A molecular atlas of plastid and mitochondrial adaptations across the evolution from chlorophyte algae to angiosperms

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Abstract

Algae and plants carry two organelles of endosymbiotic origin that have been co-evolving in their host cells for more than a billion years. The biology of plastids and mitochondria can differ significantly across major lineages and organelle adaptation likely accompanied the conquering of new ecological niches including land. Based on organelle proteome data, and the genomes of 168 phototrophic (Archaeplastida) versus a broad range of 518 non-phototrophic eukaryotes, we screened for changes in plastid and mitochondrial biology across one billion years of evolution. Taking into account 331,571 protein families (or orthogroups), we identify 31,625 protein families that are unique to primary plastid-bearing eukaryotes. 1961 and 846 protein families are predicted to operate in plastids and mitochondria, respectively. Tracing the evolutionary history of these protein families through evolutionary time uncovers the significant remodeling the organelles experienced from algae to land plants. The analyses of gained orthogroups identifies molecular adaptations of organelle biology that connect to the diversification of major lineages and facilitated major transitions from chlorophytes en route to the global greening and origin of angiosperms.

Keywords: plastid evolution, plant mitochondria, terrestrialization, organelle proteomes

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Introduction

Fewer natural phenomena have been as transformative to planet Earth as the global greening through plants [1,2]. The conquest of land rests on the emergence and expansion of the Chloroplastida, also referred to as the Viridiplantae or simply the green lineage. The Chloroplastida are made up of three phyla: chlorophytes, streptophytes and the prasinodermophytes that are thought to be the sister lineage to the two former [3]. Chloro- and prasinodermophytes are represented by algae only, whereas streptophytes are made up of algae and embryophytes, the latter uniting all land plants [3–5]. The list of key adaptations that fostered land plant expansion in a macroevolutionary context are multiple: roots, a mutualistic symbiosis with fungi, stomata, a cuticle, polyplastidy, and an expansion of many metabolite families such as flavonoids to name a few [1,3–10]. These innovations provided embryophytes with decisive fitness advantages relative to their non-terrestrial chloro-, prasinodermato- and streptophyte algal relatives [1,11].

The eponymous organelle of plants, the chloroplast, underwent various changes, too. It adapted in multiple ways to the challenges characterizing the habitat the last common ancestor of land plants (LCA) encountered. Improving a stress response was necessary to deal for instance with increased levels of ultraviolet (UV) high light stress and to cope with temperature shifts that change rapidly on land in contrast to in water [12–14]. Polyplastidy, a phenomenon that separates plastid from nuclear division, leading to cells that can harbor more than one plastid per cell, was part of being able to develop larger body plans [12,15,16]. To communicate stress and the need for component biosynthesis, an elaborate retrograde signaling evolved on the basis of messenger proteins such as GUN1 or WHIRLY [17]. In combination, these adaptations were decisive for the success of streptophytes, which is evident in the number of species they have evolved and their domination of biomass [1,18].

Plastids do not operate autonomously, but are part of an intricate metabolic network and even physically interact with other compartments such as the endoplasmic reticulum and peroxisomes [19,20]. Pronounced metabolic and physical interactions of plastids also concern the only other plant compartment of ancient endosymbiotic origin: the mitochondrion. Plant mitochondria are much less in the focus of plant research. Next to their canonical functions, they are known to be involved in immunity, lipid metabolism and other (eco)physiological processes often in crosstalk with the photosynthetic organelle [21,22]. Like plastids, mitochondria were critical in the evolution and continued adaptation of important physiological traits in the green lineage. A notable example includes malate decarboxylation in the C4 photosynthetic pathway [23] – a convergent trait that improves plant photosynthetic efficiency in warm and dry habitats [24].

In spite of the importance of these two organelles of endosymbiotic origin in coordinating their duties, the evolution of components specific to chloroplast and mitochondrial biology has not been explicitly studied in light of streptophyte evolution and in particular plant terrestrialization. Previous work has determined genes specific to certain plant clades, including valuable resources such as the "GreenCut" [25]. Such analyses, however, are not focused on organelle biology nor clustered protein families. They were also limited by a low number of archaeplastidal genomes and insufficient methods for orthology inference available at that time. Since then, genome assemblies of members from previously unsampled clades has increased manyfold [11,26–34] and more organelle proteomes and better functional annotations are available. Similarly, and concomitantly, the development of novel and accurate algorithms for orthology inference [35–38] allow to now identify critical changes and adaptations in an eco-evo context of plastid and mitochondrial biology that underpin the success of the Chloroplastida.

Here, we curate a database of protein families unique to the green lineage. We plot their evolution across the major splits in the evolutionary history of streptophytes, focusing on the biology of the two organelles of endosymbiotic origin. We report that the number of plastid- and mitochondria-associated

protein families changes most significantly at two evolutionary bifurcations: firstly, at the green lineage itself, and secondly at the split between Zygnematophyceae and embryophytes at the water to land transition. The newly recruited protein families influenced organellar processes such as carbon and lipid metabolism, information processing and organelle development. We provide an extensive catalogue of the changes the proteomes of plastid and mitochondria experienced throughout streptophyte evolution, which offers multiple angles from which to explore major evolutionary transitions such as the conquest of land and embryophyte diversification.

Results

Half of the chloroplastida protein families are unique to embryophytes

Out of a total of 12,862,035 proteins, 95% were categorized from 686 eukaryotes (Table S1A) and grouped into 331,570 orthogroups (Table S1B). From these, 31,650 were present only in chloroplastida, and classified as Green Ortho Groups (GOGs) (Fig. S1 and Table S1C-D). An examination of GOG distribution among green species revealed that around half of all GOGs were unique to terrestrial plants (Fig. 1A). Approximately 400 GOGs appeared in more than 90% of species, termed here as 'core GOGs' (Fig. 1B). For only 5% of all GOGs, a functional annotation could be identified (Fig. 1C, Table S1E). Embryophyte-specific GOGs reflected a comparable percentage of this functional ambiguity, yet maintained a consistent distribution of identified functions, except increased fraction of membrane trafficking proteins (Fig. 1D, Table S1F). Notably, for core GOGs the number is higher, with 30% having functional annotations spanning areas such as photosynthesis, mitochondrial formation, trafficking, and information processing (Fig. 1E, Table S1G). The functions of a vast majority of the GOGs remain elusive (Table S1H), numbers that mirror those of previous studies [25], however, and hence provide an excellent ground for experimental exploration.

