomal fractions. Lipid droplets were purified by two additional floatations through 15 mL of the homogenization solution containing 400 mM Suc. Cytosol and microsomes were fractionated by ultracentrifugation at 150,000g (Beckman Ti-75 rotor at 4°C in a Sorval 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 were spiked with the internal standard triptandecanoin (Tris50-TAG; 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 2.5 μL min⁻¹ into an electrospray ionization 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 three microscans per acquired scan, sheath gas of 25 arbitrary units, capillary temperature of 270°C, and capillary offset of 35 V. Acyl chains within TAGs were determined using a series of neutral loss scans for abundant fatty acids.

MudPIT Proteomics

Acetone-precipitated proteins were dissolved in 2× 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 Brilliant 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 ultra-performance liquid chromatography system and Waters Symmetry C18 peptide trap; 5 μm, 180 μm × 20 mm). Eluted peptides were sprayed directly into a Thermo Fisher Scientific LTQ-FT Ultra MS5 device, and survey scans were taken in the Fourier transform mode (25,000 resolution determined at m/z 400) with the top 10 ions in each survey scan subjected to collision-induced dissociation. Tandem mass spectra were analyzed by BioWorks Browser version 3.3.1 (Thermo Fisher Scientific) and compared with the avocado clc4 protein sequence database using Mascot version 2.3. Scaffold version 3.3.2 software was used to validate protein identifications with the ProteinProphet (Nevezhihski 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 the University of California South Coast Research and Extension Center in Irvine. Fruits were shipped overnight at 4°C to Michigan State University. Fruits were weighed, and mesocarp was isolated and stored at −80°C until used. Total RNA was extracted from 3 g of 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 12,000g for 15 min at 4°C, the aqueous phase was transferred to a fresh tube and incubated overnight with one-third volume of 8 M LiCl. Samples were then centrifuged at 12,000g for 30 min at 4°C, the pellet was resuspended in 1,000 μL of the RNeasy kit, and RNA was eluted following the manufacturer’s protocol (Qiagen). Transcriptionome data for developing mesocarp was generated using 454 and Illumina sequencing techniques. For 454 sequencing, mRNA was isolated from total RNA using Sera-Mag Oligo(dT) Magnetic Beads (Thermo Fisher 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 prelacerase protocol for directional mRNA-seq library preparation (version 1.0). A single-read, 75-cycle run was then performed on the Illumina GAIIx sequencer, following the manufacturer’s protocols. De novo assembly of reads obtained from 454 and Illumina sequencing was accomplished with Trinity RNA-Seq software version 2. Contigs obtained were identified by BLASTX annotation against The Arabidopsis Information Resource 10 database. Finally, the coding sequences for proteins of interest were verified against genome sequence data provided by the avocado genome sequencing group at the Laboratorio de Servicios Genómicos of Langebio in Cinsvestav, Mexico.

Lipid Droplet Targeting of At3g05500

The plasmid pRTL2/At3g05500-mCherry, encoding the full-length open reading frame of At3g05500 fused to the N terminus of the monomeric cherry (Prunus avium) autofluorescent protein mCherry (Shaner et al., 2004), was derived from Schmidt et al. (2010) and included At3g05500, with the exception that cells were reseeded in BY-2 growth medium rather than transformation buffer prior to bombardment. Induction of lipid droplets in BY-2 cells was carried out as follows: approximately 30 min after bombardment, cells were transferred to a culture flask containing BY-2 growth medium, the cells were then maintained at regular growth conditions for 1 h, 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 9 μM concentration at a final concentration of 0.1 μg mL⁻¹.

Confocal laser-scanning microscopy images of BY-2 cells were acquired using a Leica DM RBE microscope with a Leica 63× 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-labeling 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 512×512-pixel digital images. All fluorescence images of cells shown in individual figures are representative of more than 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 for avocado LDAP1 and LDAP2 (Fig. 4) transcripts are KF031141 and KF031142, and their nucleotide sequences will be available by the avocado genome sequencing group at the Laboratorio de Servicios Genómicos (Guadalajara, Mexico) for sharing avocado genomics information to confirm the coding sequences for LDAP1 and 2. We thank Vladimir Shulaev (University of North Texas) and Kerstin Strupat (Thermo-Fisher Scientific) for instrument access and technical support, respectively, for MS imaging experiments; Drs. Jennifer Saito and Alam Masgudul (University of Hawaii) for access to the Hevea genome browser; and Dr. Reynald Tremblay (University of Guelph) for his assistance with the phylogenetic analysis of SRPPs. Dr. Charlene Case (University of North Texas) assisted with assembly of the manuscript. Received May 31, 2013; accepted July 1, 2013; published July 2, 2013.

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