

Algal ancestor of land plants was preadapted for symbiosis

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Colonization of land by plants was a major transition on Earth, but the developmental and genetic innovations required for this transition remain unknown. Physiological studies and the fossil record strongly suggest that the ability of the first land plants to form symbiotic associations with beneficial fungi was one of these critical innovations. In angiosperms, genes required for the perception and transduction of diffusible fungal signals for root colonization and for nutrient exchange have been characterized. However, the origin of these genes and their potential correlation with land colonization remain elusive. A comprehensive phylogenetic analysis of 259 transcriptomes and 10 green algal and basal land plant genomes, coupled with the characterization of the evolutionary path leading to the appearance of a key regulator, a calcium- and calmodulin-dependent protein kinase, showed that the symbiotic signaling pathway predated the first land plants. In contrast, downstream genes required for root colonization and their specific expression pattern probably appeared subsequent to the colonization of land. We conclude that the most recent common ancestor of extant land plants and green algae was preadapted for symbiotic associations. Subsequent improvement of this precursor stage in early land plants through rounds of gene duplication led to the acquisition of additional pathways and the ability to form a fully functional arbuscular mycorrhizal symbiosis.

symbiosis | plant evolution | algae | plant-microbe interactions | phylogeny

The colonization of land by plants 450 Mya created a major transition on Earth, causing the burial of large amounts of carbon, with resultant decreases in atmospheric CO₂ leading to a dramatically altered climate. This transition provided the foundation for the majority of extant terrestrial ecosystems (1, 2). The terrestrial environment that these early plants colonized must have presented many challenges, primary among them being the acquisition of mineral nutrients. It has been suggested that the appearance of the arbuscular mycorrhizal (AM) symbiosis and other beneficial associations with fungi such as Mucoromycotina facilitated this colonization of land by improving plants' ability to capture nutrients.

Based on recent phylogenetic analyses, Zygnematales, one of the paraphyletic "advanced charophytes" (i.e., Coleochaetales, Charales, and Zygnematales), has been identified as the closest green algal relative to land plants, whereas the chlorophytes diverged much earlier (Fig. 1A) (3, 4). On the other side of this transition, bryophyte lineages (i.e., liverworts, mosses, and hornworts) are considered to be the earliest diverging land plants, although their branching order remains debated (5–7). Key

innovations present in bryophytes but not in advanced charophytes thus are good candidates for understanding the basis of land colonization by plants. In support of previous suppositions, one innovation that discriminates bryophytes from charophytes is the ability to develop beneficial associations with AM fungi (8–11). The discovery of 450-My-old fossilized spores similar to the spores of extant AM fungi supports their presence in the environment encountered by the first land plants (9). Remnants of intracellular fungal structures, likely belonging to ancestral AM fungi and to fungi belonging to the closely related Mucoromycotina, were found in the oldest plant macrofossils and suggest that one or both of these associations were instrumental for the colonization of lands (8, 12). Today, these two types of fungi also are found in, and

Significance

Colonization of land by plants was a critical event for the emergence of extant ecosystems. The innovations that allowed the algal ancestor of land plants to succeed in such a transition remain unknown. Beneficial interaction with symbiotic fungi has been proposed as one of these innovations. Here we show that the genes required for this interaction appeared in a stepwise manner: Some evolved before the colonization of land by plants and others first appeared in land plants. We thus propose that the algal ancestor of land plants was preadapted for interaction with beneficial fungi and employed these gene networks to colonize land successfully.

