New Phytologist

Forum

Letter

Arbuscular mycorrhizal conserved genes are recruited for ectomycorrhizal symbiosis

Introduction

Ectomycorrhizal (ECM) symbiosis evolved more recently than the well-studied arbuscular mycorrhizal (AM) symbiosis. AM symbiosis enables plants to take up scarce minerals from the soil through their fungal partner, by its extraradical hyphae and its highly branched intracellular structures named arbuscules. It was predicted to have a single evolutionary origin and to have facilitated the switch from an aquatic to a terrestrial lifestyle of plants *c.* 450 million years ago (Ma) (Heckman *et al.*, 2001). AM symbiosis involves a set of AM-conserved genes that have been lost in all analyzed non-AM hosts, and several of these genes have been recruited into other intracellular symbioses that evolved later, like orchid mycorrhizas, ericoid mycorrhizas and nitrogen-fixing root nodule symbioses (Radhakrishnan *et al.*, 2020).

In AM symbiosis, the fungi are accommodated in plant-derived specialized membrane compartments called peri-arbuscular membranes (PAM). Unlike this intracellular symbiosis, in the intercellular ECM symbiosis the fungi only form a mantle surrounding the root and an intercellular Hartig net between root cells. It first evolved *c.* 180 Ma (Martin *et al.*, 2017). Scattered studies indicated that ECM symbiosis involves few AM-conserved genes (Garcia *et al.*, 2015; Cope *et al.*, 2019). Here, we aim to conduct a phylogenomic study to comprehensively evaluate the recruitment of AM-conserved genes for ECM symbiosis. This can be best studied through phylogenomic analysis using ECM host species that have lost the AM symbiosis. We characterized several Fagales species with such properties and showed that they maintained the majority of AM-conserved genes, unlike other non-AM hosts. Transcriptome analysis showed that several of these maintained genes were induced in ECM symbiosis. Our results indicate that ECM formation recruits AM-derived mechanisms, which provides novel insights into the evolution of the ECM symbiosis.

Results and Discussion

To determine whether AM host conserved genes are recruited in the ECM symbiosis, ECM host species that have lost the AM symbiosis are compulsory in order to conduct a phylogenomic study. We will name them ECM-only hosts to distinguish them from the ECM- and AM-compatible (dual-mycorrhizal) hosts. Previously, 406 putative ECM host species, which are in general woody, have been reported (Werner *et al.*, 2018). They are distributed in 24 orders of flowering plants (Fig. 1a). In angiosperms, the order Fagales contains most ECM host species. We selected six recently sequenced Fagales species from different genera. As studies on the ability of forming AM/ECM symbiosis are not in all cases solid, we characterized their ability to establish an ECM and/or AM symbiosis, by using the formation of Hartig net and arbuscules as criteria. We analyzed plants, growing in their natural habitat, during a 3-yr period. This showed that 100% of the *Castanea mollissima*, *Quercus robur*, *Fagus sylvatica* and *Betula pendula* plants ($n > 50$ for each species) developed ECM, but we never observed arbuscules in them (Supporting Information Fig. S1). Furthermore, young seedlings of these species were grown in pots and were inoculated with *Rhizophagus irregularis*. At 3 months post inoculation, arbuscules were not formed (Methods S1), showing that most likely they have lost AM symbiosis and became ECM-only hosts. Twenty-two percent (11/50) of the *Juglans regia* and 14% (7/50) of the *Alnus glutinosa* plants developed ECM symbiosis, while 100% of the *J. regia* and 18% (9/50) of the *A. glutinosa* plants formed arbuscules (Figs S1, S2), indicating they are dual-mycorrhizal hosts. In these dual-mycorrhizal hosts, ECM Hartig nets were more abundant on old trees and during late seasons, but the occurrence of arbuscules in *A. glutinosa* did not show a significant correlation with tree age and season (Fig. S2).

