Biotechnology Reports xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Taraxacum brevicorniculatum rubber elongation factor TbREF associates with lipid droplets and affects lipid turn-over in yeast

Jan Niklas Bröker[a,1], Natalie Laibach[b,1], Boje Müller[b], Dirk Prüfer[a,b], Christian Schulze Gronover[b,*]

[a] University of Muenster, Institute of Plant Biology and Biotechnology, Schlossplatz 8, 48143 Münster, Germany
[b] Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schlossplatz 8, 48143 Münster, Germany

ARTICLE INFO

Article history:
Received 23 July 2018
Received in revised form 31 October 2018
Accepted 31 October 2018

Keywords:
Lipid droplets
Taraxacum brevicorniculatum
Rubber elongation factor
Latex
Saccharomyces cerevisae

ABSTRACT

A protein named TbREF that is localized on rubber particles of the rubber producing dandelion species *Taraxacum brevicorniculatum* was expressed in tobacco leaves and in yeast. TbREF fused to fluorescence proteins colocalized on globular, hydrophobic structures, most likely lipid droplets. Furthermore, triacylglycerol, sterol and total lipid content of TbREF expressing yeast was determined by photometric analyses of nile red stainings and GC–MS analyses. Therefore, yeast exposed an enhanced nile red fluorescence as well as an increased TAG and sterol content compared to wildtype and vector control. Altogether, these findings gave new insights into the putative function of TbREF that might be pushing rubber particle production due to its cytotoxic nature and/or shielding and preventing degradation of lipid droplets. Furthermore, these results highlight possible biotechnological applications regarding the accumulation of hydrophobic compounds in lipid droplet like structures.

1. Introduction

Lipids are essential substances for organisms as membrane components, signaling molecules or energy supply. In plants, triacylglycerol (TAG) is the major lipid form that is stored to ensure sufficient nutrition during growth and development, especially germination [1,2]. TAGs are hydrophobic molecules and therefore need to be stored separately from the hydrophilic cytosol in small organelles with a phospholipid monolayer, called lipid droplets [3]. In germinative tissue, e.g. seeds, TAG containing lipid droplets are associated with the structural proteins oleosin – whose expression in Arabidopsis seeds is regulated by the transcription factor leafy cotyledon 2 (LEC2; [4]) – and caleosin, which integrate into the droplet membrane [5,6]. These proteins are responsible for the stabilization of the droplets and are also important to initiate their degradation upon germination [6–8]. Furthermore, they were shown to reduce lipid droplet size in vivo and could be localized at lipid droplets when overexpressed in tobacco leaves and in yeast cells [5,9–12]. There, they also increased TAG accumulation, indicating a kingdom spanning role in the formation of lipid droplets [13]. However, plants do not only possess lipid droplets that own oleosin and caleosins as their major structural proteins but especially in vegetative tissue other kinds with different associated proteins exist [3,14]. For example in the mesocarp of avocado (*Persea americana*) and oil palm (*Elaeis guineensis*) fruits, proteins with a completely different structure from oleosin were described to localize to lipid droplets [15,16]. These proteins were homologs to small rubber particle proteins (SRPPs) and rubber elongation factor (REF) due to the shared REF domain, that was identified first on rubber particles of the natural rubber producing plant *Hevea brasiliensis* [17,18]. Rubber particles resemble lipid droplets since they consist of a hydrophobic core composed mainly by poly(*cis*-1,4-isoprene) and a phospholipid monolayer [19]. SRPPs and REF from Hevea and guayule (*Parthenium argentatum*) were shown to promote the incorporation of isopentenyl pyrophosphate into larger isoprenes [17,20,18]. Similar observations were made for SRPP homologs expressed predominantly in the latex of the dandelion species *Taraxacum brevicorniculatum*, a potential alternative natural rubber source [21]. This plant possesses five SRPP isoforms that could be localized to rubber particles [21,22]. In TbSRPP depleted dandelions, rubber content was reduced and rubber particle stability impaired, suggesting a

* Corresponding author.
E-mail addresses: j_broe08@uni-muenster.de (J.N. Bröker), n.laibach@ilr.uni-bonn.de (N. Laibach), boje.mueller@ime.fraunhofer.de (B. Müller), dpruefer@uni-muenster.de (D. Prüfer), christian.schulze.gronover@ime.fraunhofer.de (C. Schulze Gronover).
[1] Authors contributed equally to the manuscript.

https://doi.org/10.1016/j.btre.2018.e00290

function for SRPPs similar to oleosin [21]. Furthermore, the promotion of stress tolerance and an impact on the distribution and formation of artificial poly(*cis*-1,4-isoprene) bodies was shown [23]. Next to TbSRPPs, another REF homolog that is also located on rubber particles and strongly expressed in latex, was identified in *T. brevicorniculatum* [24]. Its molecular mass of about 50 kDa is twice the number of the SRPP molecular mass and RNAi silencing of this protein had a comparable effect on rubber content. However, the stability and general integrity including rubber biosynthesis associated proteins and enzymes of remaining rubber particles stayed unaffected. So TbREF has presumably a function in rubber biosynthesis that is not redundant to TbSRPP function. To get further insight into the role of this protein, TbREF was expressed in *Nicotiana benthamiana* leaves and in *Saccharomyces cerevisiae*. Then, intracellular localization of TbREF was analyzed followed by estimation of TAG and sterol content of TbREF expressing yeast cells, to get new insights into potential biotechnological applications of REF-like proteins, regarding the accumulation of hydrophobic molecules in lipid droplet like structures.

