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Letter

Ectomycorrhizal symbiosis evolved independently and by convergent gene duplication in rosid lineages

Introduction

Many land plants rely on mutualistic symbiotic associations to thrive, starting with their common ancestor associating with arbuscular mycorrhizal (AM) fungi (Rich *et al.*, 2021). Similar to AM symbiosis, multiple other intracellular symbiotic interactions have evolved in land plants, such as ericoid symbiosis in the Ericaceae and root nodule symbiosis with nitrogen-fixing bacteria in the legumes and their relatives (Strullu-Derrien *et al.*, 2018; Radhakrishnan *et al.*, 2020). Ectomycorrhizal (ECM) symbiosis contrasts with these relationships, as there is no intracellular accommodation of the symbiont inside the plant cell, but only intercellular colonization (Brundrett & Tedersoo, 2018). This symbiotic relationship is common in seed plants, mostly trees and shrubs, such as the gymnosperm pines, dicot willows and oaks (Cairney, 2000; Wang & Qiu, 2006; Tedersoo & Brundrett, 2017). The fungal partners are primarily of the Ascomycete, Basidiomycete and sporadically Zygomycota clades and evolved from a saprotrophic to a symbiotic lifestyle (Cairney, 2000; Martin *et al.*, 2016), with varying degrees of plant host specificity (Bakker *et al.*, 2004; Plett *et al.*, 2015; Lofgren *et al.*, 2021).

Efforts have been made to uncover the fungal side of this symbiotic relationship (Martin *et al.*, 2008, 2016; Kohler *et al.*, 2015; Liao *et al.*, 2016; Zhang *et al.*, 2018, 2022; Chowdhury *et al.*, 2022), but the plant side remains more elusive. Although the plant lineages involved in ECM symbiosis are known (Tedersoo & Brundrett, 2017), there has been little investigation into the evolutionary origin of ECM symbiosis in these lineages. The current hypothesis is that plants evolved the ability to engage in ECM symbiosis repeatedly and independently as a response to environmental change, but genetic evidence for this is lacking (Cairney, 2000; Wang & Qiu, 2006; Brundrett & Tedersoo, 2018). Furthermore, the genetic innovations and transcriptomic response related to this symbiosis have been studied largely in a genus or species-specific context (Liao *et al.*, 2016; Bouffaud *et al.*, 2020; Chowdhury *et al.*, 2022; Hill *et al.*, 2022), which hinders the study of their evolutionary origins. It has been proposed that most ECM plants have evolved from AM plants, potentially by repurposing genes involved in AM symbiosis for ECM symbiosis (Brundrett & Tedersoo, 2018; Li *et al.*, 2024).

In this study, we reconstruct the origin of ECM symbiosis in the rosid clade, showing at least 16 independent origins, resulting in the 17 known extant ECM rosid lineages. Moreover, comparative genomics of these lineages highlight genes involved in cell wall remodeling, which underwent duplications in a convergent manner across ECM lineages.

Results and Discussion

Ancestral state reconstruction supports multiple independent gains of ECM symbiosis in angiosperms

