Tap into non-symbiotic carbon? Exogenous myristate fuels the growth of symbiotic arbuscular mycorrhizal fungi but disrupts their carbon-phosphorus exchange with host plants

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Abstract

Arbuscular mycorrhizal fungi (AMF) form symbiotic relationships with the roots of most land plants. In this association, known as arbuscular mycorrhiza (AM), the obligate biotrophic fungi acquire host-derived (symbiotic) carbohydrates and, in return, facilitate plant uptake of mineral nutrients such as phosphate. However, the capacity of symbiotic AMF to access external non-symbiotic carbon sources remains unclear, complicating our understanding of the nutritional exchanges in AM and their ecosystem functions. In this study, we explored the direct uptake of exogenous $^{13}C_1$-labeled myristate by symbiotic AMF (*Rhizophagus irregularis*) and their growth responses using a two-compartment and a single-compartment AMF-carrot hairy root co-culture system, respectively. Additionally, we assessed the impact of exogenous myristate on the carbon-phosphorus exchange between AMF and two host species in a greenhouse experiment, using ^{13}C-neutral lipid fatty acid-isotope ratio mass spectrometry technology, alongside physiological and transcriptome analyses. Our findings reveal that symbiotic AMF can absorb external nonsymbiotic myristate, at least under the conditions tested, as evidenced by ^{13}C enrichment and transcriptional activation of putative fatty acid transport and metabolism genes in extraradical hyphae. Exogenous myristate increased both intraradical and extraradical fungal biomass, potentially due to suppressed mycorrhizal defense responses in host roots. Intriguingly, exogenous myristate reduced the mycorrhizal phosphorus benefits for both *Medicago sativa* and *Oryza sativa* and decreased their carbon allocation to symbiotic AMF, although these effects were influenced by host identity and soil phosphorus conditions. These findings advance our understanding of the nutritional interactions in AM, which are essential for comprehending and manipulating this symbiotic relationship.

Keywords : arbuscular mycorrhizal fungi (AMF), carrot hairy root, carbon-phosphorus exchange, *Medicago sativa* (alfalfa), non-symbiotic carbon (myristate), *Oryza sativa* (rice)

Introduction

Arbuscular mycorrhizal fungi (AMF) are among the most widely distributed soil microorganisms, forming symbiotic relationships with approximately 70% of terrestrial vascular plants (Brundrett and Tedersoo, 2018). In this association, known as arbuscular mycorrhiza (AM), AMF develop an extensive extraradical hyphal network in the soil, facilitating nutrient uptake for host plants, particularly phosphorus (P), and enhancing plant resilience to both biotic and abiotic stressors (Smith and Read, 2008). In return, host plants allocate 4−20% of their photosynthetically fixed carbon (C) to their fungal partners in the form of sugars and fatty acids (Helber et al., 2011; Jiang et al., 2017). This symbiotic C-P exchange, which serves as the cornerstone of the AM symbiosis (Smith and Read, 2008), not only plays a pivotal role in the biogeochemical cycling of C and P elements but also acts as a key mechanism through which AMF influence plant community structure and productivity (Johnson, 2010; Wipf et al., 2019).

As one of the most ancient symbiotic relationships, AM has undergone extensive evolutionary processes (Foster and Kokko, 2006; Rich et al., 2021), with its establishment and development intricately regulated by a set of symbiotic genes and signaling molecules influenced by biotic and abiotic conditions (Maclean et al., 2017; Shi et al., 2021). For instance, in AM plants, the two pathways for P uptake—the direct P pathway (via root hair and epidermis) and mycorrhizal P pathway (via AMF hyphae)—are coordinately regulated by the same phosphate-sensing pathway in plants (Shi et al., 2021; Das et al., 2022). Under P deficiency conditions, host plants synthesize and release strigolactones into the soil, activating the mycorrhizal P pathway by stimulating the elongation and branching of AMF hyphae (Mori et al., 2016; Yu et al., 2022). Additionally, the extent of AMF colonization and AM benefits

largely depend on the host plant species, with some being "responsive plants" that exhibit positive growth responses (e.g., alfalfa (*Medicago sativa* L.), maize (*Zea mays* L.)) and others being "nonresponsive plants" that show minimal or no growth enhancement (e.g., rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.)) (Hata et al., 2010; Cope et al., 2022).

AMF are considered obligate symbionts that rely on living host plants to complete their life cycle by producing daughter spores (Smith and Read, 2008), which presents challenges for basic research on AM and their applications in agriculture and other fields. Thus far, achieving a pure (plant-independent) culture of asymbiotic AMF has not been successful, partially due to their dependence on plant-derived C (Bravo et al., 2017; Luginbuehl et al., 2017; Kameoka et al., 2019). Genomic and transcriptomic analyses reveal that AMF lack the genes required for the biosynthesis of long-chain fatty acids, and subsequent studies have demonstrated that fatty acids are transferred from host plants to AMF as their main C source (Bravo et al., 2017; Jiang et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017). However, the molecular mechanism underlying the allocation of plant-derived fatty acids to fungi in AM symbiosis remains unclear. In plants, AMF colonization triggers the expression of lipid biosynthesis and transport genes, such as *DIS*, *FatM*, *RAM2*, and *STR2* (Gutjahr et al., 2012; Bravo et al., 2017; Keymer et al., 2017; Luginbuehl et al., 2017), with lipid transport to the AM symbiotic interface relying on the periarbuscular membrane-located transporters STR and STR2 (Zhang et al., 2010; Gutjahr et al., 2012) and fungal uptake of plant-derived lipids mediated by RiFAT1 and RiFAT2 in the model AMF species *Rhizophagus irregularis* (Brands and Dörmann, 2022).

Fatty acids and their derivatives, essential structural components and energy sources for plant, animal, and microbial cells, are commonly found in soil,

particularly in the rhizosphere, where plant-derived fatty acids are made available through root exudates and rhizodeposition (Swenson et al., 2018; Dai et al., 2022; Liu et al., 2024). Following the discovery that AMF acquire fatty acids from their hosts during symbiosis, the use of fatty acids in cultivating asymbiotic AMF has received considerable attention. Kameoka et al. (2019) demonstrated that under asymbiotic conditions, exogenous palmitoleic acid (C16:1 fatty acid) and several other fatty acids stimulated the elongation and branching of *R. irregularis* hyphae, resulting in the formation of infection-competent but morphologically and functionally incomplete spores with reduced size, lower germination rates, and decreased infection capacity compared to symbiotic spores. Additionally, Sugiura et al. (2020) showed that exogenous myristate (tetradecanoic acid, C14:0 fatty acid), a common organic acid in various plant root exudates (Fernández-Piñar et al., 2012; Luo et al., 2014; Li et al., 2017; Liu et al., 2024) and soil substrates (Swenson et al., 2018; Dai et al., 2022), can serve as a C and energy source for the asymbiotic growth of *R. irregularis*, promoting hyphal growth, the formation of branched absorbing structures, and the production of morphologically incomplete infection-competent spores. Subsequent studies revealed that combining plant hormones (strigolactone and methyl jasmonate) with myristate enhanced the efficiency of myristate as a C source in asymbiotic *R. clarus*, leading to the generation of numerous infection-competent spores that were smaller than those generated under symbiotic conditions (Sugiura et al., 2020; Tanaka et al., 2022). These discoveries have significantly progressed the pure culture of AMF and, importantly, identified myristate as the first external C source accessible to asymbiotic AMF, providing exceptional opportunities for understanding and utilizing the ecological functions of AMF in plant community regulation and global C cycling (Rillig et al., 2020).

However, the effects of external myristate on the growth and development of symbiotic AMF—the typical state of functioning AMF in nature—remain unclear. Regarding sugars, the other type of host-derived C source, symbiotic AMF may be unable to absorb them additionally through extraradical hyphae (Bücking et al., 2008; Helber et al., 2011; Sugiura et al., 2020; but see Pfeffer et al., 1999). A recent study reported that while exogenous myristate does not influence the extraradical biomass of symbiotic AMF, it enhances AMF colonization by stimulating the growth and development of extraradical hyphae. Based on this, the authors inferred that symbiotic AMF may acquire exogenous myristate (Liu et al., 2023). To date, the effects of exogenous fatty acids on AM symbiosis and their underlying mechanisms remain largely unexplored. Specifically, the capacity of symbiotic AMF to uptake myristate as an external non-symbiotic C source in the presence of plant-derived (symbiotic) C is yet to be determined. This question is crucial for understanding the symbiotic mechanisms and ecological roles of AM symbiosis (Rillig et al., 2020).

This study investigated the capacity of symbiotic AMF to absorb external non-symbiotic myristate and examined the effect of exogenous myristate on symbiotic C-P exchanges in AM. Specifically, we aimed to determine: (i) whether symbiotic AMF can take up external non-symbiotic myristate, and if so, (ii) how this additional non-symbiotic C source influences the symbiotic C allocation from plants to AMF, and (iii) whether and how fungal P transfer to the host plant is influenced by exogenous myristate. To address these questions, we performed a $^{13}C_1$-labeled myristate tracing experiment to examine the direct uptake of exogenous myristate by extraradical hyphae of AMF associated with carrot hairy roots using a two-compartment co-culture system. Additionally, we assessed the impact of varying myristate concentrations on spore germination and the intra- and extraradical growth of AMF

associated with carrot (*Daucus carota* var. *sativa* Hoffm.) hairy roots in a single-compartment co-culture system. Furthermore, we conducted a greenhouse experiment, using ^{13}C-neutral lipid fatty acid-isotope ratio mass spectrometry (^{13}C-NLFA-IRMS) technology, and physiological and transcriptome analyses, to further investigate how exogenous myristate influences the symbiotic interaction between AMF and two host plants. This work provides, to our knowledge, the first solid evidence that symbiotic AMF can absorb non-symbiotic C sources like myristate in the presence of plant-derived carbohydrates. Moreover, our findings reveal that under symbiotic conditions, exogenous myristate unexpectedly disrupts the C-P exchange in AM symbiosis.

Results

Tracing of ^{13}C in AMF extraradical hyphae and the effect of myristate on AMF colonization and marker gene expression (Exp. 1)

In the two-compartment AMF-carrot hairy root co-cultivation system (Exp. 1, Fig. 1a), exogenous myristate specifically induced the branched absorbing structures of AMF in the HC two weeks after Myr^+ treatment (Fig. 1b). The $^{13}C/^{12}C$ ratio of the MSR medium collected in RC in $^{13}C_1$-Myr (1.0966 ± 0.0012) and ^{12}C-Myr (non-labeled, 1.0967 ± 0.0032) treatments was identical, demonstrating that the exogenous myristate added in HC did not diffuse directly into RC. The $^{13}C/^{12}C$ ratio of extraradical hyphae collected in HC (1.0922 ± 0.0003%) and RC (1.0923 ± 0.0002%) in ^{12}C-Myr treatment was identical and close to the natural $^{13}C/^{12}C$ ratio. In the $^{13}C_1$-Myr treatment, the $^{13}C/^{12}C$ ratio of extraradical hyphae in HC (1.1504 ± 0.0575%) and RC (1.1026 ± 0.0055%) was significantly higher than that in the non-labeled control ($P < 0.05$, Fig. 1c), suggesting the uptake of $^{13}C_1$-Myr by extraradical hyphae.

