Heterologous expression of AtClo1, a plant oil body protein, induces lipid accumulation in yeast

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Abstract

Proteomic approaches on lipid bodies have led to the identification of proteins associated with this compartment, showing that, rather than the inert fat depot, lipid droplets appear as complex dynamic organelles with roles in metabolism control and cell signaling. We focused our investigations on caleosin [Arabidopsis thaliana caleosin 1 (AtClo1)], a minor protein of the Arabidopsis thaliana seed lipid body. AtClo1 shares an original triblock structure, which confers to the protein the capacity to insert at the lipid body surface. In addition, AtClo1 possesses a calcium-binding domain. The study of plants deficient in caleosin revealed its involvement in storage lipid degradation during seed germination. Using Saccharomyces cerevisiae as a heterologous expression system, we investigated the potential role of AtClo1 in lipid body biogenesis and filling. The green fluorescent protein-tagged protein was correctly targeted to lipid bodies. We observed an increase in the number and size of lipid bodies. Moreover, transformed yeasts accumulated more fatty acids (+46.6%). We confirmed that this excess of fatty acids was due to overaccumulation of lipid body neutral lipids, triacylglycerols and steryl esters. We showed that the original intrinsic properties of AtClo1 protein were sufficient to generate a functional lipid body membrane and to promote overaccumulation of storage lipids in yeast oil bodies.

Introduction

The recent development of proteomic approaches on purified organelles provides an abundant set of data that is now available in the scientific community in order to decipher the mechanisms of biogenesis, function and degradation of these subcellular compartments. Lipid bodies, also called oil bodies or lipid droplets, are specialized structures consisting mainly of a core of storage neutral lipids (triacylglycerols and steryl esters) surrounded by a monolayer of phospholipids, and contain a number of proteins that vary considerably with the species. Sixteen proteins were originally identified in Saccharomyces cerevisiae lipid bodies in 1999 (Athenstaedt et al., 1999). Recent advances on oleosome (plant lipid bodies) protein composition (Jolivet et al., 2004, 2006; Katavic et al., 2006), or lipid droplets from higher eukaryotes and mammalian cells (Beller et al., 2006; Cermelli et al., 2006; Bartz et al., 2007), led to the identification of c. 250 proteins associated with this compartment. The nature of these proteins revealed that, rather than inert cytosolic fat inclusion, lipid droplets are complex and dynamic organelles with a crucial role in various metabolic activities for example metabolism regulation, cell signaling and insulin response (Martin & Parton, 2006; Bostrom et al., 2007; Welte, 2007; Ducharme & Bickel, 2008).

Recently, plant oil bodies and the associated proteins have been emerging as a new target for plant biotechnology in order to improve oil production in various oleaginous seeds. Identification of molecular and cellular factors involved in biogenesis and mobilization of storage lipid bodies is then crucial and should lead to the discovery of key factors implicated in the stability of these organelles, with the aim of developing economic and efficient methods for biofuel production or agroindustries. Advances in the knowledge of lipid storage in oil bodies will also contribute towards improvement of biotechnologies using alternative and renewable sources of a lipid-rich biomass for biofuels and other biomaterials from plants to microorganisms. Understanding of yeast lipid droplet dynamics will especially facilitate an increase in the lipid production from oleaginous sources.
microorganisms for the development of single-cell oil (SCO) technology. This information will also yield benefits related to environmental preservation strategies that attempt to valorize agro-industrial wastes as substrates for microalgae (Hodaifa et al., 2008; Hu et al., 2008) and nonconventional yeasts and molds (Fickers et al., 2005; Fakas et al., 2008; Papanikolaou et al., 2008). These promising cells may accumulate up to 50% of their biomass as storage lipids.