Many different plant ECM lineages have been proposed to have originated independently (Brundrett, 2009; Tedersoo & Smith, 2013). In rosids, the main ECM lineages are Fagales, Salicaceae (Malpighiales) and Myrtoideae (Myrtales), and they also include less species-diverse clades in the orders Fabales, Rosales, Malpighiales and Malvales (Tedersoo & Brundrett, 2017). We reconstructed a phylogenetic tree of rosids based on sequences of two plastid-encoded proteins: the large subunit of ribulose-bisphosphate carboxylase (RbcL) and Maturase K (MatK) (Supporting Information Table S1), to model the evolution of the ECM trait. Using the best-performing model (All Rates Different (ARD)+kappa), we found a largely independent origin of ECM lineages, in agreement with the literature (Fig. 1; Methods S1, Table S2). The rate of non-ECM to ECM status was modeled to be higher than vice versa (0.21; 0.063), suggesting that ECM symbiosis is more frequently gained than lost. The kappa value in our model is low (*c.* 0.07), which results in little weight being put on the branch lengths for trait evolution. A clear single origin in the common ancestor of Fagales can be detected, with a loss in the Myricaceae, a group that instead associates with nitrogen-fixing bacteria of the genus *Frankia* (Huguet *et al.*, 2005). Ectomycorrhizal symbiosis in Myrtoideae has a clear single origin with a putative single loss in the common ancestor of *Pimenta* and *Myrtus*, both members of Myrteae. This single loss is consistent with the literature (Tedersoo & Brundrett, 2017), although additional losses have been suggested that can only be recovered with a denser taxon sampling of this clade (Thornhill *et al.*, 2015; Tedersoo & Brundrett, 2017).

Tedersoo & Brundrett (2017) separate four ECM lineages within Detarioideae (Fabales; Fig. 1); two of these, we estimate, originated independently: the Afzelia group (*Afzelia* and *Intsia*) and the Berlinia group (here, represented by *Brachystegia*, *Gilbertiodendron* and *Julbernardia*). We detected a single origin in the common ancestor of the other two groups (*Dicymbe* and *Cryptosepalum* + *Paramacrolobium*), followed by a loss in *Amherstia* (Fig. 1). However, the relationships among lineages of Detarioideae remain uncertain, and more data besides RbcL and MatK markers might be necessary to resolve their phylogeny (Estrella *et al.*, 2017, 2018). This is especially the case for the tribe Amherstiae, where, for example, the species *Polystemonanthus dinklagei* may be important, as it is sister to the genus *Dicymbe*, but its ECM status is unknown