Exogenous myristate significantly increased the total, hyphal, and arbuscular

colonization rates in carrot hairy roots ($P < 0.05$, Fig. 1d) but had no effect on vesicle colonization ($P > 0.05$).

qRT-PCR of the fungal marker genes in AMF extraradical hyphae collected in HC showed that exogenous myristate increased the expression levels of the fungal fatty acid transporter genes *RiFAT2* ($P < 0.05$) and *RiFAT1* ($P > 0.05$), as well as the fungal genes involved in fatty acid β-oxidation (*RiFAD1*, $P < 0.05$), palmitvaccenic acid synthesis (*RiOLE1*, $P = 0.057$), and TCA cycling (*RiCIT1*, $P < 0.05$) (Fig. 1e, f). These results suggest that exogenous myristate activated the expression of fungal genes related to fatty acid transportation and metabolism in the extraradical hyphae of AMF.

Effects of exogenous myristate on AMF spore germination, root colonization and extraradical structure development (Exp. 2)

In the single-compartment AMF-carrot hairy root co-cultivation system (Exp. 2, Fig. 2a), exogenous myristate significantly promoted AMF spore germination ($P < 0.001$, Fig. 2b), and enhanced hyphal branching and length ($P < 0.001$) before physical contact between AMF hyphae and hairy roots at four days post AMF inoculation (Fig. 2c, d). Morphological examinations showed that exogenous myristate significantly enhanced the colonization intensities of hyphae, vesicles and arbuscules in carrot hairy roots across various application levels ($P < 0.05$, Fig. 2e), with its promotional effects generally increasing at higher myristate concentrations. Exogenous myristate also significantly increased the extraradical biomass of AMF (hyphae and spore) at various application concentrations ($P < 0.05$, Fig. 2f).

Meanwhile, exogenous myristate significantly increased the density of AMF spores from eight weeks post-AMF inoculation ($P < 0.05$, Fig. 2g), and the diameter of newly produced AMF spores in mediums with exogenous myristate were identical

to those in mediums without myristate (Fig. 2h).

Effects of exogenous myristate on AMF colonization and biomass during symbiosis with alfalfa and rice (Exp. 3)

In the pot experiment (Exp. 3), all typical AMF structures (hyphae, arbuscules, and vesicles) were observed in the roots of both alfalfa and rice in AMF-inoculated groups (AM), while no AMF colonization was detected in the non-inoculated (NM) groups. Under low-P conditions, exogenous myristate significantly increased arbuscule colonization in alfalfa roots ($P < 0.05$, Fig. 3a) but did not significantly affect hyphae or vesicle colonization ($P > 0.05$, Supplementary Fig. S1a). Similarly, exogenous myristate significantly enhanced arbuscule and vesicle colonization in rice roots ($P < 0.05$, Fig. 3b, Supplementary Fig. S1b). However, under high-P conditions, exogenous myristate showed no effect on AMF colonization in either alfalfa or rice roots (Fig. 3, Supplementary Fig. S1).

The content of the AMF signature fatty acid (NLFA 16:1ω5) in AM roots (alfalfa: 143.2 ± 13.8 nmol g^{-1}; rice: 197.3 ± 11.9 nmol g^{-1}) was 10–20 times higher than in their corresponding NM roots (alfalfa: 6.1 ± 0.5 nmol g^{-1}; rice: 17.3 ± 1.2 nmol g^{-1}), suggesting that NLFA 16:1ω5 serves as a distinctive fatty acid marker for AMF in this study. The levels of NLFA 16:1ω5 in plant roots corresponded with the patterns observed in AMF colonization. Under low-P conditions, exogenous myristate significantly increased the NLFA 16:1ω5 levels in both alfalfa and rice roots ($P < 0.05$, Fig. 3c, d); however, it had no significant effect under high-P conditions. Similar trends were observed in the rhizosphere soils: under low-P conditions, exogenous myristate increased the NLFA 16:1ω5 levels in the rhizosphere soils of alfalfa ($P < 0.05$) but not rice ($P = 0.14$) (Fig. 3c, d). Under high-P conditions, myristate had no effect on the NLFA 16:1ω5 levels in the rhizosphere soil of either plant.

Effects of exogenous myristate on the transfer of plant-derived C to AMF (Exp. 3)

The ^{13}C-NLFA-IRMS experiment investigated the effect of exogenous myristate on C transfer from plants to AMF. In AM plants, a significant enrichment of ^{13}C (δ^{13}C = 1.75 ± 0.6‰) in AMF signature fatty acid (NLFA 16:1ω5) was observed compared to NM plants (δ^{13}C = −24.8 ± 2.3‰), demonstrating the transfer of newly fixed ^{13}C from plants to AMF. In alfalfa, exogenous myristate significantly reduced the plant-derived C transfer to AMF ($P < 0.001$, Fig. 4a). In rice, myristate also significantly influenced the C flow to AMF, with its effect significantly interacting with soil P level (Myr × P: $P = 0.004$, Fig. 4b): myristate significantly increased the allocation of ^{13}C to AMF under low-P conditions ($P < 0.05$) but decreased it under high-P conditions ($P < 0.01$).

qRT-PCR analysis revealed that the expressions of the symbiotic lipid transfer marker genes *STR2* (in alfalfa and rice) and the fungal genes responsible for symbiotic fatty acid *(RiFAT1* and *RiFAT2)* or monosaccharide (*RiMST2*) uptake were mostly exclusively detected in AM roots, except for a low expression level of *STR2* occasionally detected in NM roots of alfalfa (Fig. 4c–h).

In AMF-inoculated alfalfa roots, exogenous myristate significantly downregulated the expressions of the symbiotic C transfer genes *MsSTR2* and *RiMST2* ($P < 0.05$, Fig. 4c, g), while having no effects on *MsFAT1* and *MsFAT2* (Fig. 4e). In AMF-inoculated rice roots, the effects of myristate on the tested symbiotic C transfer genes were mostly significantly influenced by soil P levels ($P < 0.05$). Under low-P conditions, myristate significantly increased the expressions of *OsSTR2*, *OsFAT1*, and *OsFAT2*, while decreasing the expression of *RiMST2* ($P < 0.05$, Fig. 4d, f, h). Under high-P conditions, myristate significantly increased the expressions of *RiMST2* ($P < 0.05$) but had no effects on *OsSTR2*, *OsFAT1*, and *OsFAT2* ($P > 0.05$). In

both plant species with AMF inoculation, soil P level showed significant effects on *STR*, *RiFAT1*, and *MST2* genes ($P < 0.05$), with lower expression level of these genes under high-P compared to low-P conditions.

Effects of exogenous myristate on the activity of mycorrhizal P pathway and the MGR (Exp. 3)

In the pot experiments (Exp. 3), AMF inoculation significantly increased P concentrations in both alfalfa and rice plants ($P < 0.001$, Fig. 5a, b). Under low-P condition, exogenous myristate significantly decreased the mycorrhizal P pathway (MPR) in both alfalfa and rice ($P < 0.05$, Fig. 5c, d). Under high-P conditions, while exogenous myristate significantly decreased the MPR in alfalfa ($P < 0.05$), it had no significant effect on the MPR in rice.

The expression of the mycorrhizal P pathway marker genes in plant (*MsPT8* or *OsPT11*) and AMF (*RiPT*) was exclusively detected in the AM roots of rice and alfalfa, except for a low expression level of *OsPT11* observed in the NM root of rice under low-P and Myr^- conditions. This observation confirmed that AM symbiosis activated the mycorrhizal P pathway at the transcriptional level in both plant species (Fig. 5e, f). Exogenous myristate significantly affected the transcriptional responses of the mycorrhizal P pathway in both alfalfa and rice ($P < 0.01$). In alfalfa, the expressions of *MsPT8* and *RiPT* were downregulated by exogenous myristate under both low- and high-P conditions ($P < 0.05$, Fig. 5e). In rice, exogenous myristate similarly decreased the expression levels of *OsPT11* and *RiPT* under low-P conditions ($P < 0.05$), but not under high-P conditions ($P > 0.05$).

In alfalfa, AMF inoculation significantly increased plant biomass under both low- and high-P conditions ($P < 0.05$, Supplementary Fig. S2a), while the MGRs of alfalfa were significantly higher under high-P conditions compared to low-P

conditions ($P < 0.05$, Supplementary Fig. S2c). In rice, AMF inoculation reduced the plant biomass under high-P (but not low-P) conditions ($P < 0.05$), and the MGRs of rice were significantly lower under high-P conditions compared to low-P conditions ($P < 0.05$, Supplementary Fig. S2b, d). Exogenous myristate had no effect on plant biomass or MGR in either alfalfa or rice, whereas P levels significantly influenced MGR, exhibiting contrasting effects between alfalfa and rice ($P < 0.001$, Supplementary Fig. S2c, d).

Effects of exogenous myristate on the transcriptome of mycorrhizal roots (Exp. 3)

RNA-seq data encompassed the majority of plant genes (transcripts), with 39,944 and 33,056 genes in alfalfa and rice, respectively. Principal component analysis (PCA) of gene expression profiles in mycorrhizal roots of alfalfa and rice revealed that the first and second principal components together accounted for 32.4% and 44.1% of the variation, respectively (Supplementary Fig. S3a, b). In both alfalfa and rice, the gene expression profiles with or without myristate application were distinct under low-P but not high-P conditions, suggesting the importance of P status in myristate's effect.

Exogenous myristate induced a number of differentially expressed genes (DEGs, Myr^+ *vs*. Myr^-) in mycorrhizal alfalfa (LP: 308, HP: 137) and rice (LP: 590, HP: 855) roots (Supplementary Fig. S3c, d). Gene Ontology (GO) analysis of these DEGs revealed the enrichment in several GO terms, most of which were associated with plant stress or defense (Fig. 6a, b). These included "defense response", "response to salicylic acid", "defense response to bacterium", "peroxidase activity", "reactive oxygen species metabolic process", etc., in rice, and "glycosyltransferase activity", "alternative oxidase activity", and "heme binding" in alfalfa.