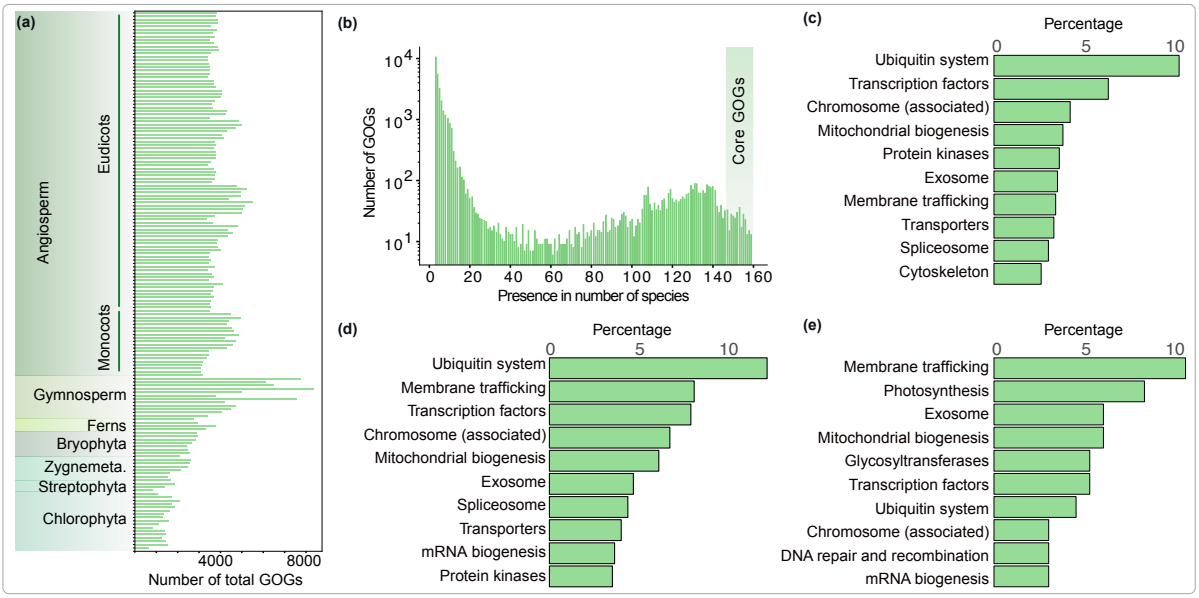

Fig. 1: Distribution and functional annotation of green orthogroups (GOGs). (a) Total number of GOGs present in each species from major Chloroplastida taxa. (b) Number of GOGs as a function of their presence across 159 Chloroplastida species. Major functional categories of 4.71% of all GOGs (c), 3.96% of the embryophyte GOGs (d) and 27.9 % of the core GOGs (e).

The origin and diversification of the Chloroplastida accompanied expansion of their organelle proteomes

To investigate changes in the proteomes of plastids and mitochondria, we curated 1961 plastid and 846 mitochondrial orthogroups (POGs and MOGs, respectively) based on published proteome data and homology-based protein clustering (Fig. S1B, Table S2A-C). In comparison to rhodophytes and glaucophytes, the green lineage has almost twice as many POGs (Fig. 2A). Within the green lineage, from the Zygnematophyceae and embryophytes onwards, plastid proteomes further expanded both in terms of the number of proteins within each POG and the number of unique POGs. The former is likely a consequence of genome duplications but the latter underscores functional divergence that followed gene duplications. The distribution of MOGs appears qualitatively similar to that of POGs (Fig. 2B, Table S2D-E). 60% of the POGs could be functionally annotated, predominantly operating in biosynthetic and other metabolic pathways such as photosynthesis (Fig. 2C, Table S2F). Around 75% of the MOGs could be functionally annotated and they predominantly operate in mitochondrial biogenesis, membrane trafficking and translation (Fig. 2D, Table S2G). Protein synthesis related proteins are abundant in both, POGs and MOGs, highlighting the biosynthetic capacity of the organelles. Proteins for mitochondrial biogenesis also appear in both. This encompasses numerous PPR and mTERF proteins (crucial for RNA editing) and proteins involved in various other information processing activities, probable to function in both organelles. Overall, the trends show that embryophytes have increased numbers of endosymbiotic organelle protein families.

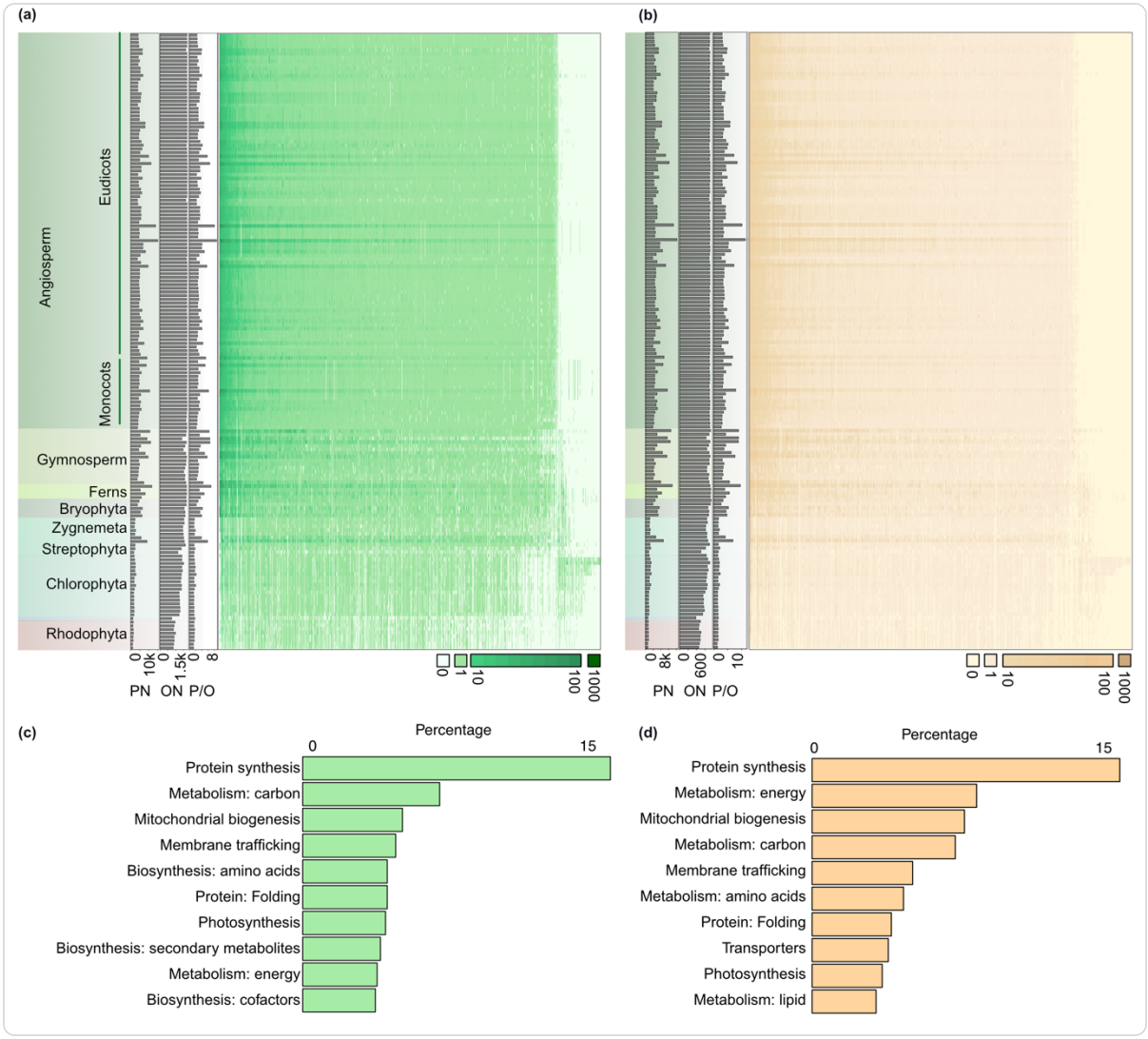

Fig. 2: Mitochondrial and plastid orthogroups across archaeplastidal species. Distribution of plastid (POGs, a) and mitochondrial orthogroups (MOGs, b). Protein copy numbers within each POG or MOG across species is shown in the heat-map as per the key on the bottom right of the heatmaps. Horizontal bars on the left side of the heatmaps show the total protein numbers (PN) likely localised to organelles, total POG or MOG numbers (ON) and distribution of protein number per OG (P/O) for a given species. Major functional categories of POGs and MOGs in (c) and (d), respectively.