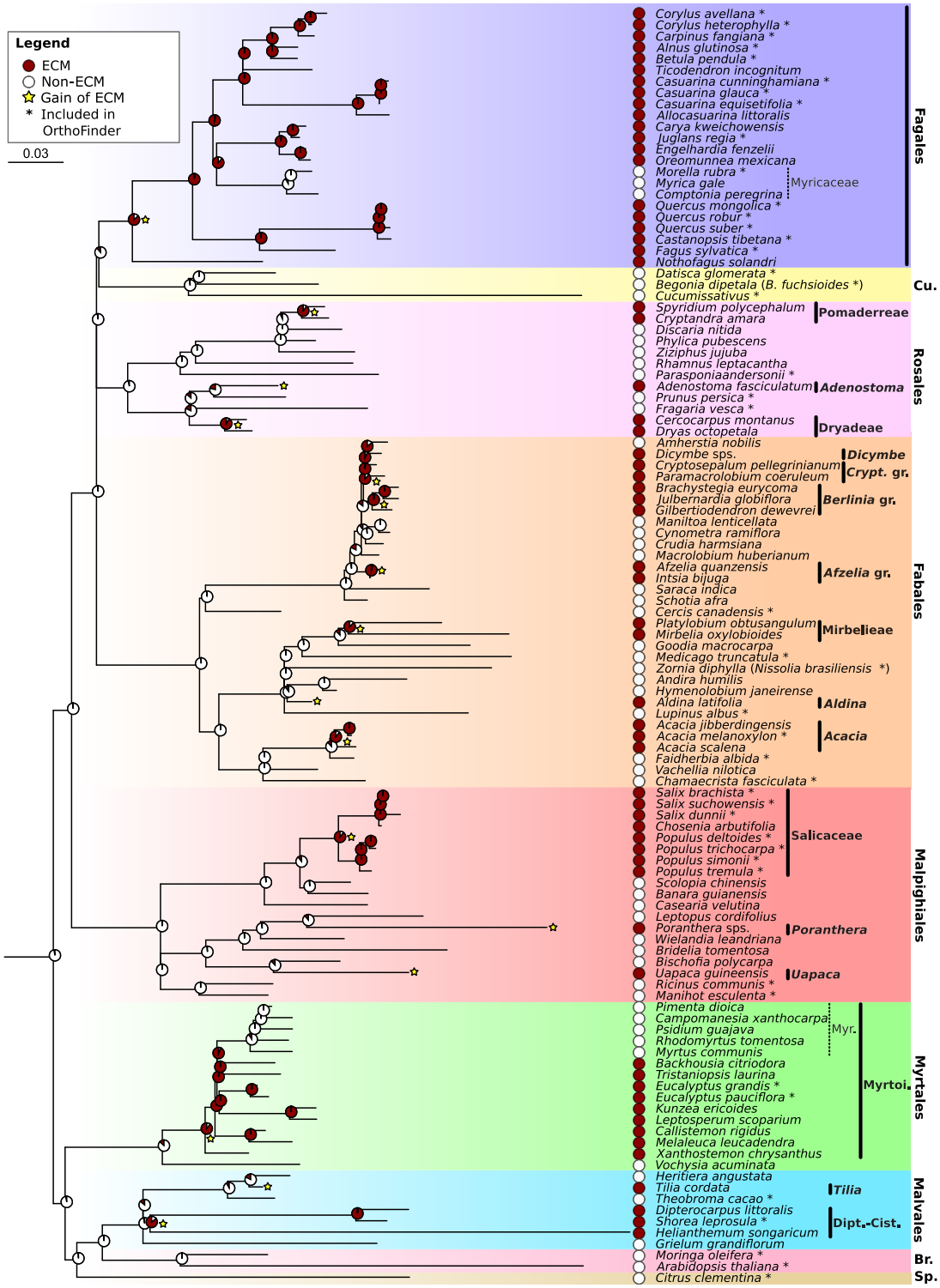

Fig. 1 Ancestral reconstruction of ectomycorrhizal (ECM) symbiosis in plant lineages of rosids. The phylogenetic tree was reconstructed based on plastid protein sequences of RbcL and MatK, reconstructed under the model Q.plant + C60 + F0 + R5. Ancestral reconstruction was based on the All Rates Different (ARD) + kappa model. The likelihood of the ECM (red) or non-ECM (white) status is shown proportionally for each node as a pie diagram, whereas the status of included rosid species is shown next to the species name. The 17 rosid ECM clades identified by Tedersoo & Brundrett (2017) are shown with thick black lines, and their names and the 16 yellow stars represent independent origins of ECM symbiosis. The two clades represented by multiple species that lost the ability for ECM symbiosis are shown with dashed lines. Asterisks denote species also included in the OrthoFinder analysis, and the species name is placed between parentheses if a different species was used. The bar represents estimated substitutions per site. Br, Brassicales; Crypt gr, Cryptosepalum group; Cu, Cucurbitales; Dipt-Cist, Dipterocarpaceae-Cistacea; Myr, Myrteae; Myrtoi, Myrtoideae; Sp, Sapindales.

(Tedersoo & Brundrett, 2017). All other rosid ECM lineages are shown to have evolved the symbiosis independently (Fig. 1). Overall, our reconstruction of ancestral states within the rosids shows that the identified ECM lineages largely evolved this symbiotic association independently.

As ancestral state reconstruction is limited by phylogenetic signal, taxon sampling and limited models for character evolution, we decided to also look for genetic signals that may indicate an ancestral origin of ECM symbiosis in rosids. With a database of 63 genomes, including 25 ECM and 19 non-ECM rosids (Table S3), we reconstructed orthologous groups associated with the common ancestral node of rosids and mined for orthogroups that are largely absent in the non-ECM rosids (Methods S1). If there would be a common ECM ancestor, convergent gene losses would be observed in the non-ECM lineages following the loss of ECM symbiosis, a phenomenon also observed following the loss of other symbioses (Delaux *et al.*, 2014; Bravo *et al.*, 2016; Griesmann *et al.*, 2018). After manual curation, we identified six genes that share an origin with the common ancestor of rosids, but have been retained mostly by ECM species, although they are still present in several genomes of non-