We subsequently analyzed the expression profiles of the component genes associated with these enriched GO terms using the RNA-seq dataset. In alfalfa,

exogenous myristate significantly downregulated several key genes involved in reactive oxygen species (ROS) balance (*MsAOX2*, *MsAOX3*) and stress tolerance (*MsUFGT*, *MsUGT73C3*, *MsUGT73C6*) under low-P conditions, as well as typical stress-responsive genes (*MsPRXP7*, *MsCYP83B1*, and gene families such as *MsG8HL*, *MsCYP71D10*, and *MsCYP736A12*) under high-P conditions (Fig. 6c). In rice, exogenous myristate downregulated several typical defense-associated transcriptional factors (*OsTGA2*, *OsTGA4*, *OsWRKY45*, *OsWRKY62*, *OsWRKY76*, and *OsbZIP79*) and marker genes (*OsPR1a*, *OsPR5L*, *OsPP2C35*, *OsACS2*, *OsDWARF4*, *OsPITA*, *OsMLO2*, *OsRH1*, and *OsCHT1*) under low-P conditions (Fig. 6d). However, most of these genes were upregulated following myristate application under high-P conditions.

Discussion

AMF are obligate biotrophic organisms that rely on host-derived (symbiotic) carbohydrates (Smith and Read, 2008). However, whether symbiotic AMF can take up external non-symbiotic organic C remains poorly understood (Rillig et al., 2020). Our study revealed ^{13}C enrichment in AMF extraradical hyphae from both HC and RC during the $^{13}C_1$-myristate tracing experiment (Fig. 1a, b), indicating that the extraradical hyphae of symbiotic AMF can absorb external non-symbiotic myristate in the presence of plant-derived carbohydrates. This is further supported by two additional observations in our study. First, exogenous myristate increased the transcription of genes associated with fatty acid transport and metabolism in extraradical hyphae (Fig. 1). Second, in our greenhouse experiment, myristate application significantly enhanced intraradical and extraradical AMF biomass, even when the symbiotic C supply from the plant was reduced (Figs. 3 and 4). These findings indicate that the extraradical hyphae of symbiotic AMF can access external

(non-symbiotic) organic C sources like myristate beyond those directly delivered by the host, at least under the conditions studied. Notably, myristate is a common root exudate component in various host and non-AMF host plants (Fernández-Piñar et al., 2012; Guelette et al., 2012; Li et al., 2017; Liu et al., 2024), and is abundant in litter and soil substrates (Swenson et al., 2018; Dai et al., 2022). Yet AMF often colonize and thrive in leaf litter and undecomposed plant material, a long-observed but poorly understood phenomenon (Went and Stark, 1968; Bunn et al., 2019). Although further research is required to determine how frequently symbiotic AMF encounter myristate and to quantify its concentration in various microenvironments, our findings suggest that in realistic environments, extraradical hyphae of symbiotic AMF could take up myristate from sources such as root exudates, decaying plant/animal material, litter, or other microbes, in addition to those symbiotic C source directly provided by the host plant.

During AM establishment, plant roots activate mycorrhizal defense responses to limit excessive C consumption caused by over-colonization (Blilou et al., 2000; Siciliano et al., 2015; Feng et al., 2019). In our study, myristate increased both intraradical and extraradical biomasses of the symbiotic AMF and promoted the formation of normal-sized secondary spores (Figs. 2 and 3), unlike the small spores induced by myristate under asymbiotic conditions (Sugiura et al., 2020). This suggests that plant roots supply additional substances to AMF beyond myristate, the identification of which is essential for the pure culture of AMF. Notably, exogenous myristate transcriptionally downregulated plant genes associated with mycorrhizal defenses under low-P conditions, including those involved in oxidative stress and salicylic acid defense responses (Fig. 6), suggesting that myristate could interfere with normal defense mechanisms in host roots to promote AMF colonization. This effect

differs from AM signals like strigolactones and quercetin, which enhance mycorrhizal defenses in plants (Sudheeran et al., 2020; Kusajima et al., 2022). Further research is needed to clarify myristate's role in regulating AMF colonization and development.

Despite enhanced mycorrhization, exogenous myristate unexpectedly reduced the mycorrhizal P response (MPR) and downregulated mycorrhizal P transporter gene expression in both alfalfa and rice, indicating a diminished mycorrhizal P benefit. This could be attributed to several factors. Firstly, AMF can modulate P transport based on the rate at which plants supply C (Kiers et al., 2011; Argüello et al., 2016). Myristate, acting as an external non-symbiotic C source, likely lowered the "price" of symbiotic C supplied by plants, leading to reduced P transfer. Secondly, exogenous myristate may interfere with AM signaling, as suggested by decreased mycorrhizal defenses, thereby limiting fungal P acquisition and delivery. This disruption might partly explain why the reduction in the mycorrhizal P response induced by exogenous myristate is primarily observed under low-P conditions. Thirdly, AMF P transport proteins expressed in arbuscular cells can re-uptake P from the symbiotic interface, creating nutrient competition between the plant and AMF (Xie et al., 2022). In our study, the increased fungal biomass with exogenous myristate suggests a higher P demand by AMF.

Meanwhile, external myristate also decreased C supply to AMF from both alfalfa and rice, except in low-P rice. This reduction may be due to AMF shifting from utilizing plant-derived C to absorbing external C (myristate), thereby reducing their competition for plant C at the AM interactional interface (Bitterlich et al., 2014). Furthermore, the decline in fungal P delivery to the host can be attributed to the functional coupling of C-P exchange in AM symbiosis (Hammer et al., 2011; Kiers et al., 2011). Notably, under low-P conditions, myristate increased the allocation of

plant-derived C to AMF in rice, underscoring the influence of both host identity and soil P status on myristate's effect. Compared to rice, AM-responsive alfalfa exhibited over tenfold higher ^{13}C transfer efficiency and *RiFAT* gene expression, along with approximately fivefold higher fungal P transporter gene expression. However, the increased rice-derived C under low-P conditions did not correspond to greater mycorrhizal P benefits, suggesting an unrewarding allocation. Overall, this study reveals that exogenous myristate disrupts the symbiotic C-P exchange. As one of the oldest coevolving species relationships (Rich et al., 2021), AM symbiosis depends on effective partner communication and reciprocal metabolic regulation for successful integration (Lanfranco et al., 2018). Our findings emphasize the importance of thoroughly understanding this ancient partnership when developing strategies to enhance AM symbiosis.

Despite these encouraging findings, our work has several limitations. First, the absorption of myristate by symbiotic AMF was observed following exogenous myristate application, which may not fully represent realistic environmental conditions. Second, only one AMF species, *R. irregularis*, was tested in our study, though AMF species may respond to myristate (or other fatty acids) in varying ways, at least under asymbiotic conditions (Kameoka et al., 2019; Sugiura et al., 2020). Furthermore, our investigation into the mechanism by which myristate disrupts C-P exchange in AM symbiosis remains relatively preliminary. Further research is therefore needed to verify and quantitatively explore the absorption of non-symbiotic myristate (or other C sources) by symbiotic AMF under natural conditions, as well as its regulatory mechanisms and ecological significance to AM symbiosis.

Conclusion

In summary, our study demonstrates that symbiotic AMF can access external organic C sources like myristate in addition to those symbiotically allocated by their host plant. Exogenous myristate promoted the proliferation of both intraradical and extraradical biomasses of symbiotic AMF, potentially due to suppressed mycorrhizal defense responses in host roots. Notably, exogenous myristate disrupted the C-P exchange in AM symbiosis, leading to reduced mycorrhizal P benefits for plants and decreased plant-derived C allocation to AMF, although these effects varied depending on host identity and soil P conditions. These findings provide novel insights into the nutritional interactions in AM symbiosis, which are essential for understanding and managing this ancient relationship.

Materials and methods

Experimental design and biological materials

Three interconnected and complementary experiments were conducted in our study. The AMF used in all experiments was *Rhizophagus irregularis* (strain: MUCL 41833), a well-studied 'model' AMF species. We investigated whether exogenous myristate could be directly absorbed by *R. irregularis* (Exp. 1) and examined its effect on the growth of *R. irregularis* (Exp. 2), where Ri T-DNA-transformed carrot roots served as the AMF host. In Exp. 3, two phylogenetically distant species—*Medicago sativa* L. cv. Sanditi (alfalfa, dicot) and *Oryza sativa* L. cv. Zhonghua 11 (rice, monocot)—were used as AMF hosts in a pot experiment to explore the mechanisms underlying the effect of exogenous myristate on AM symbiosis.

Exp. 1: $^{13}C_1$-labeled myristate ($^{13}C_1$-Myr) tracing in a two-compartment AMF-hairy root co-culture system

In Exp. 1, a $^{13}C_1$-My tracing experiment was performed to determine whether exogenous myristate can be directly absorbed by AMF (*R. irregularis*) extraradical hyphae associated with carrot hairy root in a two-compartment Petri dish system as described by St-Arnaud et al. (1996) (90 ×15 mm, see Fig. 1a). The Petri dish was divided into two compartments by a central plastic barrier. One compartment (root compartment, RC) contained 25 mL of modified Strullu–Romand (MSR) solid medium for the growth of carrot hairy root and AMF hyphae. The other compartment (hyphal compartment, HC), into which only the AMF hyphae, and not roots, were allowed to grow, contained 15 mL of solid MSR medium. Five 4 cm-long sterile carrot hairy root segments were initially co-cultivated with 200 *R. irregularis* spores in the RC in an incubator at 27°C in the dark. The Petri dishes were positioned at an angle of approximately 45 degrees (with the HC underneath) in the incubator to

facilitate hyphal growth from the RC into the HC. The roots were routinely trimmed as needed to prevent them from growing into the HC.

When dense AMF extraradical hyphae were observed in the HC (twelve weeks later), 200 μL of 0.1 mM Myr with 98% $^{13}C_1$ labeling or unlabeled (^{12}C-Myr, control) myristate was added weekly to the HC until harvest, four weeks later. During the harvest, AMF extraradical hyphae from the HC and RC were collected separately by dissolving the media in 10 mM sodium citrate and filtering through a 37 μm nylon mesh. The carrot hairy roots in the RC were harvested using sterile tweezers.

The determination of ^{13}C enrichment in fatty acids followed our previous report (Bao et al., 2019). The total ^{13}C enrichment of AMF hyphae from HC or RC were sealed in tin capsules and analyzed using an elemental analyzer coupled to an IRMS (EA-IRMS, Flash EA 2000 and Delta V Plus; Thermo Fisher Scientific, Bremen, Germany). The ^{13}C:^{12}C ratio of CO_2 from the combusted samples was determined with ± 0.2‰ precision and expressed as atom % of ^{13}C, using a sucrose standard calibrated against the Vienna Pee Dee Belemnite standard.