The increased number of POGs and MOGs in the green lineage is explained by a combination of two phenomena: (a) new gains by the green ancestor, and (b) secondary losses at the origin of rhodophytes [39]. We used ancestral state reconstruction (ASR) to resolve between these two possibilities. The branching order of the archaeplastidal lineages remains challenging [40], as sometimes glaucophytes [41] and sometimes rhodophytes come out as the sister to the other remaining archaeplastidal lineages [4,42]. Hence, we inferred Archaeplastida phylogeny for both these scenarios (Fig. S2-3) and the ASR outcomes discussed are consistent with both (Fig. S4-6). The result suggests that the plastid proteome of the last common ancestor of Archaeplastida united 1150-1200 POGs (Fig. 3A, Fig. S4, Table S3). This inferred proteome witnessed significant gains of protein families at the emergence of the green ancestor (and later speciation). 50% of these newly gained POGs could be functionally annotated (Fig. 3A, Table S4), showing that the emergence of the green lineage was accompanied by an increase in the recruitment of photosynthesis- and metabolism-related POGs, while the transition to land (Z/E and

embryophyte ancestors) added metabolism related, as well as protein synthesis- and ubiquitin-related POGs to the toolkit. The mitochondrial proteome followed a qualitatively similar trend of expansion (Fig. 3B, Fig. S5, Table S5-6). 524 MOGs trace back to the archaeplastidal ancestor, while 632 MOGs were identified at the root of angiosperms. Around 50% of the newly gained MOGs could be functionally annotated, showing that the chloroplastidal gains contribute to carbon metabolism, protein synthesis and membrane trafficking. Terrestrialization also witnessed a similar gain of MOGs, most of which function in metabolism as well as mitochondrial biogenesis.

In summary, across plant species, plastid and mitochondrial proteomes have gained a significant number of protein families reflecting the dynamic nature of organellar proteomes post-endosymbiosis [43,44]. A closer look at the function of the newly gained organelle proteins shows a wide variety of functions (e.g. lipid and carbon metabolism, information processing, development and division of organelles).

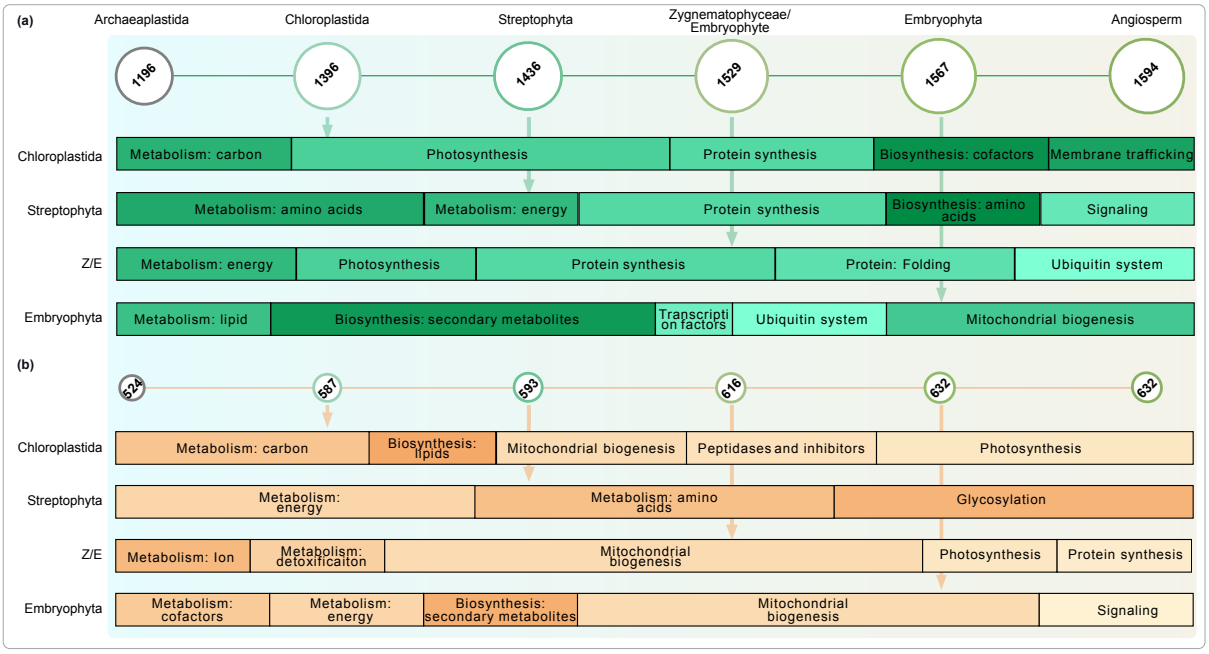

Fig. 3: Evolution of organelle proteomes in Archaeplastida. Gains in plastid (POGs, Fig. 3A) and mitochondrial orthogroups (MOGs, Fig. 3B) across the main nodes of archaeplastidal evolution. Each circle represents an ancestor, with predicted number of protein families present in that ancestor shown in the circle (also represented by the size of circles). Major gains were in the ancestor Chloroplastida, the common ancestor of Zygnematophyceae-embryophytes (Z/E) and embryophyte. Their functions are shown in the pie chart below the ancestors. The numbers of OGs across all ancestor, on a phylogenetic tree, is shown in Fig. S4-5.

Improved RNA processing and photosynthetic adaptability

RNA editing intercepts the linear information flow from mRNA to protein and is crucial for organelles to function [45,46]. Two main domains, the PPR and mTERF domain, are associated with RNA editing. We first screened for organelle orthogroups containing either of these two domains in at least 60% of all proteins within each respective orthogroup (Fig. S1C). A total of 50 POGs and 20 MOGs were found. More than 80% of them were restricted to embryophytes, only few were present in some algae (Fig. 4). A closer look revealed that most of the algal homologues lacked PPR and mTERF domains and they are hence unlikely true orthologues. More generally, this shows that any detailed interpretation regarding an inferred orthogroup's function should be supported by screening for functionally relevant

domains. True PPR or mTERF domain-containing RNA-editing proteins increased significantly in number by recruiting new orthogroups, also through adding the two domains to proteins that did not contain these in their algal ancestor. A presence-absence pattern also shows that >90% of proteins containing PPR/mTERF domains are present only in the land plants, except Chara and Klebsormidium (Fig. 4B). These proteins include, but are not limited to, OTP51 and SOT5 (present in embryophytes and Chara) as well as SOT1, SVR7, THA8, PDM4 (present only in embryophytes; Fig. S7). Target transcripts of these RNA editing factors point to the synthesis and assembly of photosynthesis-related proteins and to proteins of the thylakoid membrane (Fig. 6B). Likewise, mTERFs, which are crucial for plastid and leaf development, are also uniquely expanded in the terrestrial clade with examples of protein re-targeting across organelles [47]. The dual targeted (plastid and mitochondrion) mTERF6, unique to the land plants (Fig. S7) and the streptophyte alga *Klebsormidium*, takes part in retrograde signalling to the nucleus via ABA and imparts abiotic stress tolerance [48]. Overall, RNA editing across plants has undergone major changes and has a significant impact on photosynthesis, improvement of which was key to colonising land (Fig. 6B).