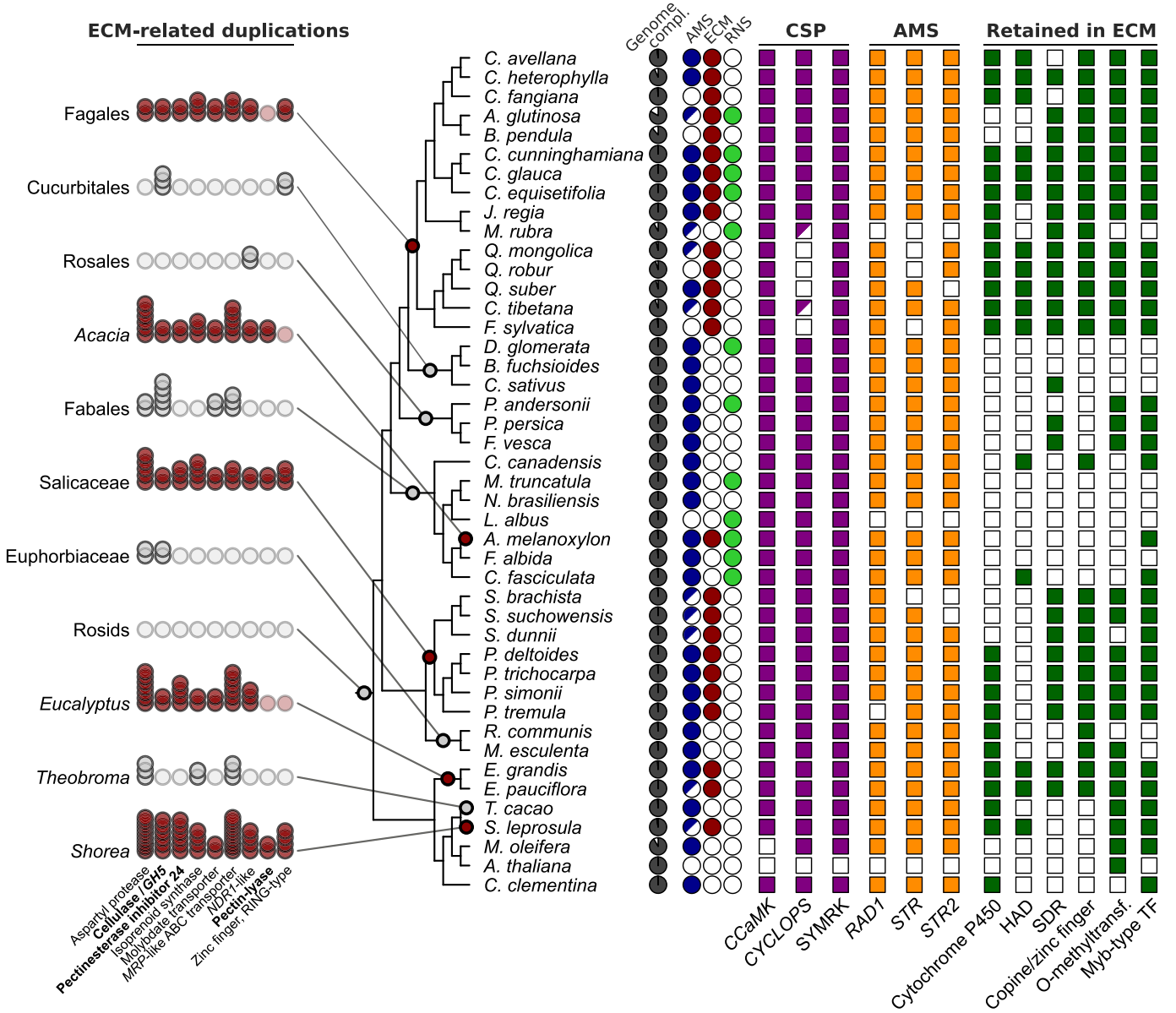

Fig. 2 Cladogram of rosid species included in the orthogroup analysis, depicting ectomycorrhizal (ECM)-specific gene duplications and retention of genes with a known or putative role in symbiosis. Phylogenetic relationships are based on Fig. 1. Duplications are shown for 11 taxonomic groups. A single faint circle means no duplications occurred, whereas two or multiple circles represent one or more duplications, and the circles are colored by the ECM (red) or non-ECM (gray) status of that node. Presence (colored) or absence (white) are shown for genes part of the common signaling pathway (CSP), specific to arbuscular mycorrhizal symbiosis (AMS) and/or root nodule symbiosis (RNS), and those retained in ECM lineages. When the AMS state of a species is uncertain, the circle is half-colored. Genes partially present are shown by a half-colored square. Duplicated genes related to cell wall remodeling are shown in bold. compl, completeness; HAD, haloacid dehalogenase-like; O-methylfransf, O-methyltransferase; SDR, short-chain dehydrogenase/reductase; TF, transcription factor.

ECM species (Figs 2, S1–S6). Part of the reason may be that the pressure to lose them is strengthened in non-ECM lineages, but not enough to result in a complete loss in these lineages. It may also be that the loss of ECM symbiosis is recent in some of these lineages. For example, a specific short-chain dehydrogenase/reductase gene was retained in non-ECM Rosales, but Rosales contains several ECM lineages; thus, the presence of this gene could be explained by the recent loss of ECM symbiosis (Fig. 2). It is however difficult to tell how many genes are expected to follow such a pattern by chance, with retention only in a small subset of lineages. In addition, none of these genes are conserved in all the ECM lineages covered by our analysis.