Exp. 2: Effects of exogenous myristate on AMF development in a single-compartment AMF-hairy root co-culture system

In Exp. 2, we evaluated the effects of exogenous myristate at varying concentrations (0.1, 0.5, 1, 5, or 10 mM) on spore germination and the development of AMF intraradical and extraradical structures associated with carrot hairy roots in a single-compartment co-culture system. Briefly, three 4 cm-long sterile carrot hairy root segments were cultivated in MSR medium with or without *R. irregularis* inoculations (200 sterilized spores per Petri dish). Potassium myristate solution (0, 0.1, 0.5, 1, 5, or 10 mM, 200 μL) was applied to the plates weekly until harvest. The germination rate of AMF spores, hyphal branching, and hyphal length were monitored

under a stereomicroscope (M250FA, Leica, Germany) and calculated using ImageJ at 4 days post-inoculation (Schneider et al., 2012). The spore density was dynamically monitored every 2 weeks starting 8 weeks post-AMF inoculation. The carrot hairy roots and culture medium were harvested at 14 weeks post-AMF inoculation. The roots and solid culture medium were carefully separated using sterile tweezers. The medium was weighed and dissolved in 10 mM sodium citrate, and the AMF collection were preliminarily separated into hyphae and spores using a blender, followed by filtration through a 250 μm and 37 μm nylon meshes, respectively, and manual selection with tweezers under a stereoscopic microscope. Extraradical AMF biomass was oven-dried at 80°C for 48 h and quantified.

Exp. 3: Effect of exogenous myristate on AM symbiosis in a pot experiment

In the pot experiment (Exp. 3), alfalfa and rice seeds were germinated for 14 days in sterilized sand before being transplanted into a substrate composed of vermiculite, perlite, and sand (3:1:1, v/v/v). To account for potential biomass differences between the two plant species, three alfalfa seedlings or one rice seedling were planted per pot, containing either 300 or 1000 mL of substrate, respectively, with or without *R. irregularis* inoculation (10 spores/mL of substrate). The plants were grown in a greenhouse under a long-day photoperiod (16 h light/8 h dark) at temperatures ranging from 20□30□. For P treatments, 10 mL Hoagland nutrient solution with either relatively low or high P level for alfalfa (25 or 100 μM KH_2PO_4, respectively, following He et al., 2020) and rice (100 or 400 μM KH_2PO_4, respectively, following Shi et al., 2021) was applied twice a week. For myristate application, 5 mL of potassium myristate solution (0.1 mM) or ddH_2O was applied near the plant roots twice a week.

The plants were harvested eight weeks post-inoculation. They were carefully

washed with tap water and divided into shoot and root portions. A portion of the fresh root samples was flash-frozen in liquid nitrogen and stored at −80°C for molecular analysis. The remaining plant samples were de-enzymed at 105°C for 30 min, dried at 60°C for 7 days, and weighed. Plant P concentration was determined using the molybdenum-antimony colorimetric method.

RNA extraction and Illumina sequencing (Exp. 3)

In Exp. 3, alfalfa and rice roots inoculated with AMF, with or without myristate application at two P levels, underwent RNA sequencing (RNA-seq) using three biological replicates (pots, totaling 24 pots). Total RNA was extracted from 100 mg of root tissue using the TRIzol R Plus RNA Purification Kit (Takara, Dalian, China) following the manufacturer's instructions. Library preparation was conducted with the NebNext Ultra RNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). Qualified libraries were sequenced on the Illumina HiSeq 4000 platform with 150-bp paired-end reads. Fastp was employed for basic statistics and quality filtering of raw data. Hisat2 was utilized to map the sequencing results to the rice reference genome (IRGSP-1.0) or the alfalfa reference genome (Zhongmu NO.1, Shen et al., 2020). Salmon quant was applied for gene expression quantification analysis.

Quantitative real-time PCR (qRT-PCR, Exp. 3)

In Exp. 3, RNA extracted from alfalfa or rice roots was reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA). qRT-PCR was performed on a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, CA, USA) with the SYBR ROX Mastermix Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Three biological replicates (samples) were utilized for each treatment, with three technical replicates per sample. The relative expression levels of genes involved in C delivery from plants to AMF (*MsSTR2*,

OsSTR2, *RiFAT1*, *RiFAT2*, and *RiMST2*) and in the mycorrhizal P pathway (*MsPT8*, *OsPT11* and *RiPT*) were analyzed, using the alfalfa *actin 2* gene (*MsACT2*), rice cycle 2 gene (*OsCyc2*) or *R. irregularis* elongation factor-1 beta gene (*RiEF-1β*,) as reference genes (Yang et al., 2012; Corrêa et al., 2014; Brands and Dörmann, 2022; Das et al., 2022). Details of all the qRT-PCR primers used are provided in the Supporting Information (Table S1).

^{13}C labeling of plants, lipid extraction and measurement of signature fatty acids (Exp. 3)

In Exp. 3, the $^{13}CO_2$ labeling procedure was conducted as described in our previous report, with minor modifications (Bao et al., 2019). Five days before harvest, alfalfa and rice plants (five randomly selected replicates) were placed in a sealed acrylic chamber containing $^{13}CO_2$ and incubated for 4 hours each.

The extraction and analysis of signature fatty acids were performed following the method described by Olsson et al. (2005), with slight modifications based on Bao et al. (2019). Fatty acid methyl esters were identified and quantified relative to an added internal standard (fatty acid methyl ester 19:0). NLFA 16:1ω5 was used as a marker for AMF in roots (Olsson and Lekberg, 2022).

Quantification of ^{13}C enrichment and excess ^{13}C in fatty acids (Exp. 3)

In Exp. 3, the ^{13}C enrichment in roots and fatty acid methyl esters was analyzed using IRMS coupled to a Trace GC Ultra gas chromatograph via the ConFlow IV interface (Thermo Fisher Scientific, Bremen, Germany), as described in our previous study (Bao et al., 2019). The chromatographic conditions matched those specified for lipid analysis, using helium (He) as the carrier gas. The effluent from the capillary column passed through an Al tube filled with CuO wires at 860°C. The δ^{13}C values were calibrated against the V-PDB using the CO_2 standard (RM 8542 (IAEA-CH-6,

sucrose)) and corrected for the additional non-labeled methyl group introduced during methanolysis. The ^{13}C enrichment was calculated by subtracting the background ^{13}C levels determined from five non-labeled controls (Olsson et al., 2005).

Quantification of AMF colonization intensity (Exps. 1, 2, and 3)

To assess AMF colonization intensity, 100 root segments (approximately 1 cm in length) were treated with a 10% KOH solution at 95°C for 10 minutes, decolorized in a 30% H_2O_2 solution at 25°C for 3 min, softened in a 1% HCl solution at 25°C for 3 min, and then stained with 0.05% Trypan Blue at 95°C for 15 min. The evenly arranged root segments were examined under 400× magnification using a light microscope (Nikon Eclipse 80i, Japan). The colonization rate was calculated using the gridline intersect method (Giovannetti and Mosse, 1980).

Statistical analysis

Mycorrhizal response (MR) was calculated based on plant biomass and plant P concentration, respectively, following Corrêa et al. (2014): MR (%) = (AM − NM) / NM × 100%, where AM denotes the observed value for the target parameter (biomass or P concentration) in mycorrhizal plants, and NM represents the mean values of nonmycorrhizal plants. Multiple comparisons among different treatments were conducted using one-way analysis of variance (ANOVA) followed by post hoc Tukey's tests (assuming equal variances). A univariate two-way ANOVA, followed by a post hoc Tukey test, was employed to assess the effects of myristate, P level, and their interactions on AMF colonization intensity and plant P concentration. The significant differences between AM and NM groups were determined using Student's t test (for normally distributed data). For RNA-seq data, the DESeq2 R package was utilized to conduct differentially expressed gene (DEG) analysis between plants with (Myr^+) and without (Myr^-) myristate application (Love et al., 2014). The *p* values

obtained were adjusted using the Benjamini-Hochberg correction to control false discovery rate. Genes (transcripts) with an adjusted *P*-value < 0.05 and $|\log_2(\text{fold change})| > 1$ identified by DESeq2 were considered as differentially expressed. The clusterProfiler method was applied for Gene Ontology (GO) enrichment analysis of DEGs (Wu et al., 2021), with GO terms showing an adjusted *p*-value < 0.05 considered significantly enriched.

Data accessibility

The RNA-seq data generated in this study have been deposited in the Sequence Read Archive at the National Center for Biotechnology Information under BioProject ID PRJNA1083641. All data required to evaluate the conclusions in the paper are included in the paper and/or the Supplementary Materials.

Author contributions

Hanwen Chen: Writing – review & editing, Investigation, Formal analysis, Methodology, Visualization. **Tian Xiong:** Investigation, Formal analysis, Data curation, Methodology. **Baoxing Guan:** Writing – original draft, Investigation, Formal analysis, Data curation, Visualization. **Jiaqi Huang:** Investigation, Resources. **Danrui Zhao:** Investigation, Resources, Visualization. **Yao Chen:** Investigation, Resources. **Haoran Liang:** Investigation, Visualization. **Yingwei Li:** Investigation, Resources. **Jingwen Wu:** Investigation, Resources. **Shaoping Ye:** Investigation, Resources. **Ting Li:** Investigation, Resources. **Wensheng Shu:** Supervision. **Jin-tian Li:** Supervision. **Yutao Wang:** Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Funding acquisition, Supervision, Project administration.

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Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Argüello A, O'Brien MJ, van der Heijden MGA, Wiemken A, Schmid B, Niklaus PA. 2016. Options of partners improve carbon for phosphorus trade in the arbuscular mycorrhizal mutualism. *Ecology Letters* **19**: 648–656.

Bao X, Wang Y, Olsson PA. 2019. Arbuscular mycorrhiza under water—Carbon–phosphorus exchange between rice and arbuscular mycorrhizal fungi under different flooding regimes. *Soil Biology and Biochemistry* **129**: 169–177.

Bitterlich M, Krügel U, Boldt-Burisch K, Franken P, Kühn C. 2014. The sucrose transporter SlSUT2 from tomato interacts with brassinosteroid functioning and affects arbuscular mycorrhiza formation. *The Plant Journal* **78**: 877–889.

Blilou I, Ocampo JA, García-Garrido JM. 2000. Induction of Ltp (lipid transfer protein) and Pal (phenylalanine ammonia-lyase) gene expression in rice roots colonized by the arbuscular mycorrhizal fungus Glomus mosseae. *Journal of Experimental Botany* **51**: 1969–1977.

Brands M, Dörmann P. 2022. Two AMP-binding domain proteins from *Rhizophagus irregularis* involved in import of exogenous fatty acids. *Molecular Plant-Microbe Interactions* **35**: 464–476.

Bravo A, Brands M, Wewer V, Dörmann P, Harrison MJ. 2017. Arbuscular mycorrhiza-specific enzymes FatM and RAM2 fine-tune lipid biosynthesis to promote development of arbuscular mycorrhiza. *New Phytologist* **214**: 1631–1645.

Brundrett MC, Tedersoo L. 2018. Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist* **220**: 1108–1115.