Rapid and efficient biological systems are needed to understand the function of the new partners of lipid bodies. One interesting approach is the exploitation of S. cerevisiae as a heterologous expression system to understand protein function and proteins of potential interest for biotechnological development. The yeast system is easy to use and inexpensive, and numerous genetic tools are available for protein studies. It has also been successfully used for deciphering the function of numerous membrane proteins from plants or mammalians (Eckart & Bussineau, 1996; Barbier-Brygoo et al., 2001; Marini et al., 2006). We chose this system to study the function of selected proteins from Arabidopsis thaliana seed lipid oleosomes identified using the proteomic approach (Jolivet et al., 2004). Oleosome proteins may be classified into three classes: structural proteins, enzymes and minor proteins. The most abundant proteins are structural low-molecular-mass amphipathic proteins called oleosins (Huang, 1996; Frandsen et al., 2001). They display a conserved architecture with three structural regions: a long central hydrophobic region flanked by two terminal hydrophilic regions. It was proposed that the conserved hydrophobic region (longest to date with c. 70 nonpolar residues) is enmeshed in the triacylglycerol matrix, forming a hairpin structure connected around a conserved central proline knot motif, also essential for correct oleosin targeting to lipid bodies (Lacey et al., 1998; Abell et al., 2004). In contrast, N- and C-terminal hydrophilic regions are less conserved and are supposed to associate with phospholipids at the lipid body surface. This original structure suggested that oleosins may be implicated in lipid body biogenesis and stabilization. It has been shown recently that oleosins effectively control oil body size and lipid accumulation in planta (Siloto et al., 2006).

Interestingly, caleosin, one of the minor proteins of seed lipid bodies, displays a sequence organization similar to that of oleosin: a central hydrophobic region with a proline knot with peripheral hydrophilic regions (Chen et al., 1999; Naested et al., 2000; Chen & Tzen, 2001). The N-terminal region, supposed to be cytosolic, contains a calcium-binding EF-hand domain, suggesting a potential calcium regulation for the protein activity (Frandsen et al., 1996). Caleosin was identified as a hemoprotein with a peroxidase capacity (Hanano et al., 2006). It shares, similar to the oleosin family, the capacity to stabilize artificial lipid bodies (Chen et al., 2004) and in vivo, it was demonstrated that during germination it plays a role in degradation of lipid storage (Poxleitner et al., 2006).

Yeasts strains have been successfully used to decipher some of the oil body protein properties and functions. Oleosin’s essential regions for lipid body targeting (Beaudoin & Napier, 2002) and A. thaliana caleosin 1 (AtClo1) peroxidase activity (Hanano et al., 2006) were evidenced using the S. cerevisiae heterologous expression system. However, the authors did not show any potential effect on lipid body morphogenesis or on total cell lipid content (Ting et al., 1997). In the present study, we used this expression system to determine the effect of caleosin functional properties on lipid body biogenesis and stabilization in vivo. We showed that AtClo1 was expressed and correctly targeted to lipid bodies in yeast. These transformed yeasts displayed an increase in the number and size of lipid bodies, providing new insights into AtClo1 function. Overall, this is the first example of a plant lipid body protein inducing lipid accumulation in yeast.

Materials and methods

Yeast strains and growth conditions

Yeast strains were transformed as described by Gietz et al. (1992). Yeast strains used throughout this study are BY 4741 (Mata his3Δ leu2Δ met15Δ ura3Δ) from Euroscarf (Frankfurt, Germany) and BY ERG6-RFP. This strain, expressing the RFP-tagged Erg6p protein, was constructed by inserting into the BY 4741 cell the REDSTAR2 coding sequence in frame at the 3’ of the ERG6 gene by homologous recombination as described in Janke et al. (2004). Each strain was transformed with either pGAL-GFP (pNBT29) (Froissard et al., 2006) or pGAL-CLO1-GFP. Cells were grown in minimal medium (YNB) containing 0.67% yeast nitrogen base without amino acids and ammonium sulfate (BD Bioscience, Heidelberg, Germany), supplemented with 5 g L⁻¹ ammonium sulfate and 0.2% casamino acids. The carbon source was 2% glucose or 2% galactose+0.02% glucose as indicated in the figure legend. Galactose induction was performed by adding 2% galactose on flasks containing cells grown overnight in 2% raffinose+0.02% glucose up to an A600 nm = 0.6. All cultures were performed in conical flasks, containing 1/5 volume of medium, and incubated at 28 °C in an orbital shaker at an agitation rate of 200 r.p.m.

Plasmid construction

DNA manipulations were performed essentially as described by Maniatis et al. (1982). To obtain the pGAL-CLO1-GFP plasmid, we performed PCR amplification of the ORF of the AtCLO1 gene using pET20b-CLO1 plasmid DNA (Purkrtova et al., 2007) as the template. The BamH1/SmaI PCR

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fragment obtained was introduced into the pNBT29 plasmid (Froissant et al., 2006) in frame with the green fluorescent protein (GFP) coding sequence, thereby creating a GFP C-terminally tagged version of AtClo1.

