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Palmitvaccenic Acid (Δ11-*cis*-hexadecenoic acid) Is Synthesized by an OLE1-like Desaturase in the Arbuscular Mycorrhiza Fungus *Rhizophagus irregularis*

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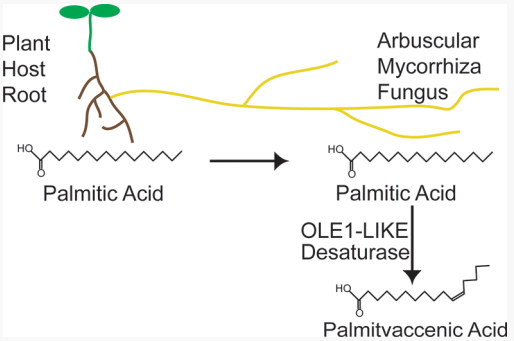
ABSTRACT: Arbuscular mycorrhiza (AM) fungi deliver mineral nutrients to the plant host in exchange for reduced carbon in the form of sugars and lipids. Colonization with AM fungi upregulates a specific host lipid synthesis pathway resulting in the production of fatty acids. Predominantly palmitic acid (16:0) and the unusual palmitvaccenic acid ($16:1^{\Delta 11cis}$) accumulate in the fungus *Rhizophagus irregularis*. Here, we present the isolation and characterization of *Ri*OLE1-LIKE, the desaturase involved in palmitvaccenic acid synthesis, by heterologous expression in yeast and plants. Results are in line with the scenario in which *RiOLE1-LIKE* encodes an acyl-CoA desaturase with substrate specificity for C15−C18 acyl groups, in particular C16. Phylogenetic analysis of *RiOLE1-LIKE*-related sequences revealed that this gene is conserved in AM fungi from the Glomales and Diversisporales but is absent from nonsymbiotic Mortierellaceae and Mucoromycotina fungi, suggesting that $16:1^{\Delta 11cis}$ provides a specific function during AM colonization.



Mycorrhiza fungi establish symbiotic interactions with plant roots. The fungi provide mineral nutrients, particularly phosphate, to the host, in exchange for reduced carbon.[1] AM fungi establish an intricate membrane system in the cytosol of the root cortex cell, the so-called arbuscule, where nutrient and carbon exchange takes place.[2] Previously, it was believed that carbon is transported to the fungus solely in the form of carbohydrates. The fact that AM fungi lack the genes for fatty acid *de novo* synthesis, together with recent evidence that plant-derived fatty acids accumulate in the fungus, demonstrated that in addition to carbohydrates, fatty acid-containing lipids are transferred from the plant to the fungus.[3−8] In line with this scenario, expression of host specific genes (e.g., *DIS*, *FatM*, and *RAM2*) involved in fatty acid and lipid synthesis is upregulated during colonization. The *DIS* gene encodes plastidial *β*-ketoacyl-acyl carrier (ACP) synthase I (KASI) that is involved in the production of palmitoyl-(16:0)-ACP.[6,a] *FatM* encodes a plastidial acyl-ACP thioesterase, with high activity for hydrolysis of 16:0-ACP.[7,8] After export from the plastids, fatty acids are converted into acyl-CoA thioesters. The RAM2 gene product produces *sn*2-acylglycerol form glycerol 3-phosphate and acyl-CoA, with a preference for 16:0-CoA.[3] Recently, methyl-myristic acid (methyl-14:0) was shown to induce spore formation of *Rhizophagus irregularis*.[9] Furthermore, supplementation of the medium with myristic acid (14:0) was sufficient to support hyphal growth in an axenic culture, and 14:0 was taken up by the fungus and metabolized.[10] Taken together, these results suggest that a 14:0- or 16:0-containing lipid most likely is transferred from the host to the fungus where it serves as a carbon source in addition to carbohydrates.

While AM fungal genomes lack the genes for fatty acid *de novo* synthesis, they do contain genes encoding fatty acid desaturases and elongases.[4] Therefore, fatty acids such as 14:0 or 16:0 can be desaturated and elongated to very long chain fatty acids in AM fungi.[11] The major fatty acids in AMF are 16:0 and $16:1^{\Delta 11cis}$ (11-*cis*-palmitvaccenic acid, $16:1^{\omega 5cis}$). $16:1^{\Delta 11cis}$ is absent from plants. It accounts for 46−78 mol % of total fatty acids in spores of AMF of the Glomales, including the families Acaulosporaceae, Glomaceae, and Gigasporaceae, albeit some species of Glomaceae (*Glomus leptotichum* and *Glomus occultum*) and Gigasporaceae (*Gigaspora albida*, *Gigaspora gigantea*, *Gigaspora margarita*, and *Gigaspora rosea*) contain no $16:1^{\Delta 11cis}$ or only traces. In addition to $16:1^{\Delta 11cis}$, *R. irregularis*, *Glomus claroideum*, and *G. rosea* spores and hyphae contain 0.2−3.3 mol % $16:1^{\Delta 9cis}$ (palmitoleic acid, $16:1^{\omega 7cis}$). Glomaceae species lacking $16:1^{\Delta 11cis}$ (*G. leptotichum* and *G. occultum*) instead contain considerable amounts (11−55%) of $16:1^{\Delta 9cis}$. Spores of *Gigaspora* spp. with small amounts of

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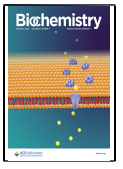

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16:1$^{\Delta 11cis}$ contain high levels (38−48%) of 18:1$^{\Delta 9cis}$ (oleic acid) and 8−15% 20:1$^{\Delta 11cis}$ (gondoic acid).[12] Oleic acid and gondoic acid are present in small amounts in spores of *R. irregularis* and other Glomaceae,[11,12] but these fatty acids are not AM fungus specific, because they are also found in noncolonized plant roots.[11,13] Further very long chain fatty acids, i.e., C20, C22, and C24, also accumulate in AM fungi, with 20:3 being the most abundant (0.2−1.5 mol % in *R. irregularis*).[11,13]

16:1$^{\Delta 11cis}$ is found in only AM fungi and is absent from plants and from most other fungi except for small amounts in the ectomycorrhiza fungi *Pisolithus tinctorius* and *Laccaria bicolor*.[14,15] 16:1$^{\Delta 11cis}$ is also found in some bacterial species, including *Cytophaga hutchinsonii* or *Lactobacillus* spp.[16,17] In some moths, 16:1$^{\Delta 11cis}$ serves as an intermediate for the synthesis of sex pheromones. In *Trichoplusia ni*, 16:1$^{\Delta 11cis}$ is chain-shortened to 14:1$^{\Delta 9cis}$ and 12:1$^{\Delta 7cis}$, the latter being reduced to the alcohol and acetylated, forming the active pheromone *cis*-7-dodecenyl acetate.[18] In the small ermine moths *Yponomeuta evonymella* and *Yponomeuta padella*, 11-*cis*-hexadecenol acetate is directly used as a sex pheromone.[19]

In AM fungi, 16:1$^{\Delta 11cis}$ accumulates in different glycerolipids, mostly in the storage lipid triacylglycerol, but it is also found in phospholipids.[4,13] Because of the rather unique presence of 16:1$^{\Delta 11cis}$ and other specific fatty acids (e.g., 20:3) in AM fungi, these fatty acids were used as lipid biomarkers for AM fungi under laboratory and field conditions.[12,20,21] In line with this scenario, the rate of colonization with *R. irregularis* correlates with the accumulation of 16:1$^{\Delta 11cis}$ in colonized roots.[22−24]