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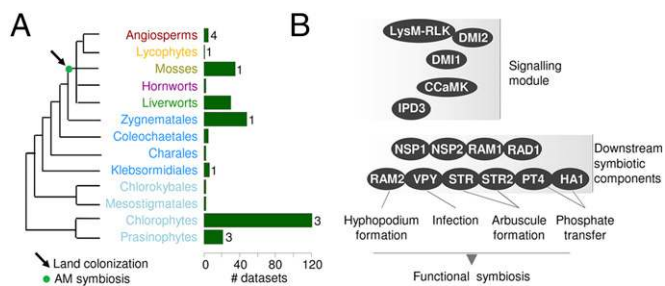


Fig. 1. The green lineage and the AM symbiosis. (A) Schematic representation of the green lineage. Light blue: chlorophytes and basal charophytes; dark blue: advanced charophytes. Green bars on the left indicate the actual number of transcriptomes and genomes included in the extended dataset used in this study. (B) Schematic representation of the symbiotic genes.

provide a growth benefit to, liverworts and hornworts (11, 13, 14). However, although Mucoromycotina fungi are restricted to a few plant species and likely evolved independently in various lineages, AM fungi colonize more than 80% of extant land plants, and their absence in a few taxa results from independent losses (15). Taken together, these lines of evidence strongly support the notion that AM fungi played an important role in the persistent colonization of land by the first Embryophyta.

Genetic and physiological studies in model legumes such as *Medicago truncatula* allowed the characterization of the steps leading to functional symbiosis in angiosperms (Fig. 1B). At the onset of the AM association, both partners initiate a chemical communication, with strigolactone signals from the plant inducing the production of a mix of diffusible signals called “Myc factors” from the fungus (16–18). The host plants detect these Myc factors that activate part of the symbiotic responses (17). The plant then allows penetration and subsequent colonization by the fungus. When the fungus reaches cortical cells in vascular plants or the inner parenchyma in liverworts (10), highly branched fungal structures surrounded by the plant plasma membrane, known as “arbuscules,” are formed (19). This specialized interface improves the transfer of nutrients and water from the AM fungi to the plant in exchange for carbohydrates (10, 19). Genetic and transcriptomic analyses in angiosperms led to the characterization of genes controlling these steps (19, 20). At the plasma membrane, a receptor complex composed of lysin motif receptor-like kinase (LysM-RLK) proteins and a malectin-like domain (MLD)-RLK (DMI2) perceives the Myc factor signals (17, 21, 22) and activates a nuclear envelope-localized potassium channel (DMI1) that initiates and maintains rapid oscillations of calcium within and around the nucleus (23). These calcium “spikes” are decoded by a calcium- and calmodulin-dependent protein kinase (CCaMK), a specific calcium-dependent protein kinase (CDPK) also regulated by calmodulin (24). Downstream transcription factors (TFs), including interacting protein of DMI3 (IPD3), which is a direct target of CCaMK, and a network of four GRAS TFs [reduced for arbuscular mycorrhization 1 (RAM1), required for arbuscule development 1 (RAD1), nodulation signaling pathway (NSP) 1, and NSP2] then activate the plant symbiotic program (25–28). Components of this program play roles at various steps: RAM2, a glycerol-3-phosphate acyl transferase (GPAT), is required for the induction of the fungal penetration structure (29). VAPYRIN controls penetration of the epidermis and arbuscule formation (30). The half-ATP-binding cassette (ABC) transporters stunted arbuscule (STR) and STR2 are critical for arbuscule development, and finally, the H⁺-ATPase HA1 and the phosphate transporter PT4 allow the uptake of phosphate in arbuscule cells (31–33). In angiosperms, loss of AM symbiosis results in the consistent loss of these genes, whose function seems to be linked specifically to AM symbiosis (15). Thus, we hypothesized that tracing the origin of these symbiotic genes would allow us to investigate the link between the appearance of AM symbiosis and land colonization.

Results

Extensive Phylogeny Indicates the Presence of Symbiotic Signaling Genes in Algae.