**Growth tests**

Cells were cultured overnight in 5 mL of minimal medium containing 2% glucose (for details, see Yeast strains and growth conditions) and were spotted on plates containing agar minimal medium with 2% galactose to induce expression of AtClo1-GFP. Plates were incubated for 72 h at 28 °C. The first drop contained 3 × 10^5 cells and each subsequent drop was diluted sixfold compared with the previous drop.

**Total yeast extracts**

Total protein extracts were prepared using the NaOH/trichloroacetic acid (TCA) lysis technique as described by Volland et al. (1994). Cells [corresponding to 1.5 UA_{600 nm} or 0.75 mg dry weight (d.w.)] were collected and resuspended into a final volume of 500 μL. Fifty microliters of 1.85 M NaOH was added to the sample and cells were disrupted by vortexing and incubating on ice for 10 min. Proteins were precipitated 10 min on ice by adding 50 μL of 50% TCA. The resulting protein pellet was resuspended in 50 μL of loading buffer containing two volumes of 2 × sample buffer [100 mM Tris-HCl, pH 6.8, 4 mM EDTA, 4% SDS (sodium dodecyl sulfate), 20% glycerol and bromophenol blue], one volume of Tris base 1 M and 2% mercaptoethanol.

**Lipid droplet fractionation**

Lipid droplets were separated by density gradients as described previously by Yu et al. (2000). Cells expressing AtClo1-GFP after 4 h of galactose induction (corresponding to 100 UA_{600 nm} or 50 mg d.w.) were harvested by centrifugation, washed with water and disrupted by vortexing (four times, for 1 min) in the presence of 0.45 mm glass beads and 0.3 mL of fat body buffer (FBB) (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA and 0.1 mM EGTA) supplemented with 10 mM DTT and protease inhibitors (Complete cocktail, Roche Diagnostics, Meylan, France). The extract was transferred to a fresh tube and the beads were washed twice with FBB. The extract and washings were pooled and spun at 3000 g for 5 min to remove cell debris. The volume of cleared extract was adjusted to 2.7 mL with FBB, mixed with an equal volume of FBB including 1.08 M sucrose and transferred into an 11-mL ultracentrifugation tube. It was overlaid sequentially with 1.8 mL each of 0.27 and 0.135 M sucrose in FBB and FBB only. After centrifugation at 50 000 g for 90 min, seven fractions of 1.5 mL were collected from the top to the bottom (floating lipid bodies in fractions 1 and 2) and the proteins were precipitated by adding 1.5 mL of 20% TCA and incubated on ice overnight. Proteins were collected by centrifugation at 10 000 g for 30 min, and resuspended in 400 μL of loading buffer.

**SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot**

Proteins were separated by SDS-PAGE using ready-to-use NuPage Novex 12% Bis-Tris gels and NuPAGE MES SDS running buffer (Invitrogen, Cergy Pontoise, France). Gels were stained with Coomassie blue (G-250) according to Neuhoff et al. (1988) or using the silver staining kit (Invitrogen). For immunoblotting experiments, proteins were transferred onto Immobilon-P PVDF membranes (Millipore, Saint Quentin en Yvelines, France) and probed with polyclonal antibodies raised against AtClo1 (Purkrtova et al., 2007), or red fluorescence protein (RFP) (Abcam, Paris, France), or monoclonal antibodies raised against GFP (Roche Diagnostics) and actin (Abcam). Primary antibodies were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G secondary antibodies and revealed using SuperSignal West Dura Extended Duration Substrate (Perbio, Bребi`eres, France). Luminescence from peroxidase activity was recorded using the LAS-3000 imaging system and MULTigauges software from Fujifilm (Saint Quentin en Yvelines, France).

**Fluorescence microscopy**

GFP and RFP fluorescences were monitored in cells collected from cultures and examined under a microscope without any treatment.

We used a Zeiss Axioplan 2 Imaging microscope (Zeiss, Le Pecq, France) equipped with fluorescence andNomarski optics and a Roper CoolSnap HQ camera coupled to a Metamorph driver (Roper Scientific, Evry, France).

