DES2 is a fatty acid Δ11 desaturase capable of synthesizing palmitvaccenic acid in the arbuscular mycorrhizal fungus *Rhizophagus irregularis*

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Arbuscular mycorrhiza (AM) fungi are oleaginous organisms, and the most abundant fatty acyl moiety usually found in their lipids is palmitvaccenic acid (16:1Δ11cis). However, it is not known how this uncommon fatty acid species is made. Here, we have cloned two homologues of lepidopteran fatty acyl-coenzyme A Δ11 desaturases from the AM fungus *Rhizophagus irregularis*. Both enzymes, DES1 and DES2, are expressed in intraradical mycelium and can complement the unsaturated fatty acid-requiring auxotrophic growth phenotype of the *Saccharomyces cerevisiae* ole1Δ mutant. DES1 expression leads almost exclusively to oleic acid (18:1Δ9cis) production, whereas DES2 expression results in the production of 16:1Δ11cis and vaccenic acid (18:1Δ11cis). DES2 therefore encodes a Δ11 desaturase that is likely to be responsible for the synthesis of 16:1Δ11cis in *R. irregularis*.

Abbreviations
16:1Δ11cis, 11-cis-palmitvaccenic acid; 18:1Δ11cis, 11-cis-vaccenic acid; AM, arbuscular mycorrhizal; CoA, coenzyme A; SCD, stearoyl-CoA desaturase; TEF1, translational elongation factor EF-1α.
metabolism. Understanding how and where 16:1 is made is therefore important to define how lipid metabolic pathways function within arbuscular mycorrhiza. Δ11 desaturases have previously been cloned from insects [13,14] and marine diatoms [15], but we are not aware of any that have been characterized in fungi. The genomes of several AM fungi have now been sequenced, including *Rhizophagus irregularis* [16], which contains 16:1Δ11Δ12Δ14 [4]. A blastp search (https://www.ncbi.nlm.nih.gov/) of the *R. irregularis* genome using known lepidopteran fatty acyl-coenzyme A (CoA) Δ11 desaturases [13,14] revealed two potential homologues (DES1 and DES2). It is problematic to test the function of these genes in AM fungi because they are not amenable to genetic modification. We therefore characterized DES1 and DES2 by heterologous expression in *Saccharomyces cerevisiae* [13] and showed that DES2 encodes a fungal Δ11 desaturase capable of synthesizing 16:1Δ11Δ12Δ14.

### Materials and methods

**Bioinformatic analysis of putative Δ11 desaturases**

A blastp search was carried out in NCBI (https://www.ncbi.nlm.nih.gov/) on the *R. irregularis* DAOM197198 genome [16] using functionally characterized Δ11 desaturase sequences from Lepidoptera [13,14] and marine diatoms [15] as queries. All returned sequences with *E* scores < 0.001 were compiled on a local server and aligned using Muscle v3.2 (EMBL-EBI, Hinxton, Cambridge, UK) [17]. Two putative fatty acyl-CoA desaturases (GenBank accession numbers EXX76018 and EXX69612) were selected for further analysis and were named DES1 and DES2, respectively. The Kyte–Doolittle hydropathy scale with an amino acid window of 19 [18] and TMHMM v2.0 (DTU Health Tech, Lyngby, Denmark) (http://www.cbs.dtu.dk/services/TMHMM/) [19] were used for hydropathy analysis and prediction of transmembrane helices (TMHs). SignalP v4.0 (DTU Health Tech, Lyngby, Denmark) (http://www.cbs.dtu.dk/services/SignalP) was used for identifying signal peptides at the N and C termini and for distinguishing these from TMHs [20]. Searches for conserved domains within protein sequences were carried out using the NCBI Conserved Domain Database (CDD) (https://www.ncbi.nlm.nih.gov/cdd) [21].