2. Materials and methods

2.1. Cloning of constructs

TbREF coding sequence was amplified from *T. brevicorniculatum* cDNA using forward primer 5`-AAA CCA TGG ATT CAG AAG AGG CTA AG-3´and reverse primer 5`-CCC GAT ATC TCA GTC ATC ATC ATC GTT CAA CC-3`. The AtLEC2 coding sequence was amplified using *A. thaliana* cDNA, forward primer 5´- AAA CCA TGG ATA ACT TCT TAC CCT TTC-3´and reverse primer 5´- AAA GCG GCC GCT CAC CAC CAC CTC AAA GTC-3´ (restriction sites are underlined). The cDNA fragments were ligated into the corresponding sites of the Gateway vector pENTR4 (Life Technologies, www.lifetechnologies.com). For the transient expression in *N. benthamiana* the Gateway-compatible vectors patTL_cerulean_ccdB and pBatTL_ccdB [25] were used for TbREF and AtLEC2, respectively. For the expression of TbREF under the control of the GAL1 promoter and fused to eCFP in yeast, plasmids pAG_GAL1 and pAG_GAL1_eCFP of the Advanced Gateway Destination Vector kit (https://www.addgene.org/) were used, respectively. The integrity of all constructs was verified by sequencing [26] on an ABI PRISM3100 Genetic Analyzer (Applied Biosystems, Foster City, USA).

2.2. Transient expression of AtLEC2 and cerulean-TbREF in N. benthamiana leaves

The AtLEC2 and cerulean constructs were introduced into *A. tumefaciens* strain GV3102 pMP90 and infiltrated into *N. benthamiana* plants as previously described [27].

2.3. Strain construction and culture conditions

INV.Sc1 yeast cells were transformed using the lithium acetate method [28]. The yeast cells were plated on minimal synthetic defined (SD) medium (Clontech, Mountain View, USA) and incubated at 30 °C. Clones were checked for integrity by colony PCR. Wildtype yeast and cells transformed with the empty vectors (pAG_GAL1 and pAG_GAL1_eCFP) served as controls.

For the expression of TbREF, a single colony was picked and inoculated into 5 ml SD medium and cultivated overnight at 30 °C on a rolling platform. From this culture, 50 ml of fresh SD medium was inoculated to a final OD_{600} of 0.1 and incubated at 30 °C shaking at 140 rpm in a 250-ml Erlenmeyer flask. When the culture reached an OD_{600} of 0.4, the medium was changed to SD medium containing galactose instead of glucose to induce gene expression and cultivated for at least 20 h.

2.4. Nile red stainings and confocal laser scanning microscopy

For CLSM leaf discs were kept in a nile red solution (1 μg/mL) and under vacuum for 10 min. to stain lipid droplets 3–5 days after infiltration. Yeast cells were stained with 1 μg/ml nile red for 10 min. Nile red, cerulean and eCFP fluorescence was visualized by confocal laser scanning microscopy, excitation wavelength 458 nm and emission wavelength 465–509 nm or 579–668 nm, respectively, using a Leica TCS SP5 confocal microscope (Leica, https://de.leica-camera.com/). Fluorescence intensity at the in the pictures indicated areas was analyzed using Fiji software (Fiji, http://fiji.sc/Fiji). The number of lipid droplets per cell was determined from 50 yeast cells, each showing eCFP and Nile red fluorescence to confer proper expression of the construct and staining of the cells, respectively. For the statistical analysis Mann–Whitney *U* test ($p < 0{,}01$) was used.

For the time course measurements OD_{600} was determined and cells adequate to an OD_{600} of 1 were harvested before induction and 3–96 h post induction at 10 min, 9200 x g, resuspended and two times washed with phosphate buffer (pH 7.3). Then, half of each sample was stained with 1 μg/ml nile red (end concentration), mixed and measured using a TECAN microplate reader and i-control™ Software (Tecan Group Ltd., http://www.tecan.com/). Nile red fluorescence was detected at two emission spectra (510–550 nm and 570–610 nm) and exited at 470 nm or 535 nm, respectively. Relative fluorescence was calculated by condensing technical replicates, subtraction of unstained samples followed by addition of all measuring points of respective spectra and normalization to the wildtype sample before induction

2.5. Western blot analysis

1 ml of yeast culture with an OD_{600} of 3 was harvested by centrifugation. The pellet was resuspended in 300 μL potassium phosphate buffer (pH 7,5) and 100 μL trichloroacetic acid (50% w/v). After incubation for 30 min. at -80 °C and centrifugation, the pellet was washed two times with ice cold aceton (80% v/v). The precipitated protein was then resuspended in 80 μL 1% SDS / 0.1 M NaOH and 20 μL 5 x SDS buffer, followed by heating at 95 °C for 5 min. Afterwards, proteins were separated on a polyacrylamide gel containing 10% SDS and transferred to nitrocellulose membranes [29]. The membranes were stained with 0.1% Ponceau S in 5% acetic acid to confirm equal loading and were then incubated with an antibody raised against GFP derivatives as eCFP (dilution 1:833, JL-8, mouse, Clontech, https://www.clontech.com/) or a TbREF-specifc antibody (dilution 1:500, [24]) each for 2 h. After washing, primary antibody was detected with secondary anti-rabbit IgG for anti-TbREF and anti-mouse IgG for GFP that are both conjugated to an alkaline phosphatase and developed with Sigma Fast™, BCIP®/NBT (Sigma, https://www.sigmaaldrich.com/)

2.6. Immunogold labeling and TEM

For immunogold labelling, yeast cells were fixed in 6% (v/v) paraformaldehyde and 0,02% glutaraldehyde overnight (4 °C), washed with PIPES buffer (Sigma, http://www.sigmaaldrich.com) and dehydrated with an increasing ethanol-series (30–100%). Embedding was carried out with an increasing lowicryl/ethanol series at −20 °C followed by polymerisation first under UV light at −35 °C and room temperature. Ultra thin slices (60–70 nm) were collected on nickel mesh grids and contrasted with 2% (w/v) uranyl acetate for 3 min and Reynolds lead citrate for 3 s [30]. Transmitted electron signals were detected on a transmission electron microscope Phillips CM10.

