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Functions of Lipids in Development and Reproduction of Arbuscular Mycorrhizal Fungi

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(Received 19 March 2022; Accepted 25 July 2022)

Arbuscular mycorrhizal fungi (AMF) form mutualistic associations with most land plants. The symbiosis is based on the exchange of nutrients: AMF receive photosynthetically fixed carbon from the plants and deliver mineral nutrients in return. Lipids are important players in the symbiosis. They act as components of the plant-derived membrane surrounding arbuscules, as carbon sources transferred from plants to AMF, as a major form of carbon storage in AMF and as triggers of developmental responses in AMF. In this review, we describe the role of lipids in arbuscular mycorrhizal symbiosis and AMF development.

Keywords: Arbuscular mycorrhizal fungi ● Fatty acids ● Lipid metabolism ● Lipids ● Plant–microbe interaction ● Symbiosis

Introduction

Arbuscular mycorrhiza (AM) is a symbiosis between most land plants and fungi of the clade Glomeromycotina (Spatafora et al. 2016). The mutualistic symbiosis is based on the exchange of nutrients: arbuscular mycorrhizal fungi (AMF) receive up to 20% of the photosynthetically fixed carbon from the plant and deliver mineral nutrients in return (Bago et al. 2000, Smith and Smith 2011). The fungi absorb these nutrients from the soil with an extended extraradical hyphal network and release them at tree-like hyphal structures, called arbuscules, into root cortex cells (Luginbuehl and Oldroyd 2017). AMF are peculiar organisms. They have not been reported to reproduce sexually. Instead, they form asexual spores, which can carry hundreds to several thousands of haploid nuclei depending on the species (Kokkoris et al. 2020). Their hyphae are coenocytic, and large numbers of nuclei share the same cytoplasm. AMF are obligate biotrophs, which is attributed to the loss of genes encoding enzymes involved in sugar and thiamin metabolism as well as fatty acid (FA) biosynthesis during coevolution with plant hosts (Tisserant et al. 2013, Wewer et al. 2014, Malar et al. 2021). It is possible that more auxotrophies will be uncovered in the future.

Lipids are important players in the symbiosis. Plants form specialized membranes, the peri-arbuscular membranes (PAMs), surrounding the arbuscules. AM fungal spores are filled with lipids likely providing the material for membrane formation and energy during germination, and species belonging to the Glomeraceae also form lipid-filled vesicles inside roots, which are regarded as storage organs. All FAs contained in fungal lipids are likely derived from host plants, although they may not be present in the fungi in the form, in which they were originally delivered, as AMF possess the enzymatic machinery for FA elongation and desaturation (Trépanier et al. 2005, Wewer et al. 2014, Brands et al. 2020, Cheeld et al. 2020). Furthermore, some lipids work as triggers that regulate AMF development. In this review, we describe the role of lipids in AM symbiosis and AMF development. Since the general lipid metabolism in plants has extensively been described (Li-Beisson et al. 2013, Luttgeharm et al. 2016, Nakamura 2017), we place a particular focus on the role of plant-derived lipids as metabolites and developmental triggers for AMF and on AM fungal lipid metabolism.

Lipid Composition of the PAM

As constituents of membranes, lipids are major contributors to the construction of the large membrane interface at the arbuscule consisting of the arbuscular membrane and the plant-derived PAM. The PAM forms in root cortex cells during arbuscule development through exocytosis to envelop the arbuscule, resulting in strong polarization of these cortex cells into a peripheral membrane and a PAM (Gutjahr and Parniske 2013, Harrison and Ivanov 2017). Both the membrane of the fungal arbuscule and the PAM are strongly curved to envelop and surround the arbuscule branches (Ivanov et al. 2019, Roth et al. 2019), and the lipid composition of these two membranes likely contributes to the curvature, membrane stability and the formation of specialized sub-domains. The exact lipid composition of and distribution within the PAM are still unknown. However, specialized domains have been observed