We included two other AM host tree species *Malus domestica* and *Parasponia andersonii* that belong to Rosales, a sister order of Fagales. Inoculation with three phylogenetically remote ECM species, *Laccaria bicolor*, *Scleroderma citrinum* and *Cenococcum geophilum* (Miyauchi *et al.*, 2020) did not form ECM in them (Fig. S3). Furthermore, another five species with well-known mycorrhizal traits, that were previously used to identify AM-conserved orthogroups (OGs) (Bravo *et al.*, 2016), were also included in our analysis. In total, we selected four ECM-only, four AM-only, four dual-mycorrhizal hosts and one nonhost (Arabidopsis) for both mycorrhizas (Fig. 1b).

The OGs across the 13 species were identified using the Orthofinder pipeline (Table S1; Dataset S1). A phylogenomic study across *c.* 50 plant species identified 72 AM-conserved OGs that were absent or mutated in all currently studied non-AM hosts (Bravo *et al.*, 2016). We focused on these 72 OGs and, in case any were not identified in ECM-only species, an extra tBLASTN was conducted by using the *J. regia* ortholog against corresponding genomes. The presence of these OGs was first analyzed in the eight AM hosts and Arabidopsis. Out of these 72, 19 could not be confirmed as AM-conserved OG. They were either present in the nonmycorrhizal Arabidopsis ($n = 2$), or not all AM hosts had these OGs ($n = 17$; Table S2; Dataset S2). This is most likely due to the different AM hosts and bioinformatic approach that we used. These 19 OGs were not included in our analysis, in order to have the