2.7. Determination of fatty acid and sterol content via GC–MS

The determination of fatty acids in yeast was based on a protocol from [31]. Therefore, yeast cells adequate to an OD_{600} of 2 were harvested for TAG extraction (10 min, 1000 × *g*), resuspended in 1 ml phosphate buffer (pH 7.3), macerated and lyophilized. Then, internal standard triheptadecanoylglycerol (18.6 mM) was added and TAGs were extracted with 1:2 n-Heptan: MeOH/NaCl (10 g/l) for 1 h at 80 °C, followed by methylation with 532 µl methanol, 273 µl toluene, 68 µl DMP and 27 µl H_2SO_4 at 70 °C overnight. Samples were completely dried and dissolved in actone. GC-MS analysis was performed on a GC–MS-QP 2010 Ultra (Shimadzu, Duisburg, Germany) equipped with a 30 cm Rtx-5MS column and two temperature gradients (50–175 °C and 175–320 °C increased by 25 °C and 4 °C min, respectively; pressure: 3905 kPa). Molecules were detected by the ion masses 43 *m/z*, 55 *m/z*, 69 *m/z*, 74 *m/z*, 83 *m/z* and 87 *m/z*. Peak integration and identification was done with LabSolution software (Shimadzu, Duisburg, Germany) and NIST library, respectively. Total ion current (TIC) of the detected fatty acid methyl ester was normalized against heptadecanoic acid methyl ester derived from the internal standard.

For sterol determination the extraction based on a protocol from [32]. Therefore, freeze-dried yeast cells together with 250 µg betulin as internal standard were saponified at 80 °C in 20 ml of KOH (6%, w/v) methanolic solution for 2 h. Evaporated methanol was replaced with water and extracted three times with 1 volume of hexane. Hexane phases were pooled, samples evaporated to dryness dissolved in 1 ml acetone overnight before being analyzed via GC-MS as described above with following differences: the column was held at 120 °C for 3 min and then developed with a temperature gradient of 15 °C/min to a final temperature of 330 °C which was held for 10 min. TIC of ergosterol and precursors was normalized against the internal standard betulin.

For the statistical analysis of the GC–MS measurements TIC values of three replicates of two colonies each were compared using Mann–Whitney *U* test ($p < 0{,}01$).

3. Results and discussion

3.1. TbREF localizes to lipid droplets in heterologous systems

Since homologs of TbREF were described to be associated with lipid droplets or similar structures [15,16], we expressed an N-terminal cerulean-TbREF fusion in tobacco leaves and an N-terminal eCFP-TbREF fusion in yeast. For the transient expression in *N. benthamiana* we additionally expressed AtLEC2 to induce enhanced lipid droplet formation in the epidermal leaf tissue. We were able to visualize the lipid droplets as small globular structures upon AtLEC2 expression and after staining with nile red, using a confocal laser scanning microscope (Fig. 1A). Lipid droplets were observed in cells infiltrated with AtLEC2 in combination with cerulean-TbREF and cerulean-ccdB, whereas the fluorescence intensities of nile red and cerulean at the indicated areas (region of interest, ROI) differ in cells expressing cerulean-ccdB, but were almost identical in the case of cerulean-TbREF, indicating the localization of TbREF to lipid droplets in *N. benthamiana* leaves.

To further evaluate, the localization of TbREF in heterologous systems we expressed the protein fused to an eCFP reporter gene under the control of the galactose inducible GAL1 promoter (GAL1P) in *S. cerevisae* INVSC1. Therefore, yeasts were cultivated under respective amino acid depriving conditions and harvested 20 h after induction. We visualized the eCFP fluorescence with a confocal laser scanning microscope simultaneously to a nile red staining of the yeast (Fig. 1B). After nile red staining small globular structures that are most probably hydrophobic lipid droplets were observed. Similar structures were detected in the eCFP-TbREF channel of eCFP-TbREF expressing yeast but not in eCFP-ccdB yeast that showed a more cellular-wide fluorescence. The fluorescence intensity at indicated areas (region of interest, ROI) in the pictures, indicate that the globular structures observed in nile red stain and eCFP channel of eCFP-TbREF expressing yeast are largely identical, as in the infiltrated *N. benthamina* leaves. Furthermore, we determined the number of lipid droplets per yeast cell and could observe an enhanced number of 6 lipid droplets per cell in eCFP-TbREF expressing cells compared to 4 lipid droplets per cell in eCFP-ccdB yeasts (Fig. 1C). For a more detailed impression of the intracellular localization of TbREF in yeast, TbREF, parallel to a vector control, was expressed in yeast and cells were fixed and embedded in lowicryl resin using gelatin capsules. Ultra-thin slices were then incubated with α-TbREF antibody, followed by immunological detection of the first antibody with a second, gold particle coupled, antibody. The yeast cells were visualized by transmission electron microscopy (TEM; Fig. 1D, arrows are indicating gold particles) and many of the gold particles observed in TbREF expressing yeast appeared close to electron lucent, round structures that are most obvious lipid droplets, as these organelles were reported to become transparent for electrons due to preparation [13,33]. However, heterologous expression of oleosin and caleosin also resulted in a localization on lipid droplets and furthermore in an accumulation of TAGs and sterols [10,13].

3.2. TbREF expressing yeasts show enhanced TAG and sterol contents

As an obvious alteration of lipid droplets could be observed in TbREF expressing yeast cells, we tested the accumulation of TAGs and other lipids after induction of TbREF expression in yeast cultures. First, relative lipid accumulation in InvSc1 wildtype, vector control (pAG424_GAL1) and TbREF expressing yeast was estimated after different time points post induction. Therefore, yeast cells were harvested and stained with nile red with parallel analysis of TbREF protein expression and cell density (OD_{600}). The content of TAGs (510–550 nm) and free lipids (570–610 nm) in wildtype and vector control yeast decreases after 20–51 h while the cell density grew continuously (Fig. 2A) [34]. TbREF expressing yeast showed an almost contrasting behavior with only little altering lipid content and inhibited growth. This changed after 51 h, when TbREF expressing yeast slowly became denser and lipids were degraded. In contrast, the lipid content of wildtype and vector control yeast increased due to the accumulation of lipid bodies in the stationary phase as has been described earlier [35]. This points towards a delay in the growth phases of the yeast upon TbREF expression. A detectable amount of TbREF was first found after 15 h (Fig. 2B), when the accumulation and growth reduction started. The signal of TbREF around 50 kDa, that matches the predicted molecular mass of the protein [24], decreased after 51 h and an additional less distinct signal with a molecular weight higher than 100 kDa appeared, indicating a correlation between growth, lipid accumulation (or preservation) and presence of a monomeric, and therefor functional, TbREF protein. The additional signals appearing after 51 h are most likely TbREF oligomers that suggest the formation of non-functional TbREF aggregates.