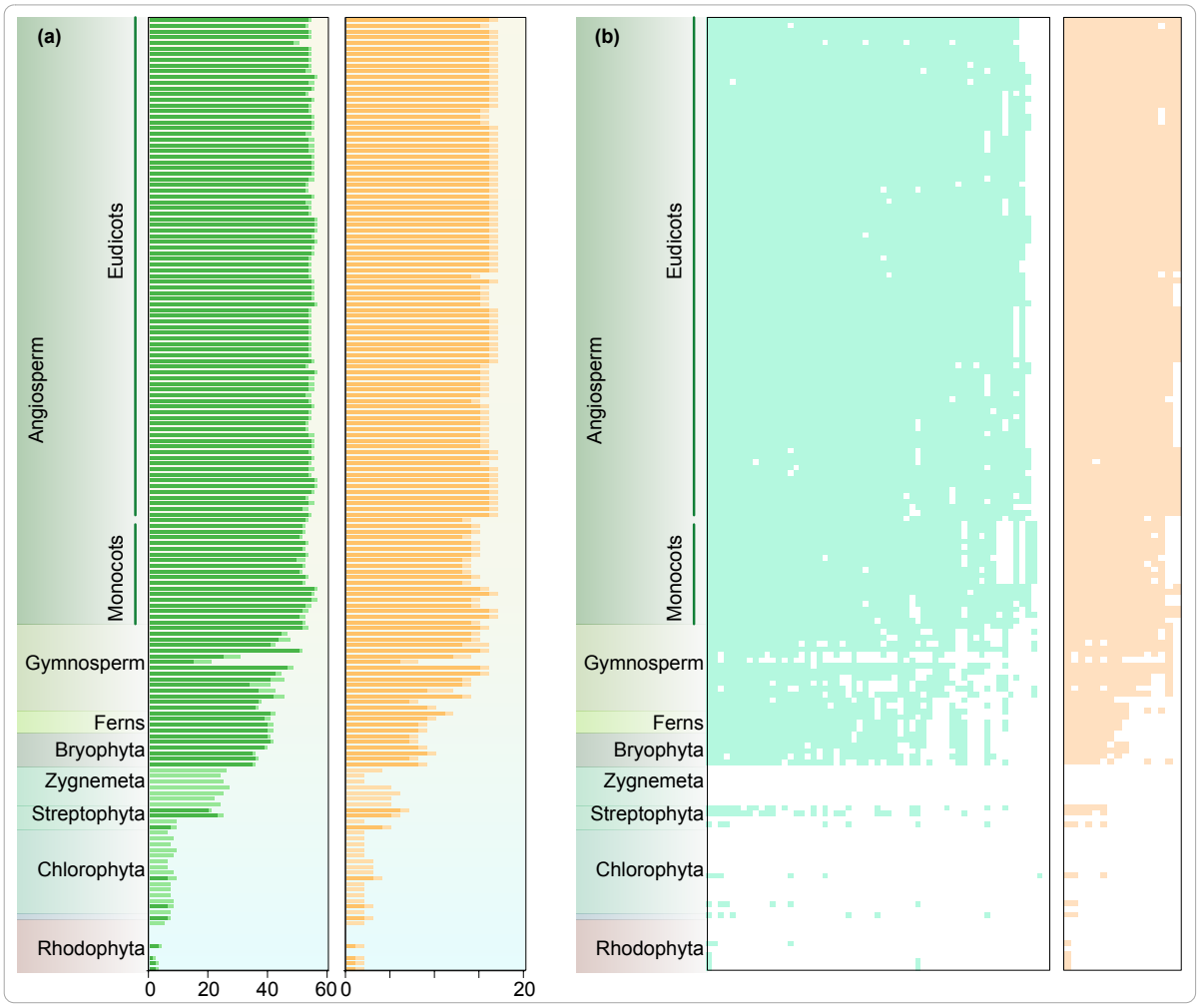

Fig. 4: Recruitment of PPR and mTERF domains in organelle proteins. (a) Number of POGs (left) and MOGs (right) where at least one protein contains a PPR/mTERF domain is shown in bars with dark shades of colors. Total number of orthogroups (regardless of presence or absence of PPR/mTERF domain in that particular spices) is shown in lighter shade. It shows the presence of the orthogroups in question in algae, but that they only later, in embryophytes, obtained PPR/mTERF domains. (b) Each cell represents an orthogroups and a coloured cell indicates the presence of a PPR or mTERF domain in the protein family (column) of a respective species (rows).

Adaptation to the terrestrial habitat and changes in plastid biochemistry

Main terrestrial stresses include draught, high (UV)light and swift temperature changes. Cutin and suberin, two of the most abundant lipid polymers on Earth [49], evolved as one countermeasure [50]. We find that cutin and suberin evolution was enabled by the embryophytic recruitment of an organelle-specific GPAT (Glycerol-3-phosphate acyltransferases) family (Fig. 5), which includes GPAT1 (mitochondrial), GPAT 4,6 and 8 of the endoplasmic reticulum [51,52]. Trafficking of these lipids across organelles was made possible by a dual targeted TGD4 [53] that appeared in the Z/E ancestor (Fig. 5). Acyl carrier thioesterases, responsible for the export of fatty acids from the plastid, acyl carrier protein desaturases (ACP-desaturase) and acyl-carrier proteins co-factors of fatty acid bio-synthesis were uniquely retained and expanded in the green lineage (Fig. S7). Duplication and divergence of ACP desaturases in embryo- and spermatophytes (Fig. S7) played an important role in regulating lipid composition shifts in response to temperature and drought, the regulation of seed oil content and development [54]. Likewise, acyl-carrier proteins also increased in copy number (Fig. S7) and adapted towards a light-induced expression and regulation of the seed fatty acid content [55,56]. These changes in organelle lipid synthesis and trafficking underpinned embryophyte adaptations to cope with draught and high temperature stress (wax biosynthesis, deposition on the layer of leaves and cuticle development), as well as seed development and germination in spermatophytes (Fig. 6D).