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by using genetically encoded reporters for phosphoinositides (PI4P and PI(4,5)P_2), diacylglycerol (DAG) and phosphatidic acid (PA) in *Medicago truncatula* roots (Ivanov and Harrison 2019). Both PA and phosphoinositides are small anionic lipids that impact a number of cellular processes such as exocytosis, endocytosis, endomembrane trafficking and signaling (reviewed in Noack and Jaillais 2020). They also affect the membrane curvature by changing the electrostatic potential of the membrane due to their negative charge (Simon et al. 2016) and may thereby contribute to the curvature of the arbuscule membrane and the PAM. PI4P and PI(4,5)P_2 can be converted to DAG via phospholipase C, while DAG and PA can be reciprocally interconverted via DAG kinase and phosphatidate phosphatase, respectively (reviewed in Noack and Jaillais 2020).
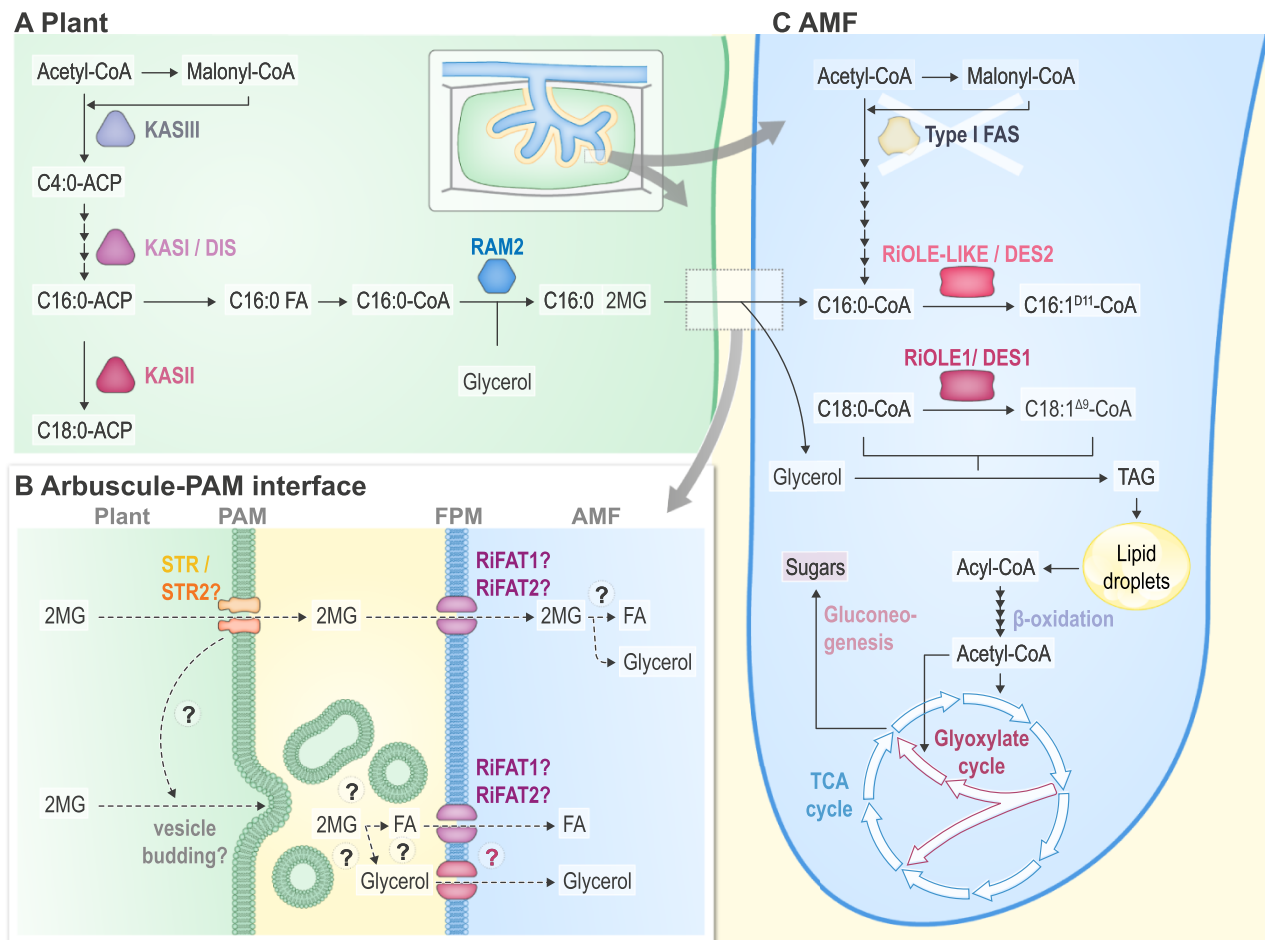
In arbuscule-containing cells, the PI(4,5)P_2 (GFP-PH$_{PLC\delta1}$) reporter and the PI4P (mCherry-PH$_{FAPP1}$) colocalized to the PAM surrounding thick and fine arbuscule branches. In addition, thick trunks that had just penetrated the cell and had not yet branched displayed dome-shaped punctae with very strong PI(4,5)P_2 reporter accumulation. These were suggested to represent possible emergence points for new hyphal branches (Ivanov and Harrison 2019). The PA marker (mCherry-PABD$_{Spo20p}$) was detected at hyphal tips, while the DAG reporter (CYS1$_{PKC\gamma}$-mCherry) was mainly detected in the cytoplasm of arbuscule-containing cells and at mobile punctae (Ivanov and Harrison 2019). The four reporters localized partially to different domains in the arbuscule trunk, indicating a variety of regions in the trunk domain of the PAM. However, they also partially colocalized at two discrete and prominent punctae at the opposite lateral sides of the base of the arbuscule trunk (Ivanov and Harrison 2019). The function of these punctate domains is unclear. But as they appear slightly bulged, the authors suggested that they could correspond to a plant-derived 'biotrophic interfacial complex (BIC)', which in interactions between the pathogenic fungus *Magnaporthe oryzae* and rice acts as a platform for the release of fungal effectors into the plant cell (Khang et al. 2010, Ivanov and Harrison 2019). Although there is no evidence yet for the role of the investigated lipids in the arbuscule-containing cell and PAM, the study by Ivanov and Harrison (2019) gave interesting first insights into the lipid landscape of the PAM.

The lipid composition likely plays an important role in determining the content and functionality of their resident proteins, which include a number of transporters and channels involved in nutrient and signal exchange between the symbionts (Wipf et al. 2019) and proteins involved in signaling (Roth et al. 2018). Glycosylated sphingolipids may play a role in PAM formation or the regulation of its protein composition, as a *M. truncatula* GLUCOSAMINE INOSITOL PHOSPHORYLCERAMIDE TRANSFERASE1 (GINT1) gene required for the glycosylation of *N*-acetyl-glucosamine-decorated glycosyl inositol phosphoryl ceramides is required for arbuscule maturation and branching (Moore et al. 2021).