The gene involved in 16:1$^{\Delta 11cis}$ production in AM fungi remained unknown. Evaluation of the *R. irregularis* genome and transcriptome revealed the presence of one sequence (*RiOLE1*) that is highly similar to the *Saccharomyces cerevisiae OLE1* (*oleic acid dependent1*) gene and a second sequence (*RiOLE1-LIKE*) that is less similar.[4] In addition, the *R. irregularis* genome contains five sequences that are highly similar to the Δ12 desaturase, Δ5 desaturase, and Δ6 desaturase sequences from *Mortierella alpina*.[4] To identify the gene involved in 16:1$^{\Delta 11cis}$ production in *R. irregularis*, we focused on the characterization of *RiOLE1* and *RiOLE1-LIKE*. Expression in the *S. cerevisiae* Δ*ole1* mutant together with fatty acid analysis and fatty acid feeding experiments revealed that *Ri*OLE1 predominantly produces oleic acid (18:1$^{\Delta 9cis}$), while *Ri*OLE1-LIKE is crucial for the synthesis of 16:1$^{\Delta 11cis}$. *Ri*OLE1-LIKE was subsequently introduced into transgenic tobacco (*Nicotiana benthamiana*) and *Camelina*, to evaluate its capacity to produce 16:1$^{\Delta 11cis}$ in a plant system, and the phylogenetic distribution of *Ri*OLE1-LIKE in symbiotic and nonsymbiotic fungi was investigated.

MATERIALS AND METHODS

Phylogenetic Analysis. To identify the desaturase sequences from *R. irregularis*, *M. alpina* desaturase sequences were retrieved from UniProtKB (uniprot.org) and used in BLASTp searches at the EnsemblFungi database (fungi.ensembl.org). For phylogenetic analysis of desaturases in symbiotic and nonsymbiotic Mucoromycota, *RiOLE1* and *RiOLE1-LIKE* sequences were used in BLASTp searches to identify orthologs in *G. rosea*, *Rhizophagus cerebriforme*, and *Rhizophagus diaphanus* genomes available at MycoCosm.[25] *Rhizophagus clarus*, *Mortierella circinelloides*, *Rhizophagus microsporus*, *Mortierella elongata*, and *Lobosporangium transversale* genomes at EnsemblFungi were surveyed for *RiOLE1* and *RiOLE1-LIKE* orthologs using BLASTp. The sequences for phylogenetic analyses were aligned using MUSCLE implemented in MEGA 7, and the alignments used to create a maximum-likelihood tree with the WAG +F nucleotide substitution model. Gamma distributed rates with invariant sites (G+I) and 1000 bootstrap iterations were performed.[26]

Expression of *RiOLE1* and *RiOLE1-LIKE* in Yeast. The cDNAs of *RiOLE1* and *RiOLE1-LIKE* were amplified from *R. irregularis* RNA isolated from colonized *Lotus japonicus* roots by RT-PCR (for oligonucleotides, see Table S1). The cDNAs were ligated into expression vector pDR196 using the restriction enzymes *Sma*I and *Xho*I.[27] The constructs were transferred into *S. cerevisiae* BY4741 (wild type, WT) and the Δ*ole1* mutant (UniProt accession code Y40963) obtained from EUROSCARF, and transformed cells selected on minimal medium lacking uracil. The growth of Δ*ole1* was supported by addition of 1 mM oleic acid.

For complementation experiments, single colonies of Δ*ole1* cells containing different constructs were used to inoculate 20 mL cultures of minimal medium with or without 1 mM oleic acid and 1% (v/v) Triton X-100, and cells were grown at 33 °C. Alternatively, the cells were spotted in a serial dilution on solid medium and grown at 28 °C for 5 days.

Fatty acid feeding experiments were performed with whole *S. cerevisiae* cells. To this end, minimal medium lacking uracil was inoculated with single colonies from plates. Cells were supplemented with 1% (v/v) Triton X-100 and 1 mM 15:0 or $^{13}C_4$-16:0 fatty acid and grown for 2 days at 28 °C.

Expression of *RiOLE1* and *RiOLE1-LIKE* in Plants. The cDNAs for *RiOLE1* and *RiOLE1-LIKE* were amplified by PCR for expression in *N. benthamiana* (for oligonucleotides, see Table S1). *RiOLE1* was cloned into vector p917RFPUBQExpr (N. Gaude, MPI Potsdam-Golm) using *Bam*HI and *Hin*dIII, and *RiOLE1-LIKE* was ligated into vector pBin35S-DsRed using *Mlu*I and *Xho*I.[28] The constructs were transferred into *Agrobacterium tumefaciens* GV3101-pMP90 and used for transient transformation of *N. benthamiana* leaves.[29] Plants with infiltrated leaves were grown at 25 °C and 60% humidity with a light intensity of 250 mmol s^{-1} m^{-2} for 4−11 days, and expression of the *DsRed* marker was evaluated with a fluorescent lamp (NightSea, Lexington, MA).

For expression in *Camelina sativa*, *RiOLE1* and *RiOLE1-LIKE* cDNAs were amplified by PCR (for oligonucleotides, see Table S1) and cloned into pBinGlyBar1 (E. Cahoon, University of Nebraska) using *Eco*RI and *Xho*I and transferred into *A. tumefaciens* GV3101. *C. sativa* accession code CAM139 (ACCID 243618; IPK, Gatersleben, Germany)[30] and a high-palmitate line expressing *CpuFatB1*[31] were transformed via vacuum infiltration.[32] T1 plants were germinated and screened by spraying the plants 5 and 12 days after germination with 0.3% (v/v) BASTA (Bayer CropScience) containing 500 μL L^{-1} Silwet L-77. Seeds from resistant plants were harvested for single-seed fatty acid analysis.

Lipid Extraction and Fatty Acid Analysis. Lipids were extracted from yeast cultures and *N. benthamiana* leaves. Yeast cultures were centrifuged at 4000*g* for 10 min, and the pellet was resuspended in 4 mL of water and boiled for 5 min. Lipids were extracted with 3 volumes of a chloroform/methanol (2:1, v/v) mixture after centrifugation at 2000*g* for 5 min and re-extracted twice with 2 volumes of chloroform each. Transformed *N. benthamiana* leaf areas were dissected and frozen in liquid nitrogen. Leaves were homogenized in a mortar under liquid nitrogen, and the leaf powder was used for lipid