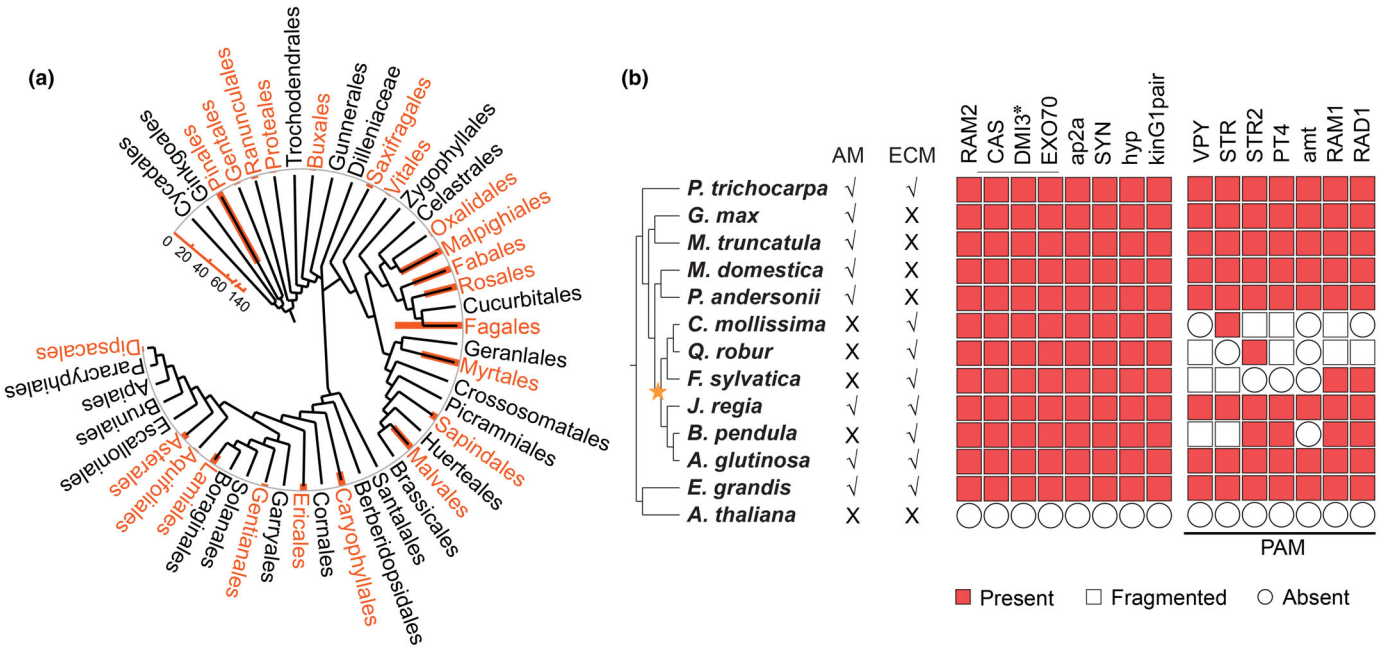

Fig. 1 Arbuscular mycorrhizal (AM)-conserved genes are maintained in ectomycorrhizal (ECM) symbiosis. (a) ECM hosts are distributed in 24 orders (orange). Trees are manually generated according to the taxonomy from NCBI. The number of hosts in each order was quantified from Werner *et al*. (2018). (b) Analysis of AM-conserved orthogroups (OGs) in 13 phylogenetically diverged plant species with various mycorrhizal traits. Four ECM-only plant hosts were characterized from Fagales order (star). Seven of the 33 AM-conserved, as well as DMI3 (asterisk) OGs were shown. Nineteen AM-conserved OGs were not maintained in all ECM-only hosts and seven of these associated with peri-arbuscular membrane (PAM) formation and functioning were shown.

criteria of presence in all AM hosts and absence in all nonhosts as strict as possible.

In the newly defined 53 AM-conserved OGs, a range of 68–85% OGs were present of which 34 (64%) in all four ECM-only species (Figs 1b, S4; Dataset S2). This is very different from all previously studied non-AM hosts in which all these OGs were lost (Bravo *et al*., 2016). Among the not fully conserved 19 OGs, *C. mollissima*, *Q. robur*, *F. sylvatica* and *B. pendula* lost 15, 15, 17 and 8 of them, respectively (Table S3). Their encoded proteins are especially involved in PAM formation and functioning (Bravo *et al*., 2016). This includes VPY involved in polar exocytosis to enable the formation of the PAM and is required for the entry of epidermal cells (Lindsay *et al*., 2022), Furthermore, PAM-located transporters like ammonium transporter AMT2 (amt), phosphate transporter PT4, lipid transporter STR/STR2, further the two GRAS transcription factors RAM1 and RAD1 which regulate the expression of the aforementioned transporter genes (Fig. 1b). VPY and amt were lost in all four ECM-only species. This is well in line with the fact that ECM symbiosis does not involve the formation of an intracellular membrane compartment, so the pressure to maintain these OGs is low. However, 34 OGs are strictly maintained, which strongly indicates that there is a high pressure to maintain them.

We hypothesized that ECM symbiosis is creating this pressure. If so, genes from the conserved OGs might be upregulated in ECM symbiosis. To verify this, we performed RNA-seq of *C. mollissima* mature ECM roots and compared it with that of control roots. Additionally, the expression of genes was also studied in a transcriptome of a mixture of flowers, seeds, stems, leaves and roots (Mix) obtained in our previous study (Xing *et al*., 2019). We showed that seven genes belonging to the 34 conserved OGs (21%) were upregulated in ECM roots, compared with control roots and Mix (Fig. 2a,b; Dataset S3), supporting that these OGs are involved in ECM symbiosis. It does not imply that the other 27 OGs are not involved. For example, if they play a role in the initiation of ECM symbiosis, they do not have to be upregulated in mature ECM roots.