As it is known that the use of nile red for the quantification of lipids in living cells might be limited, caused by kinetic anomalies of the dye itself we aimed to confirm the higher content of TAG and further hydrophobic substances via GC–MS measurements [36]. For this, we cultivated the same yeast strains again for 23 h and harvested cells for TAG and sterol extraction. Though nile red fluorescence showed an approximately 8-fold increase, the significantly enhanced TAG content, determined via GC–MS, was only doubled in TbREF expressing yeast compared to vector control yeast (Fig. 3). However, the content of ergosterol and its precursors

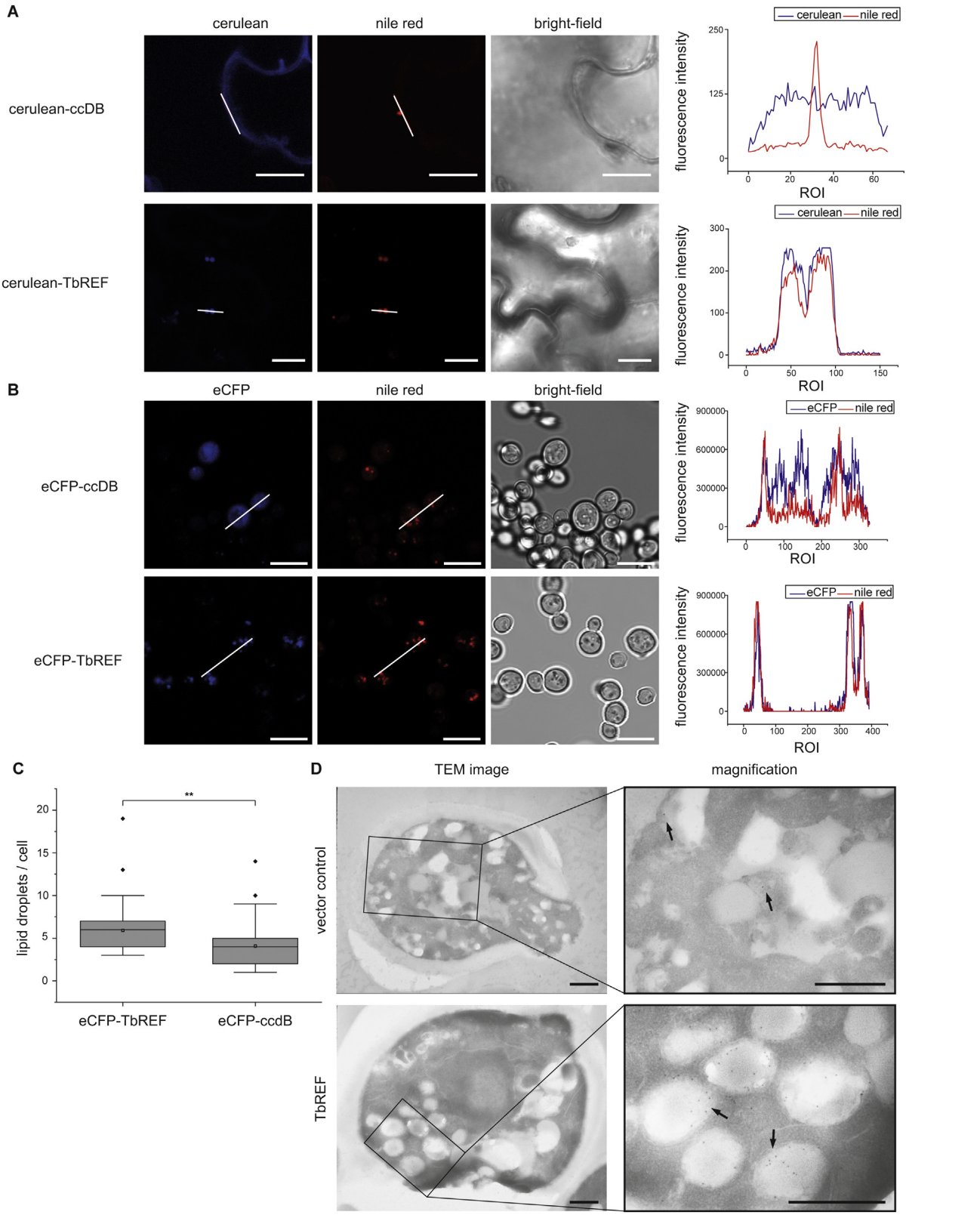

Fig. 1. Localization of TbREF in *N. benthamiana* and *S. cerevisae*. **A**. Infiltrated *N. benthamina* leaves expressing a cerulean-TbREF fusion protein and AtLEC2 (for enhanced lipid droplet formation) were stained with nile red and visualized by confocal laser scanning microscopy, excitation wavelength 458 nm and emission wavelength 465–509 nm or 579–668 nm, respectively. Cerulean-ccdB infiltrated leaves served as controls. Scale bar: 15 μm **B**. Yeast cells expressing an eCFP-TbREF fusion protein were stained with nile red and visualized by confocal laser scanning microscopy as previously described. Yeast cells transformed with pAG424_GAL1_eCFP vector served as controls. Scale bar: 7.5 μm. **C**. Determination of lipid droplets/cell in yeast cells expressing eCFP-TbREF fusion protein and ccdB-eCFP. ** = Mann–Whitney *U* test ($p < 0{,}01$) **D**. Visualization of TbREF in yeast cells via immunogold labeling. Yeast cells transformed with pAG424_GAL1_eCFP vector served as controls. Boxes indicate magnified area, arrows gold particles. Scale bar: 500 nm. ROI = region of interest.