AMF Receive Lipids from Their Host Plants

Unlike plants, AMF cannot synthesize FAs de novo. Instead, they obtain FAs from their host plants (**Fig. 1**), which is considered a major cause for their obligate biotrophy. Feeding experiments using isotope-labeled carbon sources showed that AMF cannot synthesize FAs in asymbiotic stages and in the extraradical mycelium (ERM) in symbiotic stages (Bago et al. 1999, Pfeffer et al. 1999, Trépanier et al. 2005). These results were later explained by the absence of genes encoding cytosolic type I FA synthase (FAS) from the *Rhizophagus irregularis* genome (Wewer et al. 2014), which in fungi is required to synthesize palmitic acid (C16:0 FA) from acetyl-coenzyme A (CoA) and malonyl-CoA (Wakil et al. 1983) (**Fig. 1I**). The absence of genes encoding cytosolic FAS was confirmed for genomes of six additional AM fungal species (Tisserant et al. 2013, Kobayashi et al. 2018, Maeda et al. 2018, Morin et al. 2019, Sun et al. 2019, Malar et al. 2021). This raised the possibility that AMF are unable to synthesize FAs de novo and may depend on the plant for FA delivery. Since FA synthesis consumes a large amount of ATP and nicotinamide adenine dinucleotide phosphate (Wakil et al. 1983), it makes sense that AMF afforded to lose the FA synthesis pathway once they could obtain FAs from plants. Indeed, the transport of FA-containing lipids from host plants to AMF was first suggested by lipid accumulation patterns in mycorrhizal roots of wild-type and AM-specific lipid biosynthesis mutants (Bravo et al. 2017) and then shown with two independent methods (Jiang et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017). Jiang et al. (2017) and Luginbuehl et al. (2017) expressed *UcFatB* encoding a thioesterase from *Umbellularia californica*, which terminates FA biosynthesis after 12C atoms resulting in lauric acid (C12:0 FA) in *M. truncatula* hairy roots. Since C12:0 FA neither occurs in *M. truncatula* nor in the model fungus *R. irregularis*, they could use it as a tracer to show that C12:0 FA was transferred from roots to extraradical hyphae and spores. Keymer et al. (2017) fed roots with ^{13}C-labeled glucose and used isotopolog profiling of 16:0 FA naturally occurring in roots of *Lotus japonicus* plants or *Daucus carota* hairy roots as well as extraradical hyphae and spores of *R. irregularis* (the method is explained in more detail in Keymer and Gutjahr 2018). Although the 16:0 FA isotopolog pattern differed between *L. japonicus* roots and carrot hairy roots, the fungal pattern always mirrored that of the plant, indicating that the plant determined the pattern and transferred 16:0 FA or a 16:0 FA-containing lipid to the fungus (Keymer et al. 2017).

The increased need for FAs for transfer to the fungus is satisfied by an AM-specific lipid biosynthesis pathway, which was discovered by forward and reverse genetics in model legumes (**Fig. 1A**, Bravo et al. 2016, Bravo et al. 2017, Jiang et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017, Brands et al. 2018). *DISORGANIZED ARBUSCULES* (*DIS*) encodes a β-ketoacyl-acyl carrier protein (ACP) synthase I (KASI), which acts in the plastid in concert with KASIII (biosynthesis of C4 FAs) and KASII (elongation of C16 FAs) and elongates FAs from C4 to C16 by the stepwise addition of two-carbon units from malonyl-CoA

Fig. 1 Lipid metabolism and transport in AM symbiosis. (A) An AM-specific lipid biosynthesis pathway in plants operates in arbuscule-containing cells. *DIS* is a KASI, which elongates acyl groups bound to ACP from C4 to C16 (Keymer et al. 2017). Acyl group elongation is terminated by a thioesterase FatM, which preferentially hydrolyzes 16:0-ACP into 16:0 FA (Bravo et al. 2016, Brands et al. 2018). REQUIRED FOR ARBUSCULAR MYCORRHIZATION 2 (RAM2) produces 2MGs with a preference for 16:0 FAs (Bravo et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017). (B) Hypotheses on lipid transport mechanisms from the arbuscule-containing cell into the arbuscule. STR and STR2 are two half ABCG transporters, which form heterodimers and localize to the PAM (Zhang et al. 2010). The STR/STR2 complex currently represents the best candidate exporter for 2MGs because it is required for lipid transfer to the fungus (Gutjahr et al. 2012, Bravo et al. 2017, Jiang et al. 2017, Keymer et al. 2017). Alternatively (or in addition), lipids might be transported via exocytotic vesicles (Ivanov et al. 2019, Roth et al. 2019), and STR/STR2 might be involved in triggering vesicle transport. It is unclear whether 2MGs are directly imported into AMF or hydrolyzed in the peri-arbuscular space into free FAs and glycerol or converted into other compounds before import. RiFAT1 and RiFAT2 might be involved in 2MG or FA import (Brands and Dörmann 2022). Dashed arrows and question marks indicate hypothetical transport routes and involved proteins. FPM, fungal plasma membrane. (C) Lipid metabolism in AMF. AMF cannot synthesize FAs de novo because they lack the cytosolic type I FAS (Wewer et al. 2014), and the missing biosynthetic steps are shown in light gray. Some FAs obtained from plants are desaturated (Brands et al. 2020, Cheeld et al. 2020) and/or elongated (Trépanier et al. 2005, Sugiura et al. 2020). FAs are incorporated into TAGs and preserved in lipid droplets (Bonfante et al. 1994, Bago et al. 2002). FAs are catabolized into acetyl-CoA in the β-oxidation pathway. Acetyl-CoA is oxidized in the TCA cycle to synthesize ATP or converted into C4 dicarboxylic acids in the glyoxylate cycle and subsequently into hexoses in the gluconeogenesis pathway (Bago et al. 1999, Pfeffer et al. 1999, Lammers et al. 2001, Sugiura et al. 2020).