One of the upregulated genes is *RAM2*, which is involved in lipid biosynthesis in AM symbiosis and its loss-of-function mutation impairs arbuscule branching due to the lack of lipid supply (Bravo *et al*., 2017; Luginbuehl *et al*., 2017). Therefore, we studied whether it has maintained its ancestral function in arbuscule formation. *Castanea mollissima* has three *RAM2* genes (Fig. 2b). We introduced each of the three *CmRAM2* coding sequences driven by the CaMV 35S promoter (*35S::CmRAM2*, Table S4) into a Medicago *ram2* loss-of-function mutant. This showed that all three *CmRAM2* genes could rescue the formation of arbuscules in *ram2*. Their morphology was like that seen in the wild-type (WT; Figs 2c, S5). This supported the hypothesis that in the youngest ancestor of *C. mollissima* that still could establish an AM symbiosis the ortholog of CmRAM2s was involved in lipid biosynthesis, most likely in arbuscule-containing cells. We next generated the three *CmRAM2pro::GUS* constructs (Table S4) and studied their expression pattern in WT Medicago AM roots. This showed that *CmRAM2-1* and *CmRAM2-3* were specifically expressed in arbuscule-containing cells, similar to Medicago *RAM2* (Luginbuehl *et al*., 2017), whereas *CmRAM2-2* was not expressed in AM roots (Figs 2d,e, S6). This demonstrated that *CmRAM2-1* and *CmRAM2-3* maintained their regulatory *cis*-element required for