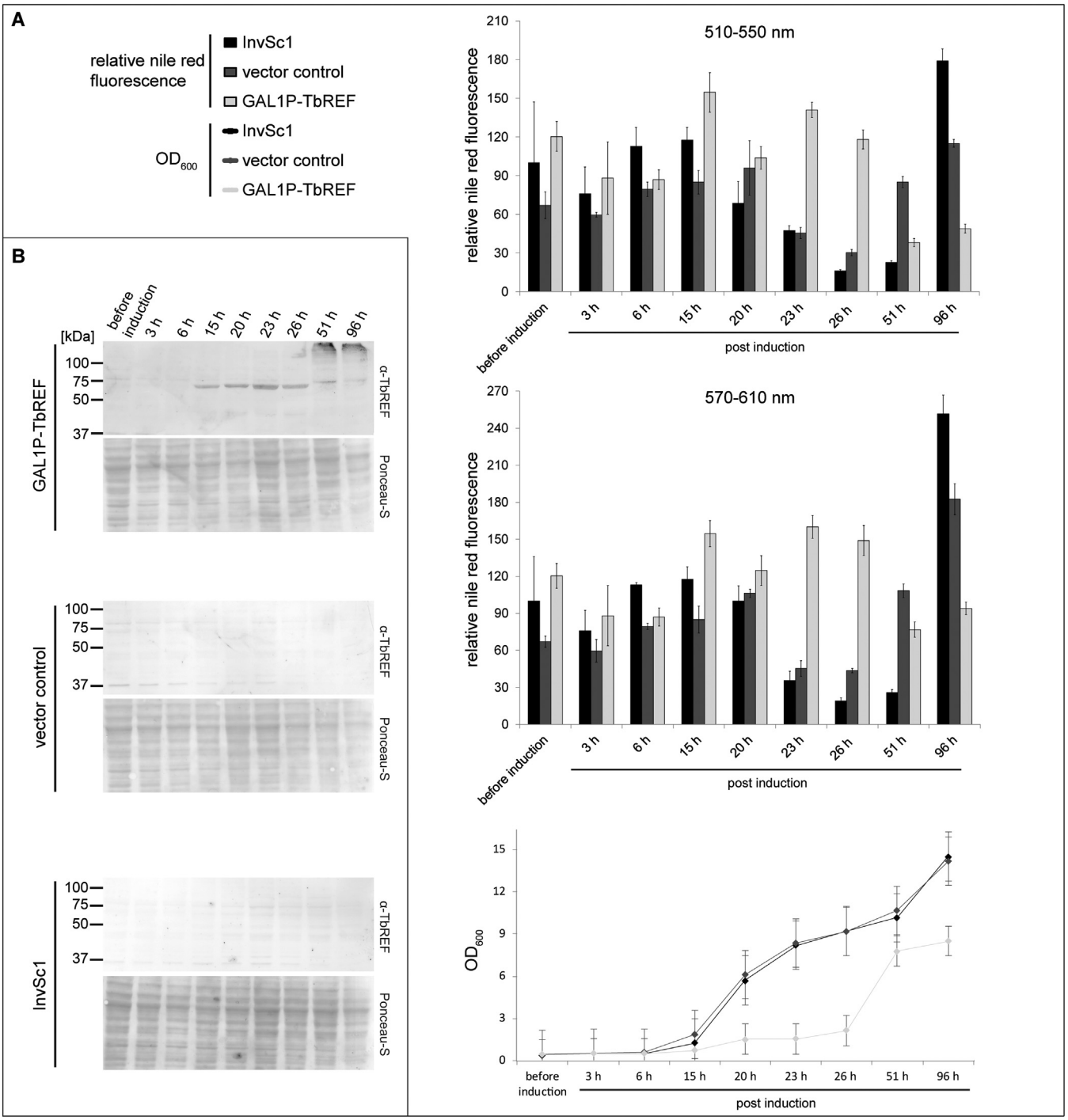

Fig. 2. Time series of lipid accumulation in TbREF expressing yeast cells. **A**. InvSc1 wildtype yeast, cells containing pAG424_GAL1 vector as a control and TbREF expressing cells were cultivated, stained and measured as described in" Materials and methods". Error bars were calculated from three replicates. **B**. Protein expression analysis of yeast cells expressing TbREF using a TbREF specific antibody. Ponceau-S stained membranes confirmed equal loading.

such as lanosterol and squalene were significantly increased in TbREF expression yeast compared to the vector control, indicating a sterol accumulation. In yeast expressing toxic proteins, these were reported to be stored in inclusion bodies and afterwards, mediated by a so far uncharacterized protein, lml2, integrated into lipid droplets [37]. These lipid droplets are described to comprise sterols as detergents for the hydrophobic protein. Due to the increase in sterol content in TbREF expression, it is thinkable that TbREF is a cytotoxic protein and might be disposed by this mechanism. However, another scenario for TbREF in yeast is suggested by the lipid accumulation over time or better the inhibited lipid degradation with delayed growth in GAL1P-TbREF yeast cells. A similar observation has been made, when the yeast TAG lipase Tgl3p was deleted [38,39]. So TbREF might also hinder lipase access to the TAGs stored in the lipid droplets and jam parts of the potentially valuable energy for growth and cell division. This underlines the biotechnological potential of the protein regarding the accumulation of hydrophobic substances in more advanced microbial cell systems that have already been optimized for the biosynthesis of higher TAG amounts [40].