Changes in starch metabolism mostly pertain to its regulation. ADP-glucose pyrophoshorylase (AGPase), an enzyme responsible for a rate-limiting step in starch metabolism, is uniquely retained in the green lineage and increased in copy number in streptophytes (Fig. S7). AGPases diverged to regulate starch metabolism under osmotic and light stress, as well as the differential regulation of starch synthesis and degradation [57–61]. Another key regulatory enzyme, PGI (phosphoglucose isomerase) evolved a distinct family (PGI1) in Zygnematophyceae (Fig. S7). It likely kickstarted the regulation of starch metabolism at the water-to-land interface and later assumed significant roles in embryophyte fatty acid content regulation and the yield of seeds [62]. PTST3 also emerged around the time of terrestrialization (Fig. S7), which evolved to regulate starch synthesis with significant impact on plastid development [63]. In contrast to the flow of carbon through glycolysis, GSM2, which originated in streptophytes (Fig. 7), shunts carbon towards the pentose-phosphate pathway and protects plastids from oxidative stress in *Arabidopsis* [64].

Emergence of sophisticated antero- and retrograde communication cascades

Communication across compartments is critical for a concerted response to environmental stimuli. Plastids are key environmental sensors that interconnect cellular metabolism with physiological requirements and stress responses, and terrestrial stressors are key triggers of plastid-to-nucleus retrograde signalling [12,13,21]. We screened for the origin and diversification of EXECUTOR, WHIRLY and SVR4, all critical organellar components of retrograde signalling. The EXECUTOR, key to regulating retrograde signalling, oxygen and light stress regulation [65–67], originated in the ancestor of the Chloroplastida and so did WHIRLY (Fig. 5); the latter underwent copy number expansion in embryophytes and likely loss in some bryophytes (Fig. S7). Divergence of these copies led to a localisation across multiple organelles and today they are crucial for maintaining functional respiration, photosynthesis and the response of mitochondria and plastids to biotic and abiotic stresses [68–70]. These two key players, EXECUTOR and WHIRLY, accompanied the origin of the Chloroplastida and additional paralogs with specific functions in the main green lineages likely aided in the conquer of land (Fig. 6B).

SVR4, a dual targeted (plastid and nucleus) recruitment of embryophytes (Fig. 5), communicates required gene expression changes needed for light-induced plastid development, thylakoid stacking and thermomorphogenesis [71,72]. In combination, this facilitates light-induced photomorphogenesis, a process key for surviving on land. An increase in the complexity of retrograde signaling was a precursor for terrestrialization [12], for instance via innovations such as the 3′-phosphoadenosine-5′-phosphate family, which facilitated the emergence of stomatal closing in land plants [73]. The recruitment and diversification of the proteins we highlight, were quintessential for responding to two major stressors more pronounced and more rapidly changing on land than in water: light and temperature (Fig. 6B).

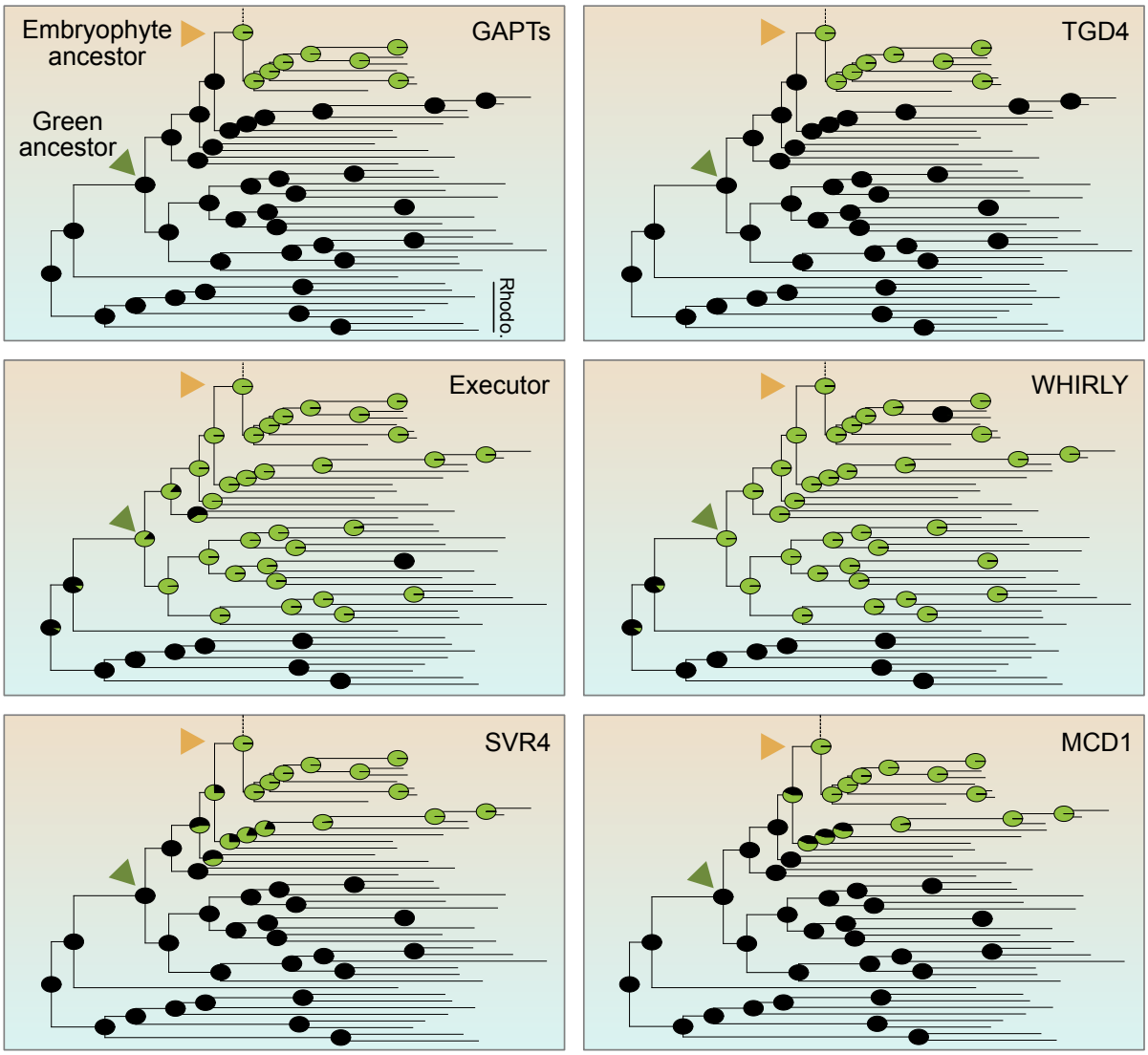

Fig. 5: Origins of key proteins involved in metabolism, communication and development. Ancestor state reconstruction (ASR) for selected lipid metabolism (GAPT and TGD4), retrograde signalling (Executor and Whirly), plastid development (SVR4) and division (MCD1) related proteins. The pie charts at each node on the tree represent probability of presence (green) or absence (black) of a protein family in that node.