A current limitation in the study of the transition from algae to embryophytes is the limited number of datasets available. To decipher the evolution of the symbiotic genes at this transition, we first combined an extended dataset spanning the entire green lineage (Fig. 1A and Dataset S1). In addition to publicly available genomes and transcriptomes, this dataset includes the newly generated deep transcriptomes of a liverwort (*Lunularia cruciata*) and an advanced charophyte complex (*Closterium peracerosum-strigosum-littorale*, hereafter *Closterium*), the draft genome of another advanced charophyte (*Spirogyra* sp.), and 249 transcriptomes of green algae, liverworts, mosses, and hornworts generated through the 1,000 Plants (1KP) project (<https://sites.google.com/a/ualberta.ca/onekp/>). This extended dataset was mined for homologs of the symbiotic genes, starting with genes belonging to the “signaling module”: *LysM-RLK*, *DMI2*, *DMI1*, *CCaMK*, and *IPD3*. In line with previous studies using PCR-based approaches, we found *LysM-RLK*, *DMI1*, *DMI2*, *CCaMK*, and *IPD3* orthologs in the bryophyte assemblies (Fig. 2 and SI Appendix, Figs. S1–S6) (10, 34). Much more surprising is the presence of homologs of most of these genes in several species across the charophyte portion of the tree (Fig. 2 and SI Appendix, Figs. S1–S6). *LysM-RLKs* are present in advanced charophytes together with *IPD3* (SI Appendix, Figs. S1 and S64). These orthologs displayed the specific features of these proteins, and phylogenetic analyses recovered them as direct orthologs of the angiosperm proteins (SI Appendix, Figs. S6B and S7). Homologs of *DMI2* also were present in a single charophyte clade, with the actual *DMI2* clade and its paralogs originating from various rounds of gene duplication (SI Appendix, Figs. S2 and S3). Finally, potential homologs of *DMI1* and *CCaMK* were found in charophytes and even chlorophytes (Fig. 2 and SI Appendix, Figs. S4 and S5). Taken together these results indicate that, with the exception of clear *DMI2* orthologs, the symbiotic signaling genes are present in extant charophytes and predate the colonization of land by plants. The presence of chlorophyte sequences with high similarity to *DMI1* and *CCaMK* suggests that these two genes may have originated even before the divergence of the chlorophytes.

The Key Components DMI1 and CCaMK Coevolved in Charophytes.

To understand better the evolutionary steps that led to the appearance of the signaling module, we looked at *DMI1* and *CCaMK* homologs in charophytes and chlorophytes in more detail. *DMI1* displays a very specific filter domain (ADAGNHA) compared with less specialized and broadly distributed potassium channels (GYGD) (23). In land plants, this sequence is highly conserved, with mutations seen only when a duplicated version compensates for the decreased activity (SI Appendix, Fig. S4) (35). Similarly, the advanced charophyte sequences also are extremely conserved with a filter domain identical to the one from *M. truncatula* *DMI1* (MtDMI1) (SI Appendix, Fig. S4). In contrast, the few chlorophyte homologs displayed a different amino acid composition (SI Appendix, Fig. S4). This result suggests that *DMI1* evolved in charophytes from a precursor stage present in chlorophytes.

Among the chlorophyte and charophyte proteins showing high homology with *CCaMK*, we found previously identified CDPKs (SI Appendix, Fig. S5) (36). One specific feature of *CCaMKs* compared with CDPKs is the presence of a calmodulin-binding domain (CBD) overlapping with the autoinhibitory domain (37). We compared the CBD of angiosperm *CCaMKs* with the corresponding region of the homologs from chlorophytes, charophytes, and bryophytes. This domain was highly conserved in most land plants, advanced charophytes, and a few chlorophytes (Prasinophytes), all belonging to the *CCaMK* clade (Fig. 2A and SI Appendix, Fig. S5). However, several chlorophyte CDPKs belonging to other clades displayed a high level of conservation of the amino acids of the CBD known to be critical for calmodulin binding (38, 39) (SI Appendix, Fig. S5). To test whether