and the production of one CO_2 per malonyl-CoA (Li-Beisson et al. 2013, Keymer et al. 2017). FA elongation is terminated by a thioesterase FatM, which preferentially hydrolyzes 16:0-ACP (Bravo et al. 2016, Brands et al. 2018). Finally, *REQUIRED FOR ARBUSCULAR MYCORRHIZATION 2* (*RAM2*) encodes a glycerol-3-phosphate ACP synthase 6 paralog producing 2-monoacylglycerols (2MGs) with a preference for 16:0-CoA and to a lesser extent for 14:0-CoA (Wang et al. 2012, Bravo et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017). The ectopic

expression of *RAM1*, a transcription factor that induces the expression of genes encoding the enzymes in the AM-specific lipid biosynthesis pathway, enhanced the accumulation of 16:0 2MGs and to a slightly lesser extent 16:0 1MGs on the root surface (Luginbuehl et al. 2021). Furthermore, the half ATP-binding cassette G (ABCG) transporter mutant *str* slightly accumulates 16:0 2MGs upon root colonization when a protocol for rapid colonization is used (Bravo et al. 2017). Taken together, these results suggest that 16:0 2MGs may be the final product of the

AM-specific lipid biosynthesis pathway that is exported to the fungus; however, this has not been directly shown. *FatM* and *RAM2* are AM-specific paralogs of housekeeping lipid biosynthesis genes and have eroded from genomes of plants that secondarily lost AM (Bravo et al. 2016). *DIS* is present in genomes of AM-competent dicotyledons and gymnosperms but not in genomes of monocotyledons, suggesting that *DIS* was lost at the base of the monocotyledon clade and members of this clade produce 16:0 FA for transfer to the fungus by means of their housekeeping KASI (Keymer et al. 2017). The promoters of all three genes are specifically active in arbuscule-containing cells. Consistent with this expression pattern and the dependence of the fungus on plant lipids, mutations in these genes disable arbuscule development, arbuscules remain stunted, root length colonization is reduced and the fungus is unable to form lipid-storing vesicles and finally dies off (Wang et al. 2012, Bravo et al. 2016, 2017, Keymer et al. 2017, Luginbuehl et al. 2017, Brands et al. 2018, Dai et al. 2022, Liu et al. 2022). Taken together, this suggests that lipids are mainly exported to the arbuscules via the PAM.

How the lipids are exported from the plant cell toward the arbuscule is currently unknown. A heterocomplex of two half ABCG transporters called STUNTED ARBUSCULE (STR) and STR2 (Zhang et al. 2010) represents currently the best candidate exporter for 2MGs or FAs (**Fig. 1B**). *str* and *str2* mutants carry stunted arbuscules similar to *dis, fatm* and *ram2* (Zhang et al. 2010, Gutjahr et al. 2012), lipid transfer is blocked in these mutants (Jiang et al. 2017, Keymer et al. 2017) and roots of an *M. truncatula str* mutant were shown to accumulate 16:0 2MGs upon colonization (Bravo et al. 2017). Furthermore, STR/STR2 has similarities with the human cholesterol transporter heterocomplex ABCG5/ABCG8 and with complexes of half ABCG transporters involved in cutin or suberin formation, which are hypothesized to transport cutin or suberin monomers including 2MGs (Sun et al. 2021, Ichino and Yazaki 2022). However, the substrate, transport activity and substrate specificity of the STR/STR2 complex remain to be demonstrated, and alternative scenarios are possible. Lipids could, for example, be transported via exocytotic vesicles (**Fig. 1B**, Ichino and Yazaki 2022). Indeed, different types of vesicles in the peri-arbuscular apoplast and club-shaped protrusions of the PAM have been observed by electron tomography (Ivanov et al. 2019, Roth et al. 2019). Interestingly, similar vesicles were also observed during suberization of the endodermal Casparian strip (de Bellis et al. 2022), making it tempting to speculate that these extracellular vesicles are involved in the secretion and transport of lipophilic metabolites. The role of the interconnected membrane protrusions from the PAM is still unclear, but the latter may act as platforms for vesicle shedding (Rilla 2021), represent traffic jams of vesicles budding off the PAM or act as membrane extensions to allow the accommodation of an increased amount of transporter proteins in the PAM (Roth et al. 2019). It is thus possible that 2MGs (or alternative transferred lipids) are not transported by STR/STR2 itself but that the ABCG transporter complex exports

a signal, which triggers vesicle-based transport or stimulates arbuscule branching thereby enabling lipid transfer.