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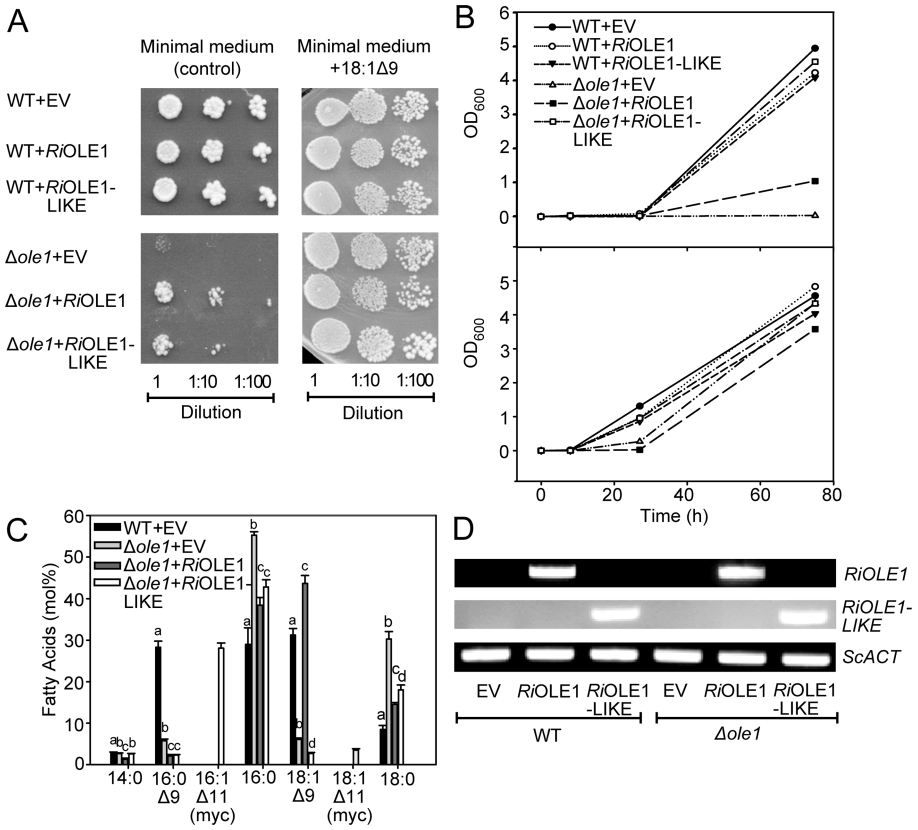

Figure 1. Complementation of yeast Δ*ole1* mutant growth by heterologous expression of *Rhizophagus* desaturases. *RiOLE1* or *RiOLE1-LIKE* cDNAs were expressed in *S. cerevisiae* WT and the Δ*ole1* mutant cells. Cells were grown in minimal medium with or without supplementation with oleic acid ($18{:}1^{\Delta 9cis}$). (A) Spotting growth assay of yeast cultures. Cells of 10-fold dilutions were pipetted onto solid medium and grown at 28 °C for 5 days. (B) Liquid cultures were inoculated from single colonies and grown at 33 °C for ≤3 days, and the OD_{600} was recorded. (C) Fatty acid composition of yeast WT and Δ*ole1* cells expressing *RiOLE1* or *RiOLE1-LIKE*. Total lipids were extracted from the harvested cells and used for the synthesis of fatty acid methyl ester that were quantified by GC. Bars show means ± the standard deviation. Letters indicate significant differences between treatments (ANOVA; post hoc Tukey; $p \leq 0.05$; $n = 3$). (D) Expression of *RiOLE1* or *RiOLE1-LIKE* in yeast WT and Δ*ole1*. RNA was isolated from WT or Δ*ole1* cells transformed with empty vector (EV), *Ri*OLE1, or *Ri*OLE1-LIKE constructs. Transcript abundance was measured by RT-PCR with primers for *RiOLE1*, *RiOLE1-LIKE*, or *ACTIN*. PCR products were separated on agarose gels and stained with ethidium bromide.

extraction with chloroform/methanol/formic acid (1:1:0.1, v/v) and 1 M KCl/0.2 M H_3PO_4 mixtures. The solvent of the lipid extracts was evaporated under N_2 gas, and the lipids were separated by solid phase extraction or directly used for synthesis of fatty acid methyl esters.

For solid phase extraction, lipids were dissolved in 1 mL of chloroform and loaded onto silica columns (1 mL bed volume; Phenomenex, Torrance, CA). Nonpolar lipids were eluted with chloroform, galactolipids with an acetone/isopropanol (1:1, v/v) mixture, and phospholipids with methanol. The solvents were evaporated with N_2 gas.

Fatty acid methyl esters were synthesized from dried total lipid extracts, solid phase extraction fractions, or single seeds of *Camelina* by incubation with 1 N methanolic HCl at 80 °C for 30 min.[33] Methyl esters were extracted with *n*-hexane and 0.9% (w/v) NaCl. *Camelina* seeds were homogenized prior to incubation with methanolic HCl. Fatty acid methyl esters from *R. irregularis*-colonized *L. japonicus* roots or *R. irregularis* extraradical mycelium (ERM) were prepared as previously described.[8] Fatty acid methyl esters were analyzed by gas chromatography with a flame ionization detector (Agilent 7890A Plus GC; Agilent, Santa Clara, CA)[4] or gas chromatography−mass spectrometry (6975C inertXL MSD with a triple-axis detector with a model 7890A gas chromatograph, Agilent) with an HP 5-MS column (Agilent J&W, 30 m, 0.25 mm diameter, 0.25 μm film). Pentadecanoic acid (15:0) was used as an internal standard.

The double bond positions were investigated after conversion of monounsaturated fatty acid methyl esters with dimethyl disulfide (DMDS) into bis(methylthio) derivatives.[34] Dimethyl disulfide derivatives were analyzed by GC−MS.

Measurement of Acyl-CoAs. Acyl-CoAs were extracted from homogenized leaf material of *N. benthamiana* (20 mg) as described previously.[35,36] For measurement in yeast, the cell pellet of a 2 mL culture was resuspended in 200 μL of extraction buffer [1 mL of isopropanol, 1 mL of 50 mM KH_2PO_4 (pH 7.2), 25 μL of acetic acid, and 40 μL of 50 mg mL^{-1} fatty acid free bovine serum albumin] and homogenized in the Precellys homogenizer with glass beads. Extracted acyl-CoAs were dissolved in solvent B [H_2O/acetonitrile (90:10, v/v) containing 15 mM NH_4OH] and separated on an RP8 column (Knauer Eurospher II, 150 mm, 3 mm) at a flow rate of 0.5 mL min^{-1} with the following gradient: 0 min, 100% solvent B; 5 min, 25% solvent A (acetonitrile containing 15 mM NH_4OH) and 75% solvent B; 11−13 min, 100% solvent A; 15−18 min, 100% solvent C [H_2O/acetonitrile/formic acid (30:70:0.1, v/v)]; 20−30 min, 100% solvent B. Eluted acyl-CoAs were quantified by MS/MS with electrospray ionization on an Agilent 6530 Q-TOF mass spectrometer. Acyl-CoAs were ionized in positive mode and quantified using the neutral

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loss of 506.9960 (characteristic of adenosine-3′-phosphate-5′-diphosphate) using 17:0-CoA as an internal standard.

RESULTS

***R. irregularis* Contains Two Genes with Sequences That Are Similar to That of the *S. cerevisiae* Acyl-CoA Desaturase OLE1.** The genome of *R. irregularis* harbors seven open reading frames with sequences that are similar to those of desaturases from *M. alpina* and *S. cerevisiae*. These genes can be divided into three clades based on the amino acid sequence relationship (Figure S1). Clade I contains sequences from *M. alpina* with specificity for Δ5 and Δ6 desaturation[37] and three *R. irregularis* genes. Clade II contains one *R. irregularis* sequence and two *M. alpina* desaturases specific for Δ12 and ω3. The *M. alpina* and *S. cerevisiae* OLE1 Δ9 desaturases belong to clade III and are the most similar with *R. irregularis* RIR 1928200 (designated *Ri*OLE1), and the second most similar sequence is RIR 2584800 (*Ri*OLE1-LIKE).