**Electron microscopy**

Cells (c. 10 UA_{600 nm} or 5 mg d.w.) were fixed for 1 h at 28 °C by adding 250 μL of 50% glutaraldehyde to 5 mL of overnight galactose-growing cells. After 5 min of centrifugation at 3000 g, cells were resuspended in 3% glutaraldehyde in phosphate buffer and fixed overnight at 4 °C. Then, cells were washed three times with 0.1 M sodium cacodylate, pH 7.4, three times with water, incubated in 1% KMnO₄ for 2 h on ice and again washed three times in water. Subsequently, cells were treated with 2% aqueous uranyl acetate for 1 h at 4 °C and washed with water. Cells were gradually dehydrated in ethanol, and in propylene oxide, and embedded in Epon containing 2% dimethylaminoethanol (Delta Microscopies, Labège, France). Thin sections (70 nm) were cut, stained with lead citrate and examined in a Zeiss EM902 transmission electron microscope (Zeiss) at 80 kV. Micrographies...
were acquired using MegaView III CCD camera and analyzed using ITEM software (Eloise SARL, Roissy en France, France).

**Lipid analysis**

Lipids from yeast cells were extracted as described by Folch et al. (1957). Cells (corresponding to 200 UA 600 nm or 100 mg d.w.) were harvested by centrifugation and washed with water. Five milliliters of chloroform/methanol ratio 2/1 (v/v) was added to the pellet and the cells were disrupted by vortexing for 5 min using glass beads (0.45 mm). After a 1-h incubation with shaking, the extract was centrifuged for 5 min at 500 g and the supernatant was recovered in a new tube and mixed with 2.5 mL of 0.9% NaCl. The organic phase (lower phase) was collected after centrifugation at 500 g for 5 min and washed three times with chloroform/methanol/water 3/48/47 (v/v/v) (Schneiter & Daum, 2006b). The organic solvents were evaporated under a stream of N2 and lipids were resolubilized in 100 mL of chloroform/methanol 2/1 (v/v). Lipids (20 μL) were separated by thin-layer chromatography (TLC) on HPTLC silica-coated aluminum plates (Merck, Fontenay Sous Bois, France) using successively two mobile phases, petroleum ether/diethyl ether/acetic acid 10/10/0.4 (v/v/v) and petroleum ether/diethyl ether 49/1 (v/v), until the solvent front reached about 15 and 1 cm from the top of the plate, respectively (Athenstaedt et al., 1999; Schneiter & Daum, 2006a). Lipid classes were visualized using the MnCl2 charing method. A silica plate was incubated for 1 min in a solution containing 120 mL of methanol, 120 mL of water, 0.8 g of MnCl2 and 8 mL of sulfuric acid and then heated in an oven at 100 °C until the dark lipid spots appeared. Lipid identification was based on migration obtained for lipid standards (Sigma-Aldrich, Saint Quentin Fallavier, France). Lipid staining was recorded using the LAS-3000 imaging system and MULTIGAUGE software from Fujifilm.

**Fatty acid analysis**

Transmethylation of acylated and free fatty acids was performed as described in Browse et al. (1986). Cells (corresponding to 50 UA 600 nm or 25 mg d.w.) were collected by centrifugation, washed with water and freeze dried for 72 h. The pellet was disrupted by vortexing in the presence of 0.45 glass beads and 2 mL of 2.5% (v/v) sulfuric acid in methanol. Heptadecanoic acid (Sigma-Aldrich) was added (100 μg for each sample) as an internal standard for quantification. The samples were heated for 90 min at 80 °C. Fatty acid methyl esters (FAME) were extracted by addition of 3 mL of water, 0.9 mL of hexane and with vigorous shaking. Samples of the organic upper phase were separated by GC using a GC3900 chromatograph (Varian, Les Ulis, France) with a Factor Four VF-23 ms 30 m × 0.25 mm capillary column (Varian). The carrier gas was helium at an inlet pressure of 1 mL min⁻¹. The column temperature program started at 40 °C for 1 min, ramping to 120 °C at 40 °C min⁻¹, holding for 1 min at 120 °C, ramping to 210 °C at 3 °C min⁻¹ and holding for 10 min at 210 °C. Identification of FAME peaks was based on the retention times obtained for standards (Sigma-Aldrich). The quantification was performed by flame ionization detection at 270 °C. The total amount of fatty acids was calculated from the ratio between