Overall, the retention of these genes does not clearly support an ancestral origin of this symbiosis, and in light of the ancestral state reconstruction, the occurrence of ECM symbiosis in rosids is still best explained by multiple independent origins.

ECM evolution is linked with convergent gene duplications

The independent emergence of traits may result either from completely different or from convergent genetic pathways. Convergent evolution occurs through the co-option of existing genes or following gene duplication and may result in similar molecular responses (Satterlee *et al.*, 2024).

First, we reasoned that convergence toward the ability to engage in ECM symbiosis should be detected at the transcriptomic level; thus, we looked for shared transcriptomic responses to ECM fungi in three host species: *Quercus robur*, *Populus tremula* × *P. tremuloides* and *Eucalyptus grandis* (Bouffaud *et al.*, 2020; Chowdhury *et al.*, 2022; Hill *et al.*, 2022). Comparing the overlap in deregulation of genes during ECM symbiosis, we found a set of 537 orthogroups that were deregulated in all three species (Methods S1; Fig. S7; Table S4). This number of shared differentially expressed orthogroups was higher than would have been expected by chance (mean: 359, *P*-value: 0.00001), which was especially notable considering different symbionts and time points tested in the three studies included in this analysis. Exclusion of orthogroups that were also deregulated in response to chitin in *M. truncatula* and *A. thaliana* (Feng *et al.*, 2019; Bjornson *et al.*, 2021), datasets that mimic a generic response to fungi, still resulted in a set larger than expected (observed: 447, sampled mean: 327, *P*-value: 0.00001). This shows that, at least from a transcriptomic perspective, there is a similar response to the presence of ECM fungi.