Lipid Metabolism in AMF

The mechanisms by which AMF import lipids are unknown. Labeling experiments showed that AMF can import both acyl and glycerol moieties of 2MGs exported from plants (Luginbuehl et al. 2021) (**Fig. 2A**); however, it is unclear whether 2MGs would be directly imported or hydrolyzed in the peri-arbuscular space into free FAs and glycerol or be converted into other compounds before import (**Fig. 1B**). Brands and Dörmann found two homologs of yeast FA transporter FAT1 in the *R. irregularis* genome. *RiFAT1* and *RiFAT2* exhibited higher expression in intraradical mycelium (IRM) than in ERM, making it tempting to speculate that they could be involved in the uptake of FAs or 2MGs delivered by the plant. Indeed, when heterologously expressed in a yeast *fat1* mutant, *RiFAT1* and *RiFAT2* enhanced the uptake of isotope-labeled C14:0 and C16:0 FAs and 2MGs (Brands and Dörmann 2022), supporting the hypothesis that *RiFAT1 RiFAT2* may mediate lipid import during the symbiosis (**Fig. 1B**), although their functions in the IRM have not been assessed. An ortholog of MG lipase highly expressed in the arbuscules might be involved in the hydrolysis of 2MGs (Kameoka et al. 2019a). Although the majority of 2MGs resulting from the AM-specific 2MG biosynthesis are decorated with a C16:0 (Keymer et al. 2017, Luginbuehl et al. 2017, 2021), AMF can import not only C16:0 FA but also other FAs (or FA-containing lipids): when C12:0 FA biosynthesis was enhanced in genetically modified host plants, the ratio of C12:0 FA and C12:0 in triacylglycerol (TAG) was increased in AMF (Jiang et al. 2017, Luginbuehl et al. 2017, Rich et al. 2021). ^{13}C-labeled myristic acid (C14:0 FA), C16:0 FA and fluorescently labeled C12:0 FA and C16:0 FA derivatives were also imported into AMF in asymbiotic hyphae derived from germinating spores (Sugiura et al. 2020). These results suggest that AMF can import at least FAs with a chain length between C12 and C16.

Although AMF cannot synthesize FAs de novo, they modify the imported long-chain FAs. Many AMF species, including the model species *R. irregularis*, produce a unique unsaturated FA, palmitvaccenic acid (C16:1$^{\Delta 11cis}$ FA) (Graham et al. 1995, Bentivenga and Morton 1996, Olsson and Johansen 2000, Wewer et al. 2014) (**Fig. 2B**). Since C16:1$^{\Delta 11cis}$ FA has been found in only a limited number of organisms other than AMF such as some moths, bacteria and two ectomycorrhizal fungal species (Hofmann and Tausig 1955, Walker 1969, Bjostad and Roelofs 1983, Da Rocha Campos et al. 2008, Reich et al. 2009, Ding et al. 2014), it is used as a lipid biomarker for AMF (Graham et al. 1995, Olsson et al. 1997, Van Aarle and Olsson 2003, Ngosong et al. 2012). The ratio of AMF hyphae and spores can be estimated from the ratio of phospholipids and neutral lipids containing C16:1$^{\Delta 11cis}$ groups because the ratio of the plasma membrane, composed of phospholipids, is higher in hyphae than in spores and that of stored neutral
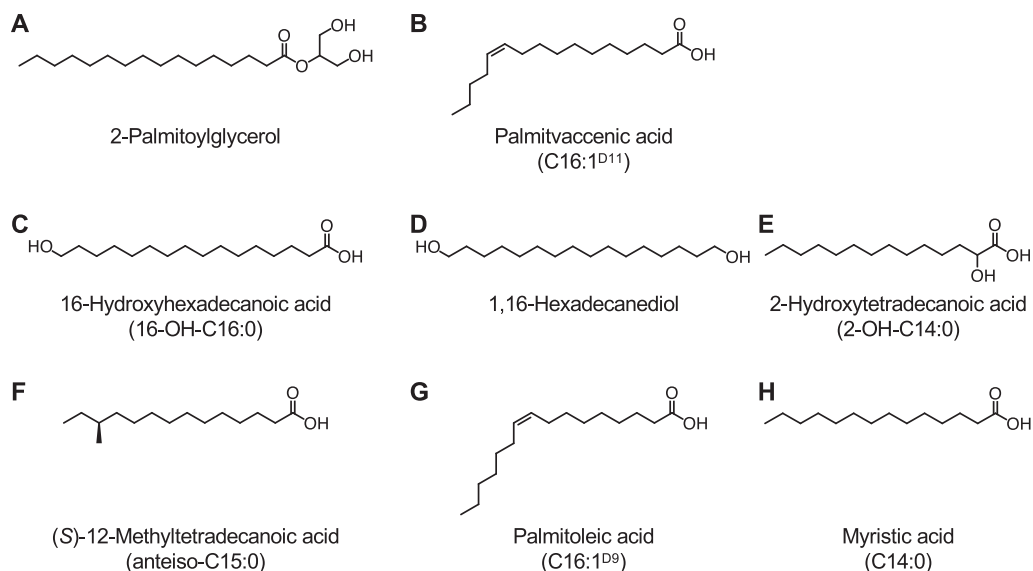
Fig. 2 Chemical structures of lipids that have functions in AMF development. (A) 2-Palmitoylglycerol (C16:0 MG). (B) Palmitvaccenic acid (C16:1$^{\Delta11}$ FA). (C) 16-Hydroxyhexadecanoic acid (16-OH-C16:0 FA). (D) 1,16-Hexadecanediol. (E) 2-Hydroxytetradecanoic acid (2-OH-C14:0 FA). (F) (S)-12-methyltetradodecanic acid (anteiso-C15:0 FA). (G) Palmitoleic acid (C16:1$^{\Delta9cis}$ FA). (H) Myristic acid (C14:0 FA).