**Expression of DES1 and DES2 in *S. cerevisiae***

The open reading frames of *DES1* and *DES2* were codon-optimized for expression in *S. cerevisiae* by Genscript, synthesized and supplied in the pUC-57 vector. *DES1* and *DES2* were then excised using BamHI and SalI restriction sites and ligated into pHEY1 [22], for expression under the constitutive translational elongation factor EF-1α (*TEFI*) promoter. pHEY-DES1, pHEY-DES2 and pHEY-EVC (empty vector) were transformed into *S. cerevisiae* [23] wild-type (WT) strain DTY-11a (MATα, leu2-3, leu2-12, trp1–1, can–1–100, ura3–1, ade2–1) and ole1Δ knockout strain AMY–3Δ (MATα, ole1Δ::LEU2, trp1–1, can–1–100, ura3–1, ade2–1) [24], and colonies were selected on synthetic dextrose (SD) minimal medium agar plates lacking uracil. The SD minimal medium used for selection of yeast transformants and culture cultivations consisted of 6.9 g L⁻¹ yeast nitrogen base without amino acids (Formedium, Hunstanton, Norfolk, UK), 1.92 g L⁻¹ yeast synthetic dropout medium supplements minus uracil (Sigma-Aldrich, St. Louis, MO, USA), 40 mg L⁻¹ of adenine (Sigma-Aldrich) and 20 g L⁻¹ glucose (Sigma-Aldrich) as sole carbon source. 10-mL cultures were grown overnight in SD minimal medium to optical density of 0.5–1 at 600 nm and then used to inoculate in 100 mL of SD minimal media to a starting OD600 of 0.1. The 100-mL cultures were then incubated at 30 °C and shaken at 200 r.p.m. for 72 h. ole1Δ cultures were supplemented with 1 mm odd- or even-chain monounsaturated fatty acids (MUFA) dissolved in 1% (v/v) tergitol (Sigma-Aldrich). ole1Δ was also grown on SD minimal medium agar plates containing fatty acids.

**Lipid extraction and analysis**

Cultures were normalized for cell volume based on OD600 measurements and the cells were pelleted by centrifugation at 2400 g, the supernatant discarded, and the pellets frozen in liquid nitrogen and stored at −80 °C. Heptadecanoic acid (17:0) was added to the cell pellets to provide an internal standard (IS). Fatty acid methyl esters (FAMEs) were then prepared from the cell pellets by transmethylation in 1 mL of methanol/toluene/dimethoxypropane/H₂SO₄ (66 : 28 : 2 : 1 by volume) at 80 °C for 40 min, before 0.5 mL hexane and 1 mL KCl (0.88% w/v) were added and the contents were vortexed and centrifuged, and the upper hexane phase was transferred to a fresh vial. Extraction with hexane was repeated twice to ensure extraction of all FAMEs and the three extracts pooled. The FAMES were dried down under N₂ and reconstituted in 0.5 mL hexane. 50 µL hexane were combined with 5 µL 50 mg·mL⁻¹ iodine in diethyl ether and 50 µL DMSO and were vortexed and heated at 40 °C for 15 h. Then, 5 µL 5% (w/v) sodium thiosulfate and 200 µL hexane were added, vortexed and centrifuged to separate the phases. The hexane layer was removed, dried under N₂ and
reconstituted in 50 µL heptane for analysis by GC-MS. Separation of FAMEs and DMDS adducts was performed by 6890N Network GC System (Agilent Technologies, Santa Clara, CA, USA) fitted with a 30 m × 0.25 mm, 0.25 µm film thickness, HP1-MS-UI capillary column (Agilent Technologies). FAME/DMDS adducts (1 µL) were injected (splitless) at 280 °C and He used as the carrier gas (0.6 mL·min⁻¹) at a constant flow. The oven program was as follows: 70 °C (1 min), 40 °C·min⁻¹ ramp to 150 °C, 4 °C·min⁻¹ ramp to 300 °C (2 min), 325 °C (18 min). For FAME/DMDS adduct identification, GC was coupled to a 5975B mass selective detector (Agilent Technologies) with a 3.5-min solvent delay, on constant scan mode 42–500 m/z.