Then, we investigated the potential contribution of gene duplication to the convergent evolution of ECM symbiosis. To do so, we looked into gene duplication events that had occurred independently in the ancestral nodes of the five ECM rosid lineages covered in our genome analysis: Fagales, Salicaceae, Myrtoideae, Dipterocarpaceae-Cistacea and *Acacia* (Methods S1). Using OrthoFinder, we detected 61 orthogroups with duplication events in the groups represented by multiple genera: the common ancestors of the Salicaceae and the Fagales. Among these 61 orthogroups, several are frequently duplicated across the whole phylogeny. To further identify relevant candidates in this list, we selected those that matched one of the following three additional criteria: at least one of the duplicated genes is deregulated in the three ECM species with available RNAseq data (Table S4); a high proportion (> 1/7) of duplications co-occur with the gain of ECM symbiosis; or more than half of the duplications occurred in any node with ECM status. This filter resulted in the shortlisting of 27 orthogroups, which after manual curation through single gene phylogenetic analyses resulted in a final selection of nine genes (Figs S8–S16). These genes are duplicated in a minimum of three of the five ancestral nodes with ECM status and not frequently duplicated in a set of nodes with non-ECM status (Fig. 2; Table S5).

Strikingly, three out of the nine candidate genes have functions associated with cell wall remodeling: a pectinesterase inhibitor, a pectin methylesterase and a cellulase (Fig. 2). Modification of the plant cell wall is a common feature in intra- and intercellular plant–fungi mutualistic symbioses (Su, 2023). In particular, extensive pectin modifications have been observed during the interaction between ECM fungi and their hosts, and a part of these modifications is mediated by the fungal partner (Chowdhury *et al.*, 2022). However, comparative phylogenomics of the fungal symbionts have revealed that ECM fungal lineages have a reduced set of plant cell wall degrading enzymes, a genomic reduction that occurred in a convergent manner (Kohler *et al.*, 2015). The convergent expansion of gene families encoding cell wall modifying enzymes in plant lineages that evolved the ability to engage in ECM symbiosis might reflect a shift in the distribution of this functionality from the symbiont to the host, which may represent a common principle for the evolution of ECM symbiosis.

Convergent co-option of the common symbiosis pathway for ECM symbiosis may have occurred in rosids

Besides gene duplication, convergent evolution originates from the co-option of existing genes and pathways. In the context of ECM symbiosis, it has been proposed that its evolution in Fagales was facilitated by the co-option of the common signaling pathway (CSP) (Li *et al.*, 2024), a module of several proteins essential for the establishment of intracellular symbiosis (Parniske, 2008). This hypothesis, supported by reverse genetic analyses in poplar (Cope *et al.*, 2019), is based on the conservation of those genes in certain ECM plant species, which have, in parallel, lost the ability to engage in any form of intracellular symbiosis. Indeed, the loss of this ability has been correlated, in many plant lineages, with the loss of associated genes by relaxed selection (Delaux *et al.*, 2014; Bravo *et al.*, 2016; Radhakrishnan *et al.*, 2020). Thus, the retention of these genes may reflect the evolution of another selection pressure, possibly by ECM symbiosis (Li *et al.*, 2024). Similarly, genes have been found to be specifically dedicated to the ability to engage in AM symbiosis. The retention of one of such genes in ECM Fagales, the Glycerol 3-Phosphate Acyltransferase RAM2, an enzyme essential for the functioning of the AM symbiosis (Wang *et al.*, 2012), also indicates that it might have been co-opted for ECM symbiosis in that lineage (Li *et al.*, 2024).

To determine whether the convergent evolution of ECM symbiosis relied more broadly on the co-option of these genes, we conducted targeted phylogenies on the CSP genes *SYMRK*, *CCaMK* and *CYCLOPS*, and on the AM symbiosis-specific genes *RAD1*, *STR* and *STR2* (Methods S1, Figs S17–S22). Only three of