lipids is higher in spores than in hyphae (Olsson et al. 1997). Recently, *RiOLE1-LIKE/DES2* has been identified as an acyl-CoA Δ11 desaturase in *R. irregularis*. *Rhizophagus irregularis* contains two homologs of the acyl-CoA Δ9 desaturase gene, *RiOLE1/DES1* and *RiOLE1-LIKE/DES2*. RiOLE1/DES1 desaturates acyl-CoA, especially stearoyl-CoA (C18:0-CoA), at position Δ9, similar to its orthologs in yeast and many other organisms. On the other hand, RiOLE1-LIKE/DES2 desaturates acyl-CoA, especially C16:0-CoA, at position Δ11 (Brands et al. 2020, Cheeld et al. 2020) (**Fig. 1C**). AMF can also elongate long-chain FAs. Two homologs of FA elongase in *Saccharomyces cerevisiae* were found in the *R. irregularis* genome (Kameoka et al. 2019a), although their enzymatic activities have not been examined. Feeding experiments showed that at least C14–C18 FAs are elongated to C16–C20 FAs in AMF (Trépanier et al. 2005, Sugiura et al. 2020) (**Fig. 1C**). Since AMF also have C22 and C24 FAs (Trépanier et al. 2005, Wewer et al. 2014), these FAs must also be synthesized in AMF through FA chain elongation.

Most FAs are incorporated into TAGs and are preserved in lipid droplets (**Fig. 1C**). TAGs account for about 40–90% of stored carbon in AMF (Beilby and Kidby 1980, Bécard et al. 1991, Gaspar et al. 1994, Bago et al. 2000, Wewer et al. 2014) and are dominantly synthesized in IRM, transported to ERM and eventually stored in spores (Bécard et al. 1991, Gaspar et al. 1994, Pfeffer et al. 1999, Bago et al. 2002). Lipid droplets are thought to be important for TAG preservation and transport (Bonfante et al. 1994, Bago et al. 2002). Kobae et al. (2014) showed that two phylogenetically distant AMF species, *R. irregularis* and *Paraglomus occultum*, generate lipid droplets in the IRM including mature or senescent arbuscules. Of note, the accumulation of lipid droplets was observed to coincide with arbuscule collapse, suggesting the importance of arbuscule turnover for lipid droplet generation or alternatively for recycling of arbuscule membrane lipids by the fungus (Kobae et al. 2014). The lipid droplets are then transported in the cytoplasm toward the ERM (Bago et al. 2002, Kobae et al. 2014). FA modifications may contribute to controlling the physical features of lipid droplets. Normally, about 60–70% of the FA groups in fungal TAGs are unsaturated (Beilby and Kidby 1980, Bécard et al. 1991, Olsson and Johansen 2000, Wewer et al. 2014), and lipid droplets are in a liquid state. However, when *R. irregularis* is grown in C14:0 FA-containing media, it exhibits a higher ratio of C14:0 groups in TAGs and abnormal lumpy lipid droplets that appear to be in a more solid state presumably due to stronger intermolecular forces of saturated FAs (Sugiura et al. 2020), implying that FA desaturation may contribute to the fluidity of lipid droplets.

AMF use the stored FAs to produce energy and synthesize saccharides. FAs are catabolized into acetyl-CoA in the β-oxidation pathway (**Fig. 1C**). Acetyl-CoA is oxidized in the tricarboxylic acid (TCA) cycle to synthesize ATP (**Fig. 1C**) or to convert it into C4 dicarboxylic acids in the glyoxylate cycle (**Fig. 1C**) and subsequently into hexoses in the gluconeogenesis pathway (**Fig. 1C**). The orthologs of all enzymes required for these pathways are conserved and expressed in AMF (Lammers et al. 2001, Wewer et al. 2014, Kameoka et al. 2019a, Wendering and Nikoloski 2022). The stimulation of ATP production by FA treatment suggests the significance of the β-oxidation and the TCA cycle for ATP synthesis in AMF (Sugiura et al. 2020). Furthermore, labeling experiments showed the conversion of FAs, acetate, or glycerol into trehalose, glycogen and chitin (Bago et al. 1999, Pfeffer et al. 1999, Lammers et al. 2001, Sugiura et al. 2020), demonstrating that β-oxidation, glyoxylate cycle and gluconeogenesis are active in AMF.

Lipids Stimulate the Growth and Branching of AM Fungal Hyphae

In addition to the functions of FAs as carbon sources, some FAs and FA derivatives work as triggers of developmental changes in AMF. For example, it has been suggested based on phenotypes of *M. truncatula ram2* mutants that cutin monomers including 16-hydroxyhexadecanoic acid (16-OH-C16:0 FA) (**Fig. 2C**) and 1,16-hexadecanediol (**Fig. 2D**) activate the formation of hyphopodia by *R. irregularis* (Wang et al. 2012). However, more recent studies have shown that *RAM2* synthesizes lipids for transfer to the fungus as described above (Jiang et al. 2017, Keymer et al. 2017, Luginbuehl et al. 2017). Careful reassessment is required to confirm that the role of *ram2* in hyphopodium formation is caused by reduced cutin monomers and is not a secondary effect of disrupted lipid transfer at the arbuscule.