Bücking H, Abubaker J, Govindarajulu M, Tala M, Pfeffer PE, Nagahashi G, Lammers P, Shachar-Hill Y. 2008. Root exudates stimulate the uptake and metabolism of organic carbon in germinating spores of *Glomus intraradices*. *New Phytologist* **180**: 684–695.

Bunn RA, Simpson DT, Bullington LS, Lekberg Y, Janos DP. 2019. Revisiting the "direct mineral cycling" hypothesis: arbuscular mycorrhizal fungi colonize leaf litter, but why? *The ISME Journal* **13**: 1891–1898.

Cope KR, Kafle A, Yakha JK, Pfeffer PE, Strahan GD, Garcia K, Subramanian S, Bücking H. 2022. Physiological and transcriptomic response of *Medicago truncatula* to colonization by high- or low-benefit arbuscular mycorrhizal fungi. *Mycorrhiza* **32**: 281–303.

Corrêa A, Cruz C, Pérez-Tienda J, Ferrol N. 2014. Shedding light onto nutrient

responses of arbuscular mycorrhizal plants: nutrient interactions may lead to unpredicted outcomes of the symbiosis. *Plant Science* **221–222**: 29–41.

Dai H, Zhang X, Zhao B, Shi J, Zhang C, Wang G, Yu N, Wang E. 2022. Colonization of mutualistic mycorrhizal and parasitic blast fungi requires OsRAM2-regulated fatty acid biosynthesis in rice. *Molecular Plant-Microbe Interactions* **35**: 178–186.

Das D, Paries M, Hobecker K, Gigl M, Dawid C, Lam HM, Zhang J, Chen M, Gutjahr C. 2022. PHOSPHATE STARVATION RESPONSE transcription factors enable arbuscular mycorrhiza symbiosis. *Nature Communications* **13**: 1–12.

Feng F, Sun J, Radhakrishnan GV, Lee T, Bozsóki Z, Fort S, Gavrin A, Gysel K, Thygesen MB, Andersen KR, Radutoiu S, Stougaard J, Oldroyd GED. 2019. A combination of chitooligosaccharide and lipochitooligosaccharide recognition promotes arbuscular mycorrhizal associations in *Medicago truncatula*. *Nature Communications* **10**: 5047.

Fernández-Piñar R, Espinosa-Urgel M, Dubern JF, Heeb S, Ramos JL, Cámara M. 2012. Fatty acid-mediated signaling between two Pseudomonas species. *Environmental Microbiology Reports* **4**: 417–423.

Foster KR, Kokko H. 2006. Cheating can stabilize cooperation in mutualisms. *Proceedings of the Royal Society B: Biological Sciences* **273**: 2233–2239.

Giovannetti M, Mosse B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* **84**: 489–500.

Guelette BS, Benning UF, Hoffmann-Benning S. 2012. Identification of lipids and lipid-binding proteins in phloem exudates from *Arabidopsis thaliana*. *Journal of Experimental Botany* **63**: 3603–3616.

Gutjahr C, Radovanovic D, Geoffroy J, Zhang Q, Siegler H, Chiapello M, Casieri L,

An K, An G, Guiderdoni E, Kumar CS, Sundaresan V, Harrison MJ, Paszkowski U. 2012. The half-size ABC transporters STR1 and STR2 are indispensable for mycorrhizal arbuscule formation in rice. *The Plant Journal* **69**: 906–920.

Hammer EC, Pallon J, Wallander H, Olsson PA. 2011. Tit for tat? A mycorrhizal fungus accumulates phosphorus under low plant carbon availability. *FEMS Microbiology Ecology* **76**: 236–244.

Hata S, Kobae Y, Banba M. 2010. Interactions between plants and arbuscular mycorrhizal fungi. *International Review of Cell and Molecular Biology* **281**: 1–48.

Helber N, Wippel K, Sauer N, Schaarschmidt S, Hause B, Requena N. 2011. A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *Glomus sp* is crucial for the symbiotic relationship with plants. *The Plant Cell* **23**: 3812–3823.

Jiang Y, Wang W, Xie Q, Liu N, Liu L, Wang D, Zhang X, Yang C, Chen X, Tang D, Wang E. 2017. Plants transfer lipids to sustain colonization by mutualistic mycorrhizal and parasitic fungi. *Science* **356**: 1172–1175.

Johnson NC. 2010. Resource stoichiometry elucidates the structure and function of arbuscular mycorrhizas across scales. *New Phytologist* **185**: 631–647.

Kameoka H, Tsutsui I, Saito K, Kikuchi Y, Handa Y, Ezawa T, Hayashi H, Kawaguchi M, Akiyama K. 2019. Stimulation of asymbiotic sporulation in arbuscular mycorrhizal fungi by fatty acids. *Nature Microbiology* **4**: 1654–1660.

Keymer A, Pimprikar P, Wewer V, Huber C, Brands M, Bucerius SL, Delaux PM, Klingl V, Röpenack-Lahaye EV, Wang TL, Eisenreich W, Dörmann P, Parniske M, Gutjahr C. 2017. Lipid transfer from plants to arbuscular mycorrhiza fungi. *eLife* **6**: e29107.

Kiers ET, Duhamel M, Beesetty Y, Mensah J, Franken O, Verbruggen E, Fellbaum CR, Kowalchuk GA, Hart MM, Bago A, Palmer TM, West SA, Vandenkoornhuyse P, Jansa J, Bücking H. 2011. Reciprocal rewards stabilize cooperation in the mycorrhizal symbiosis. *Science* **333**: 880–882.

Kusajima M, Fujita M, Soudthedlath K, Nakamura H, Yoneyama K, Nomura T, Akiyama K, Maruyama-Nakashita A, Asami T, Nakashita H. 2022. Strigolactones modulate salicylic acid-mediated disease resistance in *Arabidopsis thaliana*. *International Journal of Molecular Sciences* **23**: 5246.

Lanfranco L, Fiorilli V, Gutjahr C. 2018. Partner communication and role of nutrients in the arbuscular mycorrhizal symbiosis. *New Phytologist* **220**: 1–10.

Li S, Xu C, Wang J, Guo B, Yang L, Chen J, Ding W. 2017. Cinnamic, myristic and fumaric acids in tobacco root exudates induce the infection of plants by *Ralstonia solanacearum*. *Plant and Soil* **412**: 381–395.

He H, Wu M, Guo L, Fan C, Zhang Z, Su R, Q Peng, J Pang, Lambers H. 2020. Release of tartrate as a major carboxylate by alfalfa (*Medicago sativa* L.) under phosphorus deficiency and the effect of soil nitrogen supply. *Plant and Soil* **449**: 169–178.

Liu X, Feng Z, Zhang W, Yao Q, Zhu H. 2023. Exogenous myristate promotes the colonization of arbuscular mycorrhizal fungi in tomato. *Frontiers in Plant Science* **14**: 1250684.

Liu Y, Zhang H, Wang J, Gao W, Sun X, Xiong Q, Shu X, Miao Y, Shen Q, Xun W, Zhang R. 2024. Nonpathogenic *Pseudomonas syringae* derivatives and its metabolites trigger the plant “cry for help” response to assemble disease suppressing and growth promoting rhizomicrobiome. *Nature Communications* **15**: 1907.

Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology* **15**: 550.

Luginbuehl LH, Menard GN, Kurup S, Van Erp H, Radhakrishnan GV, Breakspear A, Oldroyd GED, Eastmond PJ. 2017. Fatty acids in arbuscular mycorrhizal fungi are synthesized by the host plant. *Science* **356**: 1175–1178.

Luo Q, Sun L, Hu X, Zhou R. 2014. The variation of root exudates from the hyperaccumulator *Sedum alfredii* under cadmium stress: metabonomics analysis. *PLoS ONE* **9**: e115581.

MacLean AM, Bravo A, Harrison MJ. 2017. Plant signaling and metabolic pathways enabling arbuscular mycorrhizal symbiosis. *The Plant Cell* **29**: 2319–2335.

Mori N, Nishiuma K, Sugiyama T, Hayashi H, Akiyama K. 2016. Carlactone-type strigolactones and their synthetic analogues as inducers of hyphal branching in arbuscular mycorrhizal fungi. *Phytochemistry* **130**: 90–98.

Olsson PA, van Aarle IM, Gavito ME, Bengtson P, Bengtsson G. 2005. ^{13}C incorporation into signature fatty acids as an assay for carbon allocation in arbuscular mycorrhiza. *Applied and Environmental Microbiology* **71**: 2592–2599.

Olsson PA, Lekberg Y. 2022. A critical review of the use of lipid signature molecules for the quantification of arbuscular mycorrhiza fungi. *Soil Biology and Biochemistry* **166**: 108574.

Pfeffer PE, Douds Jr DD, Becard G, Shachar-Hill Y. 1999. Carbon uptake and the metabolism and transport of lipids in an arbuscular mycorrhiza. *Plant Physiology* **120**: 587–598.

Rich MK, Vigneron N, Libourel C, Keller J, Xue L, Hajheidari M, Radhakrishnan GV, Le Ru A, Diop SI, Potente G, Conti E, Duijsings D, Batut A, Le Faouder P, Kodama K, Kyozuka J, Sallet E, Bécard G, Rodriguez-Franco M, Ott T,

Bertrand-Michel J, Oldroyd GED, Szövényi P, Bucher M, Delaux PM. 2021. Lipid exchanges drove the evolution of mutualism during plant terrestrialization. *Science* **372**: 864–868.

Rillig MC, Aguilar-Trigueros CA, Anderson IC, Antonovics J, Ballhausen MB, Bergmann JBM, Chaudhary VB, Deveautour C, Grünfeld L, Hempel S, Lakovic M, Lammel DR, Lehmann A, Lehmann J, Leifheit EF, Liang Y, Li E, Lozano YM, Manntschke A, Mansour I, Oviatt P, Pinek L, Powell JR, Roy J, Ryo M, Sosa-Hernández MA, Veresoglou SD, Wang D, Yang G, Zhang H. 2020. Myristate and the ecology of AM fungi: significance, opportunities, applications and challenges. *New Phytologist* **227**: 1610–1614.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**: 671–675.

Shen C, Du H, Chen Z, Lu H, Zhu F, Chen H, Meng X, Liu Q, Liu P, Zheng L, Li X, Dong J, Liang C, Wang T. 2020. The chromosome-level genome sequence of the autotetraploid alfalfa and resequencing of core germplasms provide genomic resources for alfalfa research. *Molecular Plant* **13**: 1250–1261.

Shi J, Zhao B, Zheng S, Zhang X, Wang X, Dong W, Xie Q, Wang G, Xiao Y, Chen F, Yu N, Wang E. 2021. A phosphate starvation response-centered network regulates mycorrhizal symbiosis. *Cell* **184**: 5527–5540.e18.