the included genomes are lacking *RAD1*, *STR1* and *STR2*. Two of these are *Arabidopsis thaliana* and *Lupinus albus*, both known to lack mycorrhizal (both AM and ECM) symbioses. The other is *Morella rubra*, which has multiple observations of AM fungi listed on the FungalRoot database (Soudzilovskaia *et al.*, 2020, 2024). Moreover, other members of the genus *Myrica*/*Morella* are considered to be both ECM and AM species (Urgiles *et al.*, 2014; Teste *et al.*, 2020). It is possible that the loss of AM symbiosis is species-specific, although we cannot rule out that the genes were simply not recovered in the genome assembly (BUSCO completeness 92.2%). More interestingly, the AM symbiosis-specific genes were mostly retained in the ECM species that lost AM symbiosis: *C. fangiana* and *B. pendula* retained all three genes, whereas *Q. robur* and *F. sylvatica* retained two of the three genes (Fig. 2). Similarly, among the CSP genes, *SYMRK* and *CCaMK* were found in all ECM species, including those that lost AM symbiosis (Fig. 2). The same pattern was observed for CYCLOPS with the notable exception of Fagaceae and the genus *Morella*, in which the gene was either not detected or only partially recovered. Although this may be attributable to low-quality genomes, the observation that these potential losses are restricted to a single clade might deserve further investigation. For the retained genes, we also investigated the expression level during ECM symbiosis. Common signaling pathway and AM symbiosis-specific genes were generally not deregulated, except for one copy of *CYCLOPS* in *P. tremula* × *P. tremuloides* and the *STR* gene in *E. grandis* (Table S4). Altogether, this suggests there may be co-option of CSP and AM symbiosis-specific genes to independent instances of ECM symbiosis, or that the losses of AM symbiosis in ECM-only lineages are relatively recent.

The combination of ancestral state reconstruction, comparative phylogenomics and transcriptomics, and single-gene phylogeny conducted here does not support an ancestral gain of ECM symbiosis in rosids, but rather the independent evolution of this symbiotic ability. These independent events, involving different fungal lineages (Kohler *et al.*, 2015), occurred in a convergent manner by gene co-option and duplications leading to the emergence of a similar transcriptional response.

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Competing interests

None declared.

Author contributions

PMD conceived and coordinated the project. FvB, YB, CP, MEB and CL carried out the bioinformatic analyses. MB, JK and PMD coordinated the analyses. FvB, YB and PMD wrote the manuscript with input from all authors. FvB and YB contributed equally to this work.

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Data availability

Code used for this work is available on GitLab (https://gitlab.com/fabianvanbeveren/ecm/) and phylogenies and alignments can be found on Figshare (doi: 10.6084/m9.figshare.27241689). Genome data used is listed in Table S3 and sources for the RbcL and MatK sequences in Table S1.

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Supporting Information

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

Figs S1–S6 Single gene phylogeny of cytochrome P450s (S1), SDR-like proteins (S2), Zinc fingers (S3) and HAD-like proteins

(S4), O-methyltransferases (S5) and Myb-type transcription factors (S6) that are largely retained in ECM lineages.

Fig S7 Random sampling approach testing the significance of the number of orthogroups with genes deregulated in three ECM plant species.

Figs S8–S16 Single gene phylogeny of MRP-like ABC transporters (S8), aspartyl proteases (S9), NDR-like proteins (S10), isoprenoid synthases (S11), pectinesterase inhibitor 24s (S12), GH5 cellulases (S13), molybdate transporters (S14), ring-type zinc fingers (S15) and pectin lyases (S16) that are convergently duplicated in ECM lineages.

Figs S17–S19 Single gene phylogenies of the CSP genes SYMRK (S17), CCaMK (S18) and CYCLOPS (S19).

Figs S20–S22 Single gene phylogenies of AM symbiosis-related genes RAD1 (S20), STR1 (S21) and STR2 (S22).

Methods S1 Materials and Methods used in this manuscript.

Table S1 Sources of the MatK and RbcL protein sequences.

Table S2 Results of the tested models for ancestral state reconstruction.

Table S3 Genome statistics and sources of all included species.

Table S4 Differential expression of genes and orthologous groups.

Table S5 Number of duplications for 11 different taxonomic groups for nine genes frequently duplicated in ECM lineages.

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