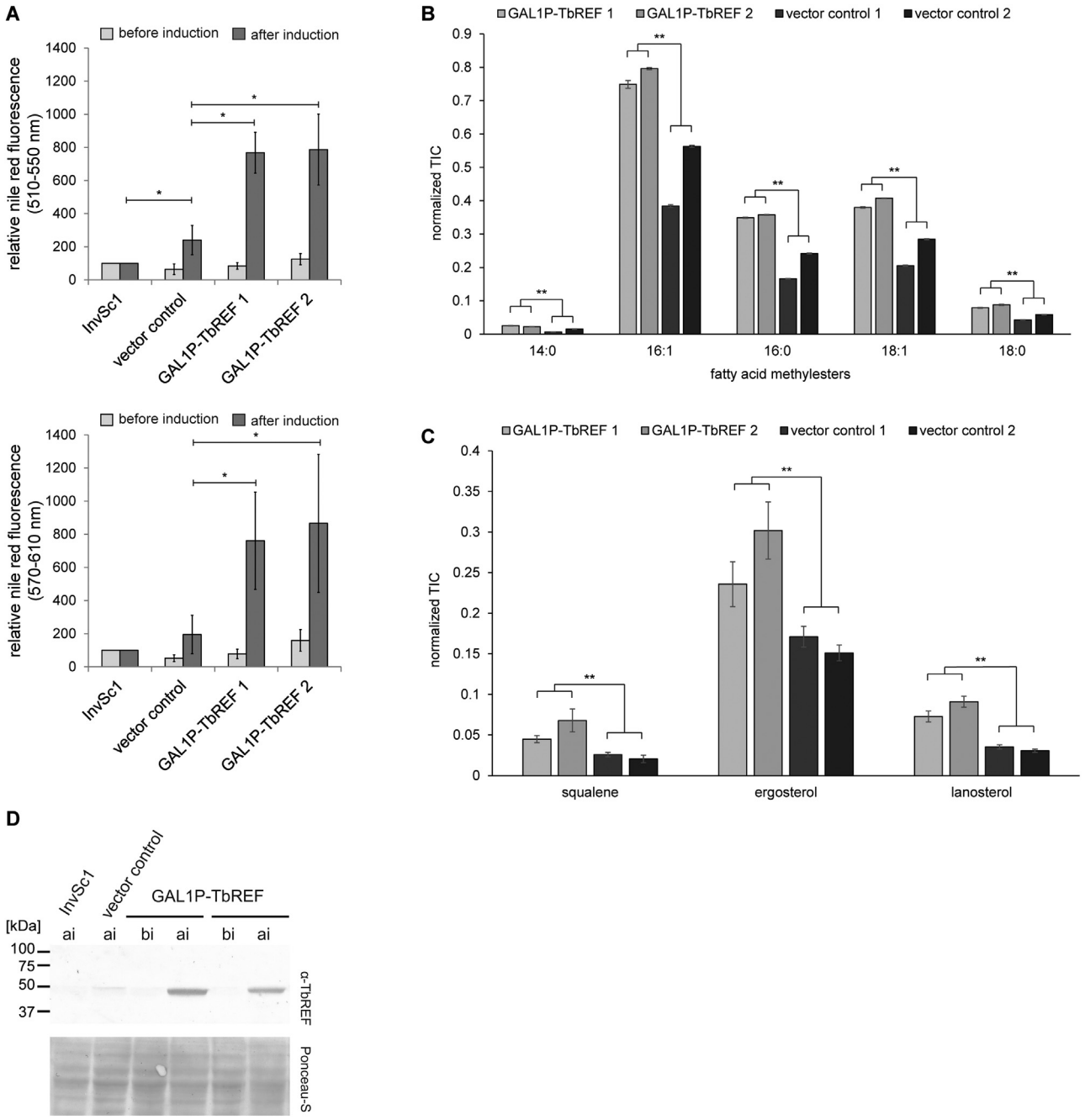

Fig. 3. TAG and sterol accumulation in two TbREF expressing yeast colonies 23 h post induction. **A**. Nile red staining of InvSc1 wildtype yeast, cells containing pAG424_GAL1 vector as a control and TbREF expressing cells, before and after induction. * = Mann–Whitney *U* test ($p < 0{,}05$) **B**. TAG measurements of TbREF expressing yeast cells via GC–MS. ** = Mann–Whitney *U* test ($p < 0{,}01$) **C**. Sterol content of TbREF expressing yeast cells. ** = Mann–Whitney *U* test ($p < 0{,}01$) **D**. Confirmation of TbREF expression via western-blot analysis using a TbREF specific antibody. bi = before induction; ai = after induction.

4. Conclusions

By the expression of AtLEC2 in combination with a cerulean-TbREF fusion protein in *N. benthamiana* leaves and an eCFP-TbREF fusion protein in yeast we could show that TbREF attaches to nile red stainable, globular and hydrophobic structures, most likely lipid droplets. This observation was further supported by the immunogold labeling of TbREF expressing yeast cells. Furthermore, we were able to show that the protein enhances the number of lipid droplets per cell, reduces cell growth and leads to lipid body conservation in yeast. In laticifers of rubber producing plants, this properties might increase rubber particle formation and hence rubber production to protect the cells from cytotoxic effects. On the other hand, the protein might shield rubber particles during their ripening at the endoplasmic reticulum and also after budding from lipases and other harmful molecules to prevent their degradation and agglomeration. Here it was shown that the localization of TbREF to storage organelles like lipid droplets is also adaptable to other plant species and organs, as well as to non-plant expression systems. Therefore, TbREF might be useful for biotechnological applications, since TAGs and in general plant derived hydrophobic molecules are of great economic interest, as the prevention of their degradation before extraction is an

important issue [14]. Oleosin for example has to be biotechnologically modified before it is resistant to lipoxygenases, so TbREF might be a possible alternative [14,41]. In general expression of stabilizing proteins might already increase the yield, in plants or other expression systems like yeast or bacteria. As already discussed and shown for SRPP homologs, protection of artificial oil bodies might be another suggestive application [14,23].

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The technical assistance of Daniela Ahlert (Institute of Plant Biology and Biotechnology, Münster) and Ursula Malkus (Institute for Medical Physics and Biophysics, Münster) is kindly acknowledged. Jan Niklas Bröker was funded by a grant (FKZ 031B0014D) of the Federal Ministry of Education and Research, Germany.