Siciliano I, Amaral Carneiro G, Spadaro D, Garibaldi A, Gullino ML. 2015. Jasmonic acid, abscisic acid, and salicylic acid are involved in the phytoalexin responses of rice to *Fusarium fujikuroi*, a high gibberellin producer pathogen. *Journal of Agricultural and Food Chemistry* **63**: 8134–8142.

Smith SE, Read DJ. 2008. *Mycorrhizal symbiosis*. Academic press, London.

St-Arnaud M, Hamel C, Vimard B, Caron M, Fortin JA. 1996. Enhanced hyphal

growth and spore production of the arbuscular mycorrhizal fungus *Glomus intraradices* in an in vitro system in the absence of host roots. *Mycological Research* **100**: 328–332.

Sudheeran PK, Ovadia R, Galsarker O, Maoz I, Sela N, Maurer D, Feygenberg O, Oren Shamir M, Alkan N. 2020. Glycosylated flavonoids: fruit's concealed antifungal arsenal. *New Phytologist* **225**: 1788–1798.

Sugiura Y, Akiyama R, Tanaka S, Yano K, Kameoka H, Marui S, Saito M, Kawaguchi M, Akiyama K, Saito K. 2020. Myristate can be used as a carbon and energy source for the asymbiotic growth of arbuscular mycorrhizal fungi. *Proceedings of the National Academy of Sciences of the United States of America* **117**: 25779–25788.

Swenson TL, Karaoz U, Swenson JM, Bowen BP, Northen TR. 2018. Linking soil biology and chemistry in biological soil crust using isolate exometabolomics. *Nature Communications* **9**: 19.

Tanaka S, Hashimoto K, Kobayashi Y, Yano K, Maeda T, Kameoka H, Ezawa T, Saito K, Akiyama K, Kawaguchi M. 2022. Asymbiotic mass production of the arbuscular mycorrhizal fungus *Rhizophagus clarus*. *Communications Biology* **5**: 43.

Went FW, Stark N. 1968. The biological and mechanical role of soil fungi. *Proceedings of the National Academy of Sciences of the United States of America* **60**: 497–504.

Wipf D, Krajinski F, van Tuinen D, Recorbet G, Courty PE. 2019. Trading on the arbuscular mycorrhiza market: from arbuscules to common mycorrhizal networks. *New Phytologist* **223**: 1127–1142.

Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z, Feng T, Zhou L, Tang W, Zhan L, Fu X, Li

S, Bo X, Yu G. 2021. ClusterProfiler 4.0: A universal enrichment tool for interpreting omics data. *The Innovation (Camb)* **2**: 100141.

Xie X, Lai W, Che X, Wang S, Ren Y, Hu W, Chen H, Tang M. 2022. A SPX domain-containing phosphate transporter from *Rhizophagus irregularis* handles phosphate homeostasis at symbiotic interface of arbuscular mycorrhizas. *New Phytologist* **234**: 650–671.

Yang S, Grønlund M, Jakobsen I, Grotemeyer MS, Rentsch D, Miyao A, Hirochika H, Kumar CS, Sundaresan V, Salamin N, Catausan S, Mattes N, Heuer S, Paszkowski U. 2012. Nonredundant regulation of rice arbuscular mycorrhizal symbiosis by two members of the phosphate transporter1 gene family. *The Plant Cell* **24**: 4236–4251.

Yu H, He Y, Zhang W, Chen L, Zhang J, Zhang X, Dawson W, Ding J. 2022. Greater chemical signaling in root exudates enhances soil mutualistic associations in invasive plants compared to natives. *New Phytologist* **236**: 1140–1153.

Zhang Q, Blaylock LA, Harrison MJ. 2010. Two Medicago truncatula half-ABC transporters are essential for arbuscule development in arbuscular mycorrhizal symbiosis. *The Plant Cell* **22**: 1483–1497.

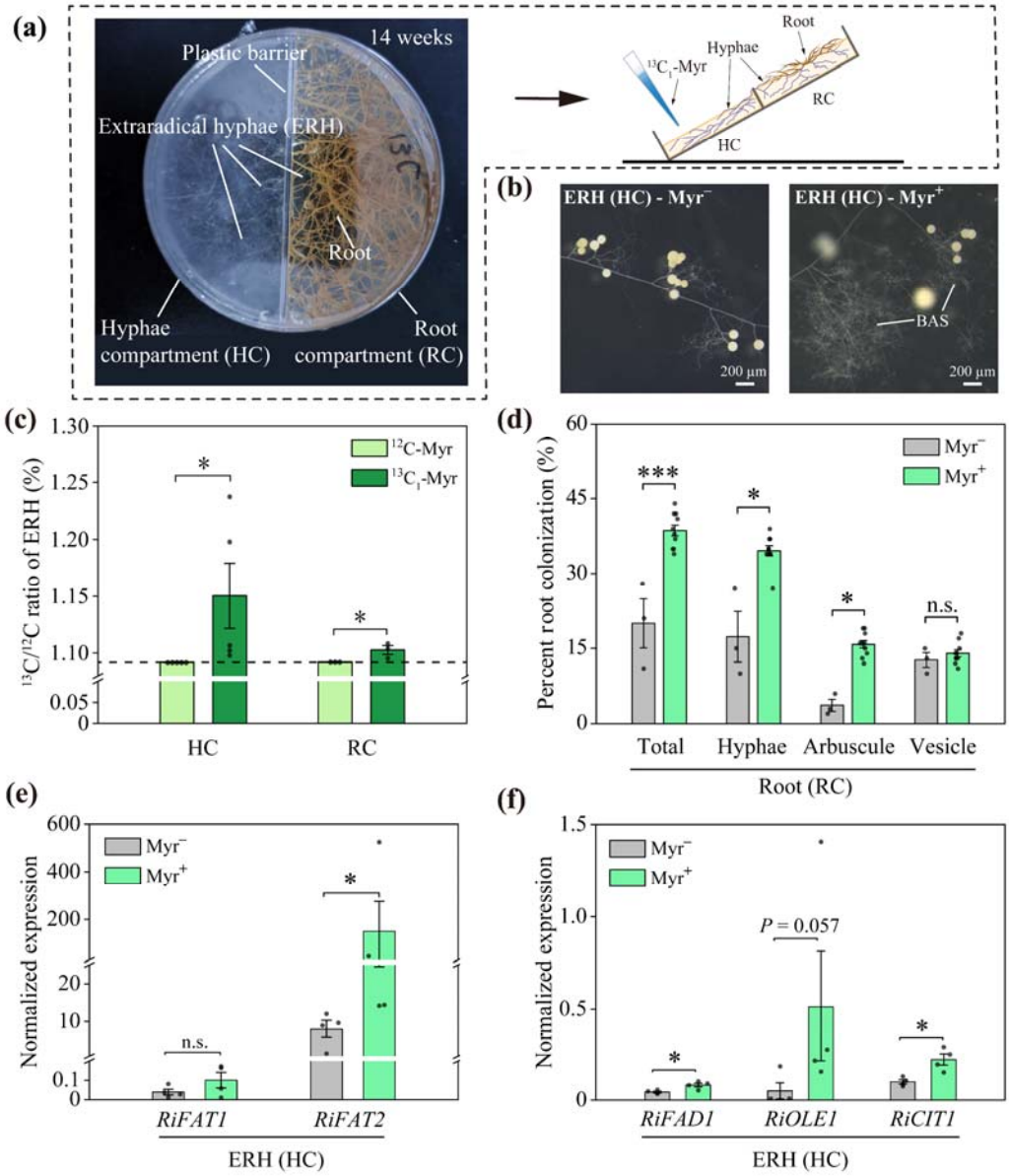

Fig. 1 Uptake of exogenous myristate by extraradical hyphae (ERH) of arbuscular mycorrhizal fungi (AMF) under symbiotic conditions and its effects on mycorrhization and expression of fungal marker genes for fatty acid uptake and metabolism. **(a)** Schematic illustration of the two-compartment AMF-carrot hairy root co-cultivation system and the addition of myristate to the hyphae compartment (HC). Carrot hairy roots colonized by *Rhizophagus irregularis* were grown in the root compartment (RC). Starting from the twelfth week, 0.1 mM of $^{13}C_1$-labeled ($^{13}C_1$-

Myr), non-labeled (^{12}C-Myr) myristate, or ddH_2O (Myr^-, control) was added weekly into the HC with well-developed AMF hyphae until harvest. **(b)** Typical branched absorbing structures (BAS) were observed exclusively in the HC treated with exougenous myristate (Myr^+). **(c)** $^{13}C/^{12}C$ ratio of the harvested ERH from HC or RC (n = 3−5) when $^{13}C_1$-labeled ($^{13}C_1$-Myr) or non-labeled (^{12}C-Myr, background) myristate was added to HC. The background $^{13}C/^{12}C$ ratio of extraradical hyphae in HC (1.0922 ± 0.0003, n = 5) and RC (1.0923 ± 0.0002, n = 3) was identical. The exogenous myristate added in HC cannot diffuse directly into RC, as evidenced by the identical $^{13}C/^{12}C$ ratio of MSR medium collected in RC in $^{13}C_1$-Myr (1.0966 ± 0.0012, n = 3) and ^{12}C-Myr (1.0967 ± 0.0032, n = 3) treatments. **(d)** AMF percent root colonization in carrot hairy roots from the RC when HC was treated with myristate (Myr^+) or ddH_2O (Myr^-) (n = 3−10). **(e-f)** Normalized expression (relative to *RiEF-1β*) of AMF genes potentially involved in fatty acid uptake (*RiFAT1* and *RiFAT2*) and metabolism (β-oxidation (*RiFAD1*), palmitvaccenic acid synthesis (*RiOLE1*), and TCA cycle (*RiCIT1*) in AMF ERH collected from HC with myristate (Myr^+) or ddH_2O (Myr^-) treatments (n = 4). Means ± SE, *, **, and *** indicate statistical significance at the 0.05, 0.01, and 0.001 probability levels (Student's *t*-test), respectively.