![Fig. 1. AtClo1-GFP is expressed in yeast Saccharomyces cerevisiae. (a) Evaluation of AtClo1-GFP toxicity by growth test experiments for yeast. BY cells transformed with either pGAL-GFP (empty vector) or pGAL-CLO1-GFP, were grown on glucose- (noninducing medium) or galactose- (inducing medium) containing plates. (b) Immunoblot analysis of AtClo1-GFP. Total protein extracts from BY pGAL-CLO1-GFP cells cultured in glucose- or galactose-containing medium were prepared, resolved by SDS-PAGE and analyzed by immunoblotting using a polyclonal antibody against AtClo1. (c) Time-course analysis of AtClo1-GFP expression. By cells transformed with pGAL-CLO1-GFP were grown to the midexponential phase in a raffinose-containing medium. Galactose was then added to induce AtClo1-GFP expression. At the indicated times after galactose addition, protein extracts were prepared, resolved by SDS-PAGE and analyzed by immunoblotting using the anti-GFP antibody to detect the fusion protein and anti-actin antibody as the loading control.](image-url)
the sum of FAME peak areas and the heptadecanoic acid methyl ester peak area.

**Results**

**AtClo1-GFP is properly expressed in yeast**

In order to follow AtClo1 expression in yeast cells, a GFP C-terminally tagged version of the protein, a powerful tool for monitoring the intracellular fate of heterologous (Wieczorke et al., 2003; Maresova & Sychrova, 2006) or endogenous membrane proteins (Froissard et al., 2007; Stawiecka-Mir-ota et al., 2007), was created. AtCLO1 was under the control of the galactose-inducible GAL promoter in order to check the potential toxicity of AtClo1-GFP in yeast. Cell growth tests were performed on plates. BY4741 cells expressing pGAL-GFP or pGAL-CLO1-GFP were spotted onto plates containing glucose or galactose as the carbon source. The size of colonies was weakly altered by the presence of AtClo1-GFP when growth was performed on galactose-containing medium (Fig. 1a), but no toxicity was evidenced. The expression of the AtClo1-GFP protein (presence and apparent molecular mass) was analyzed by immunoblot experiments using the polyclonal serum raised against the N-terminal hydrophilic domain of the protein as described elsewhere (Purkrtova et al., 2007). One band at 55 kDa was present in the galactose-cultured cell extract, but not in the glucose-culture one, corresponding to the proper fusion protein (Fig. 1b). As this anti-caleosin serum also reacted with several yeast proteins, we confirmed that the 55-kDa band detected was indeed AtClo1-GFP using antibodies raised against GFP. We also determined by time-course experiments that the highest level of AtClo1-GFP expression was obtained after an overnight induction (Fig. 1c).

**AtClo1-GFP is targeted to lipid bodies in yeast**

An experiment conducted in planta revealed the localization of AtClo1 at the periphery of seed lipid bodies (Poxleitner et al., 2006). Our previous immunoblot experiments revealed the expression of AtClo1-GFP in yeast cells, but it was necessary to demonstrate the correct targeting of the protein to lipid bodies before investigating the function of the protein. As Erg6p, a delta(24)-sterol C-methyltransferase, is localized specifically inside the lipid bodies, this protein was selected as a specific marker for colocalization experiments (Gaber et al., 1989). We introduced at the chromosomal locus of the ERG6 gene the RedStar2 coding sequence a brighter fast-maturing version of DsRed (Janke et al., 2004), leading to the expression of an RFP C-terminal-tagged version of Erg6p. This strain was transformed with the pGAL-GFP or the pGAL-CLO1-GFP plasmids. Experiments were first conducted on cells fully induced for AtClo1-GFP (overnight galactose-containing medium cultures), but due to the high level of expression, the protein was present in all the sucrose gradient fractions and the cells were too bright for observation of fluorescence punctuate staining under the microscope (data not shown). To reduce the level of AtClo1-GFP, the induction was started after overnight growth in a raffinose-containing medium. After 4 h of galactose induction, cells were collected for sucrose gradient fractionation and for observations under the microscope. Lipid bodies from cells expressing Erg6-RFP and AtClo1-GFP were purified by centrifugation on sucrose gradient density (Fig. 2a) and were recovered in fractions 1 and 2 (upper fractions) of the gradient as revealed by the presence of the 69 kDa Erg6-RFP protein on Western immunoblot experiments (Fig. 2c). AtClo1-GFP was mostly present in fractions 1 and 2, indicating the localization of the protein in lipid bodies (Fig. 2c). Faint staining was also detectable in fractions 3 and 4, indicating that AtClo1-GFP localization was not entirely restricted to lipid bodies (protein on their way to lipid bodies) or could be linked to the high expression of the protein in the cells. Indeed, AtClo1-GFP was one of the most abundant proteins in the lipid bodies (Fig. 2b).