Symbiotic signaling events are abolished in a *Medicago ccamk-1* mutant (24). If CpCCaMK is a bona fide CCaMK, expressing it in the *ccamk-1* mutant should rescue its symbiotic defects. To test this hypothesis, CpCCaMK, the endogenous MtCCaMK gene, and two CDPKs from Chlorophytes (*CvCDPK3* and *CrCDPK1*) were expressed in the *ccamk-1* mutant using *Agrobacterium*-mediated root transformation (40). Wild-type and *ccamk-1* seedlings transformed with empty expression vectors were used as controls. After 6 wk of incubation with the AM fungus *Rhizophagus irregularis*, none of the *ccamk-1* roots transformed with the empty vector or the chlorophyte CDPKs were colonized (Fig. 2C). In contrast, *ccamk-1* roots transformed with CpCCaMK or MtCCaMK were colonized at a level similar to that seen in wild-type plants transformed with the empty vector (Fig. 2C). Internal hyphae, vesicles, and arbuscules were present in these roots, and expression of the symbiotic phosphate transporter *MtPT4* was detected, suggesting a full complementation of the mutant phenotype (Fig. 2D–F). Taken together these results confirm that an advanced charophyte protein, CpCCaMK, is fully functional in a symbiotic context.

Interestingly, a few chlorophytes (prasinophytes) and basal charophyte sequences also were recovered in the CCaMK clade (Fig. 2A). To determine if a functional CCaMK evolved even before the advanced charophytes, we expressed two of them in the *ccamk-1* mutant and conducted mycorrhization assays. Roots transformed with CCaMK, from the prasinophyte *Nephroselmis pyriformis* (*NpCCaMK*), did not display any colonization event, and the expression of *MtPT4* was not induced (Fig. 2C–E). In contrast, roots expressing CaCCaMK from the basal charophyte *Chlorokybus atmophyticus* showed events of internal colonization and even arbuscules (Fig. 2C and D). However, the colonization rate was extremely low, and the expression of *MtPT4* was induced only slightly (Fig. 2C and E). Closer inspection confirmed that most hyphopodia that formed on the *ccamk-1* roots expressing CaCCaMK aborted, whereas they led to internal hyphae and arbuscules in roots transformed with MtCCaMK or CpCCaMK (Fig. 2F). The partial complementation of the *ccamk-1* mutant achieved by CaCCaMK suggests that an additional layer of functionalization occurred after the divergence of the basal charophytes. Even if the CBD of CaCCaMK is predicted to be functional, and the key amino acids are present, we cannot exclude the possibility that the ability to bind calmodulin is affected. The CBD of CpCCaMK was swapped with the CBD from CaCCaMK, and the ability of the resulting chimeric protein (Cp-CaCBD) to bind calmodulin was assayed as previously. Calmodulin binding of Cp-CaCBD was reduced compared with the native CpCCaMK but was not abolished (SI Appendix, Fig. S9A and B). Additionally, when expressed in a *ccamk-1* mutant, this chimeric gene was able to complement mycorrhizal defects to a level close to that achieved with CpCCaMK (SI Appendix, Fig. S9C–F). Thus, even though the difference in calmodulin-binding activity may explain part of the differences observed between CaCCaMK and CpCCaMK, other innovations must be present in CCaMK from advanced charophytes. In addition to the conservation of the CBD, the formation of the hydrogen-bond network might be affected in basal charophyte and chlorophyte CCaMKs. To test this hypothesis, we first determined the conservation of the autophosphorylation site. Although land plant CCaMKs and most advanced charophyte CCaMKs display an amino acid that can be phosphorylated at the position orthologous to Thr-271 (either threonine or serine), the basal charophyte and chlorophyte sequences from the CCaMK clade displayed other amino acids (SI Appendix, Fig. S10A). Because nearby residues could play a similar role, we built structural models for the CCaMK from two basal charophyte species, CaCCaMK and *Mesostigma viride* CCaMK (MvCCaMK). Neither the predicted CaCCaMK nor the MvCCaMK structure has serine or threonine in the region of interest, and thus neither can be phosphorylated (SI Appendix, Fig. S10C and D).