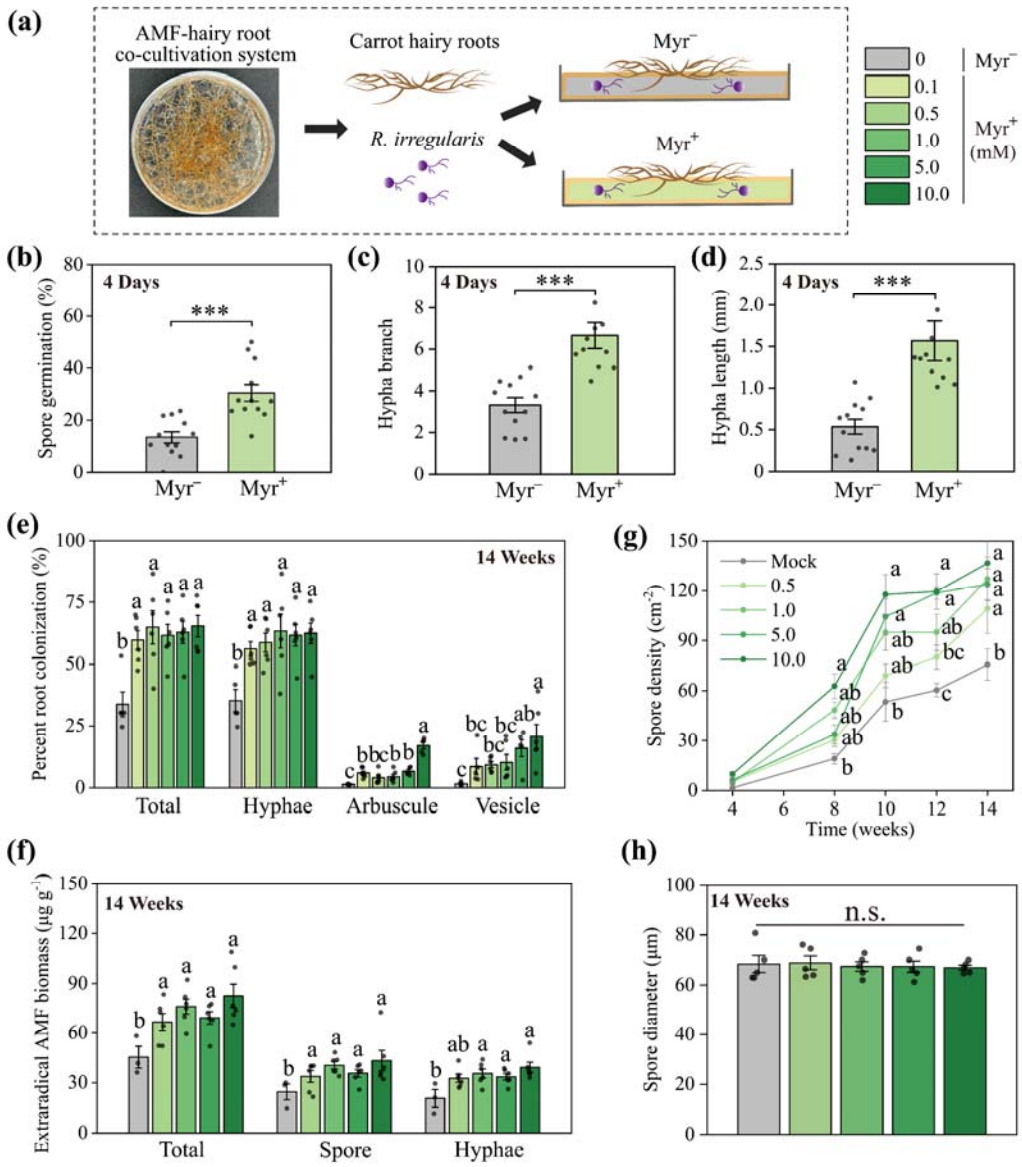

Fig. 2 Effects of exogenous myristate on the development of intraradical and extraradical structures of arbuscular mycorrhizal fungal (AMF) in the single-compartment AMF-carrot hairy root co-cultivation system. **(a)** Schematic illustration of the single-compartment AMF-hairy root co-cultivation system and the addition of myristate (Myr^+) or not (Myr^-). **(b)** Spore germination rate, **(c)** hyphal branching, and **(d)** extraradical hyphal length in the co-cultivation system with (Myr^+) or without (Myr^-) myristate treatment at 4 days post-inoculation (dpi) (n = 11−12). **(e)** AMF percent root colonization in carrot hairy roots and **(f)** biomass of the AMF extraradical

structures (per gram of medium, n = 3−6). **(g)** Dynamics of AMF spore density over time (per square centimeter, n = 3−5); and **(h)** AMF spore diameter at harvest with 0, 0.1, 0.5, 1, 5, or 10 mM myristate treatment under symbiotic conditions (n = 5, calculation from > 900 spores in each replicate). Means ± SE; different letters indicate significant differences at $P < 0.05$ level (Tukey's post hoc test); *** indicates statistical significance at the 0.001 probability level (Student's *t*-test).

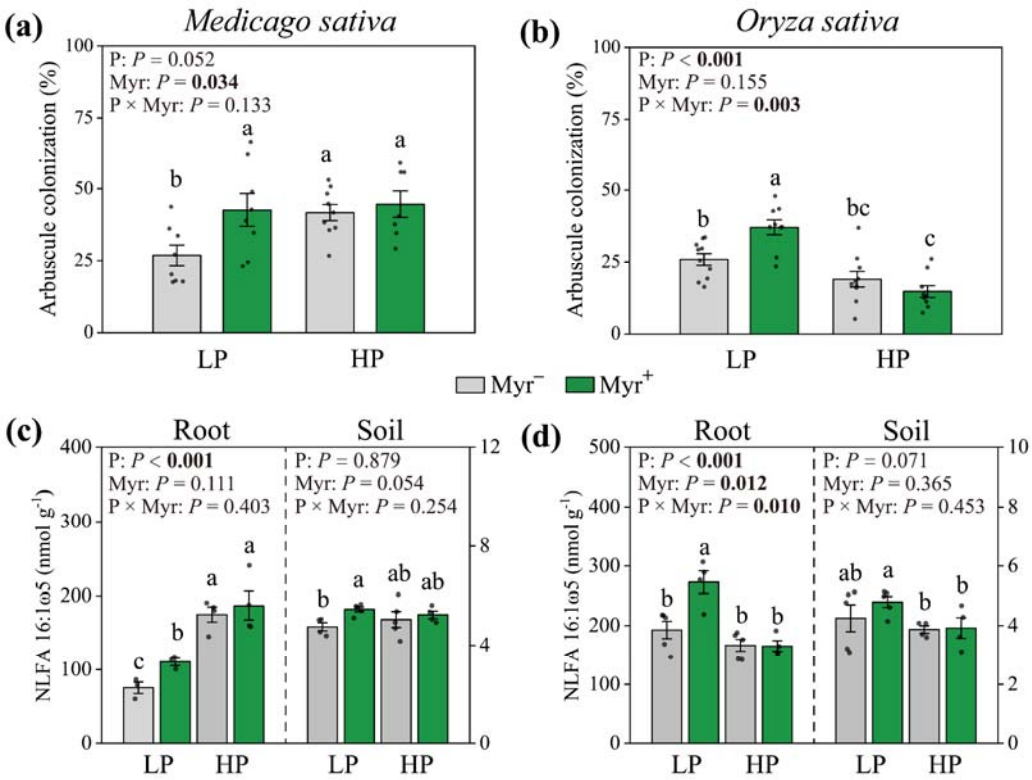

Fig. 3 Effects of exogenous myristate on arbuscular colonization and the intraradical and extraradical biomass of arbuscular mycorrhizal fungi (AMF) associated with *Medicago sativa* or *Oryza sativa* under low (LP) or high (HP) phosphorus conditions. **(a, b)** Arbuscule colonization in *M. sativa* and *O. sativa* roots treated with 0.1 mM myristate (Myr^+) or ddH_2O (Myr^-) (n = 10; Total, vesicle, and hyphal colonization rates are provided in Supplementary Figure S1); **(c, d)** AMF biomass, indicated by the AMF signature fatty acid (NLFA 16:1ω5), in AMF-colonized roots and rhizosphere soil of *M. sativa* and *O. sativa* treated with myristate (Myr^+) or ddH_2O (Myr^-) (n = 5). Means ± SE; different letters indicate significant differences at the $P < 0.05$ level (Tukey's post hoc test).

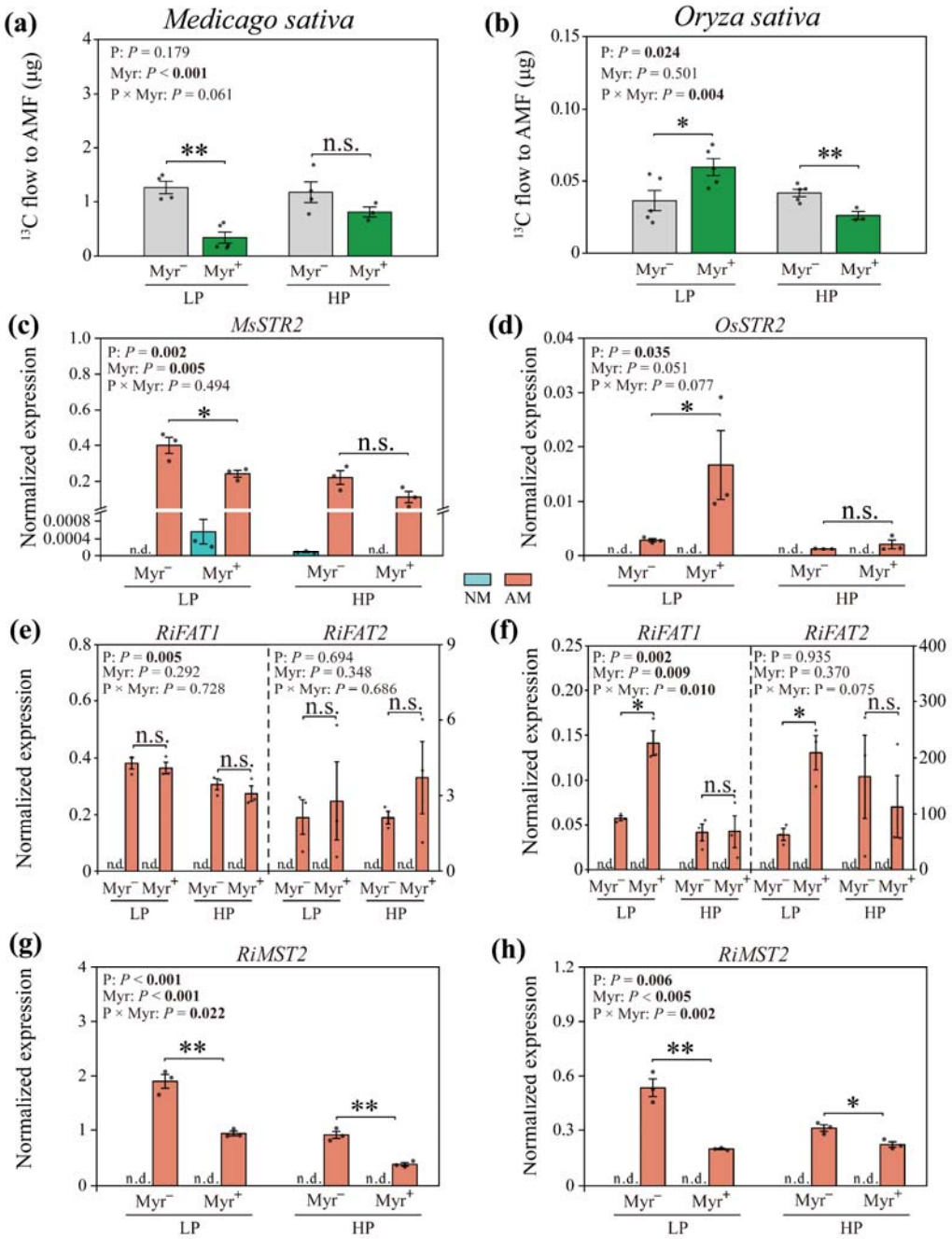

Fig. 4 Effects of exogenous myristate on the symbiotic carbon (C) allocation from *Medicago sativa* and *Oryza sativa* to arbuscular mycorrhizal fungi (AMF) and the expression profiles of mycorrhizal C transportation genes. **(a, b)** Plant ^{13}C flow to the AMF signature fatty acid (NLFA 16:1ω5) in AMF-colonized roots of *M. sativa* and *O. sativa* treated with 0.1 mM myristate (Myr^+) or ddH_2O (Myr^-) under low (LP) and