Results

Identification of putative Δ11 desaturases

To identify candidate Δ11 desaturases from AM fungi, we performed a blastp search of the *R. irregularis* DAOM197198 genome [16] using characterized insect [13,14] and marine diatom [15] protein sequences. Two genes designated *DES1* and *DES2* (GenBank accession numbers EXX76018 and EXX69612, respectively) were identified that encode proteins that share substantial (>35%) sequence identity with the archetypal palmitoyl (16:0)-CoA Δ11 desaturase from *Trichoplusia ni* (GenBank accession number AAD03775) [13]. Comparison of the amino acid sequences (Fig. 1) revealed that *DES1* and *DES2* contain a membrane desaturase-like conserved domain (cl00615) [21] which includes three His-box motifs, characteristic of desaturases and essential for their catalytic activity [27]. In addition, *DES1* and *DES2* also contain a cytochrome b5-like haem-binding conserved domain (cl34968) [21] at their C terminus, featuring a HPGG motif (Fig. 1) that is characteristic of the fusion between desaturases and cytochrome b5 [28]. Both insect and mammalian fatty acyl-CoA desaturases lack cytochrome b5-like C-terminal extensions, but they are present in fungal counterparts such as the *S. cerevisiae* Δ9 desaturase Ole1p [24,29]. TMHs predicted by the Kyte–Doolittle hydropathy scale [18] and TMHMM [19] were in qualitative agreement (Fig. S1), and both algorithms placed the N and C termini of *DES1* and *DES2* in the cytosol, consistent with the topology of mammalian stearoyl (18:0)-CoA desaturases (SCDs) [30]. Four TMHs were identified (Fig. S1), which are typical features of membrane-bound desaturases [30] and are consistent with the crystallographic structures of SCDs [31,32].

Expression of *DES1* and *DES2* in *R. irregularis*

To investigate whether *DES1* and *DES2* are expressed in *R. irregularis*, we analysed a RNA-sequencing data set that includes structures from both asymbiotic and symbiotic stages of the AM fungal life cycle such as germ tubes, runner hyphae, intraradical mycelium, arbuscules, branched absorbing structures and immature and mature spores [33]. A search for the corresponding transcripts of *DES1* and *DES2* within this data set revealed that both genes are expressed in all seven AM fungal structures, but *DES2* appears to be

![Conserved regions of Rhizophagus irregularis DES1 and DES2 aligned with Trichoplusia ni Δ11 desaturase and other functionally characterized fatty acyl-CoA desaturases from Mus musculus and S. cerevisiae.](https://doi.org/10.1002/fpls.15770)
The more strongly expressed of the two genes, particularly in intraradical mycelium, arbuscules and spores (Table 1). A desaturase responsible for producing 16:1^11cis in R. irregularis should be expressed in these structures since this fatty acyl moiety is most abundant in triacylglycerol that accumulates first in lipid droplets that form in the intraradical mycelium proximal to arbuscules [2,34].

### Functional analysis of DES1 and DES2 by expression in S. cerevisiae

To test the enzymatic function of DES1 and DES2, we transformed WT S. cerevisiae and the desaturation-deficient ole1Δ knockout strain [24,29] with the high-copy-number plasmids pHY-DES1 and pHY-DES2, designed to express the two genes under the control of the strong constitutive TEF1 promoter [22]. The ole1Δ strain is completely deficient in fatty acid desaturation and can only grow on media that are supplemented with exogenous long-chain unsaturated fatty acids [24,29]. A plate test of ole1Δ harbouring either pHY-DES1 or pHY-DES2 showed that cell growth could be rescued by expression of DES1 or DES2 (Fig. 2), suggesting that both proteins can function as desaturases [24,29].