Taken together, these results strongly suggest that a fully functional CCaMK evolved in the common ancestor of charophytes

and land plants in a three-step process: (i) duplication of an ancestral CDPK before the divergence of the chlorophyte–streptophyte lineages; (ii) evolution of a CBD in one of the paralogs; and (iii) emergence of additional layers of regulation, including the autophosphorylation site, that allow the formation of an inhibitory hydrogen-bond network. Further experiments with additional CCaMK from Prasinophytes and basal charophytes would help elucidate the remaining uncertainty in this proposed model. Interestingly, the evolution of CCaMK mirrored the evolution of DMI1, which acquired its elaborate filter domain in advanced charophytes. Together with the presence of LysM-RLK and IPD3 in advanced charophytes, these data support the evolution of the signaling module before colonization of land by embryophytes.

Downstream Symbiotic Genes Evolved in Land Plants. In addition to the signaling module, establishing AM symbiosis in angiosperms requires a network of downstream GRAS transcription factors (NSP1, NSP2, RAM1, and RAD1) and six other components involved at various stages during the mycorrhization process (RAM2, VAPYRIN, STR and STR2, HA1, and PT4). Given the presence of the signaling module in charophytes, we hypothesized that downstream genes also might predate land colonization. We searched for potential orthologs in the extended datasets. GRAS proteins were found only in advanced charophytes and liverworts (Fig. 3A and SI Appendix, Fig. S11). A maximum-likelihood tree was built with the recovered sequences to distinguish between homologs and orthologs of the four symbiotic GRAS proteins (SI Appendix, Fig. S11). Based on this reconstruction, three clades, supported by low bootstrap values, encompassing

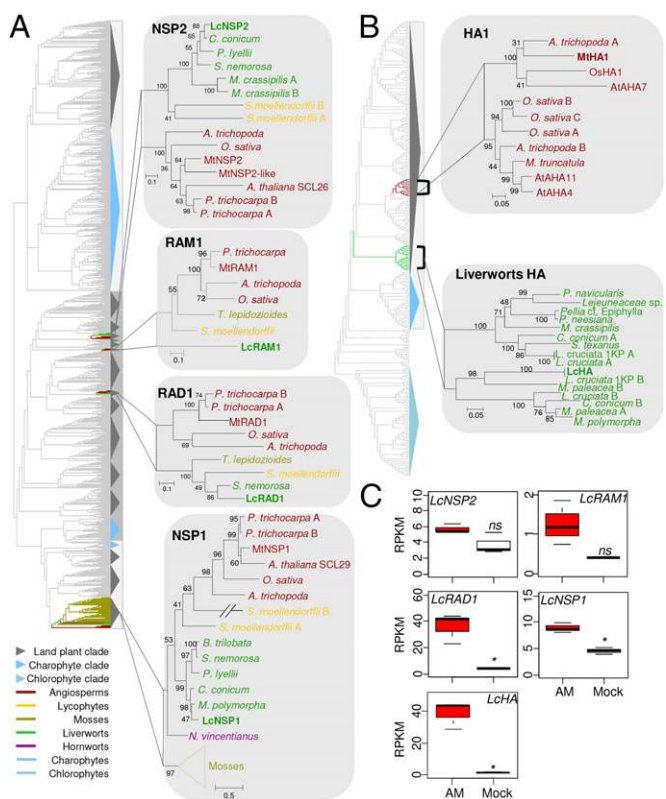


Fig. 3. Multiple paths for the evolution of symbiotic genes in basal land plants. (A) Maximum-likelihood phylogenetic tree of NSP2, RAM1, RAD1, and NSP1 extracted from the GRAS tree on the left. These four clades originate from land plant-specific duplications. (B) Maximum-likelihood phylogenetic tree of HA1 and liverworts HA extracted from the H^+ -ATPase tree (available in SI Appendix, Fig. S17). (C) Differential expression of LcNSP2, LcRAM1, LcRAD1, LcNSP1, and LcHA in mock-treated (Mock) or colonized (AM) *Lunularia cruciata*. * $P < 0.05$. AM-inducible H^+ -ATPase evolved independently in angiosperms and liverworts.