Expression of the two genes was recorded in *Daucus carota* roots growing in a tissue culture that were infected with *R. irregularis* (+myc, intraradical mycelium, IRM) and extraradical mycelium (ERM). Semiquantitative RT-PCR with total RNA revealed that expression of *RiOLE1* and *RiOLE1-LIKE* is similar in IRM and ERM, even though *RiOLE1-LIKE* shows stronger overall expression (Figure S2A). Therefore, expression of *RiOLE1* or *RiOLE1-LIKE* is not upregulated in the fungus during AM colonization, but the two genes are constitutively expressed.

To study the biochemical function of *R. irregularis RiOLE1* and *RiOLE1-LIKE*, the two cDNAs were transferred into the fatty acid-auxotroph yeast Δ*ole1* mutant. This yeast mutant is deficient in the 18:0-CoA Δ9 desaturase and therefore can grow only after addition of unsaturated fatty acids such as oleic acid ($18{:}1^{\Delta 9cis}$) to the medium. Introduction of *Ri*OLE1 or *Ri*OLE1-LIKE resulted in complementation of the growth deficiency of Δ*ole1* (Figure 1A,B). The yeast Δ*ole1* mutant was used to examine the fatty acid desaturation products after heterologous expression of *RiOLE1* and *RiOLE1-LIKE* by GC (Figure 1C). Expression of *RiOLE1-LIKE* led to the accumulation of the mycorrhiza signature fatty acids, 11-*cis*-palmitvaccenic acid ($16{:}1^{\Delta 11cis}$) and vaccenic acid ($18{:}1^{\Delta 11cis}$) (Figure 1C and Figure S2A). On the other hand, expression of *RiOLE1* resulted in a large increase in oleic acid ($18{:}1^{\Delta 9cis}$) content. Expression of the two desaturases in Δ*ole1* led to a slight decrease in the level of palmitoleic acid ($16{:}1^{\Delta 9cis}$), and *RiOLE1* expression slightly affected the myristic acid (14:0) content. However, no desaturation product of 14:0 (14:1) was detected in any yeast strain.

RiOLE1 and *RiOLE1-LIKE* gene expression in the different yeast cultures was assessed by semiquantitative RT-PCR (Figure 1D). *RiOLE1* and *RiOLE1-LIKE* were expressed to the same extents in the recombinant yeast cultures, indicating that the differences in fatty acid desaturation are due to different activities or substrate preferences of the enzymes.

Determination of the Double Bond Position of Mycorrhiza Signature Fatty Acids. The double bond position of unsaturated fatty acids can be determined after covalent modification of double bonds with dimethyl disulfide followed by GC−MS analysis, giving rise to peaks with specific retention times and fragmentation patterns. Therefore, total lipids isolated from the different yeast Δ*ole1* strains were converted into methyl esters and derivatized with dimethyl disulfide (Figure 2). Four monounsaturated fatty acids were present in the total fatty acid extracts (Figure S3). Their fragmentation patterns allowed the identification of the double bonds at Δ9 of palmitoleic acid ($16{:}1^{\Delta 9is}$) and oleic acid ($18{:}1^{\Delta 9cis}$) (Figure 2A,C) and Δ11 for palmitvaccenic acid ($16{:}1^{\Delta 11cis}$) and vaccenic acid ($18{:}1^{\Delta 11cis}$) (Figure 2B,D).

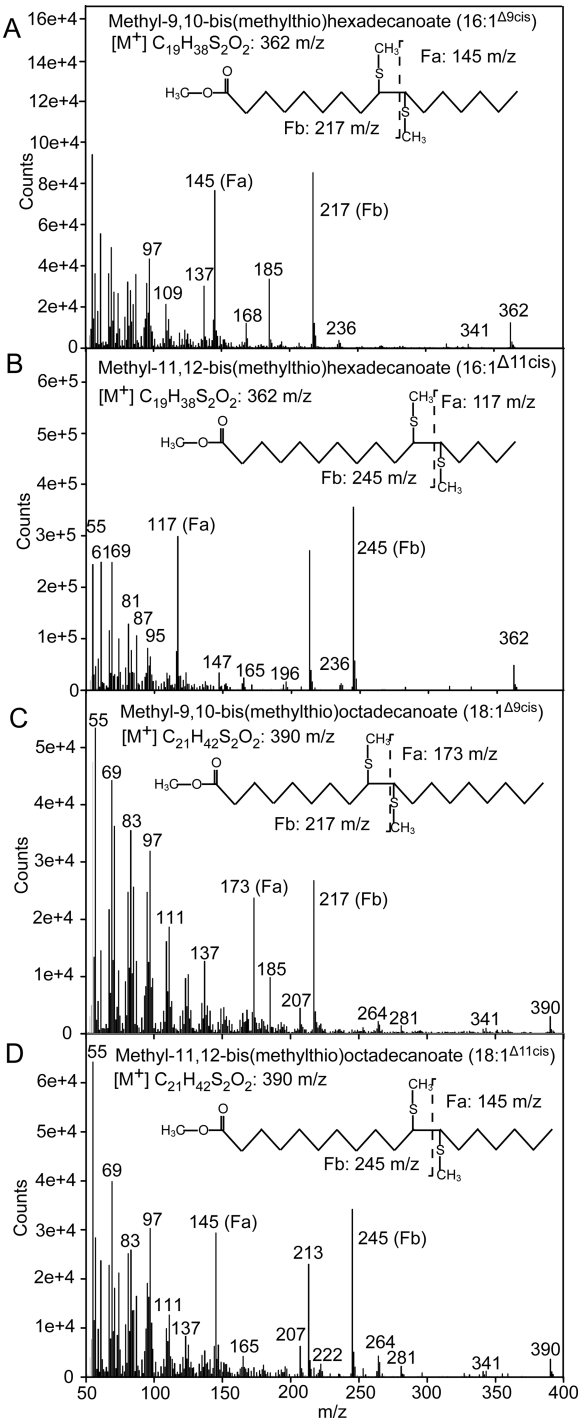

Figure 2. Determination of double bond position in monounsaturated fatty acids isolated from *RiOLE1*- or *RiOLE1-LIKE*-expressing yeast. Lipids were isolated from yeast Δ*ole1* mutant cells expressing *RiOLE1-LIKE* and fatty acid converted into methyl esters. Double bonds were derivatized with dimethyl disulfide, and the products analyzed by GC−MS (for chromatograms, see Figure S3). Mass spectra and structures are shown for (A) $16{:}1^{\Delta 9cis}$, (B) $16{:}1^{\Delta 11cis}$, (C) $18{:}1^{\Delta 9cis}$, and (D) $18{:}1^{\Delta 11cis}$. F_a, fragment a; F_b, fragment b.

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While palmitoleic acid and oleic acid accumulated in *RiOLE1*-expressing cells, palmitvaccenic acid and vaccenic acid were abundant in *RiOLE1-LIKE* cells (Figure S3).