References

[1] S.-C. Lung, R.J. Weselake, Diacylglycerol acyltransferase: a key mediator of plant triacylglycerol synthesis, Lipids 41 (2006) 1073–1088.
[2] J. Nielsen, Systems biology of lipid metabolism: from yeast to human, FEBS Lett. 583 (2009) 3905–3913, doi:http://dx.doi.org/10.1016/j.febslet.2009.10.054.
[3] K.D. Chapman, J.M. Dyer, R.T. Mullen, Biogenesis and functions of lipid droplets in plants thematic review series: lipid droplet synthesis and metabolism: from yeast to man, J. Lipid Res. 53 (2012) 215–226, doi:http://dx.doi.org/10.1194/jlr.R021436.
[4] N. Che, Y. Yang, Y. Li, L. Wang, P. Huang, Y. Gao, C. An, Efficient LEC2 activation of OLEOSIN expression requires two neighboring RY elements on its promoter, Sci. China C: Life Sci. 52 (2009) 854–863, doi:http://dx.doi.org/10.1007/s11427-009-0119-z.
[5] A.H. Huang, Oleosins and oil bodies in seeds and other organs, Plant Physiol. 110 (1996) 1055.
[6] J.T. Tzen, A. Huang, Surface structure and properties of plant seed oil bodies, J. Cell Biol. 117 (1992) 327–335.
[7] A. Liavonchanka, I. Feussner, Lipoxygenases: occurrence, functions and catalysis, J. Plant Physiol. 163 (2006) 348–357, doi:http://dx.doi.org/10.1016/j.jplph.2005.11.006.
[8] M. Rudolph, A. Schlereth, M. Körner, K. Feussner, E. Berndt, M. Melzer, E. Hornung, I. Feussner, The lipoxygenase-dependent oxygenation of lipid body membranes is promoted by a patatin-type phospholipase in cucumber cotyledons, J. Exp. Bot. 62 (2011) 749–760, doi:http://dx.doi.org/10.1093/jxb/erq310.
[9] S.K. Gidda, S. Park, M. Pyc, O. Yurchenko, Y. Cai, P. Wu, D.W. Andrews, K.D. Chapman, J.M. Dyer, R.T. Mullen, Lipid droplet-associated proteins (LDAPs) are required for the dynamic regulation of neutral lipid compartmentation in plant cells, Plant Physiol. 170 (2016) 2052–2071, doi:http://dx.doi.org/10.1104/pp.15.01977.
[10] N. Jacquier, S. Mishra, V. Choudhary, R. Schneiter, Expression of oleosin and perilipins in yeast promotes formation of lipid droplets from the endoplasmic reticulum, J. Cell. Sci. 126 (2013) 5198–5209, doi:http://dx.doi.org/10.1242/jcs.131896.
[11] T.L. Shimada, T. Shimada, H. Takahashi, Y. Fukao, I. Hara-Nishimura, A novel role for oleosins in freezing tolerance of oilseeds in *Arabidopsis thaliana*, Plant J. 55 (2008) 798–809, doi:http://dx.doi.org/10.1111/j.1365-313X.2008.03553.x.
[12] T. Wahlroos, J. Soukka, A. Denesyuk, R. Wahlroos, T. Korpela, N.J. Kilby, Oleosin expression and trafficking during oil body biogenesis in tobacco leaf cells, Genesis 35 (2003) 125–132, doi:http://dx.doi.org/10.1002/gene.10172.
[13] M. Froissard, S. D'andréa, C. Boulard, T. Chardot, Heterologous expression of AtClo1, a plant oil body protein, induces lipid accumulation in yeast, FEMS Yeast Res. 9 (2009) 428–438, doi:http://dx.doi.org/10.1111/j.1567-1364.2009.00483.x.
[14] N. Laibach, J. Post, R.M. Twyman, C.S. Gronover, D. Prüfer, The characteristics and potential applications of structural lipid droplet proteins in plants, J. Biotechnol. 201 (2015) 15–27, doi:http://dx.doi.org/10.1016/j.jbiotec.2014.08.020.
[15] S.K. Gidda, S.C. Watt, J. Collins-Silva, A. Kilaru, V. Arondel, O. Yurchenko, P.J. Horn, C.N. James, D. Shintani, J.B. Ohlrogge, Lipid droplet-associated proteins (LDAPs) are involved in the compartmentalization of lipophilic compounds in plant cells, Plant Signal. Behav. 8 (2013) e27141, doi:http://dx.doi.org/10.4161/psb.27141.
[16] P.J. Horn, C.N. James, S.K. Gidda, A. Kilaru, J.M. Dyer, R.T. Mullen, J.B. Ohlrogge, K.D. Chapman, Identification of a new class of lipid droplet-associated proteins in plants, Plant Physiol. 162 (2013) 1926–1936, doi:http://dx.doi.org/10.1104/pp.113.222455.
[17] M.S. Dennis, D. Light, Rubber elongation factor from *Hevea brasiliensis*. Identification, characterization, and role in rubber biosynthesis, J. Biol. Chem. 264 (1989) 18608–18617.
[18] S.K. Oh, H. Kang, D.H. Shin, J. Yang, K.-S. Chow, H.Y. Yeang, B. Wagner, H. Breiteneder, K.-H. Han, Isolation, characterization, and functional analysis of a novel cDNA clone encoding a small rubber particle protein from *Hevea brasiliensis*, J. Biol. Chem. 274 (1999) 17132–17138.
[19] K. Cornish, Similarities and differences in rubber biochemistry among plant species, Phytochemistry 57 (2001) 1123–1134.
[20] I.J. Kim, S.B. Ryu, Y.S. Kwak, H. Kang, A novel cDNA from Parthenium argentatum Gray enhances the rubber biosynthetic activity in vitro, J. Exp. Bot. 55 (2014) 377–385, doi:http://dx.doi.org/10.1093/jxb/erh039.
[21] A. Hillebrand, J.J. Post, D. Wurbs, D. Wahler, M. Lenders, V. Krzyzanek, D. Prüfer, C.S. Gronover, Down-regulation of small rubber particle protein expression affects integrity of rubber particles and rubber content in *Taraxacum brevicorniculatum*, PLoS One 7 (2012)e41874, doi:http://dx.doi.org/10.1371/journal.pone.0041874.