In species belonging to the genus *Gigaspora*, but not in *R. irregularis*, hydroxy FAs trigger hyphal growth, suggesting that they work as signals. Nagahashi et al. showed that 2-hydroxytetradecanoic acid (2-OH-C14:0 FA) (**Fig. 2E**) and 2-hydroxydodecanoic acid (2-OH-C12:0 FA) stimulate hyphal elongation and branching (Nagahashi et al. 2010, Nagahashi and Douds 2011). In contrast, 2-hydroxydecanoic acid (2-OH-C10:0 FA), 2-hydroxyhexadecanoic acid (2-OH-C16:0 FA) and 3-hydroxytetradecanoic acid (3-OH-C14:0 FA) did not stimulate hyphal growth (Nagahashi and Douds 2011), suggesting a structure-specific recognition of hydroxy FAs by AMF. Compounds presumed as 2-hydroxy FAs were found from carrot root exudates by MS analysis (Nagahashi et al. 2010). In addition, strigolactones (SLs) in root exudates induce hyphal branching of AMF (Akiyama et al. 2005). While SLs induce higher-order hyphal branching, 2-hydroxy FAs increase the branching points on the primary hyphae (Nagahashi and Douds 2011). Since carrot root exudates stimulate both types of branching (Nagahashi and Douds 2011), *Gigaspora* species may enhance hyphal branching in response to 2-hydroxy FAs in root exudates as well as to SLs to increase the chances of contact with host roots.

FAs Stimulate AM Fungal Spore Formation in the Absence of a Host

After the discovery of lipid transfer from plants to AMF, the dependence on plant-derived FAs was considered a major basis of their obligate biotrophy. However, Hildebrandt et al. (2006) showed that AMF species in the order Glomerales, including *R. irregularis*, can form a small number of spores when co-cultured with the Gram-positive bacterium, *Paenibacillus validus*, in the absence of a host plant. In addition, the fungi responded to *P. validus* with intensive hyphal branching resulting in densely packed hyphal coils. Although the spores formed by co-culture with *P. validus* were smaller than the spores resulting from symbiosis, they were able to germinate and colonize roots (Hildebrandt et al. 2002, 2006). Activity-guided fractionation

of supernatant from *P. validus* culture led to the discovery that (*S*)-12-methyltetradodecanic acid (anteiso-C15:0 FA) (**Fig. 2F**), a branched-chain FA, can induce hyphal branching and the formation of a small number of asymbiotic spores by *R. irregularis* (Kameoka et al. 2019b). Since several types of branched-chain FAs are produced by a wide range of bacteria (Kaneda 1991), the activities of a number of them were examined. FAs branching at C11 or C12 had the strongest hyphal branch-inducing activities, while FAs branching at C13 or C14 had weaker or no activities, respectively (Kameoka et al. 2019b). This structure–activity relationship implies that these FAs work as signaling compounds, along with a possible function as carbon sources. Extensive testing of FAs revealed that three straight-chain FAs, C16:1$^{\Delta 11cis}$ FA (**Fig. 2B**), palmitoleic acid (C16:1$^{\Delta 9cis}$ FA) (**Fig. 2G**) and C14:0 FA (**Fig. 2H**), also induce hyphal branching and spore formation in *R. irregularis* (**Fig. 3**) (Kameoka et al. 2019b, Sugiura et al. 2020, Tanaka et al. 2022) [note that Kameoka et al. (2019b) did not detect the activity of C14:0 FA, presumably because the hyphal branching-inducing activities of FAs were examined by paper disk diffusion assay for the first screening, in which the activity of C14:0 FA was not significant]. C12:0 FA stimulates hyphal elongation but no spore formation (Sugiura et al. 2020). While C16:1$^{\Delta 11cis}$ FA shows spore-inducing activity to the same degree as anteiso-C15:0 FA, C16:1$^{\Delta 9cis}$ FA and C14:0 FA have a much stronger activity (Kameoka et al. 2019b, Sugiura et al. 2020). Interestingly, these FAs induce spore formation in *R. irregularis* and *Rhizophagus clarus* in the order Glomerales, but not in *Gigaspora margarita* belonging to the order Diversisporales, although C14:0 FA slightly enhances the hyphal growth in *G. margarita*. The range of species whose spore formation is induced by FAs is comparable to that of species whose spore formation is induced by co-culture with *P. validus* (Hildebrandt et al. 2006, Kameoka et al. 2019b, Sugiura et al. 2020, Tanaka et al. 2022).