Heterologous Expression of *RiOLE1* and *RiOLE1-LIKE* in Transgenic Plants. To characterize the two *R. irregularis* desaturases in a nonfungal background, *Agrobacterium* harboring vectors with the *RiOLE1* and *RiOLE1-LIKE* cDNAs were infiltrated into *N. benthamiana* leaves for transient expression (Figure S4). Expression of *RiOLE1* resulted in an increase in the level of oleic acid in nonpolar lipids, galactolipids, and phospholipids, accompanied by an increased 18:2 content in nonpolar lipids and phospholipids (Figure S4A). *RiOLE1-LIKE* expression in *N. benthamiana* leaves led to the accumulation of $16{:}1^{\Delta 11cis}$, particularly in neutral lipids and phospholipids but not galactolipids (Figure S4B). In the experiment shown in Figure S4, *N. benthamiana* leaves were analyzed 4 days after *Agrobacterium* infiltration. When lipids were measured 11 days after infection, small amounts of $16{:}1^{\Delta 11cis}$ also accumulated in the galactolipids. Therefore, lipid backbones with this unusual fatty acid are also transported from the endoplasmic reticulum to the chloroplast. These results are in agreement with the data obtained from heterologous expression in yeast and demonstrate that *RiOLE1* and *RiOLE1-LIKE* encode Δ9 and Δ11 desaturases, respectively.

It is known that unusual fatty acids such as $16{:}1^{\Delta 11cis}$ are excluded from plant membrane lipids, but they can accumulate in the seed oils of many plants.[38] We therefore expressed *RiOLE1-LIKE* using a seed specific promoter in *Camelina* to study the fatty acid composition of the oil in mature seeds. Fatty acid measurement showed that $16{:}1^{\Delta 11cis}$ accumulated to a level of 0.03−0.08 mol % in single transgenic *RiOLE1-LIKE*-expressing *Camelina* seeds (Table S2), while it was undetectable in WT seeds. In addition, the content of $20{:}1^{\Delta 11}$ increased from 12.7 to 17.5−20.5 mol % when *RiOLE1-LIKE* was expressed. The very small amount of $16{:}1^{\Delta 11cis}$ in transgenic *Camelina* seeds might be due to the low availability of the substrate, 16:0, because *Camelina* seeds mostly accumulate C18 fatty acids. We therefore introduced the *RiOLE1-LIKE* construct into a high-16:0-containing *Camelina* line.[31] This line carries an overexpression construct for the acyl-ACP thioesterase CpuFatB1 from *Cuphea pulcherrima*, resulting in the termination of fatty acid synthesis at C16 and in the accumulation of 16:0 in the seed oil from ∼11% to ∼42% (Table S2). Expression of *RiOLE1-LIKE* in transgenic CpuFatB1 seeds resulted in an increase in the palmitvaccenic acid content to 0.24−0.82 mol % (Table S2). Likewise, the content of $20{:}1^{\Delta 11}$ was slightly increased, albeit the total amounts were smaller compared with those of *Camelina* wild type (CAM139) transformed seeds. Taken together, *RiOLE1-LIKE* expression in the seeds of *Camelina* resulted in the accumulation of $16{:}1^{\Delta 11cis}$, albeit in small amounts, and the amount was increased but still <1 mol % in a high-palmitic acid *Camelina* line.

Addition of Exogenous Fatty Acids to Yeast. To examine the fatty acid substrate specificity of *Ri*OLE1 and *Ri*OLE1-LIKE in more detail, fatty acids were added to growing yeast cultures. We first added a stable isotope-labeled 16:0 fatty acid ($^{13}C_4$-16:0) to follow the incorporation of the label into the different desaturation and elongation products. When $^{13}C_4$-16:0 was supplied to the yeast cells, the $^{13}C_4$ label accumulated in all fatty acids in WT, but its level was strongly reduced in $16{:}1^{\Delta 9cis}$ and $18{:}1^{\Delta 9cis}$ in the Δ*ole1* mutant where

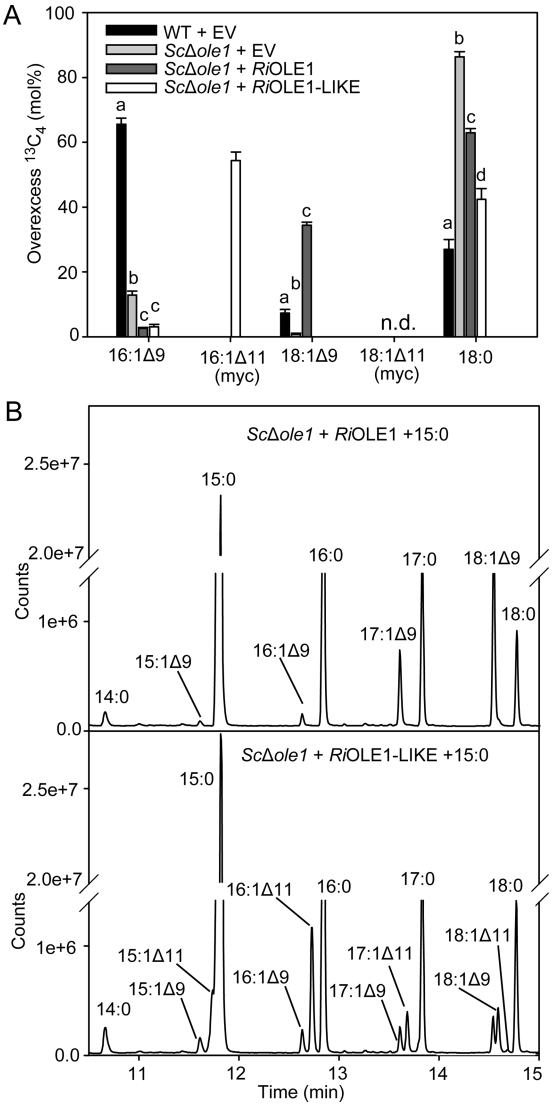

Figure 3. Desaturation of exogenously supplied fatty acids in yeast cells expressing *Rhizophagus* desaturases. *S. cerevisiae* cells (WT, Δ*ole1*) expressing *RiOLE1* or *RiOLE1-LIKE* were grown for 3 days at 28 °C in minimal medium supplemented with (A) 1 mM $^{13}C_4$-16:0 or (B) 1 mM 15:0. Lipids were extracted from cell pellets and used for fatty acid analysis of methyl esters by GC−MS. (A) The amount of overexcess $^{13}C_4$ accumulation (mol %) was quantified in the different fatty acid methyl esters. Letters indicate significant differences among treatments (ANOVA; post hoc Tukey; $p \leq 0.05$; $n = 3$). Bars represent means ± the standard deviation. n.d., not detected; EV, empty vector. (B) GC chromatograms showing methyl esters of yeast lipids after 15:0 feeding.

18:0 accumulated instead (Figure 3A). Upon expression of *RiOLE1* and *RiOLE1-LIKE* in Δ*ole1*, the level of accumulation of the $^{13}C_4$ label in $16{:}1^{\Delta 9cis}$ was even further decreased. In addition, oleic acid became abundantly labeled when *RiOLE1* was expressed in Δ*ole1*, while expression of *RiOLE1-LIKE* in Δ*ole1* led to the most abundant accumulation of the $^{13}C_4$ label in $16{:}1^{\Delta 11cis}$ (54.44 mol %) and none in $18{:}1^{\Delta 9cis}$.

Next, we added the odd chain fatty acid pentadecanoic acid (15:0) to Δ*ole1* cells expressing *RiOLE1* or *RiOLE1-LIKE*. 15:0 was taken up by the cells and elongated to margaric acid (17:0), and both 15:0 and 17:0 were desaturated to $15{:}1^{9cis}$ and $17{:}1^{9cis}$, respectively, in Δ*ole1* cells expressing *RiOLE1* (Figure 3B). When *RiOLE1-LIKE* was expressed in Δ*ole1*, 15:0

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and 17:0 were converted into two new peaks that were tentatively identified as $15{:}1^{\Delta 11cis}$ and $17{:}1^{\Delta 11cis}$, respectively, on the basis of their mass spectra and retention times.