Fatty acid methyl ester analysis of lipids from WT S. cerevisiae cells [24,29] expressing DES1 revealed that there was no change in the molecular species that were produced (Fig. 3). However, there was a significant (P > 0.05) increase in the relative abundance of oleic acid (18:1^11cis), as compared to the EVC (Fig. 3; Table S1). By contrast, DES2 expression in WT cells led to the appearance of two major new molecular species of fatty acyl moiety (Fig. 3), which GC-MS analysis indicated were isomers of 16:1 (m/z 268) and 18:1 (m/z 296). Further analysis of the double bond positions by extraction of the molecular ions of DMDS adducts [25] revealed the characteristic fragment ions of 16:1^9cis (m/z 117, 245) and 11-cis-vaccenic acid (18:1^11cis) (m/z 145, 245) (Fig. S2). Small amounts of 13-cis-octadecenoic (18:1^13cis) (m/z 117, 273) were also detected (Fig. 3; Table S1; Fig. S2). Further analysis of the fatty acyl composition of ole1Δ cells expressing DES1 or DES2 confirmed that with the substrates that are available, DES1 preferentially produces 18:1^9cis over 16:1^9cis, whereas DES2 produces 16:1^11cis and to a lesser extent 18:1^11cis (Fig. 3; Table S1).

In WT S. cerevisiae cells, trace amounts of 16:1^11cis and 18:1^11cis were also detected (Table S1). 16:1^11cis is known to be a product of 9-cis-myristoleic acid (14:1^9cis) elongation by Elo1p [35], and 18:1^11cis is most likely an elongation product of 16:1^9cis. 16:1^11cis elongation is also likely to explain the small amounts of 18:1^13cis detected in both WT and ole1Δ cells expressing DES2. To test this hypothesis, ole1Δ cells expressing DES2 were supplemented with 16:0 or 18:0 free fatty acids to increase the respective amounts of substrate available for desaturation. The addition of 16:0 resulted in a significant increase in 16:1^11cis and 18:1^13cis (P > 0.05), which is consistent with a precursor–product relationship (Table S1). Addition of 18:0 resulted in a significant increase in 18:1^11cis (P > 0.05), but not in 18:1^13cis (Table S1), suggesting that these MUFAs are not products of the same substrate. Taken together, these data suggest that the 18:1^13cis is not a direct product of 18:0 desaturation, but of 16:1^11cis elongation.

### Discussion

Our data show that R. irregularis contains two desaturases that share sequence similarity with fatty acyl-CoA Δ11 desaturases from Lepidoptera [13], but also possess a cytochrome b5-like C-terminal extension characteristic of their fungal Δ9 counterparts [24,29]. DES1 and DES2 are both expressed in the intraradical mycelium where 16:1^11cis and 18:1^11cis are produced (Fig. 1). Further analysis of the fatty acyl composition of ole1Δ cells expressing DES1 or DES2 confirmed that with the substrates that are available, DES1 preferentially produces 18:1^9cis over 16:1^9cis, whereas DES2 produces 16:1^11cis and to a lesser extent 18:1^11cis (Fig. 3; Table S1). In WT S. cerevisiae cells, trace amounts of 16:1^11cis and 18:1^11cis were also detected (Table S1). 16:1^11cis is known to be a product of 9-cis-myristoleic acid (14:1^9cis) elongation by Elo1p [35], and 18:1^11cis is most likely an elongation product of 16:1^9cis. 16:1^11cis elongation is also likely to explain the small amounts of 18:1^13cis detected in both WT and ole1Δ cells expressing DES2. To test this hypothesis, ole1Δ cells expressing DES2 were supplemented with 16:0 or 18:0 free fatty acids to increase the respective amounts of substrate available for desaturation. The addition of 16:0 resulted in a significant increase in 16:1^11cis and 18:1^13cis (P > 0.05), which is consistent with a precursor–product relationship (Table S1). Addition of 18:0 resulted in a significant increase in 18:1^11cis (P > 0.05), but not in 18:1^13cis (Table S1), suggesting that these MUFAs are not products of the same substrate. Taken together, these data suggest that the 18:1^13cis is not a direct product of 18:0 desaturation, but of 16:1^11cis elongation.