![Fig. 2. AtClo1-GFP is present in the lipid body floating fraction. (a and b) BY cells transformed with either pGAL-GFP or pGAL-CLO1-GFP were cultured overnight in a raffinose-containing medium. AtClo1-GFP synthesis was induced for 4 h by adding galactose to the medium. Cells were disrupted and subcellular fractions were separated protein on a sucrose density gradient. Aliquots of the various fractions were resolved by SDS-PAGE and the proteins were revealed by Coomassie blue staining (a) or silver staining (b). (c) Protein fractions were also analyzed by immunoblotting for the presence of AtClo1-GFP and Erg6p-RFP using, respectively, anti-GFP and anti-RFP antibodies. F1 and F2, floating fractions containing lipid bodies; F3 and F4, midzone; and F5, F6 and F7, cytosol. Microsomes were present in the pellet (not shown).](image-url)
The cells were also observed under a microscope equipped with Nomarski optics and for GFP and RFP fluorescences. Cell observations confirmed the proper localization of Erg6p-RFP to lipid bodies, clearly visible with Nomarski optics (Fig. 3a and b). The same staining, ring-like structures, at the surface of lipid bodies, was also evidenced for GFP fluorescence corresponding to the AtClo1 fusion protein (Fig. 3c). These observations demonstrated that the correct targeting of the protein as its insertion at the surface of lipid bodies was similar to the localization observed for the endogenous AtClo1 reported in A. thaliana seeds by Poxleitner et al. (2006).

AtClo1-GFP induction in yeast promotes lipid accumulation

Because the morphological modifications observed on lipid bodies after AtClo1-GFP expression could lead to lipid accumulation, we analyzed the lipid content of AtClo1-GFP-expressing yeasts. We first focused our investigation on cell fatty acids, which provided a rapid overview of the total cell lipid content. After overnight induction in a galactose-containing medium, cells were processed to obtain FAME, and the organic extract was separated by GC to determine the FAME composition. We observed a significant accumulation of lipids in cells expressing AtClo1-GFP (59.1 ± 0.56 μg FAME mg⁻¹ d.w.; n = 3) when compared with the control cells (40.3 ± 0.69 μg FAME mg⁻¹ d.w.; n = 3), leading to a statistically significant increase (P < 0.001) in the total fatty acid content of 46.6%. According to the retention times obtained from FAME standards, we also determined the fatty acid composition of yeast with major species, C16:1, C18:1 and C16:0, and minor species, C18:0, C14:1, C14:0, C12:0 and C10:0. The expression of AtClo1-GFP did not modify the FAME composition of yeast (Fig. 5).

To determine the nature of the lipids accumulated after AtClo1-GFP expression, cellular lipids were analyzed by TLC after Folch extraction. We decided to quantify storage neutral lipid, triacylglycerols and steryl esters, lipids specifically stored in lipid bodies. Lipids from cells expressing AtClo1-GFP were analyzed and compared with those extracted from the control strain expressing only free GFP. Quantification of each class of lipids revealed a significant variation in cells expressing AtClo1-GFP for lipids specific for lipid bodies (i.e. storage...
lipids: triacylglycerols and seryl esters) and not for other cellular lipid classes such as free fatty acids, phospholipids and sterols (Fig. 6a). We observed that the impact on storage was most significant for neutral lipids, 2- and 2.8-fold increases for steryl esters and triacylglycerols, respectively, and less significant for synthesis intermediates such as diacylglycerols and monoacylglycerols (Fig. 6b).