Acyl-CoA Composition in Yeast Cells and Plants Expressing *R. irregularis* Desaturases. Acyl-CoAs are important intermediates of fatty acid metabolism, and they are the substrate for the OLE1 desaturase from *S. cerevisiae*. Acyl-CoAs were quantified in yeast cells and *N. benthamiana* leaves expressing *RiOLE1* or *RiOLE1-LIKE* by LC−MS/MS (Figure 4). In this method, acyl-CoAs containing different

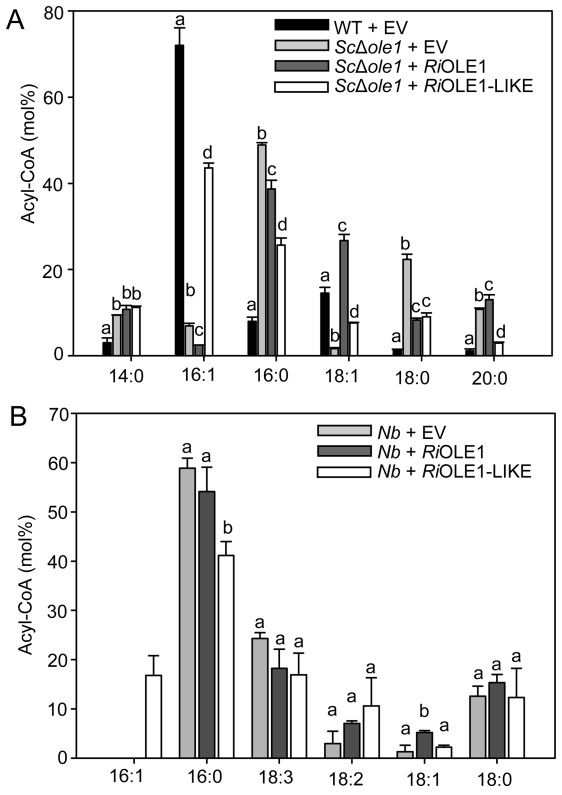

Figure 4. Acyl-CoA composition in transformed yeast cells and *N. benthamiana* leaves expressing *Rhizophagus* desaturases. Acyl-CoAs were measured by LC−MS/MS. (A) Acyl-CoAs in yeast cells (WT, Δ*ole1*) expressing the empty vector (EV) control, *RiOLE1*, or *RiOLE1-LIKE*. (B) Acyl-CoAs in *N. benthamiana* leaves transiently expressing the EV control, *RiOLE1*, or *RiOLE1-LIKE*. Letters indicate significant differences among treatments (ANOVA; post hoc Tukey; $p \leq 0.05$). Bars represent means ± the standard deviation ($n = 3$). *Sc*, *S. cerevisiae*; *Nb*, *N. benthamiana*; n.d., not detected.

numbers of carbon atoms or double bonds in the acyl chain can be separated and quantified using an internal standard. However, it is not possible to separate isomeric monounsaturated acyl-CoAs containing the double bond at different positions. In yeast Δ*ole1*, the levels of 16:1-CoA and 18:1-CoA decreased while 16:0-CoA, 18:0-CoA, and 20:0-CoA accumulated, compared to WT (Figure 4A). Expression of *RiOLE1-LIKE* in Δ*ole1* led to a very strong increase in the amount of 16:1-CoA (43.6 mol %) and to a smaller increase in the amount of 18:1-CoA (7.6 mol %), while the amount of 20:0-CoA (2.9 mol %) was decreased. On the other hand, the amount of 18:1-CoA increased most predominantly when *RiOLE1* was expressed (26.7 mol %).

Expression of *RiOLE1-LIKE* in *N. benthamiana* leaves led to the accumulation of 16:1-CoA (16.8 mol %) accompanied by a decrease in the amount of 16:0-CoA (41.1 mol %) that was not detected in control leaves (Figure 4B). Expression of *RiOLE1* led to the increase in the amount of 18:1-CoA to 5.2 mol %, which remained low in the control (1.3 mol %) and *RiOLE1-LIKE* (2.2 mol %). Taken together, these results are in agreement with a substrate/product relationship of 18:0-CoA/18:1-CoA and 16:0-CoA/16:1-CoA for *Ri*OLE1 and *Ri*OLE1-LIKE, respectively, suggesting that *Ri*OLE1 and *Ri*OLE1-LIKE are acyl-CoA desaturases.

The *OLE1-LIKE* Genes Are Conserved in AM Glomeromycotina but Not in Nonsymbiotic Mucoromycota. AM fungi of the Glomeromycotina likely evolved from saprotrophic Mucoromycota.[39] To study the evolution of *Ri*OLE1 and *Ri*OLE1-LIKE sequences and their distribution in the different fungal phyla, the two desaturase sequences from *R. irregularis* were used to identify orthologs in other Glomeromycotina as well as nonmycorrhizal Mucoromycota using protein Blast searches. Protein sequences of several fungal species were retrieved and used to assemble a phylogenetic tree (Figure 5). Two major clades were identified, one clade that compromises sequences similar to *Ri*OLE1-LIKE that were present in all five representatives of the Glomeromycotina. These sequences were more similar to each other than to the sequences organized in the second clade representing the sequences highly similar to *Ri*OLE1. Sequences similar to *Ri*OLE1 were found in all six Glomeromycotina species and in the nonsymbiotic Mortierellaceae (*M. alpina*, *M. elongata*, and *L. transversale*) as well as in the nonsymbiotic Mucoromycotina (*M. circinelloides* and *R. microsporus*). Thus, OLE1-LIKE is conserved in symbiotic Glomales and Diversisporales but not in nonsymbiotic fungi, suggesting that it evolved to fulfill a specific function in AM fungi.

DISCUSSION

***Ri*OLE1 and *Ri*OLE1-LIKE Are Front-End Desaturases.** Desaturases are classified on the basis of their ability to recognize the methyl (ω) or carboxyl (Δ) end of fatty acids for insertion of the double bond.[40] The double bond position of fatty acids can be identified after derivatization with dimethyl disulfide. Four monounsaturated fatty acids were detected in Δ*ole1* cells expressing *RiOLE1-LIKE*, i.e., $16{:}1^{\Delta 9cis}$, $16{:}1^{\Delta 11cis}$, $18{:}1^{\Delta 9cis}$, and $18{:}1^{\Delta 11cis}$ (Figure 2 and Figure S3). $16{:}1^{\Delta 11cis}$ could in principle be derived from front-end (Δ11) or methyl-end (ω5) desaturation. However, $18{:}1^{\Delta 11cis}$ must be derived from Δ11 desaturation. This fatty acid cannot be the product of $16{:}1^{\Delta 11cis}$ elongation by addition of two carbons at the carboxyl end, which would have resulted in $18{:}1^{\Delta 13cis}$ production. Similarly, ω5 desaturation of 18:0 by *RiOLE1-LIKE* would also have resulted in the production of $18{:}1^{\Delta 13cis}$. Likewise, upon *RiOLE1* expression in Δ*ole1*, only $16{:}1^{\Delta 9cis}$ and $18{:}1^{\Delta 9cis}$ were produced. While $18{:}1^{\Delta 9cis}$ could in principle be produced via Δ9 or ω9 desaturation, $16{:}1^{\Delta 9cis}$ is clearly derived from Δ9 desaturation of 16:0, because ω9 desaturation would result in $16{:}1^{\Delta 7cis}$ production, and elongation of a putative $14{:}1^{\Delta 7cis}$ precursor is not likely as it was not detected.