### Table 1. Transcript abundance of DES1 and DES2 in different structures of Rhizophagus irregularis. RNA-sequencing data are derived from Kameoka et al. [33] and are expressed as mean log2 FPKM (fragments per kilobase of exon per million reads mapped).

| Structure | GT | RH | IRM | ARB | BAS | IS | MS |
|-----------|----|----|-----|-----|-----|----|----|
| DES1      | 5.88 | 7.66 | 6.80 | 7.90 | 6.80 | 8.22 | 7.22 |
| DES2      | 3.04 | 6.51 | 7.99 | 10.93 | 9.30 | 11.68 | 13.71 |

AR, arbuscules; BAS, branched absorbing structures; GT, germ tubes; IRM, intraradical mycelium; IS, immature spores; MS, mature spores; RH, runner hyphae.
(C16 and C18) suggests that both are front-end desaturases that count carbon atoms from the carboxyl terminus for insertion of the double bond. The structural basis of chain length specificity has been studied previously in fatty acyl-CoA desaturases [31]. The substrate binding channel of Mus musculus SCD1 is capped by Tyr104, which is located on the second transmembrane helix and blocks access of acyl chains longer than C18 [31,38]. DES1 also possess Tyr in the corresponding position, while DES2 possesses a less bulky Cys residue (Fig. 1). One helical twist above Tyr104 in MmSCD1, and therefore facing the binding pocket, is Ala108 [31]. Mutant analysis suggests that when the Ile residue present at this position in MmSCD3 is substituted for Ala, the substrate preference of MmSCD3 changes from C16 to C18 [31]. Ile has a bulkier side chain than Ala and may therefore shorten the substrate channel [31]. DES1 has Gly in this position (Fig. 1), which has a small side chain. DES2 has Met in this position (Fig. 1), which has a slightly larger side chain. The residues occupying these positions might therefore explain why both DES1 and DES2 accept a C18 substrate.

Although 16:1\text{\textsubscript{11cis}} is highly abundant in R. irregularis, the levels of 18:1\text{\textsubscript{11cis}} are much lower [4,12]. Given that DES2 can synthesize both MUFAs in S. cerevisiae, it is possible that the predominance of 16:1\text{\textsubscript{11cis}} in R. irregularis is the result of substrate availability rather than acyl chain length specificity [39]. It is thought that
Fig. 3. GC-FID analysis of FAMEs derived from lipid extracts of WT or ole1Δ cultures harbouring pHEY vectors, either as EVC or containing DES1 or DES2. For ole1Δ + pHEY1-EVC, an odd-chain MUFA supplement (15:1Δ10cis) was used to complement the ole1Δ phenotype and the 17:1Δ12cis is an elongation product of 15:1Δ10cis. 17:0 was added to all lipid extracts before transmethylation to provide an IS. The individual GC-FID traces are representative of three replicates.
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R. irregularis receives fatty acyl moieties from its host plant that are mainly C16 [5–9] and so this substrate is likely to be most abundant. However, it is also conceivable that 16:1Δ11cis might be preferentially incorporated into triacylglycerol, owing to the activities of lipid assembly and remodelling enzymes that are present in R. irregularis but have yet to be characterized [4]. R. irregularis also contains a comparatively low level of 18:1Δ9cis [4,12] that is likely to be produced by DES1, given its activity in S. cerevisiae. In addition to R. irregularis, 16:1Δ11cis is present in many Glomeromycota and putative orthologues of DES2 can also be found in the R. diaphanous, R. clarus, R. cerebriforme and Gigaspora rosea genomes [40], but not in those of nonmycorrhizal fungi. Interestingly, G. rosea is one of the species from the family Gigasporaceae that does not contain 16:1Δ11cis [11,12]. It is therefore possible that G. rosea DES2 either has a different activity (i.e. is not a ∆11 desaturase) or is not expressed. At present, it is not known why many Glomeromycota make 16:1Δ11cis and some do not. The identification of DES1 and DES2 may help in future studies to better understand the physiological role of the different molecular species of MUFAs found in AM fungi.

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Author contributions

PJE conceived the research. HC, GB and FB performed the research and analysed the data; and HC and PJE wrote the paper.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Fatty acid composition of WT or ole1A cultures expressing DES1 or DES2.