charophyte and land-plant sequences can be recovered (*SI Appendix*, Fig. S11). However, the number of GRAS proteins per species is much higher (*Dataset S2*), suggesting the occurrence of multiple rounds of duplication at various levels. The presence of bryophyte sequences at the base of the NSP1, NSP2, RAM1, and RAD1 clades suggests that they originate from duplication events that occurred in the last common ancestor of land plants (Fig. 3A). Half-ABC transporters of the G subfamily were found in chlorophytes and charophytes, but direct orthologs of STR and STR2, which function in the cells of angiosperms containing arbuscules, evolved in land plants only via duplication (*SI Appendix*, Figs. S12 and S13A). No VAPYRIN or GPAT homologs were found in either chlorophytes or charophytes, but clear orthologs were found in liverworts (*SI Appendix*, Figs. S14–S16). Finally, orthologs of PT4 and HA1 were found only in vascular plants (Fig. 3B and *SI Appendix*, Figs. S17–S19). In addition to their symbiotic function identified by genetics, *NSP1*, *RAM1*, *RAD1*, *RAM2*, *STR*, and *STR2* are up-regulated during symbiosis in angiosperms, whereas *NSP2* is down-regulated (25, 26, 29, 32, 41). To test if their orthologs identified in liverworts displayed the same expression pattern, which would suggest a conserved function in symbiosis, we sequenced the transcriptomes of *Lunularia* in association with the AM fungus *R. irregularis* and compared them with noncolonized *Lunularia* thalli. A total of 509 contigs were found to be significantly up-regulated (fold change >2, false-discovery rate <0.05) (*Dataset S3*), including the orthologs of *NSP1*, *RAD1*, *RAM2*, *STR*, and *STR2* (Fig. 3C and *SI Appendix*, Fig. S12B). The ortholog of *RAM1* was slightly, but not significantly, up-regulated, and the expression of *LcNSP2* was not affected (Fig. 3C).

Taken together, these results indicate that the downstream symbiotic components very likely appeared in first land plants via two processes, one being the duplication of existing gene families, with one of the paralogs being recruited for symbiotic function—as suggested by the expression pattern—and the other being the appearance of new proteins such as VAPYRIN and RAM2. Interestingly, these two proteins display domains found in many other protein families (29, 30, 42, 43) and thus likely originate from domain shuffling. However, several components and regulations are missing in basal land plants, such as the orthologs of PT4 and HA1 and the down-regulation of *NSP2* during mycorrhizae. In legumes, down-regulation of *NSP2* is mediated by microRNA 171h (mir171h) and allows plants to control the level of root infection (41). This microRNA and the corresponding target sequence in *NSP2* are not present in basal land plants, providing an explanation for the absence of down-regulation (41). The absence of mir171h suggests that clade-specific innovations evolved after the appearance of AM symbiosis to fine-tune the association in a new developmental context (gametophyte vs. root sporophyte). The absence of PT4 and HA1 is more surprising, given that the transfer of phosphate to the host plant is an ancestral feature of the association (11). To determine if a nonorthologous pair of symbiotic PT and H⁺-ATPase could be present in liverworts, we mined the *Lunularia* transcriptome and found one PT (*LcPT*) and one HA (*LcHA*) strongly up-regulated during mycorrhizae (Fig. 3C and *SI Appendix*, Fig. S19B).

Discussion

Identifying the innovations that led to the colonization of land by plants has been a long-lasting effort in plant and evolutionary biology. It requires genomic and transcriptomic data from a large and phylogenetically diverse panel of species across charophyte green algae and land plants. The charophyte and bryophyte transcriptomes generated by the 1KP project together with the deep transcriptomes generated for *Closterium* and *Lunularia* and the draft genome of *Spirogyra* sp. allowed us to address such questions. It must be noted that, until multiple additional genomes from charophyte and chlorophyte species have been sequenced, the presence of genes not detected in transcriptomes cannot be ruled out. However, when a similar dataset for liverworts and other bryophytes with an even lower coverage of the species space was used, all the symbiotic genes were recovered,

in line with results obtained in the study of other processes (44–46) and lending support to our conclusions.