Recruitment of new proteins and changes in organelle development

The coordination of tissue and plastid development is linked to ensure an appropriate response to biotic and abiotic factors, especially in morphologically complex plants [74–76]. Polyplastidy is a land plant trait [77] and known molecular determinants include MinD, MinE, ARC3 and the FtsZ proteins [16,75]. Our data supports that MULTIPLE CHLOROPLAST DIVISION SITE 1 (MCD1), a core component of the plastid division machinery [78], originated in the ancestral embryophyte (Fig. 5). The cotyledon chloroplast biogenesis factor CYO1 and the transcriptionally active chromosome factor 7 (TAC7) are core components of thylakoid biogenesis and the plastid translation machinery, respectively. Both originated in the streptophyte ancestor (Fig. S7) and, in *Arabidopsis*, play key roles in chloroplast, cotyledon, thylakoid and leaf development [79–81]. Lastly, CRUMPLED LEAF (CRL), a protein residing in the outer plastid membrane, emerged during terrestrialization, too (Fig. S7), likely for regulating plastid division and securing correct plastid inheritance during embryogenesis [82,83].

Crucial for plastid biogenesis, especially in light of an expanding proteome, is the import of proteins. The membrane GTPase TOC159 is essential for chloroplast biogenesis via the selective recognition and import of the photosynthetic proteins [84] and is unique to the green lineage (Fig. S7). The membrane recruitment of this protein requires TOC75, of which a special variant evolved in the green ancestor after the duplication of OEP80 [14]. The copy number of TOC159 expanded from the Zygnematophyceae onwards (Fig. S7), hinting at its functional diversification. Unlike in the chlorophyte alga *Chlamydomonas*, land plant TOC159 homologues possess an N-terminal acidic domain that gets phosphorylated to alter substrate specificity [84,85]. Furthermore, TOC159, along with TOC132 and TOC120, play important roles in regulating plastid lipid synthesis and membrane fluidity [86–88]. Further on the course of evolution, the J-domain-containing protein TOC12 [89] was likely recruited in the ancestral embryophyte for supporting the import machinery at the intermembrane space (Fig. S7). The terrestrial habitat demands a highly efficient and fluid import of proteins, for example upon high light and other abiotic stresses [14,90]. The expansion of the TOC/TIC system in the embryophyte ancestor reflects how the organelle dealt with an ever-increasing diversity of substrates that were required to be processed.

Discussion

The conquest of land by a streptophyte alga and the propagation of plants (Fig. 6A) was pivotal in the transformation of the terrestrial habitat and it laid the foundation for the concomitant colonization of land by animals [1,2]. Throughout the hundreds of millions of years of this endeavor, both plant organelles of endosymbiotic origin underwent a multitude of molecular adaptations, hereby evolving into the plastid and mitochondrion of modern plants. We identified 31,650 protein families unique to the green lineage, approximately 50% of which are unique to embryophytes. It demonstrates an expansion and divergence of protein families around plant terrestrialization, in line with a recent study that identified around 10,000 duplications at the birth of embryophytes [91].

Expansion of proteins families is evident in both organellar proteomes at the origin of the green lineage itself and at the water-to-land transition. The gain of protein families at the origin of the green lineage needs to be treated with caution due to the documented genetic bottleneck that characterizes rhodophyte origin [92–96] and the sparse availability of glaucophyte genome data. Some of the newly recruited protein families at the origin of the green lineage might rather be explained by a loss in rhodophytes and a retention in the chloroplastidal ancestor instead of a gain. Regardless, this has a little bearing on the biological significance of a given protein family with respect to the overall increase in complexity of organelle biology – both concerning the variety as well as the number of proteins targeted to plastids

and mitochondria – throughout streptophyte evolution that affected the organelles metabolic, informational and developmental complexity, and facilitating the conquest of land (Fig. 6).

Changes in organelle lipid biochemistry contributed to one of the key adaptations in land plants: cuticles. Land plant GPATs (Glycerol-3-phosphate acyltransferases, crucial to lipid synthesis for cutin and suberin) contribute to increased hydrophobicity and water retention in embryophytes [50] and show activity distinct from algae [97,98]. Our analyses underpin origins of organelle specific GAPTs (GAPT 1,4,6,8) to the ancestor embryophyte, of which, deleting GPAT4 and GPAT8 distorts cuticles and increases water loss by several fold [51,52]. In parallel, lipid trafficking was mediated by the recruitment or divergence of proteins such as TGD4 and acyl carrier thioesterases, which contributed to wax biosynthesis and deposition on leaves, cuticle development, thylakoid membrane stacking [53], seed development and germination [54]. As for starch metabolism, the archaeplastidal ancestor likely stored starch in the cytosol [99], but the red and green lineage experienced different fates from there on. Rhodophytes continued to store starch in the cytosol in the form of Floridean starch [100], while in the green lineage, particularly in complex plants, more localized control of starch synthesis and degradation was facilitated by changes in regulatory proteins (eg AGPase). Together, organelle metabolism evolved to serve key roles in the synthesis, regulation and trafficking of lipids involved in wax coating to prevent water loss in the land plant ancestor, as well as synthesis and storage of starch (Fig. 6D).

RNA processing and editing is a crucial component of information processing and overall functionality of plant organelles [45,46]. Changes in RNA editing are evident from the origin of the green lineage itself, where RNAse-P (tRNA maturation) was replaced by protein only RNAse P or PROPs [101,102]. Subsequent expansion of PROPs in embryophytes (Fig. S7) led to organelle-localised copies of which some are essential for maintaining organelle morphology, functions and plant viability [103]. RNA editing of C to U is not found in algae, however, and editing sites in embryophytes are unlike those of any other eukaryote, suggesting they emerged independently [46]. Of the many RNA-editing proteins we find that were gained during terrestrialization, known targets are transcripts involved in photosynthesis and stress tolerance-related transcripts, both key to colonising the land (Fig. 6B). For instance, THA8, PDM4, SVR7 and SOT1 edit transcripts such as ycf2 and ycf3, and contribute to thylakoid development and biogenesis [104], the generation of photosynthetic complex proteins, granal stacking, embryo [105] and plastid development [106,107]. OTP51 and SOT5 edit transcripts related to chlorophyll synthesis, photosynthesis and thylakoid membranes (ycf3, TRNK and RPL2) [108–110], whereas DOG1 is important to high temperatures response and chloroplast development[111]. This elaborate RNA processing in organelles, especially plastids, appears to serve photosynthesis (and thylakoid) related transcripts. It is feasible that by benefitting photosynthesis, organelle RNA editing continued to be positively selected for and was expanded.

One adaptation towards efficient photosynthesis, where RNA editing also plays a key role, are grana [74]. The evolutionary origins of grana remain elusive, along with the underlying developmental pathways involved in regulating its formation and maintenance [74,112,113]. Grana are observed in embryophytes and some Zygnematophyceae (e.g. the *Cosmarium* genus) [114], but not chlorophytes such as *Chlamydomonas* [115]. We noticed a patchy distribution of grana morphology associated proteins such as CURT1, RIQ1 and RIQ2 (Fig. S7), with both RIQs being present in all streptophytes and some chlorophytes but excluding *Chlamydomonas*. In light of the many key adaptations in Zygnematophyceae discussed here and elsewhere [11,116], we speculate that grana originated in streptophytes and were important in conquering land through photosynthesis optimization, in particular with respect to photosystem repair and the separation of the photosystems and the ATP synthase [117,118].