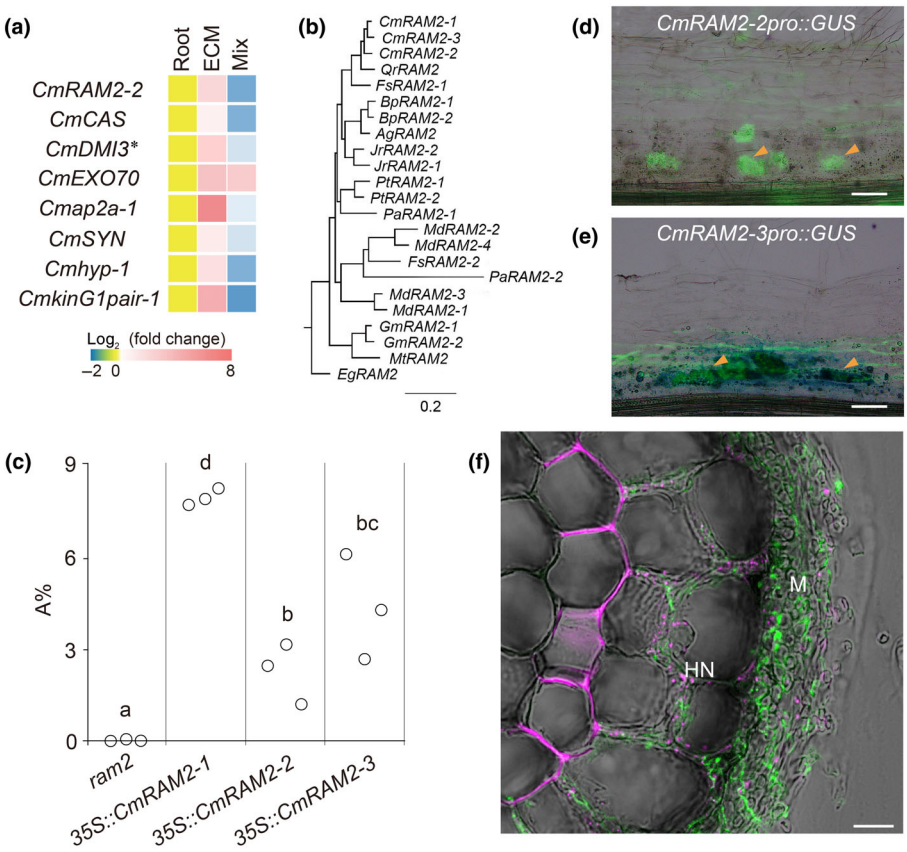

Fig. 2 Arbuscular mycorrhizal (AM)-conserved orthogroups (OGs) are involved in ectomycorrhizal (ECM) symbiosis. (a) Expression of seven AM-conserved genes, as well as *DMI3* (asterisk), were up-regulated in ECM roots of *Castanea mollissima*. Fold change is for the RPKM (Reads Per Kilobase per Million mapped reads) from corresponding samples relative to that in the root. (b) Phylogeny of *RAM2*. Phylogenetic tree was constructed by using orthologous protein sequences identified in the 13 interesting species. Scale bar represents substitution per amino acid. (c) Percentage of arbuscule abundance in the total root system (A%) in Medicago *ram2* mutants and mutants expressing *35S::CmRAM2-1/2/3*. Roots were collected at 4 wk postinoculation. Circles represent the value from each independent experiment (in one experiment ≥ 10 transgenic roots were used for each construct). Letters indicate significant differences (one-way ANOVA and Tukey HSD ($P < 0.05$)). (d, e) Expression pattern of *CmRAM2-2pro::GUS* (d) and *CmRAM2-3pro::GUS* (e) in Medicago AM roots. Bright-field and fluorescent view were merged. Arrowheads, arbuscule-containing cells. (f) Presence of lipid droplets in Hartig net. The image shown was the cross-section of a 3-month-old *C. mollissima* ECM root stained by Nile red (magenta) and WGA488 (green). Representative images were shown ($n = 20$ in c and d, or 5 in f). Bars: (d, e) 50 μm; (f) 10 μm.

expression in arbuscule-containing cells, whereas this was lost in *CmRAM2-2*. Given that *CmRAM2-2* is upregulated in mature ECM roots and the other two *CmRAM2s* are not (Datasets S2, S3), this implies that *CmRAM2-2* possesses a new *cis*-element, which enables its expression in ECM roots.

Whether *CmRAM2-2* is involved in lipid biosynthesis in ECM roots remains to be demonstrated. In AM symbiosis, RAM2 functions in the endoplasmic reticulum to synthesize specific lipid molecules, which are transported to the fungus (Bravo *et al.*, 2017). Consistent with such a function, we observed an accumulation of lipid droplets, which were visualized by Nile red staining, in the Hartig net (Fig. 2f). This might be the result of lipid secretion by the host. This is in line with the study that ECM symbiosis leads to changes in the composition of fungal lipids (Laczko *et al.*, 2004). Host-provided lipids might be used as building blocks, when the fungus extensively grows, for example, when the mantle and the Hartig net are formed.

CAS (*Castor*) and *DMI3/CCaMK* were present in the ECM-only hosts and upregulated in *C. mollissima* ECM roots (Figs 1b, 2a). Although the latter OG was not identified to be AM-conserved (Lévy *et al.*, 2004; Bravo *et al.*, 2016), it has been shown to be essential for AM symbiosis in all studied species. In poplar, knockdown of these two genes reduced the formation of both Hartig net and arbuscules (Cope *et al.*, 2019). As poplar is dual-mycorrhizal, it could not be concluded that ECM symbiosis contributed to the pressure to maintain these genes. Our observation that these two genes are conserved in the ECM-only species underlines the importance of them in the ECM symbiosis (Fig. 1b).

Arbuscules were not observed in the four ECM-only hosts neither under field nor under laboratory conditions. Inoculation of more AM fungal species under laboratory conditions and the study of arbuscule formation at different time points could add weight to the argument that the four species become ECM-only. Furthermore, *c.* 36% AM-conserved genes, for example, *PT4*, *STR1/2* and *RAM1* that are essential for arbuscular formation, were not conserved in the four species. Together, this strongly indicates that they have lost AM symbiosis. However, the aforementioned genes were still present in one or two of the four species (Fig. 1b); likely they await mutations or are involved in other biological processes.