These conclusions are corroborated by the fatty acid analysis of yeast Δ*ole1* cultures after 15:0 feeding (Figure 3). In this experiment, the levels of accumulation of $15{:}1^{\Delta 9}$ and $17{:}1^{\Delta 9}$ were strongly increased after expression of *RiOLE1* compared with the control. Expression of *RiOLE1-LIKE* in Δ*ole1* cells resulted in the accumulation of $15{:}1^{\Delta 11}$ and $17{:}1^{\Delta 11}$ from 15:0. Taken together, these data demonstrate that *Ri*OLE1 and

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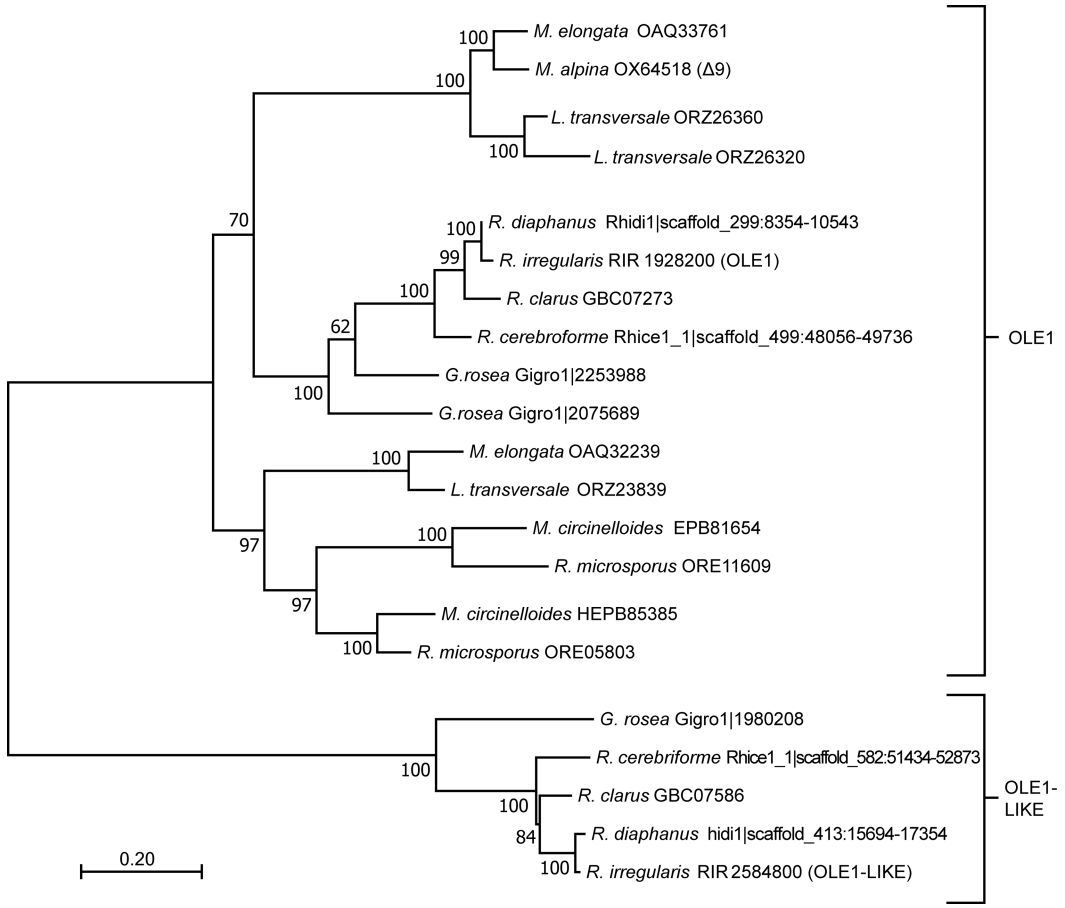

Figure 5. Phylogenetic tree of OLE1 and OLE1-LIKE sequences from Mucoromycota. OLE1 and OLE1-LIKE from *R. irregularis* were used to identify orthologs in species of other Glomeromycotina, Mortierellomycotina, and Mucoromycotina.[25] Amino acid sequences were aligned using MUSCLE with MEGA 7 and a maximum-likelihood tree constructed with 1000 bootstrap iterations as depicted on the nodes.

*Ri*OLE1-LIKE are front-end desaturases that count carbon atoms from the carboxyl terminus for insertion of the double bond at positions Δ9 and Δ11, respectively.

***Ri*OLE1 and *Ri*OLE1-LIKE Likely Are Acyl-CoA Desaturases with Specificities for Long Chain Fatty Acids.** *Ri*OLE1 and *Ri*OLE1-LIKE show sequence similarity with the yeast acyl-CoA desaturase *Sc*OLE1 (Figure S1) and can functionally replace *Sc*OLE1 in the Δ*ole1* mutant, strongly promoting growth in the absence of exogenous unsaturated fatty acids (Figure 1). In similar approaches, desaturases from rat or *Rhodosporidium* with a sequence similar to that of *Sc*OLE1 were characterized.[41,42] Expression of *RiOLE1* and *RiOLE1-LIKE* leads to alteration of the acyl-CoA composition in yeast and plants with a clear substrate/product relationship (Figure 4). While *RiOLE1* expression resulted in an increase in the level of 18:1-CoA and a decrease in the level of 18:0-CoA, expression of *RiOLE1-LIKE* caused an increase in the level of 16:1-CoA and a reduction in the level of 16:0-CoA, both compared with Δ*ole1*. Therefore, *Ri*OLE1 and *Ri*OLE1-LIKE likely are acyl-CoA desaturases.

Addition of exogenous 15:0 to the transformed yeast resulted in the accumulation of the respective desaturation products [15:1$^{\Delta 11cis}$ and 17:1$^{\Delta 11cis}$ for *RiOLE1-LIKE*; 15:1$^{\Delta 9cis}$ and 17:1$^{\Delta 9cis}$ for *RiOLE1* (Figure 3B)], indicating that the two enzymes are to a certain degree promiscuous in their substrate specificity and accept a range of long chain (C15−C18) fatty acids. Feeding of $^{13}C_4$-labeled 16:0 revealed the accumulation of the $^{13}C_4$ label in all endogenous yeast fatty acids (Figure 4A). Yeast *Sc*OLE1 can desaturate 16:0 and 18:0.[41] When $^{13}C_4$-16:0 was offered, the $^{13}C_4$ label accumulated in 18:0 (27.0 mol %) in yeast WT. This was sufficient as a substrate for *Sc*OLE1 to produce $^{13}C_4$-18:1$^{\Delta 9cis}$, even though the level of accumulation of this product reached only 7.4 mol %. Using the same conditions, no $^{13}C_4$ label was found in 18:1$^{\Delta 11cis}$ in Δ*ole1* expressing *RiOLE1-LIKE*, even though sufficient substrate was available (42.5 mol % $^{13}C_4$-18:0). Therefore, *Ri*OLE1-LIKE has much poorer activity on 18:0 than yeast *Sc*OLE1, and consequently, *Ri*OLE1-LIKE has a strong preference for the 16:0 substrate (Figure 1C). 18:0 desaturation in *R. irregularis* is probably foremost conducted by *Ri*OLE1, in accordance with the accumulation of $^{13}C_4$-18:1$^{\Delta 9cis}$ (34.4 mol %), while only minute amounts of $^{13}C_4$-16:1$^{\Delta 9cis}$ (2.6 mol %) were detected upon *RiOLE1* expression. Functional differentiation of acyl-CoA desaturases was reported before for mice where four desaturases exist; three act on 16:0-CoA and 18:0-CoA, and one is specialized for acyl-CoAs with <16:0 fatty acids.[43]