This expansion of an organelle proteome necessitates improving the capacity to import proteins. Changes in some import receptors within the green lineage and in targeting sequences at its origins are known, with phosphorylation likely emerging as a key regulator for sorting the newly expanded proteome differentially to plastid and mitochondria (Fig. 6C) [14,119]. Despite such adaptations, protein sorting is never perfect and some mistargeting might be positively selected for. A regulated distribution of newly recruited proteins (e.g. WHIRLY, TGD4, mTERF6; Fig. 6B) to multiple organelles (with distinct organellar functions) hints at adaptive values of this apparent mis-sorting. How many of newly recruited proteins get 'mis-sorted' owing to biological adaptability versus stochasticity remains to be explored together with obtaining a more comprehensive picture of (regulatory) mechanisms associated with sorting in general.

Embryophyte cells target proteins not to a single plastid, but many simultaneously. The presence of multiple plastids per cell, polyplastidy, evolved in embryophytes, likely through changes in plastid fission and a decoupling of organelle fission from the cell cycle [15,16]. We find that MCD1 a core regulator of the plastid division proteins FtsZ2 and ARC3, emerged in the embryophyte ancestor, which corroborates the idea of a mono- to polyplastidy switch during terrestrialization [16,77,120,121]. A change in the copy number of plastids also requires a mechanism that maintains a functional organelle to cell volume ratio and resource allocation (Fig. 6C). The REDUCED CHLOROPLAST COVERAGE (REC) protein is involved in such a mechanism in *Arabidopsis* [122] and the phylogenetically related protein FRIENDLY regulates the distribution of mitochondria, also in plants and non-photosynthetic organisms [123,124]. REC and FRIENDLY share almost all of their domains. How they exactly function and differentiate between the two organelles remains elusive. From what we can tell, FRIENDLY emerged during eukaryogenesis and the origin of mitochondria. REC we trace back to the streptophyte ancestor (Fig. S7) and after a likely duplication event of FRIENDLY. We speculate that the origin of REC helped to establish polyplastidy, which itself supports larger body plans and the diversification of different plastid types [15]. Lastly, an increase in organelle copy number also requires an overall increase in the capacity to synthesize proteins. The largest fraction of organelle proteins operate in tRNA, amino acid and ribosomal biosynthesis and undergird the biosynthetic capacity of organelles, an adaptation strategy akin to their bacterial ancestor [125,126].

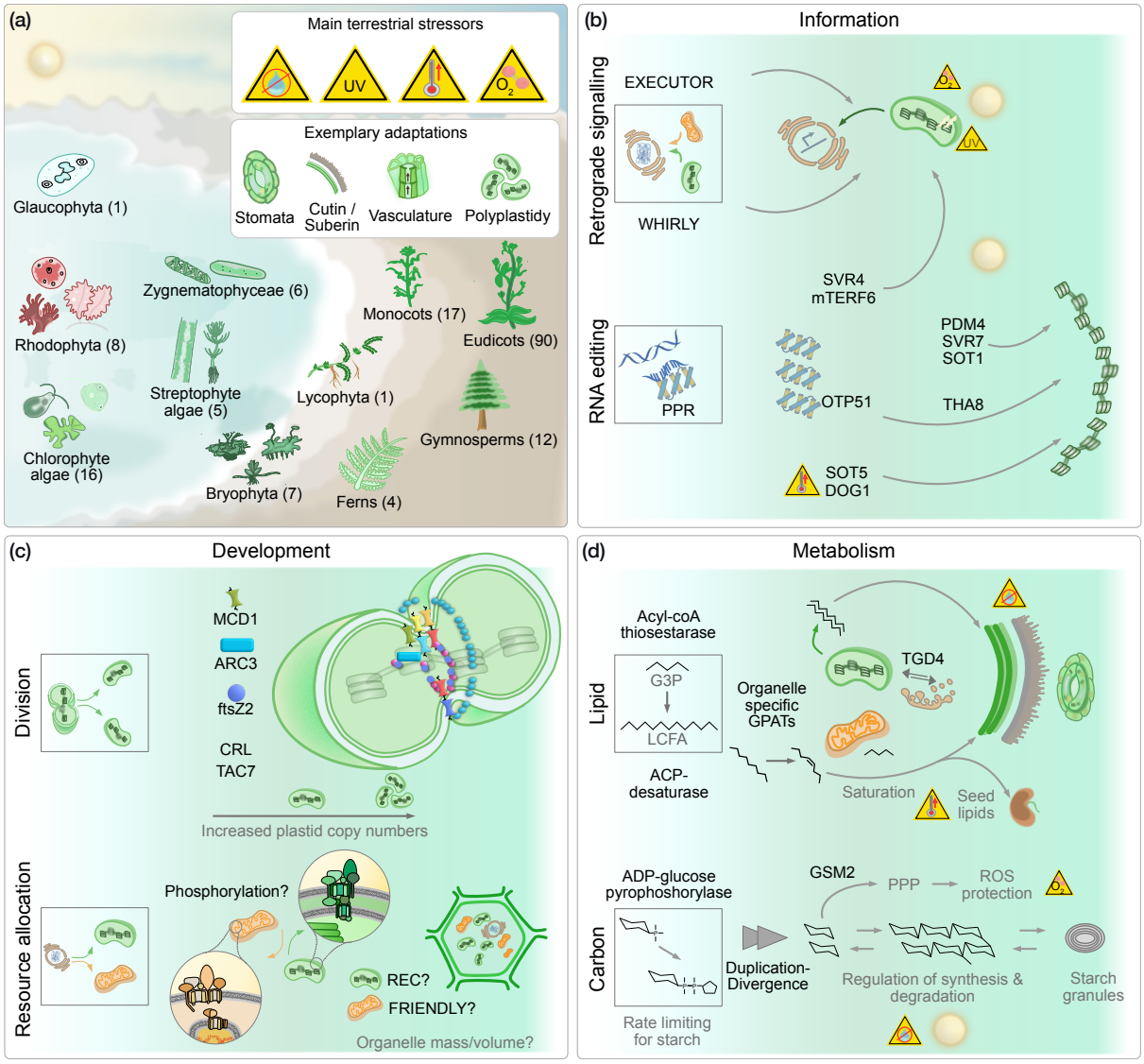

Fig. 6: The global greening and endosymbiotic organelles. (a) After the endosymbiotic origin of the plastid, three aboriginal lineages emerged that form the Archaeplastida: the glaucophytes, rhodophytes and chlorophytes. From the latter, streptophyte algae evolved, including the zygnematophyceae, that represent the algal sister clade to land plants (embryophytes). Abiotic stresses encountered during terrestrialization (water scarcity, high UV, swiftly altering temperatures and higher levels of O_2) selected for adaptive features such as stomata and a cutin layer. The numbers in parenthesis indicate the number of genomes from each major group we screened. Recruitment of new organelle proteins improved three key aspects of organelle biology in light of terrestrialization: (b) information processing, (c) development and (d) metabolism. Details for each tile are discussed in the main text.