In conclusion, we characterized ECM-only hosts and showed that they maintained 64% of the AM-conserved OGs, whereas all previously studied non-AM hosts have lost them all. Future analyses on a larger number of non-AM hosts that engage only with ECM symbiosis could show that this is a common feature of such plants. In mature ECM roots, eight maintained genes are upregulated (Fig. 2a). Furthermore, seven genes belonging to the conserved OGs have moderate expression levels in root and Mix samples (Dataset S3). Similar to this, 16 Medicago genes belonging to the conserved OGs also have moderate expression levels in control roots (Dataset S3), based on Medicago gene expression atlas (https://medicago.toulouse.inrae.fr/MtExpress). This indicates that they can have a role in other nonsymbiotic processes. However, it is probable that such processes are shared between AM hosts and non-AM hosts, so it is unlikely that this is the reason why they are only conserved in AM hosts studied up until now. The massive maintenance of AM-conserved genes in ECM-only hosts, together

with their relatively strong upregulation in ECM roots, support the hypothesis that these maintained AM-conserved genes play a role in ECM symbiosis.

The ECM symbiosis of *C. mollissima* (Fagales) and poplar (Malpighiales) most likely evolved independently, as indicated by a scattered distribution of host species in the phylogenetic tree (Fig. 1a). However, at least some identical AM-conserved genes are recruited as *CAS* and *DMI3* are shared by ECM symbiosis of them. It will be interesting to study whether the other AM-conserved genes, that are conserved in *C. mollissima*, are also recruited in the ECM symbiosis of poplar.

Acknowledgements

We thank Francis Martin for critical reading. Also thanks to Yuxiang Cheng, Junpei Zhang, Katharina Pawlowski, Annegret Kohler and Jarkko Salojarvi for providing essential materials and methods. This work was funded by the National Natural Science Foundation of China (32002019) to HL and by the R&D Program of Beijing Municipal Education Commission (KZ20231002035) to QC.

Competing interests

None declared.

Author contributions

TB, QC and HL conceived the project. HL, YG, ZZ, HZ, YW, MW, XZ, JY, QL and LQ performed experiments. HL, YG and ZZ analyzed data and wrote the manuscript. TB and QC supervised the research and revised the manuscript. HL, YG and ZZ contributed equally to this work.

ORCID

Ton Bisseling https://orcid.org/0000-0001-5494-8786
Qingqin Cao https://orcid.org/0000-0002-1550-7440
Huchen Li https://orcid.org/0000-0002-5388-4124
Qian Li https://orcid.org/0000-0002-0239-9153
Ling Qin https://orcid.org/0000-0002-9814-5771

Data availability

Data supporting this work are available in the NCBI (https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA786529) and in Table S1.

Huchen Li[1,2†], Yueyang Ge[1,2†], Zhiyong Zhang[1†], Haolin Zhang[1], Yiyang Wang[1], Mingdong Wang[1], Xin Zhao[1], Jundi Yan[2], Qian Li[1], Ling Qin[1], Qingqin Cao[1]* and Ton Bisseling[1,2]*

[1]College of Plant Science and Technology, Beijing University of Agriculture, Beijing, 102206, China;
[2]Department of Plant Sciences, Cluster of Plant Development Biology, Wageningen University, Droevendaalsesteeg 1, 6708 PB, Wageningen, the Netherlands

(*Authors for correspondence: email: ton.bisseling@wur.nl; caoqingqin@sina.com)
[†]These authors contributed equally to this work.

References

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.

Bravo A, York T, Pumplin N, Mueller LA, Harrison MJ. 2016. Genes conserved for arbuscular mycorrhizal symbiosis identified through phylogenomics. *Nature Plants* **2**: 1–6.

Cope KR, Bascaules A, Irving TB, Venkateshwaran M, Maeda J, Garcia K, Rush TA, Ma C, Labbé J, Jawdy S *et al.* 2019. The ectomycorrhizal fungus *Laccaria bicolor* produces lipochitooligosaccharides and uses the common symbiosis pathway to colonize *Populus* roots. *Plant Cell* **31**: 2386–2410.

Garcia K, Delaux P-M, Cope KR, An J-M. 2015. Molecular signals required for the establishment and maintenance of ectomycorrhizal symbioses. *New Phytologist* **208**: 79–87.

Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB. 2001. Molecular evidence for the early colonization of land by fungi and plants. *Science* **293**: 1129–1133.

Laczko E, Boller T, Wiemken V. 2004. Lipids in roots of *Pinus sylvestris* seedlings and in mycelia of *Pisolithus tinctorius* during ectomycorrhiza formation: changes in fatty acid and sterol composition. *Plant, Cell & Environment* **27**: 27–40.

Lévy J, Bres C, Geurts R, Chalhoub B, Kulikova O, Duc G, Journet EP, Ané JM, Lauber E, Bisseling T *et al.* 2004. A putative Ca^{2+} and calmodulin-dependent protein kinase required for bacterial and fungal symbioses. *Science* **303**: 1361–1364.

Lindsay PL, Ivanov S, Pumplin N, Zhang X, Harrison MJ. 2022. Distinct ankyrin repeat subdomains control VAPYRIN locations and intracellular accommodation functions during arbuscular mycorrhizal symbiosis. *Nature Communications* **13**: 5228.

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.

Martin FM, Uroz S, Barker DG. 2017. Ancestral alliances: plant mutualistic symbioses with fungi and bacteria. *Science* **356**: eaad4501.

Miyauchi S, Kiss E, Kuo A, Drula E, Kohler A, Sánchez-García M, Morin E, Andreopoulos B, Barry KW, Bonito G *et al.* 2020. Large-scale genome sequencing of mycorrhizal fungi provides insights into the early evolution of symbiotic traits. *Nature Communications* **11**: 5125.

Radhakrishnan GV, Keller J, Rich MK, Vernié T, Mbadinga Mbadinga DL, Vigneron N, Cottret L, Clemente HS, Libourel C, Cheema J *et al.* 2020. An ancestral signalling pathway is conserved in intracellular symbioses-forming plant lineages. *Nature Plants* **6**: 280–289.

Werner GDA, Cornelissen JHC, Cornwell WK, Soudzilovskaia NA, Kattge J, West SA, Toby KE. 2018. Symbiont switching and alternative resource acquisition strategies drive mutualism breakdown. *Proceedings of the National Academy of Sciences, USA* **115**: 5229–5234.

Xing Y, Liu Y, Zhang Q, Nie X, Sun Y, Zhang Z, Li H, Fang K, Wang G, Huang H *et al.* 2019. Hybrid *de novo* genome assembly of Chinese chestnut (*Castanea mollissima*). *GigaScience* **8**: giz112.

Supporting Information

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

Dataset S1 Orthofinder analysis across 13 plant species.

Dataset S2 Analysis of 72 previously identified AM-conserved OGs and DMI3 OG.

Dataset S3 Expression analysis of chestnut genes in control roots, ECM roots and a mix sample, and relative expression of Medicago genes in control and AM roots.

Fig. S1 Mycorrhizal traits in Fagales.

Fig. S2 Collection time and estimated plant ages of dual-mycorrhizal species in the field study.

Fig. S3 *Malus domestica* and *Parasponia andersonii* are not ECM hosts.

Fig. S4 Functional categories of 33 AM-conserved OGs and DMI3 that are conserved in ECM-only hosts.

Fig. S5 *Castanea mollissima RAM2* genes complement Medicago *ram2* AM phenotype.

Fig. S6 Expression pattern of *CmRAM2-1pro::GUS* in Medicago AM roots.

Methods S1 Materials and methods used in this manuscript.

Table S1 List of 13 species used for phylogenomic analyses.

Table S2 Seventeen previously identified OGs were not AM-conserved.

Table S3 Nineteen newly defined AM-conserved OGs were mutated differently in ECM hosts.

Table S4 Primers used in this study.

Please note: Wiley is not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.

Key words: common symbiotic signaling, evolution, lipids, mycorrhizas, phylogenomics, trees.

Received, 3 January 2024; accepted, 19 February 2024.