***RiOLE1* Expression in Plants Results in a Low Level of Palmitvaccenic Acid Production.** Expression of *RiOLE1* in plants (*N. benthamiana* leaves and *Camelina* seeds) led to a very low level of production of 16:1$^{\Delta 11cis}$ (Table S1 and Figure S4). In plant seeds, lipid backbones like lysophosphatidic acid can be synthesized via the Kennedy pathway by two successive acyltransferases. A low proportion of lysophosphatidic acid is hydrolyzed by a phosphatase and used directly for

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triacylglycerol production, while a large proportion of lipid backbones is employed for phosphatidylcholine (PC) synthesis in *Arabidopsis* seeds and tobacco leaves.[44,45] The predominant proportion of triacylglycerol is synthesized from PC-derived diacylglycerol, not from diacylglycerol directly produced via the Kennedy pathway.[44,45] In addition, fatty acids from the acyl-CoA pool are exchanged between the CoA and PC pools by the forward and reverse reactions of lysophosphatidylcholine acyltransferase (LPCAT).[46] Therefore, unusual fatty acids like $16:1^{\Delta 11cis}$ need to be channeled through the PC pool, which is known to cause a major bottleneck.[44,47] This zigzag pathway between the CoA and PC pools for the incorporation of unusual acyl groups into triacylglycerol can explain the low level of accumulation of $16:1^{\Delta 11cis}$ in transgenic *N. benthamiana* leaves and *Camelina* seeds.

OLE1-LIKE Evolved in AM Fungi. OLE1-LIKE is conserved in the Glomerales *R. clarus*, *R. irregularis*, *R. diaphanus*, *R. cerebriforme*, and *G. rosea* (Diversisporales) but not in the other nonsymbiotic, nonmycorrhizal Mucoromycota (Figure 5). The Mucoromycota species contain only OLE1 orthologs. Characteristic of Glomeromycotina are their large genomes with high numbers of duplicated genes, compared to Mortierellomycotina and Mucoromycotina.[25] Therefore, the *OLE1-LIKE* gene could originate from gene duplication of *OLE1* and neofunctionalization in Glomeromycotina and might have evolved to fulfill a specific function during the obligate mutualistic AM symbiosis. The expression pattern of *RiOLE1-LIKE* in IRM and ERM (Figure S2A) is consistent with the occurrence of $16:1^{\Delta 11cis}$ in nonpolar storage lipids and membrane-forming phospholipids in IRM and ERM, even though considerably less $16:1^{\Delta 11cis}$ is found in the IRM.[13]

Possible Functions of $16:1^{\Delta 11cis}$ in AM Fungi. Previously, overexpression of the *S. saccharomyces* Δ9 desaturase (*Sc*OLE1) or of an insect Δ11 desaturase, which resulted in the accumulation of $16:1^{\Delta 9cis}$ or $16:1^{\Delta 11cis}$ in transgenic *Nicotiana tabacum* leaves, respectively, led to the increase in the levels of lipoxygenase products, in particular *cis*-3-hexenal.[48] The same effect was observed when the two fatty acids were exogenously applied to *N. tabacum* leaves, indicating that these fatty acids stimulated the lipoxygenase pathway, thereby contributing to enhanced resistance against pathogens. In line with this finding, overexpression of *Sc*OLE1 in tomato or eggplant increased the resistance against powdery mildew (*Erysiphe polygoni*) and Verticillium wilt (*Verticillium dahliae*),[49,50] and $16:1^{\Delta 9cis}$ inhibited the growth of *V. dahliae*. Therefore, it is possible that monounsaturated C16 fatty acids, in particular $16:1^{\Delta 11cis}$, protect AM fungal spores and hyphae in the soil against attack by bacteria or other fungi.

Furthermore, the unusual $16:1^{\Delta 11cis}$ fatty acid could be involved in recognition of the AM fungi by the plant host. Membrane lipids, e.g., ergosterol, derived from pathogenic fungi can be recognized by the plant host and can elicit defense responses.[51,52] Similarly, certain molecular species of 18:2-, 18:1-, and 16:0-containing phosphatidylcholine and phosphatidylethanolamine from female *Sogatella furcifera* eggs are elicitors in plant−pathogen interactions.[53] *R. irregularis* alters its phospholipid fatty acid composition by suppressing di-16:1- and 24:1-containing molecular species when growing intraradically.[4] This acyl editing in IRM might suggest that the fungus regulates the $16:1^{\Delta 11cis}$ content in the membrane phospholipids presented to the host plant. Thus, $16:1^{\Delta 11cis}$ might be perceived by the host as a symbiotic signature to avoid the host's immune response. Therefore, $16:1^{\Delta 11cis}$ biosynthesis could have evolved in Glomeromycota for survival in the hostile soil environment during asymbiotic growth, and it might be beneficial during host perception and symbiotic growth. Further studies are required to deconvolve the potential function of $16:1^{\Delta 11cis}$ in AM fungi. These experiments might include the study of the effects of application of $16:1^{\Delta 11cis}$ to plants, bacteria, or fungi and the analysis of the consequences of downregulation of *RiOLE1-LIKE* expression in *R. irregularis* during symbiotic interactions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biochem.0c00051.

Phylogenetic tree of desaturases from *R. irregularis*, *S. cerevisiae*, and *M. alpina* (Figure S1), expression of *RiOLE1* and *RiOLE1-LIKE* and accumulation of palmitvaccenic acid (16:1Δ11cis) in plant roots colonized with *R. irregularis* (Figure S2), determination of double bond positions in monounsaturated fatty acids from yeast cells expressing *RiOLE1* or *RiOLE1-LIKE* (Figure S3), fatty acid composition of lipid fractions from *N. benthamiana* leaves transiently expressing *Rhizophagus* desaturases (Figure S4), oligonucleotides used for cloning and RT-PCR (Table S1), and quantification of fatty acids in transgenic *Camelina* seeds expressing the *R. irregularis* desaturase *RiOLE1-LIKE* (Table S2) (PDF)

Accession Codes

The UniProtKB codes for the *R. irregularis* *Ri*OLE1 and *Ri*OLE1-LIKE sequences are A0A015N9Y4 and A0A015JJF9, respectively.

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Notes

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■ ABBREVIATIONS

ACP, acyl carrier protein; AM, arbuscular mycorrhiza; CoA, coenzyme A; ERM, extraradical mycelium; IRM, intraradical mycelium; WT, wild type.

■ ADDITIONAL NOTE

[a]Fatty acids are abbreviated as X:Y, where X depicts the number of carbon atoms and Y the number of double bonds in the acyl chain.

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