[22] T. Schmidt, A. Hillebrand, D. Wurbs, D. Wahler, M. Lenders, C.S. Gronover, D. Prüfer, Molecular cloning and characterization of rubber biosynthetic genes from *Taraxacum koksaghyz*, Plant Mol. Biol. Rep. 28 (2010) 277–284, doi:http://dx.doi.org/10.1007/s11105-009-0145-9.
[23] N. Laibach, S. Schmidl, B. Müller, M. Bergmann, D. Prüfer, C. Schulze Gronover, Small rubber particle proteins from *Taraxacum brevicorniculatum* promote stress tolerance and influence the size and distribution of lipid droplets and artificial poly(cis-1,4-isoprene) bodies, Plant J. 93 (2018) 1045–1061, doi:http://dx.doi.org/10.1111/tpj.13829.
[24] N. Laibach, A. Hillebrand, R.M. Twyman, D. Prüfer, C. Schulze Gronover, Identification of a *Taraxacum brevicorniculatum* rubber elongation factor protein that is localized on rubber particles and promotes rubber biosynthesis, Plant J. 82 (2015) 609–620, doi:http://dx.doi.org/10.1111/tpj.12836.
[25] J. Epping, N. van Deenen, E. Niephaus, A. Stolze, J. Fricke, C. Huber, W. Eisenreich, R.M. Twyman, D. Prüfer, C. Schulze Gronover, A rubber transferase activator is necessary for natural rubber biosynthesis in dandelion, Nat. Plants 1 (2015) 15048, doi:http://dx.doi.org/10.1038/NPLANTS.2015.48.
[26] F. Sanger, S. Nicklen, A.R. Coulsen, DNA sequencing with chainterminating inhibitors, Proc. Natl. Acad. Sci. U. S. A. 74 (1977) 5463–5467.
[27] B. Müller, G.A. Noll, A.M. Ernst, B. Rüping, S. Groscurth, R.M. Twyman, L.M. Kawchuck, D. Prüfer, Recombinant artificial forisomes provide ample quantities of smart biomaterials for use in technical devices, Appl. Microbiol. Biotechnol. 88 (2010) 689–698, doi:http://dx.doi.org/10.1007/s00253-010-2771-4.
[28] R.D. Gietz, R.H. Schiestl, High-efficiency yeast transformation using the LiAC/SS carrier DNA/PEGmethod, Nat. Protoc. 2 (2007) 31–34, doi:http://dx.doi.org/10.1038/nprot.2007.13.
[29] H. Towbin, T. Staehlin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[30] E.S. Reynolds, The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, J. Cell Biol. 17 (1963) 208–212.
[31] N. Ruiz-López, E. Martínez-Force, R. Garcés, Sequential one-step extraction and analysis of triacylglycerols and fatty acids in plant tissues, Anal. Biochem. 317 (2003) 247–254, doi:http://dx.doi.org/10.1016/S0003-2697(03)00139-8.
[32] S. Rodriguez, J. Kirby, C.M. Denby, J.D. Keasling, Production and quantification of sesquiterpenes in *Saccharomyces cerevisiae*, including extraction, detection and quantification of terpene products and key related metabolites, Nat. Protoc. 9 (2014) 1980–1996, doi:http://dx.doi.org/10.1038/nprot.2014.132.
[33] D. Binns, T. Januszewski, Y. Chen, J. Hill, V.S. Markin, Y. Zhao, C. Gilpin, K.D. Chapman, R.G. Anderson, J.M. Goodman, An intimate collaboration between peroxisomes and lipid bodies, J. Cell Biol. 173 (2006) 719–731, doi:http://dx.doi.org/10.1083/jcb.200511125.
[34] P. Greenspan, E.P. Mayer, S.D. Fowler, Nile red: a selective fluorescent stain for intracellular lipid droplets, J. Cell Biol. 100 (1985) 965–973.
[35] L. Sandager, M.H. Gustavsson, U. Stahl, A. Dahlqvist, E. Wiberg, A. Banas, M. Lenman, H. Ronne, S. Stymne, Storage lipid synthesis is non-essential in yeast, J. Biol. Chem. 277 (2001) 6478–6482, doi:http://dx.doi.org/10.1074/jbc.M109109200.
[36] U. Pick, T. Rachutin-Zalogin, Kinetic anomalies in the interactions of Nile red with microalgae, J. Microbiol. Methods 88 (2012) 189–196, doi:http://dx.doi.org/10.1016/j.mimet.2011.10.008.
[37] O. Moldavski, T. Amen, S. Levin-Zaidman, M. Eisenstein, I. Rogachev, A. Brandis, D. Kaganovich, M. Schuldiner, Lipid droplets are essential for efficient clearance of cytosolic inclusion bodies, Dev. Cell 33 (2015) 603–610, doi:http://dx.doi.org/10.1016/j.devcel.2015.04.015.
[38] K. Athenstaedt, G. Daum, Tgl4p and Tgl5p, two triacylglycerol lipases of the yeast *Saccharomyces cerevisiae* are localized to lipid particles, J. Biol. Chem. 280 (2005) 37301–37309, doi:http://dx.doi.org/10.1074/jbc.M507261200.
[39] K. Athenstaedt, G. Daum, The life cycle of neutral lipids: synthesis, storage and degradation, Cell. Mol. Life Sci. 63 (2006) 1355–1369, doi:http://dx.doi.org/10.1007/s00018-006-6016-8.
[40] P.G. Teixeira, F. David, V. Siewers, J. Nielsen, Engineering lipid droplet assembly mechanisms for improved triacylglycerol accumulation in *Saccharomyces cerevisiae*, FEMS Yeast Res. 1 (2018) 18, doi:http://dx.doi.org/10.1093/femsyr/foy060.
[41] N.J. Roberts, R.W. Scott, S. Winichayakul, M. Roldan, Modified oil encapsulating proteins and uses thereof. Google Patents (2015).