**Discussion**

In this study, we investigated the functional properties of the caleosin AtClo1, a protein anchored at the lipid body surface by heterologous expression in *S. cerevisiae*. We used a GFP-tagged version of caleosin to obtain information about the fate of the protein in the cell. We proved the correct targeting of AtClo1 to lipid body by colocalization experiments with an endogenous lipid body marker. Caleosin and other interfacial proteins from plant lipid bodies display noncanonical structures for integral membrane proteins with a long central hydrophobic region surrounded by two hydrophilic C- and N-terminal regions involved in the stability of oil bodies. Previous *in vitro* experiments on caleosin revealed the importance of the hydrophobic central part for the protein targeting into artificial oil bodies (Chen & Tzen, 2001). Oleosins or caleosin homologues have not been described in *S. cerevisiae* and no functionally equivalent molecules have been identified in lipid bodies in this organism to date. Indeed, it appeared that the yeast cellular machinery was capable of recognizing intrinsic information contained in the central part of the protein, most probably in the hydrophobic region, and this information was sufficient to properly target caleosin to lipid bodies.
AtClo1-GFP alone, a minor protein of the plant lipid body, was shown to be capable of inducing a proliferation of lipid droplets leading to the specific accumulation of storage lipids (triacylglycerols and steryl esters). Morphological observations of yeast expressing caleosin-GFP revealed modifications of the ultrastructural organization of cell compartments. Lipid bodies appeared clustered around the proliferation of membranes, where mitochondria were also recruited. Lipid bodies and their constituents, triacylglycerols, phospholipids and oleosins, were all synthesized or inserted on the cytosolic face of the endoplasmic reticulum (Martin & Parton, 2006). We could hypothesize the reorganization of the cell with an exacerbated endoplasmic reticulum network dedicated to the production of lipids for lipid body filling and budding from the endoplasmic reticulum. Mitochondria were also involved in triacylglycerol metabolism (Athenstaedt & Daum, 2006) and could also be recruited in the close vicinity of lipid bodies for neutral lipid synthesis and degradation. This subcellular organization around lipid bodies was observed in differentiating 3T3-L1 cells where the lipid droplets, endoplasmic reticulum and mitochondria formed ‘constellations’ that may reflect the interplay of lipid-metabolizing enzymes in these organelles (Novikoff et al., 1980).

The significant modifications in the lipid metabolism of a nonoleaginous yeast (from 40.3 to 59.1 μg of FAME mg⁻¹ d.w. corresponding to a 46.6% increase of the total lipid content without fatty acid profile modification), induced by the AtClo1-GFP protein, proved that the interfacial properties of caleosin are sufficient to generate an overproduction or an overaccumulation of storage neutral lipids. We observed that in these experiments, AtClo1-GFP was not fully expressed in all the cells due to the utilization of the inducible GAL promoter. It is certainly possible to further increase storage of neutral lipids in cells using another promoter for AtClo1 expression as the strong GPD promoter.

We demonstrated that caleosin organization in the membrane generated specialized interface with the lipid body identity. This proliferation of lipid body packaging led to a specific increase of neutral lipids, revealing potentially a novel function for caleosin in lipid body filling during seed maturation, a function currently assigned to oleosins only (Siloto et al., 2006). To date, we do not know whether this...
effect on lipid storage was due to a defect in neutral lipid mobilization or due to an increase in their synthesis or a combination of both. We propose that caleosin could modify lipid body membrane function by impairing lipase accessibility to lipid bodies, leading to a defect in storage lipid degradation. We also hypothesized that a lipid over-accumulation could be a consequence of a global increase of lipid production with modifications on carbon fluxes and enzyme regulations.

These observations are important for biotechnological development with the aim to increase the oil content in renewable lipid-rich biomasses, i.e. oleaginous plants and microorganisms (SCO technologies), which are powerful new targets for biofuel and biomaterial production. The modification of interfacial properties of lipid bodies could lead to a global modification of lipid storage in these cells. Such observations were made directly in plants; in A. thaliana, a decrease in lipid storage was observed when oleosins were depleted by RNA interference (Siloto et al., 2006). In maize, the same conclusions were obtained using the oleosin/oil ratio (Ting et al., 1996). One major difference was observed in yeast where expression of caleosin leads to an increase of oil body size. This is not the case in plants where the size of oleosomes increases with the reduction of the oleosin content. This could have been because the steric constraints in mature seeds were subcellular structures tightly packed by the cytosolic compression to maintain a high surface-to-volume ratio that would facilitate enzymatic reactions during germination.

We conclude from this study that the yeast system is a powerful tool for the fundamental knowledge of lipid body dynamic by the study of functional properties of lipid body proteins identified by MS with an unknown function in lipid body biogenesis and regulation. Using this approach, we demonstrated here that AtClo1 had the capacity to organize the membrane with lipid body identity and to promote neutral lipid overaccumulation. The exploitation of this unicellular simple model could lead to attractive prospects for engineering of lipid bodies from microorganisms to plant biotechnological development.

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