Experimental evolution and phylogenetic studies have identified three steps toward the stable acquisition of a trait: potentiation, actualization, and refinement (47, 48). Here, we identified the genetic innovations linked to these steps that lead to functional AM symbiosis in land plants. We propose that potentiation occurred in a common ancestor of charophytes and land plants via mutations in CDPK and DMI1 precursors present before the divergence of chlorophytes (Fig. 4). MLD-RLKs, LysM-RLKs, IPD3, and nonsymbiotic GRAS TFs also evolved in the streptophyte lineage from as yet unknown ancestors. So far, there is no evidence of symbiotic associations between AM fungi charophytes. These data argue for a clear absence of correlation between the appearance of the signaling module in an ancestor of charophytes and land plants and the appearance of AM symbiosis in the first land plants. Strigolactones produced by plants to activate AM fungal metabolism also are present in advanced charophytes (49). In addition to their symbiotic function, these compounds are plant hormones controlling numerous developmental traits, and a hormonal role seems conserved in charophytes (49, 50). Similarly, a nonsymbiotic function of the signaling module in charophytes cannot be ruled out. Given that green algae can form specific associations with bacteria and fungi (51, 52), another exciting hypothesis would be that this module might be required for symbiotic associations in charophytes and would have been recruited for AM symbiosis by the first land plants. In early land plants, actualization took place with the diversification of GRAS transcription factors and half-ABC transporters via gene duplication together with the acquisition of the other downstream symbiotic genes. Whether these steps occurred together or as successive events remains an open question. Finally, refinement is an often ongoing process, with multiple paths for optimization of a trait (47, 48) linked to specificity of every organism. Independent recruitments of symbiotic nutrient transport systems in liverworts and angiosperms reflect this process (Fig. 4).

Other gene regulatory networks playing major roles in vascular plants are thought to have originated in early land plants (53).

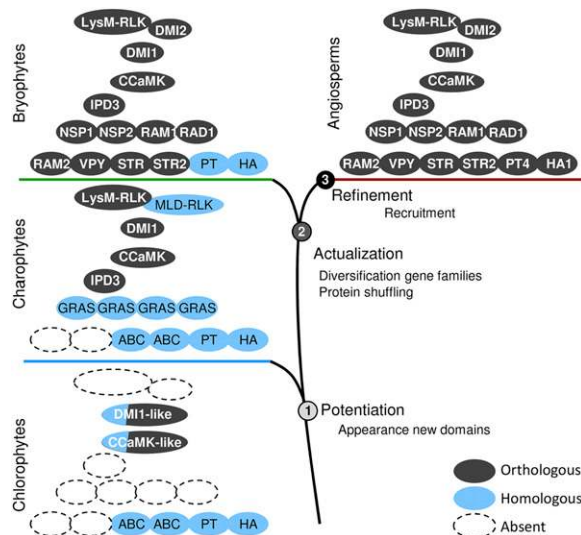


Fig. 4. Stepwise acquisition of symbiotic genes in the green lineage. The appearance of new domains in advanced charophytes led to the emergence of new protein families and to the evolution of the symbiotic signaling module via the neofunctionalization of existing proteins (DMI1 and CCaMK). Most of the downstream symbiotic genes evolved in basal land plants via gene duplication in these newly evolved gene families (i.e., STR) or by combining existing domains in new proteins (i.e., VAPYRIN). Finally, genes originating from lineage-specific duplications were recruited independently to fulfill their symbiotic functions (PT and H⁺-ATPase).

Mining advanced charophyte genomes and transcriptomes is a powerful approach to identify precursor stages of these networks and to confirm the role played by charophyte ancestors in the colonization of land. As we showed for symbiotic genes, actualization of these networks in early land plants, potentially via massive gene duplications, could have been a major enhancer of the successful colonization of land by plants.

Materials and Methods

Details of materials and methods are available in [SI Appendix](#).

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