high (HP) phosphorus conditions (n = 5). (**c**−**h**) Normalized expression of mycorrhizal C transportation genes in AMF-colonized (AM) and non-colonized (NM) roots treated with 0.1 mM myristate (Myr^+) or ddH_2O (Myr^-) (n = 3). The mycorrhizal C transportation genes include the plant symbiotic fatty acid transportation gene *STR2* **(c, d**), the fungal fatty acid transporters (*RiFAT1*, *RiFAT2*; **e, f**), and the monosaccharide transporter (*RiMST2*; **g, h**). The normalized expression of *M. sativa*, *O. sativa*, and AMF genes was calculated relative to *MsACT2*, *OsCyc2*, and *RiEF-1β*, respectively. Two-way ANOVA in each histogram applies only to AM roots. Means ± SE; * and ** indicate statistical significance at the 0.05 and 0.01 probability levels (Student's *t*-test), respectively.

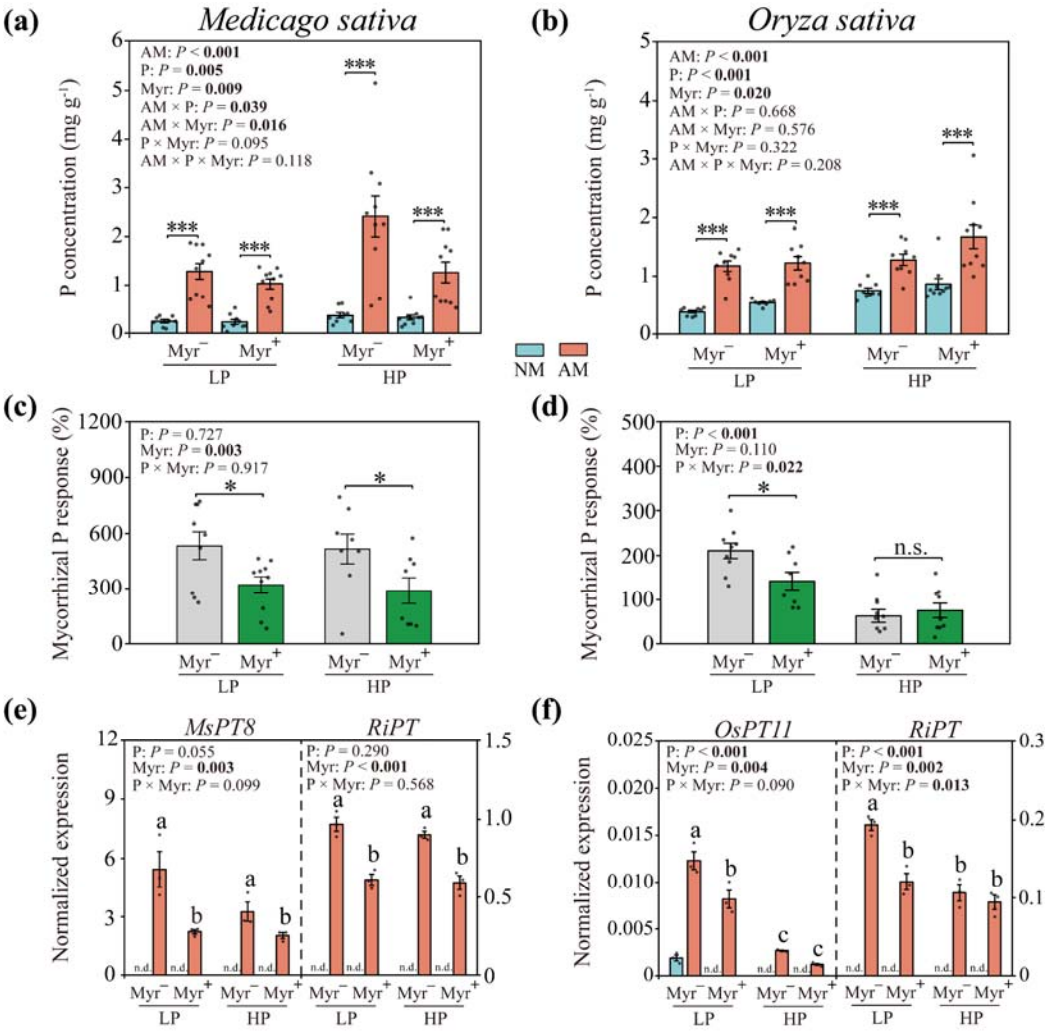

Fig. 5 Effects of exogenous myristate on plant phosphorus (P) concentration, mycorrhizal P response (MPR), and expression of mycorrhizal P pathway marker genes in *Medicago sativa* and *Oryza sativa*. **(a, b)** Plant P concentration (n = 10); **(c, d)** MPR (n = 10); and **(e, f)** relative expression of *MsPT8*, *OsPT11*, and *RiPT* (n = 3) of *M. sativa* and *O. sativa* roots under low (LP) and high (HP) phosphorus conditions with 0.1 mM myristate (Myr^+) or ddH_2O (Myr^-) treatments. AM, inoculated with *Rhizophagus irregularis*; NM, non-inoculated. The normalized expression of *M. sativa*, *O. sativa*, and AMF genes was calculated relative to *MsACT2*, *OsCyc2*, and *RiEF-1β*, respectively. Two-way ANOVA in each histogram (c−f) applies only to AM roots. Means ± SE; different letters indicate significant differences at $P < 0.05$ level

(Tukey's post hoc test). * and *** indicate statistical significance at the 0.05 and 0.001 probability levels (Student's *t*-test), respectively.

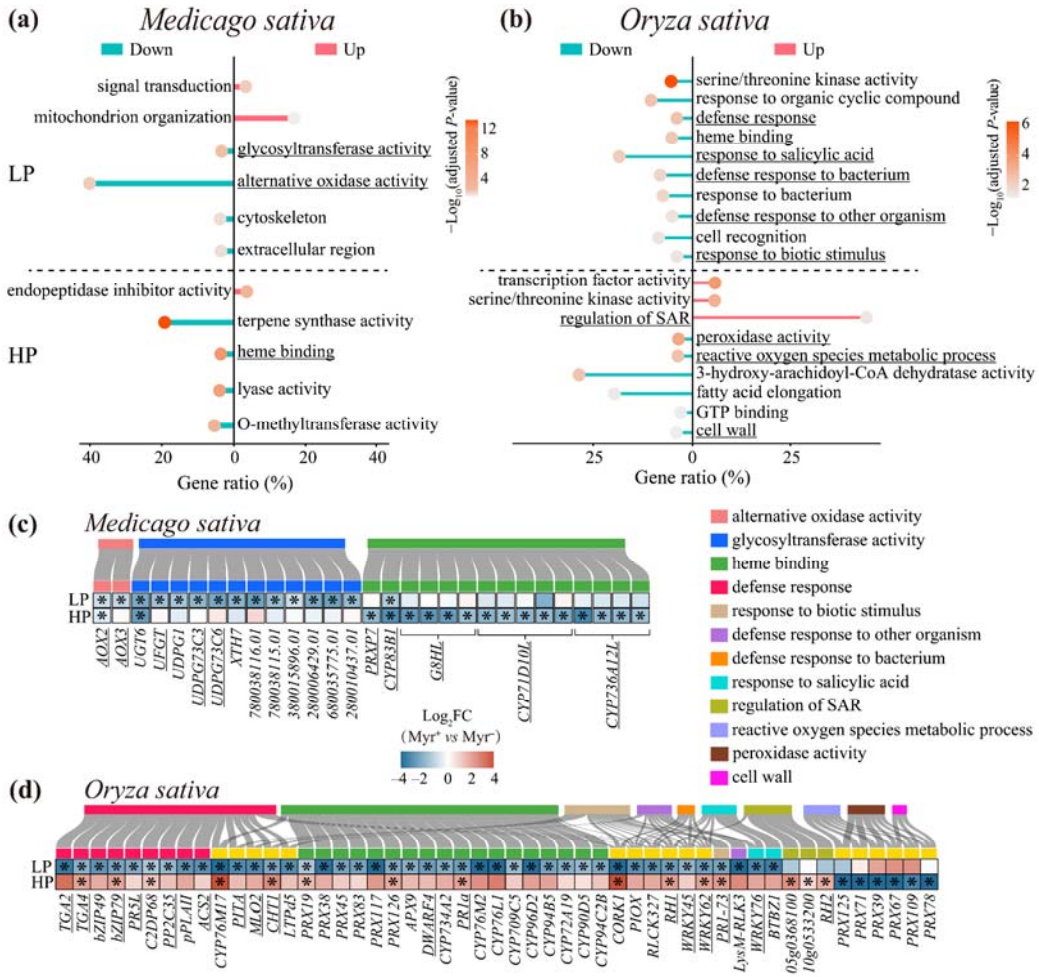

Fig. 6 Effects of exogenous myristate on the transcriptome of *Medicago sativa* and *Oryza sativa* roots inoculated with arbuscular mycorrhizal fungi (AMF) under low (LP) and high (HP) phosphorus conditions. **(a, b)** Significantly enriched gene ontology (GO) terms (P_{adj} < 0.05) for the differentially expressed genes (DEGs) between myristate (Myr^+) and ddH_2O (Myr^-) treatments. Underlines denote stress- or defense-related GO terms. **(c, d)** Relative expression of genes associated with stress- or defense-related GO terms between Myr^+ and Myr^- treatments. Underlined are known stress- or defense-related transcription factors or marker genes. Genes associated with multiple GO terms are marked in yellow. * indicate statistical significance between Myr^+ and Myr^- treatments at the 0.05 probability level (Benjamini-Hochberg approach).