The accommodation of the early mitochondrial endosymbiont was key to the emergence of the endomembrane system and complex eukaryotic traits including mito- and autophagy [127–129]. Our analyses show that the integration of a subsequent endosymbiont, the plastid, coincided with the emergence of proteins that work for the endomembrane system. Particularly, changes in the ubiquitin system were prominent around terrestrialization, when polyplastidy in the green lineage also emerged (Fig. 3). Ubiquitination is key to proteosome-mediated degradation and is performed chiefly by a family of proteins called E3 ubiquitin ligases which are important in land plants also for

photomorphogenesis[130]. RING (Really interesting new gene) E3 ligases contribute to growth, development and stress response via also mediating protein-protein interactions [131–134]. We trace a number of RING finger (and related) proteins to terrestrialization (Fig. S7) that include, but are not limited to, *DAL1* and *DAL2* (for *Drosophila* DIAP1 like 1 and 2), KEG (Keep on going), and NIP1 and NIP2. *DAL1* and *DAL2* play a key role in regulation of programmed cell death [135], peroxisome and chloroplast biogenesis [136–138]. KEG contributes to stress mitigation [139,140], while NIP1 and NIP2 play a role in plastid development by docking plastid RNA polymerase to the thylakoid membrane [141]. The regulated degradation of plastids and other changes in the endomembrane system were key to housing multiple plastids per cell and we find many more recruitments broadly affiliated with the endomembrane system, with no well characterised functions. Exploring the functions of these proteins will add valuable insights into the cell biological changes that endosymbiosis stipulates.

In closing, although experimentally reported plant plastid and mitochondrial proteomes are scarce, we were able to generate a first comprehensive molecular atlas of the changes of plastid and mitochondrial protein families in the evolution of the green lineage. Ancestral state reconstruction (ASR) allows to map the organelle transformations that facilitated the major transitions such as terrestrialization and which will improve with every new proteome that is added. By inferring plastid and mitochondrial proteomes for 168 species, we set testable expectations for new proteomes to come and provide a solid database, where origins and across species orthologues of any known (organelle) protein can be searched easily (Table S2B-C). We identify numerous mitochondrial protein recruitments, the physiological roles and adaptive values of which help to better understand plant mitochondrial biology. For plastid proteins, we infer their functions and physiological importance based on the extensively studied *Arabidopsis* system. Utilizing an advanced orthology search technique [37], we postulate that orthologues of *Arabidopsis* are likely to exhibit similar functions in other species. Our methodologically robust approach offers various changes in evolution, associated in particular with terrestrialization, that can now be experimentally explored across selected models and with a focus on less-well studied streptophyte algal and bryophyte species [142,143].

Conclusions

Endosymbiotic organelles have a distinct place in the evolutionary tapestry of life. Through the combination of organelle proteome data and phylogeny, we trace the evolution of mitochondria and plastids over a span of a billion years of plant evolution by inferring their proteomes for over a hundred Archaeplastida species. Our comprehensive molecular atlas identifies main changes in their metabolism, communication, information processing and biogenesis. Key adaptations in plant organelles fostered the emergence of wax and cutin (see organelle lipid synthesis and transport), improved the photosynthetic yield (see organelle RNA editing and grana) and the response to abiotic stressors (see inter-organelle communication), and mediated the transition from mono- to polyplastidy (see division and volume control). By connecting the molecular adaptations of mitochondria and plastids to macroevolutionary trends, we show how important changes in organelles of endosymbiotic origin were for the speciation that gave rise to the Chloroplastida and later the origin of land plants from a charophyte algal ancestor.

Material and Methods

Curation of green orthogroups (GOGs). Input protein sequences from 686 proteomes (from KEGG and Phytozome, Table S1A) were clustered using Orthofinder version 2.5.4 [37], after all vs all blasts were conducted (E-value cutoff 10e-10) using diamond blast version2.011 [35]. From orthogroups (OGs) recovered, OGs with at least 3 Chloroplastida species green species and less than 3 species other than Chloroplastida were annotated as green orthogroup (GOGs). Schematic in Fig. S1A.

Curation of plastid and mitochondria orthogroups (POGs and MOGs). 4,915,150 proteins from 168 Archaeplastida species were clustered using Orthofinder as described above. Orthogroups that contained at least one experimentally verified organelle protein from any one of the four experimentally verified organelle proteome of *C. reinhardtii* [144], *P. patens* [145] , *Z. mays* [146], *A. thaliana* [146], were annotated as organelle (plastid and mitochondria) orthogroups. Schematic in Fig. S1B.

Functional annotation of orthogroups. The source of >90% species was Kyoto Encyclopedia of Genes and Genomes (KEGG), which included KEGG orthology identification (KOID) for protein sequences. For all proteins within each GOG, KOIDs were retrieved and the most frequent KOID (i.e. majority rule) was annotated to each GOG (Fig. S1C). From the assigned KOIDs, their KO BRITE functional category was assigned to each GOG. KOIDs for POGs and MOGs were retrieved the same way. For each KOID, the pathway names and BRITE categories at various level of resolutions were used for assigning functional categories manually to each OG. Manual assignment was necessary since BRITE names included a large fraction of categories such as 'enzymes' and 'exosomes'. These were either not very informative or were misleading as many of 'exosome' annotated proteins took part in protein synthesis or folding. Lastly, for OGs or proteins discussed with respect to their physiological relevance, the functions were retrieved from the literature (cited in the text).

Inference of ancestral states. A phylogeny of Archaeplastidal species was inferred based on all genes conserved in all species, using 'Species tree inference from all genes (STAG)' method [147], as a part of orthofinder analysis. STAG infers a species tree by taking greedy consensus of gene trees from each protein family (including that of multigene families). This phylogeny was rooted using minimal ancestral deviation [148] which places Rhodophyta as the sister to all others. Independently, the same unrooted phylogeny was manually rooted using FigTree (v1.4.4) [149] such that Glaucophyta were at the base. Ancestor state of presence and absence of organelle protein families across nodes, were inferred using Phytool [150] package 0.7.80. Based on character state at the tips of the tree, Phytool inferred Bayesian posterior probabilities under a single rate model [151,152] of the character state across nodes of the tree. All OGs that were present in major ancestors of plant groups with probability higher than 0.75 and absent in the preceding ancestor, were considered as newly recruited in that lineage. OGs or proteins discussed with respect to its physiological role in a given clade, their absence outside the group was verified in our copy number database as well as on homologue database available on TAIR.

Searching for potential RNA editing POGs and MOGs. Hidden Markov models (HMM) of PPR and mTERF domains were downloaded from pFAM [153] with the IDs: PF01535, PF12854, PF13041, PF13812, PF02536. Each of these HMMS was used as a query to search against the full sequences of all proteins within each POG and MOG. If a given OG had more than 60% of individual proteins containing PPR or mTERF, the OG was annotated as RNA editing OG. Origin of such OGs were traced using ASR as described above.

Author contributions

PKR: Conceptualization; Experimental design; Methodology; Investigation, Data curation; Formal analysis; Visualization; Writing - original draft, review and editing. AIM: Methodology, Investigation, Formal analysis; Writing - original draft, review and editing SBG: Conceptualization; Project administration; Funding acquisition; Resources; Supervision; Visualization; Writing - original draft, review and editing.

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