The possibility of complimenting the FA auxotrophy of AMF by FAs in the culture medium bears great potential for in vitro propagation of AMF (**Fig. 3**). The media must contain spore-inducing FAs, such as C16:1$^{\Delta 9cis}$ FA and C14:0 FA (Kameoka et al. 2019b, Sugiura et al. 2020). In addition to inducing spore formation, C14:0 FA also increased the biomass of AMF, implying that AMF prefer C14:0 FA to C16:1$^{\Delta 9cis}$ FA as a carbon source. Indeed, ^{13}C-labeled C14:0 FA in the media was converted into TAGs and saccharides in AMF (Sugiura et al. 2020). Furthermore, it was suggested that C14:0 FA may be required for the myristoylation of proteins required for fungal functioning (Sugiura et al. 2020). C16:0 FA co-applied with C14:0 FA more strongly enhances AMF growth than C14:0 FA treatment alone and exogenously applied C16:0 FA is incorporated into fungal TAGs (Sugiura et al. 2020), indicating that it can also act as a carbon source in spite of not showing spore-inducing activity. Tanaka et al. further improved the medium to enable the mass production of spores of *R. clarus* and *R. irregularis* (Tanaka et al. 2022). To this end, they added an organic nitrogen source and thiamine that cannot be produced by AMF (Tisserant et al. 2013), as well as the plant hormones SLs and jasmonic acid, both of which were
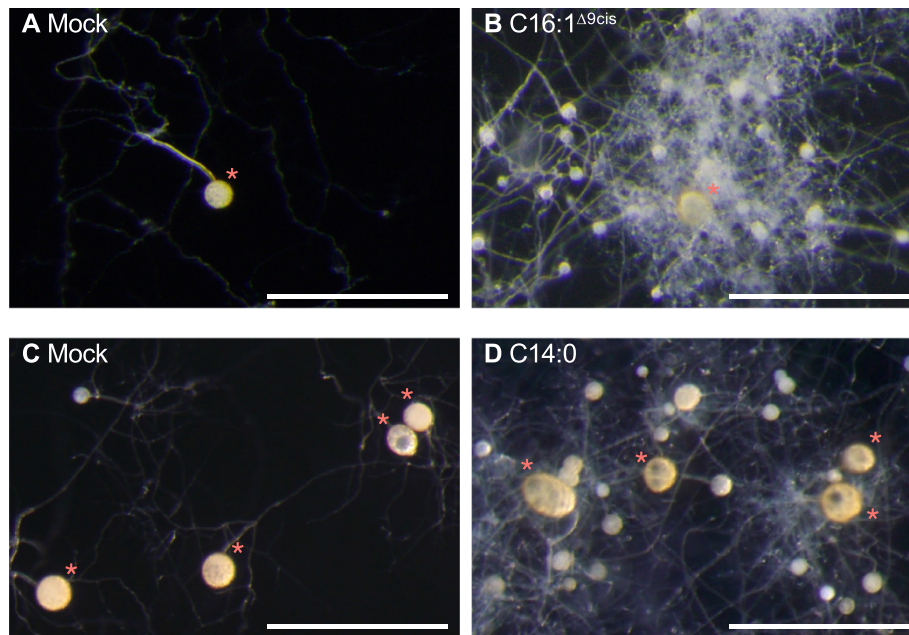
Fig. 3 Spore formation by AMF in axenic culture. *Rhizophagus irregularis* cultured in a modified M medium (A), a modified M medium with 100 μM C16:0 FA (B), a T medium without C14:0 FA (C) and a T medium that contains 500 μM C14:0 FA (D) [(A, B) Kameoka et al. 2019b, (C, D) Tanaka et al. 2022]. Asterisks indicate mother spores. Bar = 500 μm.

previously described to stimulate fungal growth (Akiyama et al. 2005, Nagata et al. 2016). They also showed that the asymbiotically produced spores can promote the growth of Welsh onions (Tanaka et al. 2022), demonstrating their utility for application. However, the growth of AMF and the production of spores are slower in axenic culture as compared to conventional culture methods in the presence of plants or root organ culture. Furthermore, the asymbiotically produced spores are smaller than those produced during the symbiosis, which appears to cause lower germination and colonization rates (Kameoka et al. 2019b, Sugiura et al. 2020, Tanaka et al. 2022). Although the possibility to induce large amounts of spores asymbiotically represents a great and long-awaited breakthrough in the field (Tanaka et al. 2022), an improvement in the method by optimizing the concentrations and combination of FAs and other nutrients and adding other compounds that promote AMF growth and spore production is required for the commercial mass production of AMF inoculum. Furthermore, asymbiotic propagation of AMF is currently limited to two species, *R. irregularis* and *R. clarus*, while species of the genus *Gigaspora* do not produce spores in response to the tested FAs. More work is required to understand whether combinations of other compounds or specific FAs can induce spores in *Gigaspora* species. Nevertheless, the novel protocols provide already several important advantages for research. For example, this method can reduce the labor for preserving AMF isolates belonging to the permissive species. Furthermore, large amounts of AMF mycelia and spores can be produced, which can be desirable for different experimental applications such as detecting low-abundance transcripts or proteins or recording transcriptomic proteomic or metabolomic responses of the fungus to different environmental conditions or molecules (in the absence of the plant).

Currently, it is unclear whether ERMs of AMF respond to FAs also under natural conditions. Since roots exude C14:0 FA and C16:0 FA (Badri and Vivanco 2009, Zhu et al. 2016, Li et al. 2017, Rillig et al. 2020) as well as SL and jasmonate (Nagata et al. 2015, 2016), *Rhizophagus* species may respond by hyphal branching to the cocktail of these compounds in the rhizosphere. *Gigaspora* species may specifically respond to another set of FAs, for example, the 2-hydroxy FAs at least with hyphal branching. AMF carry many different soil bacteria on the surface of their hyphae (Emmett et al. 2021). These bacteria may induce hyphal branching by releasing branched-chain FAs, for example, to increase the fitness of their fungal host and the hyphal surface area they can colonize. It is unclear whether FAs in combination with exuded plant hormones as well as possibly bacteria-released thiamin also induce spore formation in soils because relatively high concentrations (0.1–1 mM) of FAs are required for spore induction in vitro; spore induction by root exudates or bacteria under natural conditions has not yet been reported. In vitro induction of spores may be caused by unnaturally high concentrations of FAs. However, it is well possible that *Rhizophagus* species exposed to plant or bacterial exudates produce spores in the absence of symbiosis or that spore formation of root-colonizing fungi is further stimulated by FAs in these exudates. Further research is required to reveal whether asymbiotic spores are also formed in natural soils in response to specific FAs and whether this has any ecological significance.

Author Contributions

H.K. prepared **Figures 2** and **3**, and provided a sketch for **Figure 1** with input from C.G. Both authors wrote and revised the manuscript text.

Disclosures

The authors have no conflicts of interest to declare.

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